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Paratuberculosis

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PROGRAM AND ABSTRACTS

8th International Colloquium on Paratuberculosis

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The History of Paratuberculosis

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In the mid-1820's, a debilitating intestinal disease of cattle was noticed with increasing frequency, but it was not well described and its cause was unknown. For 70 years, the disease was just considered one of many uncharacterized diseases of unknown etiology. In those 70 years however, the tubercle and "avian tubercle" bacillus had been identified and tuberculosis had become a familiar disease in humans and cattle. In 1894 a cow from the Odenberg region of Germany was presented for post-mortem examination to Dr. H. A. Johne of the Veterinary Pathology Institute in Dresden as a suspected case of intestinal tuberculosis.

Dr. Johne and his fellow, Dr. L. Frothingham from Boston, described the lesions of the disease, noting the abundant acid-fast bacilli in the intestinal tract. The diagnosis of intestinal tuberculosis seemed possible, however the organism failed to grow and a sample of the infected tissue containing the organisms failed to cause disease in guinea pigs, thus eliminating tuberculosis as the cause. Johne and Frothingham suspected that the disease might be caused by the avian tubercle bacillus and proposed the name pseudotuberculous enteritis; in other words, sham or false tuberculosis.

Following the description by Johne and Frothingham, reports from all over the world began to document just how widespread was the disease. The disease also caught the attention of Bang, who, after examination of tissues, concurred that it was not caused by the tubercle tuberculosis. Bang proposed the disease be called paratuberculosis; i.e., resembling tuberculosis. Bang also noted that infected animals failed to respond to tuberculin antigens (PPD), but did respond to similar preparations prepared from the avian tubercle bacillus. These observations were rapidly confirmed and the use of diagnostic tests emerged. Within just 20 years of the first description of the disease by Johne and Frothingham, the disease was recognized throughout Europe and the United States.

Just a few years later, in 1910, Twort succeeded in isolating the causative agent. This was not accomplished by any sort of planned laboratory experimentation, but rather in many respects through a laboratory accident. Twort proposed the name *Mycobacterium enteriditis chronicae pseudotuberculosis bovis johne.*

Although Twort received full recognition for this achievement, another scientist also deserves much of the credit. Holth succeeded in isolating the causative agent during the same year and completely independent of Twort. His achievement was by experimental design rather than by chance and was more attuned to the work of others. Holth recognized the work of Bang and others and shared the belief that this organism may not be a unique species. He simply called his isolate the paratuberculous bacillus. Unfortunately, Holth's efforts and achievements were lost in history.

With the isolation of the causative agent, and the extraction of its crude antigens, the gateway was open for a host of experiments into the biology and pathogenicity of this organism and the development of diagnostic assays including complement fixation, agglutination, skin testing, and others. In a series of transmission experiments, Vallee and Ringard in 1923 observed that subcutaneous inoculation did not result in disease. These findings rapidly led to the development of a subcutaneously inoculated vaccine.

Also in 1923, the first edition of the Bergey's Manual of Determinative Bacteriology was published and adapted the nomenclature of Bang and Holth, officially naming the disease paratuberculosis and its causative agent *Mycobacterium paratuberculosis.*

In 1922, the scientific community started requesting that the disease be controlled and reported. By the mid-1920's, it was well recognized that this disease was distributed worldwide, spreading rapidly, and something needed to be done to control it. Between 1922 and 1935, no fewer than a dozen countries were calling for some sort of control measures. Even the World Health Organization called for its classification and regulation as it had for brucellosis. These outcries for control were essentially ignored, even though the disease was now being classified and referred to a "common" disease of cattle.

Throughout the 1930's to the present, there have only been a few discoveries or reports that have had a major impact on paratuberculosis that this author considers of historical significance. This is not to detract from the tremendous efforts made during this period by numerous investigators and the contributions they have made to our understanding of this disease. It is the combined contributions of all these investigators that has had significance, rather than a single effort.

During this time period, almost any conceivable experiment or observation was reported. Every imaginable diagnostic test was exhaustively evaluated, control measures were defined and implemented, susceptibility of laboratory animals, transmission, vaccination, resistance, wildlife reservoirs, etc., were all extensively studied. In fact, anything you can imagine was evaluated in one form or another, particularly in the earlier years of the 1930's and 1940's.

In 1939, a mycobactin-independent laboratory strain of *M. paratuberculosis* was deposited with the American Type Culture Collection as the working strain of this species and was designated ATCC 12227. This strain, also known as "strain 18" was widely used in paratuberculosis research and considered the "type strain" until 1968. Despite its withdrawal from the ATCC in 1968, it continued to be used in paratuberculosis research. It is now known that the infamous "strain 18" was a laboratory contaminant and is actually a strain of *M. avium* serovar 2. As a result, many studies and data presented between the period 1920 (when strain 18 was first described) and through the 1970's are simply invalid and serve to confuse and corrupt our knowledge of *M. paratuberculosis*. Strain 18 deserves a place in history for setting science back for many many years.

As previously stated, there was a major boom to the paratuberculosis field starting in the 1920's form which several conclusions may be drawn, namely:

Diagnostic tests do not work. When evaluated in a clean environment, i.e., non-endemic areas, most diagnostic tests perform at acceptable levels, but when evaluated within an endemic area, like an infected herd, the tests fail miserably. That situation has not changed. Although in more recent years, statistics have been used to increase the specificity and sensitivity of diagnostic tests, they really do not work much better than they did 50 years ago. Furthermore, no currently used diagnostic test has been put through the rigorous and extensive evaluations of the past. History shows that diagnostic tests provide broad indications of infection in populations of animals and cannot reliably separate individual infected from non-infected animals within endemic areas. This is no different form the diagnostic expectations for tuberculosis and leprosy.

Eradication/control programs do not work. Whether conducted on a state/country level or on an individual farm, paratuberculosis cannot be eliminated. Certainly there are exceptions and there might be some individual successes, but the resources of the past are not available today to conduct the type of experiments needed to truly and authoritatively document elimination. History shows that elimination, i.e., creation of a disease-free state, cannot be achieved once a herd becomes infected and the term "control" needs to be used in the broader context meaning simply to reduce infection and keep the disease "in check".

Things have changed little since then and what has been learned in the past has only been corroborated and expanded upon in more recent years. Certainly there has been a better understanding, some improvements, and overall progress, but few reports have created scientific landmarks. The few exceptions with long-lasting impact on the field are noted below.

In June 1983, Richard Merkal of the National Animal Disease Center in Ames Iowa organized a meeting to bring together researchers from around the world involved in the study of paratuberculosis. This meeting was the first devoted exclusively to this disease and was called The International Colloquium on Research in Paratuberculosis. It was a small group but represented most of the major researchers in the field at the time.

In 1984, *M. paratuberculosis* was isolated from human patients with Crohn's disease providing the first physical evidence of a link between *M. paratuberculosis* in cattle and Crohn's disease in humans. Although

similarities between these 2 diseases were noted as early as the mid 1800's and, from time to time, reports appeared bringing forth those similarities, it took physical evidence to stir sufficient controversy to warrant a place in history. This controversy continues to this date, as it will likely for many years to come – the politics of science promises to ensure just that.

In 1988, Marie Thorel and Dick Merkal organized a second meeting in Alfort France specifically devoted to research on paratuberculosis. This meeting, named the Second International Colloquium on Paratuberculosis, is where and when the concept of this association was envisioned and proposed. In 1989, the International Association was officially formed, incorporated, and began to solicit membership.

Also in 1989, a species-specific insertion sequence of *M. paratuberculosis*, known as IS900, was identified by 2 independent groups. This discovery provided the first definitive, non-subjective, means for the identification of this organism. It also led to improved diagnostics and means of identifying the species.

In 1990, Marie Thorel and co-workers published their findings on a matrix analysis of *M. paratuberculosis* and several species of *M. avium* and concluded that *M. paratuberculosis* belonged to the *M. avium* complex. These authors proposed to rename *M. paratuberculosis* as a subspecies of *M. avium*, specifically *M. avium* subsp. *paratuberculosis*. With the publication of this proposed nomenclature in the International Journal of Systematic Bacteriology, *M. avium* subsp. *paratuberculosis* (commonly abbrevieated as MAP) became the official and formal name of the organism. Few understood (or understand) the politics of the International Working Group on Mycobacterial Taxonomy (IWGMT) and, although it took many years for the name MAP to gain wide acceptance, most investigators now use the official name *M. avium* subsp. *paratuberculosis* rather than the prior name, *M. paratuberculosis*.

This author rejects the MAP nomenclature and, under rights provided by the International Committee on Systematics of Prokaryotes, never has and never will use the now accepted nomenclature or its abbreviation "MAP". When the International Committee on Systematics of Prokaryotes and other taxonomic groups reject pathogenicity and phenotyes as criteria for speciation in other bacterial species, then and only then will this author accept *M. avium* subsp. *paratuberculosis* or MAP as the recognized and formal name of the causative agent of paratuberculosis, i.e., *M. paratuberculosis*.

So, after 100 years and well over 3,000 publications, what can we learn and how can such information be used for the future?

Control. The time for regulatory control has passed. The opportunity existed in the 1920's, but this opportunity has been missed. The infection is far too widespread and endemic, and the costs too great, to even consider such an endeavor. It would be like trying to regulate mastitis. History documents that mandatory and voluntary control programs have all failed -the insidious and endemic nature of the disease ensures its ever-lasting presence.

On an individual farm basis, control within the context of reducing overall infection is a worthwhile endeavor, but history shows that eradication is not a realistic goal. Like human leprosy, unless continuously treated the disease will eventually come back again. It is only a matter of time. Within that context, vaccination is a viable alternative.

Although this author has opposed vaccination in the past, it now appears to be a realistic option. Although vaccination, within the context of available vaccines, does not lead to disease eradication, it does help with control of the infection. It is no better or worse than human BCG vaccination against tuberculosis. It is not a magic wand it is certainly not perfect, but it does help within endemic areas where there are few other realistic options.

Diagnostics. History clearly documents that they simply cannot identify all infected animals. Looking back in history and trying to compare "apples-to-apples", it appears that although diagnostic tests have improved over the years, the amount of improvement is not great. More importantly, history shows that no diagnostic test for any infectious disease has ever worked in an endemic area, at least within the context of detecting sub-clinical infections. Enough already!

Certainly there is room for improvement, but with improvement comes increased cost and diminished use. Available diagnostic tests need to be used, and used exclusively, for their appropriate applications. Namely, identification of infection in non-endemic areas and identification of disease within endemic areas. Uses outside this limited scope of their application, is a never ending battle of futility.

There is much to learn from history. It is there for us all to review and learn, learn from past achievements as well as failures. It is only up to you whether or not you choose to take advantage of it.

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Vaccination strategies against human tuberculosis

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Tuberculosis is one of the biggest infectious disease killers worldwide, a situation worsened by the advent of the HIV epidemic and the emergence of multi-drug resistant strains of *M. tuberculosis*. Although the current TB vaccine (BCG) is efficient against severe childhood forms of tuberculosis, it has a limited effect on development of the predominant pulmonary form of the disease in adults. Therefore, for a number of years large resources have been invested in the identification of candidate molecules for a new generation of TB sub-unit vaccines. With the complete genome of *M. tuberculosis* available, antigen discovery has taken a leap forward and applies highly efficient post genomic approaches based on proteomics and antigen/epitope identification "in silico". This has resulted in the identification of a large number of antigens within the last 6-8 years, many of them with potential in TB vaccines. Many of these molecules are encoded by genes within a few immuno-dominant gene families. The multiple members within these families may serve as a source of antigen variation and result in pathogen escape from the host immunesystem. The next phase of this work has now started: putting the most relevant molecules back together as fusion molecules and cocktails. This requires careful monitoring of aspects such as immunodominance, recognition in different populations as well as the influence of different adjuvants and delivery systems. The establishment of these highly defined effective vaccines allows detailed studies of the optimal route, as well as prime-boost regimes for expression of immunity in the lung and enables further attempts to determine correlate markers of protection in TB. The most advanced of these vaccines such as the fusion between ESAT6 and Ag85B have been evaluated in a range of animal models including non-human primates and are now in the clinical trial pipeline. For these vaccines to be successfully implemented in future vaccination programmes it is necessary to understand the immunological background for the failure of BCG and optimize the vaccines for their ability to boost the immuneresponse primed by BCG, environmental mycobacteria or already established latent TB.

Antigens encoded by the dormancy regulon of *Mycobacterium tuberculosis* are preferentially recognized by T cells from individuals with latent infection

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Recently, the so-called dormancy regulon of *Mycobacterium tuberculosis* was identified. Comprised of 48 genes, protein expression is noted in vitro during hypoxia and low-dose nitric oxide stimulation. Tubercle bacilli are thought to encounter these conditions in vivo during the onset of immune responses and during subsequent persistence in immune competent hosts. In this study, human immune responses to the 25 most strongly induced dormancy regulon-encoded proteins or candidate latency antigens were assessed, using *M. tuberculosis* specific T cell lines and peripheral blood mononuclear cells (PBMC) from *M. tuberculosis* infected persons, including tuberculosis (TB) patients and latently infected individuals, as well as uninfected controls. Nineteen of the 25 tested antigens were recognized by PBMC from *M. tuberculosis* infected individuals, which demonstrates that the dormancy regulon is indeed expressed during natural *M. tuberculosis* infection in humans. Interestingly, IFN- production in response to the entire group of latency antigens was significantly higher in latently infected individuals than in TB patients ($P < .01$). In particular, four antigens were able to induce strong IFN- responses in latently infected individuals. Overall, latency antigens are preferentially recognized by T cells from individuals with latent *M. tuberculosis* infection suggesting that immune responses against these antigens contribute to controlling latent infection.

Use of risk analysis in the control of bovine tuberculosis and paratuberculosis

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Effective control and prevention of infectious diseases requires an understanding of the relative importance of the different possible pathways of infection introduction and maintenance in a population. Risk analysis provides an appropriate framework for a structured and standardised approach for dealing with such problems. The methodology is defined in the OIE Animal Health Code as well as the Codex Alimentarius, and is widely used to deal with disease risks and their management in association with international trade of livestock and their products. The risk analysis methodology consists of the components of hazard identification, risk assessment, risk management and risk communication. The hazard identification leads to a specific risk question, such as 'what is the risk that a dairy herd currently free from MAP becoming endemically infected with MAP during a year'. The associated risk assessment should then be divided into release, exposure and consequence assessments. The emphasis throughout the whole process is on transparency and risk communication, and both can be enhanced by producing diagrammatical representations of the relevant risk pathways. The assessment can be performed using qualitative or quantitative approaches, the latter requiring specific quantitative information in order to be able to conduct simulation analyses. Risk management involves the development of control strategies influencing the most important or most easily modifiable pathways identified during the risk assessment. Risk communication should be applied throughout the whole process of risk analysis, and it includes communication with scientists, farmers, policy makers and other stakeholders. In fact, the impact of the whole process depends strongly on the effectiveness of the risk communication strategies employed. A number of risk assessments have been conducted for TB and MAP. For example, the European Food Safety Authority conducted a risk assessment of the risk transmission of MAP through bovine semen (Anon 2004a). The risk of introduction of MAP into a dairy herd through infected rabbits has been assessed by Guitian et al (unpublished). A risk analysis of the impact of transmission of TB to cattle from badgers in the UK was conducted by Gallagher (2005). The risk analysis methodology is described in Anon (2004b, c).

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Evaluation of intervention strategies for control of Johne's Disease in Switzerland

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ABSTRACT

In 2004 the Animal Population Health Institute (APHI) at Colorado State University collaborated with the Swiss Federal Veterinary Office (SFVO) to devise scientifically sound methods for Johne's disease (JD) surveillance and control in Switzerland. Herd level sensitivity, herd level specificity, and cost were modeled for the application of whole-herd ELISA, herd-subset ELISA, whole-herd fecal culture (FC), and pooled FC. The results of our model suggest that none of the testing strategies evaluated in this study could be used to accurately classify herds by their *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection status in Switzerland if the within herd prevalence of JD is equal or lower than 5%. Management strategies aimed at reducing the exposure of susceptible calves to contaminated manure would be more cost-effective at controlling the infection. In herds with prevalence levels higher than 10%, intervention strategies based on fecal culture could be used to accurately classify MAP-infected herds. Pooled fecal culture is the least costly of the two culture-based intervention strategies evaluated in this study

Key words: Johne's disease, control program, herd sensitivity, herd specificity

INTRODUCTION

The prevalence of Johne's disease (JD) in Switzerland is not well described. Although several small scale studies have attempted to assess its prevalence, especially in dairy cattle, the limited sample size (Ludi et al. 2004, Giacometti et al. 1995, Corti and Stephan 2002), inadequate geographic distribution of sampled cases, and the poor sensitivity and/or specificity of the methods used to detect infection (Meylan et al 1994, Meylan et al. 1995, Stark et al. 1997, Corti and Stephan 2002) have hampered efforts to estimate the national prevalence accurately.

While it is mandatory to report clinical cases of JD, the low number of cases registered annually by the Swiss Federal Veterinary Office (SFVO) suggests that the disease might be under-reported. An average of as few as seven cases per year was registered by the SFVO between 1995 and 2003.

The objective of this study was to evaluate several testing strategies for the detection of MAP-infected dairy farms, and to provide recommendations for the testing strategies and/or management practices. This model would form the basis for controlling JD in Switzerland.

MATERIALS AND METHODS

General Assumptions
The Swiss dairy cattle population was categorized into three major groups according to the spectrum of infection that may be present. Group 1 herds included farms with apparently healthy animals and no records of clinical disease, Group 2 herds included farms from which at least one animal had available records from culture of tissues collected as part of a proposed abattoir survey, and Group 3 herds included farms for which at least one cow with clinical disease had been officially reported or detected by the owner. This study focused mainly on Group 1 herds.

All dairy herds were assumed to have 33 cattle, the median herd size in Switzerland (Ludi et al., 2004). Other general assumptions for this model were that the herds were closed (no cattle purchased from other farms), and that only cattle older than 36 months and in initial stages of infection or latent infection were included for testing. The herd-level sensitivity (HSE) and herd-level specificity (HSP) for each testing strategy were calculated for three values of within-herd prevalence: 5%, 10% and 15%.
Model assumptions related to the costs of testing were: (1) The cost of the veterinarian's visit without any intervention was €18.15 (28.58 CHF) (Weber et al. 2004) and, (2) The Euro vs. Swiss Francs (CHF) exchange rate was assumed to be €1 = 1.575 CHF.
 All calculations were performed in Microsoft Office Excel 2003.

Test Strategies and specific assumptions
The type of testing strategy proposed varied depending upon the cattle group. For Group 1, herd sensitivity and specificity, practicality, and cost of testing were evaluated for each of four testing strategies: (1) Herd Subset Testing with serum ELISA (2) Whole Herd Testing using serum ELISA, (3) Whole Herd Testing using Fecal Culture (FC) and (4) Pooled FC Testing.

The assumptions for each testing strategy were:

(1) Herd Subset testing with ELISA: (a) Cattle subset tested: n=16 (50 % of the herd), (b) Sensitivity (SE) of ELISA in this group: 10 % (Weber et al. 2004), (c) Specificity (SP) of ELISA in this group: 98.9% (Whitlock et al. 2000 (f) Cost of the test: 25 CHF per sample tested (g) Cost of veterinarian: €2.27/animal (3.575 CHF/animal) Weber et al. (2004).

(2) Whole Herd testing with ELISA: (a) SE of ELISA in this population = 10% (Weber et al. 2004), (b) SP of ELISA in this population = 98.9% (Whitlock et al. 2000), (c) Cost of the test: Purquier Kit 25 CHF per sample run in duplicates (Dr Wittenbrink, personal communication, Zurich, 2004), (d) Cost of veterinarian: € 2.27/animal (3.575 CHF/animal). Weber et al. (2004)

(3) Whole herd testing by FC: (a) SE of fecal culture in this population = 40% (Wells et al. 2002), (b) SP of fecal culture in this population = 99.9% , (c) Testing costs: 35 CHF/sample (Zurich JD reference Laboratory, Dr Wittenbrink, personal communication, 2004), (d) Cost of veterinarian: €2.27/animal (3.575 CHF/animal) Weber et al 2004

(4) Pooled FC from the whole herd:(a) Fecal pools are conformed by pooling feces from 5 cows of similar age/lactation number group. If the pool is positive, all individual samples are re-tested (van Shaik et al, 2003; Weber et al. 2004), (b) SE of pooled fecal culture in this population = 36% (Weber et al. 2004), (c) SP of fecal culture in this population = 99.9%, (d) Cost of the test: 35 CHF/pool, (e) Cost of veterinarian: Collection of feces and pooling €2.72/animal (4.28 CHF/animal) (Weber et al. 2004).

Calculation of HSE and HSP in Group 1 Herds
HSE, or the probability that an infected herd yielded at least one positive test result, was calculated for each of the four testing strategies listed above. A herd was considered MAP-infected if at least one cow tested positive. The procedure used to calculate HSE depended on whether the whole herd or a herd subset was tested.

(a) Calculating HSE when Testing a Herd Subset
For testing with herd subsets, HSE was calculated as the sum of the probability of detecting false negatives in the sample (true positive individuals that go undetected) plus the probability of detecting uninfected animals that test positive (false positives). That is,
HSE = [P (selecting *n* truly infected animals) * P(truly infected animals test positive)] + [P(selecting *n* uninfected animals) * P(uninfected individuals test positive)]
The hypergeometric distribution function was used to calculate the probability of selecting n truly infected individuals from a sample withdrawn without replacement (Berry and Lindgren 1996). This approach allowed incorporates both the probability of detecting a test positive cow in the herd and the probability that an infected cow was selected in the sample.

(b) Calculating HSE when Testing the Whole Herd

If the whole herd was tested, HSE was calculated according to the following equation:

$$HSE = 1-(1-AP)^{\text{number of individuals tested}}$$

Where AP or apparent prevalence = (1-Sp) + (Se + Sp -1)* True Prevalence.

Calculation of HSP
HSP was defined as the probability that all animals in a non-infected herd test negative, and was calculated with the following equation:

$$HSP = Sp^n \text{ , where n = number of tests}$$

The total probability of detecting a positive reactor among non-infected cattle (false positive rate) was calculated as (1 – HSP).

Test Strategies for Classification of Infection Status in Group 2 Herds
One proposed strategy to increase information about national level prevalence of JD in Switzerland was a proposal to collect three tissue samples from randomly selected adult cattle at the main abattoirs in Switzerland. These tissues would be cultured isolate MAP, and if isolates were made, the farm of origin would be classified as infected.

Farms with at least one animal participating in the abattoir survey with tissue culture information were included in Group 2. Farms in which MAP was not detected after culture of tissues from cattle sent to abattoir would be included in Group 1 (unless there are cattle with clinical disease in the operation, in which case the herd will be classified as Group 3).

Test Strategies for Infection Status Classification in Group 3 Herds
Group 3 would consist of herds with at least one clinical case compatible with JD reported by private veterinarians or owners. Few herds would be expected to participate in this category mainly due to under-reporting of clinical cases of JD. Briefly, a herd with at least one animal with clinical signs compatible with JD would be classified as infected if MAP were isolated from the clinically affected animal after two attempts. Even In the absence of MAP isolation the affected animal/s would be slaughtered. Culture of tissues from the affected animal/s would attempt to confirm MAP infection. If the results of tissue culture were negative the herd would be monitored for a period of two years before it could be certified as infection-free. Herd monitoring would include screening for infection twice a year.

Table 1. HSE, HSP and costs of four test strategies for classificaiton of herd status i Group 1 herds

Testing Strategy	Prevalence	HSE Hypergeometric distribution	HSE $1-(1-AP)^n$	HSP	Cost in CHF
ELISA Herd Subset[1]	5%	0.20	n/a	0.84	457.2[1]
	10 %	0.29	n/a	0.84	
	15%	0.32	n/a	0.84	
Whole Herd ELISA	5%	0.40	0.40	0.69	971.56
	10 %	0.55	0.48	0.69	
	15%	0.61	0.56	0.69	
Whole Herd Fecal Culture	5%	0.43	0.50	0.96	1301.56
	10 %	0.81	0.75	0.96	
	15%	0.90	0.87	0.96	
Pooled Fecal Culture[3]	5%	0.39	0.47	0.96	414.95[4]
	10 %	0.77	0.71	0.96	
	15%	0.86	0.84	0.96	

[1]This assumes that only 16 animals out of a herd of 33 are tested; [2] This assumes laboratory costs of testing plus veterinarians fee for bleeding 16 cattle; [3] This assumes 6 pools of 5 cows and 1 pool of 3 cows for the 33 cow herd; [4] This cost relates to detecting infection in one pool; additional costs apply to detecting infection in individual animals.

RESULTS

Group 1 Herds
HSE, HSP, and costs of each test strategy proposed for Group 1 herds are shown in Table 1.

Group 2 Herds
The cost of tissue culture is estimated to be 35 CHF per tissue. Thus the total cost for classification of infection status of a Group 2 herd could be as low as 105 CHF, i.e. the cost of culturing tissues from one animal collected at abattoir.

To our knowledge the sensitivity (Se) of tissue culture for isolation of MAP has not been reported in the literature. However, it has been suggested that the Se of tissue culture is higher than the SE of FC, especially if there are granulomatous lesions compatible with JD (Dr. Wittenbrink, personal communication). The Sp of tissue culture is estimated to be close to 100%.

Group 3 Herds
The proposed strategy for these herds with at least one clinically affected animal includes two attempts to isolate the bacteria through fecal culture from the sick animal(s). The sensitivity of fecal culture from clinically infected animals has been reported to be as high as 75% to 80% with almost complete specificity (99.9%-100%) (Whitlock et al.2000).

The costs associated with classification of MAP infection status in Group 3 herds could be as low as the cost of culturing one fecal sample (35 CHF). Assuming a within-herd prevalence of 5% or less, the cost could include the testing and slaughter of one clinically infected cow (and its replacement), the expense of tissue culture for isolation of MAP (105 CHF), and the expense of herd monitoring for a two year period with one of the testing strategies described for Group 1 herds.

DISCUSSION

This study modeled different testing strategies that might be used if Switzerland were to undertake a JD certification and control program. Major emphasis was given to the detection of MAP infection in herds with apparently healthy cattle (Group 1).

The probability of correctly classifying a herd as infected by using ELISA on a herd subset (n=16) was less than 32% for all three hypothetical prevalence levels, suggesting that this strategy is ineffective for small herds of apparently healthy cattle. The low Se of the ELISA in the first stages of infection (10-15%) explains the poor ability of this strategy to correctly detect herds in initial stages of MAP infection. While it is expected that the HSE would be higher when the test is applied to highly infectious cattle or continuous shedders, this scenario is unlikely for the majority of the Swiss dairy farms. Although low cost and short turnaround time are significant advantages of this test, its low HSE and HSP make it unsuitable for a program in Group 1 herds.

The ELISA test of a whole herd almost doubled the probability of infection detection when compared with testing a herd subset. Nevertheless, the HSE of this strategy in the most likely prevalence scenario for Switzerland, i.e. 5% prevalence or less, is still low (40%) with a high rate of false positive test results. Although practical and rapid, whole herd ELISA surveillance is inadequate for a herd certification program under Swiss dairy production conditions. Because of the small size of the Swiss herds and the expected low prevalence of JD, testing strategies such as serology, characterized by low sensitivity and imperfect specificity, will not succeed in accurately classifying herds by their MAP-infection status in Switzerland.

Strategies based on fecal culture, instead, might provide a better classification of herd status in geographical areas where the disease clusters at high prevalence levels. When comparing the model's results for individual FC and pooled FC from the whole herd, the HSEs were similar at 5%, 10% and 15% prevalence levels. However, but the cost of individual IF is almost 4 times higher than that of pooled fecal

culture.   The HSE of both culture-based tests reaches values between 70% to 80% at prevalence levels of 10% and 15%. One major advantage of fecal culture, both from individual and pooled feces, is the almost perfect specificity of the test (low false positive rate). Nevertheless, the long turnaround time of fecal culture testing poses a serious drawback in the implementation of a plan for control for the disease. Rapid testing techniques for detection of MAP, such as PCR of fecal pools or environmental samples could be used as a first line of screening while culture results are pending. In this way, effective management strategies such as prompt separation of shedders from the rest of the herd could be implemented to prevent infection transmission to susceptible animals.

 Management strategies that lead to preventing the fecal-oral transmission of MAP to young susceptible animals, such as pasteurization of colostrums or milk administered to young animals, are also valuable for the control of other fecal pathogens such as *Salmonella* and *E. coli*.

The accurate classification of the MAP infection status of herds from which at least one animal was confirmed infected by either culture of feces or tissues depends on the sensitivity of the culture technique. The sensitivity of fecal culture varies depending upon the stage of disease and the bacterial load present in the sample (Whitlock et al. 2000). Although the sensitivity of tissue culture has not been widely reported, a personal communication with a microbiologist from the JD reference laboratory in Zurich (Dr. Wittenbrink) leads us to think that is higher than that of fecal culture. Long turnaround time for test results is one of the major issues associated with the use of culture-based detection methods. Rapid turnaround time for test results makes molecular tests for detection of MAP in clinical specimens a suitable alternative for confirmation of infection status in Group 3 herds.

CONCLUSIONS

For herds where the prevalence of JD is 5% or less, as is expected to be the case for the majority of herds in Switzerland, none of the aforementioned testing strategies in this model proved to be cost-effective for the accurate classification of herds by infection status. Control methods based on preventing the infection of susceptible young animals would be more effective than testing and culling test-positive adults.

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Paratuberculosis control in the Netherlands: the target and an overview of activities

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ABSTRACT

As in many other countries *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection is present in Dutch cattle. The government together with the livestock industry are working to control the infection. The control program is based on existing knowledge about the pathogen plus epidemiological and economical modelling of different intervention scenarios' costs vs. benefits.

A new control program will be introduced in the Netherlands, based on individual ELISA on blood and milk. The program's key control issues are preventive measures and biosecurity. The aim of the new program is to reduce MAP contamination in milk. An overview of the Dutch approach is given.

Key words: Paratuberculosis, cattle, dairy, control program, ELISA, biosecurity, prevention, milk.

INTRODUCTION

As in many other countries *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is present in cattle in the Netherlands. The prevalence at herd level is estimated at about 30%. Of 46,000 herds, 24,000 are dairy operations milking 1.6 million cows with an average annual production of 7,800 kg. To control Johne's disease and its drain on farm profitability, in 2000 the national Dutch Paratuberculosis Program was started. The livestock industry and the Government worked together on this program as outlined in Fig. 1.
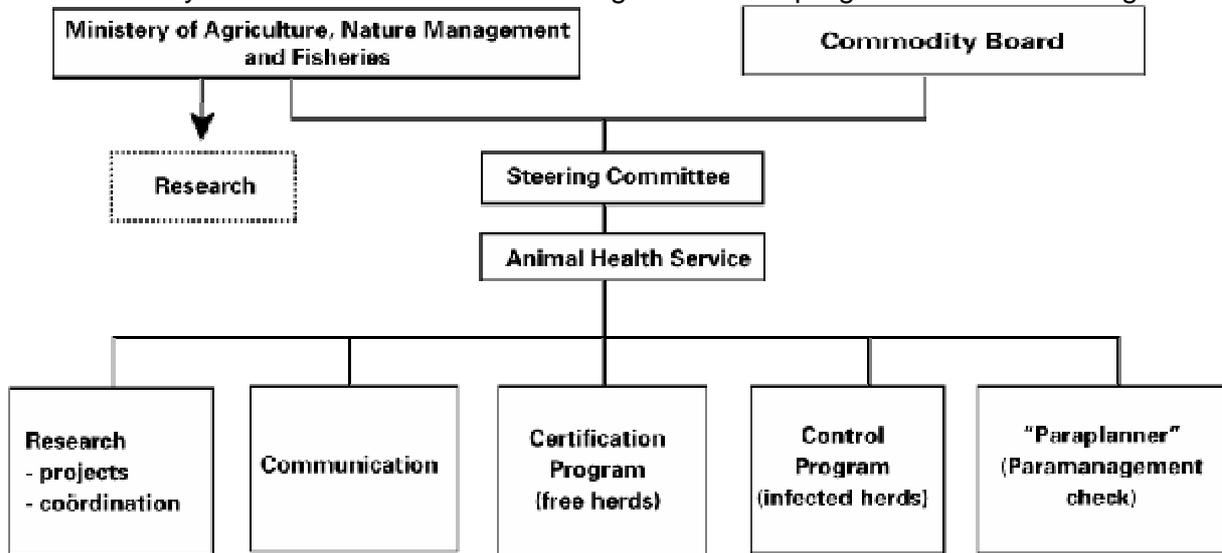

Fig. 1. Management structure for the Dutch Paratuberculosis Program (PPN)

The steering committee consists of representatives of the Ministry of Agriculture, Nature Management and Fisheries, the Ministry of Human Health, The Commodity Board, The National Dairy Organisation (NZO), the Animal Health Organisation and the Dutch farmers organisation (LTO) with this last providing the chairman. The Animal Health Service (Dutch abbreviation, GD) is responsible for executing the program.

The program includes research projects, herd certification and control activities for infected herds. As an important preventive tool, the program offers dairy farmers "Paraplanner", a risk assessment plan that includes the guidelines for prevention of MAP infection.

Effective communication is important and as such has its own place and budget in the program. The costs of the program for activities such as certification, control and Paraplanner are directly charged to the farmer. There is public funding for the project management, communication and research.
Experience gained since the start of the program emphasises the importance of infection prevention.
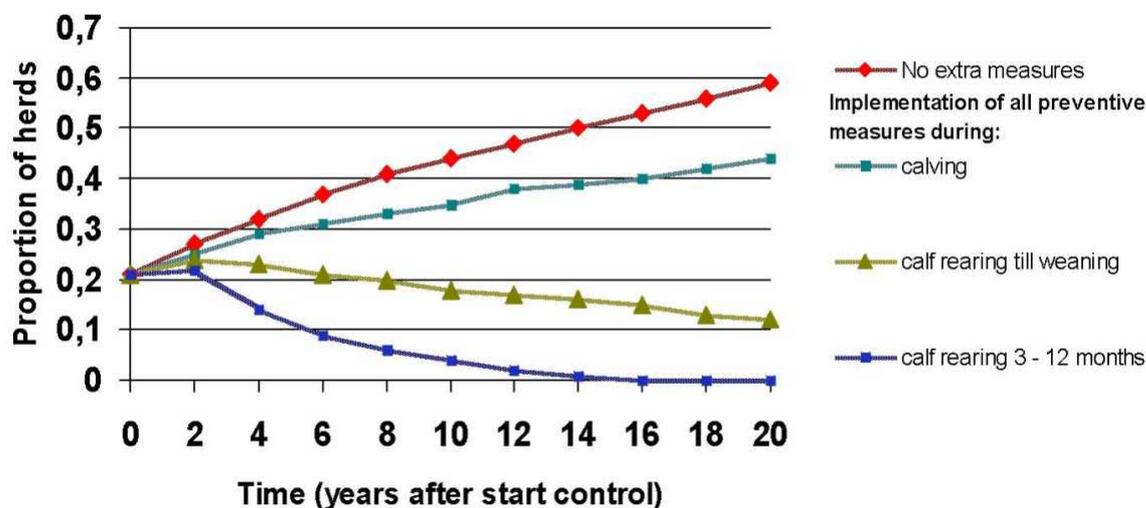
Fig. 2. Effects of preventive measures to limit within herd transmission (Groenendaal et al. 2003)

In the Dutch program, preventive management methods (Paraplanner) and certification of infection-free herds are tools used to minimize transmission of paratuberculosis within and between herds.
New ideas for controlling paratuberculosis, based on research results, have enabled the PPN steering committee to open the way for a new, cost effective and user friendly program.

New program techniques
In a new approach, the program's aim is to limit the concentration of MAP in dairy products of which, economically, milk is the most important product. Diagnostic tests are used to monitor levels of infection in the herd. The objective is to reduce the MAP bacterial count in the bulk milk tank. The target is fewer than 10^3 MAP bacteria per kg milk in the tank. Pasteurisation eliminates up to 10^6 MAP per kg milk and thus should be able to eliminate the target concentration with a large margin of safety. To encourage farmers to take part, the program has to be cost effective and farmer friendly. Thirty six different scenarios for herd testing have been compared. Variables were: herd diagnostics at intake (ELISA or faecal culture), surveillance (ELISA or faecal culture, every one or two years), control schemes for infected herds (ELISA or faecal culture, different frequency), open vs. closed herds, no or improved preventive management methods. The optimal choice was an ELISA-based program using individual blood or milk samples. Dairy farmers with cattle that test positive have the choice to use either individual blood ELISA or individual faecal culture to detect infected cattle (especially heavy shedders of MAP). Culling these animals, and putting in place good preventive measures, limits transmission of the infection within the herd.

Three component program (classification, surveillance for test-negative herds, control for test-positive herds)
The program consists of three diagnostic screenings: the first test is for the classification of the herd, the second is for surveillance of the negative testing herds and the third for the control of herds that test positive.

Component 1: The classification of herds is based on individual blood ELISA of all animals over 3 years of age or individual milk ELISA on all lactating animals. Assuming a prevalence of paratuberculosis in 30% of herds at the start of the new program, the range in MAP bacteria concentration in the bulk milk tank is

shown in Fig. 3. The serostatus of a herd is related to the number of MAP in bulk tank milk; a small proportion of herds test false negative or false positive.
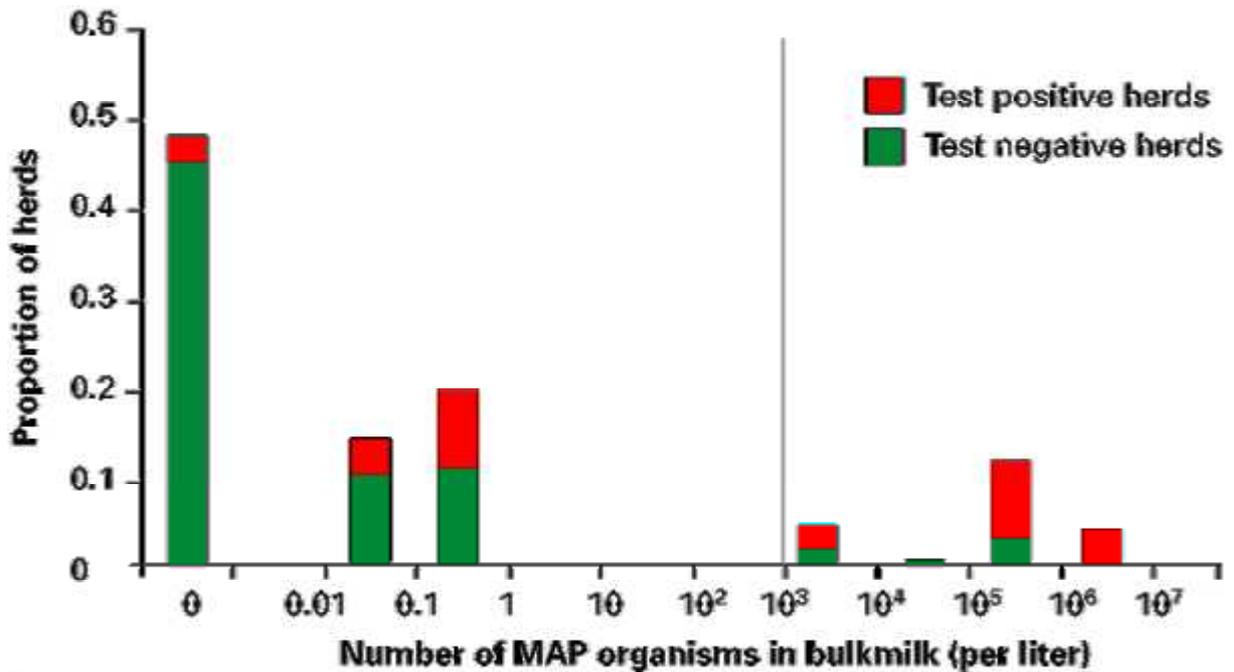
Fig. 3 Test positive vs. test negative herds at intake by the number of MAP organisms/liter in the bulk milk tank (Weber et al. 2005)
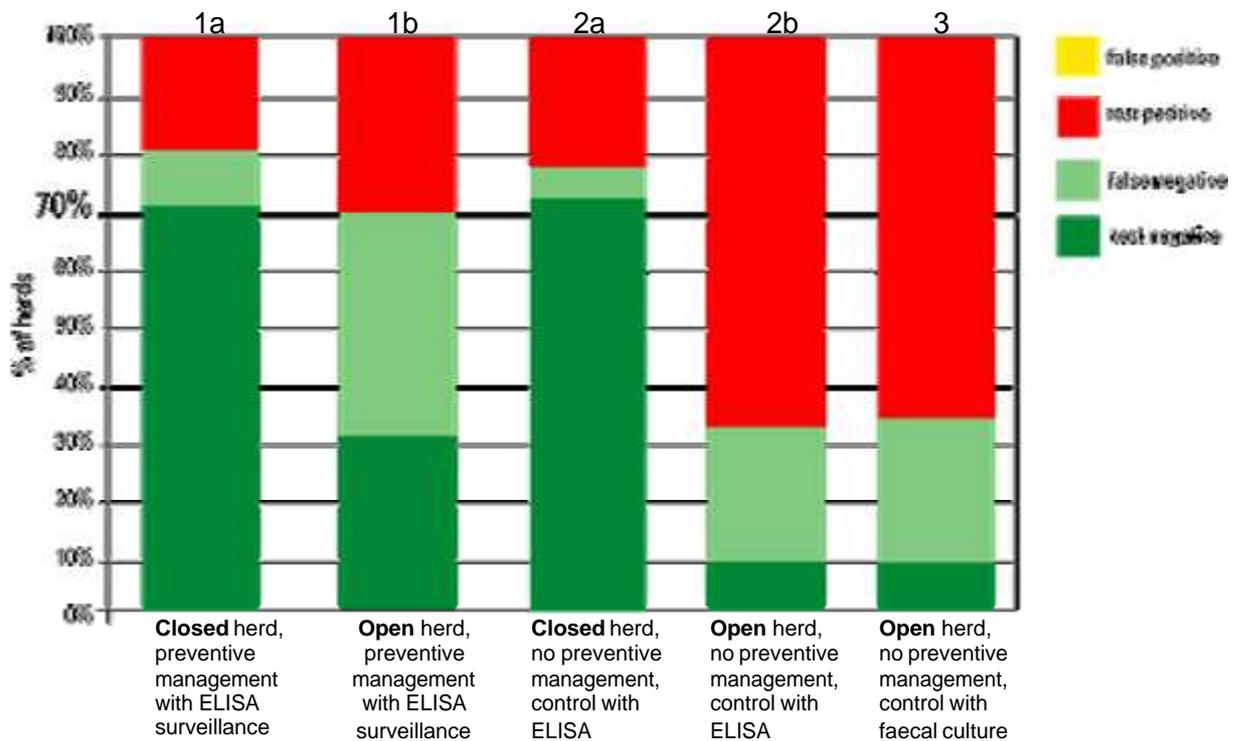
Fig. 4. Expected herd status after 8 years of program (van Roermund et al. 2005)

Component 2: Monitoring of test negative herds is based on individual blood ELISA every 2 years on all animals over 3 years old. Individual milk ELISA for all lactating animals, once every 2 years, is also an option. In the future, even more user-friendly options like bulk milk ELISAs may be developed.

Component 3: For infection control in herds that test positive, there are three options:

- individual blood ELISA on all animals of over 3 years, once a year
- individual milk ELISA on all lactating cows, once a year
- individual faecal culture, once every two years, on all animals over 2 years of age.

A particular objective is to detect animals that contribute substantially to the contamination of the environment and milk and to cull these animals and their lastborn calves.

DISCUSSION

The new Dutch Paratuberculosis Program is expected to stabilize the number of infected herds if the ELISA testing scheme is combined with preventive management. In open herds paratuberculosis will almost certainly occur regardless of the test and cull methods. In this program, an intake testing scheme based on faecal culture did not perform significantly better in reducing prevalence than did ELISA-based intake schemes. Fig. 4 shows the expected infection status of the national herd 8 years after the start of the program.

Fig. 5 clearly shows that herds testing negative at intake with closed herd management can achieve $<\sim 10^3$ MAP per litre. The costs to achieve this vary from € 0,001 to € 0,007 per litre, depending on whether or not prevention measures are taken. Preventive management is more expensive than no prevention but improves biosecurity for other herd health problems as well.
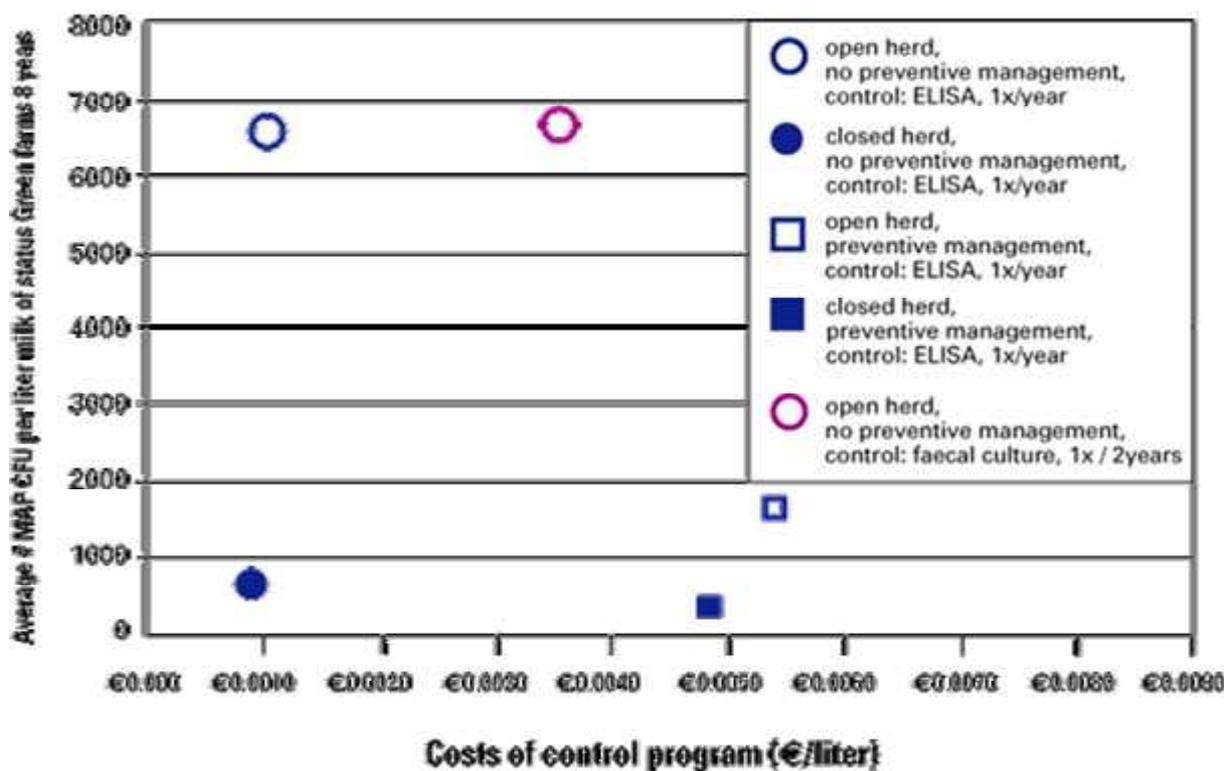
Fig. 5. Costs and expected results of control program (van Roermund et al. 2005)

CONCLUSION

The serostatus of the herd and the concentration of MAP in the bulk milk tank are correlated. ELISA screening (individual blood or milk samples) is a practical tool for monitoring the herd status for paratuberculosis. The models show that the new Dutch ELISA-based Paratuberculosis program leads to a stabilisation of the number of test-negative herds if it is combined with control measures, closing the herd as much as possible and culling infected cows and their lastborn calves. A closed herd is necessary to reduce MAP concentration to $<\sim 10^3$ per litre. The costs of the program range from € 0.001 to € 0.007 per

litre milk and rely upon the effectiveness of herd management. The program has four major advantages: it is cost effective, farmer friendly, simple to manage and easy to communicate. In all of the models, the importance of preventive management measures is clearly demonstrated.

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Increasing involvement of herd owners in controlling paratuberculosis through assurance based trading

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ABSTRACT

Australia's Johne's disease program has largely focused on voluntary herd/flock certification, on the regulatory control of infected herds and flocks and on restrictions on movements.

The national program has now developed new approaches that should eventually involve most livestock producers in managing Johne's disease. These aim to be more equitable and to encourage disease prevention and management.

Different livestock industries have taken varied approaches to controlling paratuberculosis. In the sheep industry, the *Assurance Based Credits* scheme is based on a formal quantitative risk assessment. All flocks and consignments of sheep may be scored between 0 and 10 depending on the background prevalence of infection in the region, test results and vaccination history. The dairy industry has applied a herd scoring system from 0 to 10, the *Dairy Assurance Score,* based on the results of herd testing and zone classification. In the two schemes, progeny can achieve higher scores through vaccination and calf rearing management respectively. Paratuberculosis is endemic in these industries, so herds or flocks that do not take action to manage paratuberculosis are given a low score. The schemes also provide pathways for improving scores by controlling infection. The goat industry is also developing a scoring system.

In contrast, Johne's disease is rare in beef cattle and alpaca herds in Australia. New approaches in these industries classify herds that have had little or no contact with dairy cattle or other high-risk animals as *Beef Only* and *Q-Alpaca*. Herd owners are encouraged to manage their biosecurity to protect the advantageous trading positions enjoyed by these low-risk statuses.

Owners of herds and flocks with these scores or statuses are now declaring them in writing at the point of sale on nationally standardised animal health statements. Compliance is being monitored by audit.

Keywords: Johne's disease, paratuberculosis, risk, assurance, declaration.

INTRODUCTION

Control of Johne's disease (JD) in Australia has been based on zoning areas according to disease prevalence and risk, market assurance of tested negative herds and flocks and regulatory controls on known infected and suspect herds and flocks. The development and implementation of Australian Johne's Disease Market Assurance programs have been described at a previous colloquium (Allworth and Kennedy 1999; Kennedy and Allworth 1999). These programs are based on a high level of biosecurity, negative herd testing and audit. The regulatory control of known infected herds and flocks has been largely based on quarantine agreements requiring on-farm control of disease and movements. These strategies have been generally successful in protecting the zones that have little or no JD and have allowed individuals to find replacement animals with high confidence that they are free of infection. In the alpaca industry, aggressive control of an initial outbreak has resulted in no further cases for several years. In contrast, in 2003 OJD was found to have been established in sheep flocks in the previously OJD (ovine JD or OJD) Free Zone and in 2000 BJD (bovine JD or BJD) was found to be spreading in the red deer industry. In the dairy and sheep

industries, JD caused by cattle- and sheep-type strains of MAP has continued to spread in south-eastern Australia.

Animal Health Australia conducted comprehensive reviews of the strategies with the respective industry and government stakeholders between 2002 and 2004. This input resulted in new approaches for most affected species (Kennedy et al.2002b). At that time large numbers of dairy herds and sheep flocks were suspected of being infected but were not involved in managing JD and in fact were spreading it. Unwitting buyers had no means of determining if flocks or herds were infected and were often unaware that a non-assessed herd was a potential risk for infection.

One of the main recommendations of the reviews was to involve all producers in the management of Johne's disease. The great majority of producers are located in the endemic regions, and while they have herds and flocks not known to be infected, they have been encouraged to manage the risk of JD spreading to their herds and flocks. A minority of risk-averse producers had already implemented additional management practices and had joined the Market Assurance Programs while another minority were the managers of herds and flocks that were known to be infected.

Principles of assurance based trading
Assurance based trading encompasses the elements of risk analysis at the herd and flock level.

- *Risk assessments* include the history and background prevalence of infection in the region or sector of the industry and testing.

- *Risk management* in the herd or flock includes farm biosecurity management and specific disease prevention activities such as vaccination and calf/lamb rearing.

- The infectious risk that animals from the herd or flock represent is *communicated* through a written and signed declaration of an assurance score either for the whole herd or flock or for groups of animals within it. This is supported by broader education in the respective industries.

Assurance Based Credits for sheep
The "ABC" scheme for sheep, implemented in 2004, is based on a quantitative assessment of the risk that sheep will spread OJD (Sergeant et al., 2003). Sheep flocks are eligible for credits under two categories (background prevalence of infection in the area and flock testing) and groups of sheep within the flock can gain additional credits through vaccination and further testing to a total maximum of 10 (Figure 1).

Much of the data and tools used in the ABC scheme were developed during the six-year National OJD Control and Evaluation Program (Allworth et al.2002a). Surveillance of slaughter sheep at abattoirs is funded by the Australian sheep industry and the results of applying a standardised inspection and histological procedure (Allworth et al.2002b) are used to model the area prevalence of infection (Sergeant and Baldock, 2002). Where a sufficiently large representative sample of sheep from the flock is examined by abattoir surveillance and found negative, the flock is eligible to claim credits for 12 months. Pooled faecal culture of 350 sheep, in pools of 50, is now the standard flock test used in the Market Assurance Program Australia and for assessment of non-MAP flocks (Whittington et al.2000). This testing method gives 98% confidence of detecting a 2% prevalence of OJD in a flock. Controlled trials in three heavily infected flocks have demonstrated that vaccination with a killed, oil-adjuvanted vaccine (Gudair®, CZV Spain) have shown that it is highly effective in preventing clinical disease and reducing shedding of MAP in infected sheep flocks (Eppleston et al. 2005).

OJD ASSURANCE BASED CREDIT (ABC) POINTS
Select one line from each category if applicable then add for total credit rating

CATEGORY A[1]: Flock of origin – Location and Description
The flock is located in:

	Possible Credits	Credits for this consignment
a very low prevalence area and is not suspected or known to be infected	5	
a low prevalence area and is not suspected or known to be infected	3	
a medium prevalence area and is not suspected or known to be infected	1	
a high prevalence area	0	
any area and is suspected or known to be infected[3]	0	

CATEGORY B[1]: Testing
The flock is:

in the MAP and has had 3 Sample Tests[2]	4	
in the MAP and has had 2 Sample Tests[2]	3	
in the MAP and has had 1 Sample Test[2]	2	
not in the MAP but has had a negative 350 PFC test within the last 12 months	2	
not in the MAP but is eligible for Abattoir 700 status	2	
not in the MAP but is eligible for Abattoir 200 status	1	
a tested low prevalence flock	1	

CATEGORY C[1]: Vaccination

the flock is a second generation approved vaccinate flock	4	
the sheep are approved vaccinates born into an approved vaccinate flock	3	
the sheep are approved vaccinates from an approved vaccinate flock	2	
the sheep are approved vaccinates	1	

CATEGORY D: Consignment Sheep – Risk Assessment
The sheep are:

sourced from an infected flock but are certified low-risk by a MAP approved veterinarian	1	

**TOTAL CREDIT RATING FOR THIS CONSIGNMENT OF SHEEP
UP TO A MAXIMUM OF 10 CREDITS** [ ]

[1], [2], [3] – see notes on reverse of this form.

Figure 1. The Assurance Based Credit Scheme for Australian sheep flocks, 2004.
Note: explanatory notes are on the reverse side of the form. For details, see Animal Health Australia's website: www.animalhealthaustralia.com.au.

Dairy BJD Assurance Score
Under the CattleMAP and national rules for control of BJD, there are several existing herd statuses based on whether the herds have tested negative, are in low-risk zones or are known to be infected and have progressed in disease control programs. These have been consolidated into the herd score, with 10 being the highest level of assurance for herds at the top level of the CattleMAP or in Free Zone. Scores 1 to 6 are for herds with a history of infection that are undertaking increasingly successful control as evidenced by the seroprevalence at herd tests. Calves reared under audited calf rearing programs are entitled to additional credits and the industry is currently considering whether the herd score can also be advanced by compliance with long-term calf rearing management. Acknowledging that BJD is common in the dairy industry of the south-east, plans are underway to demote untested (non-assessed) herds from an interim

score 3 to zero once an inexpensive bulk herd test becomes available. This reinforces the concept that non-assessed populations of animals provide little or no assurance of JD-free animals in infected regions.

Beef Only

Surveillance of the beef industry in Australia has detected very little BJD in beef herds that do not have contact with dairy herds in south-eastern Australia (Table 1). Most of the known infection has been related to dairy contact although some notable purebred beef herds have been endemically infected and have sold infected cattle to others. The generally low-risk nature of the beef sector was recognised in 2004 by a new assurance category based on biosecurity alone. Owners of herds in which cattle are electronically identified under the National Livestock Identification Scheme and that have no recent contact with dairy cattle (unless they are from MAP assured herds) can declare their cattle to be *Beef Only*. This status allows cattle from the endemic south-east access to some markets and assured herds without the need for testing.

Table 1. The prevalence of known infected cattle herds in Australia by Zone and State, June 2004 (source: Animal Health Australia)

Zone	State	Dairy herds		Beef herds	
		Number	Apparent prevalence %	Estimated number	Apparent prevalence %
Free	Western Australia	311	0	4,500	0
Protected	Northern Territory	0	0	500	0
	Queensland	966	0.1	50,000	0
	New South Wales	879	2.5	68,000	0.01
	South Australia	0	0	200	0
	Flinders I. Tasmania	0	0	100	0
	SubTotal	1,845	1.2	118,800	0.008
Control	New South Wales	335	17.9	11,000	0.24
	South Australia	447	8.5	9,800	0.08
	Victoria	6,340	16.3	30,000	0.29
	SubTotal	7,122	15.9	50,800	0.24
Residual	Tasmania	564	3.9	2,000	0.35
AUSTRALIA	Total	9,842	11.9	176,100	0.08

Q-Alpaca

The success of infection control efforts and the AlpacaMAP in the 1990's, plus the subsequent absence of confirmed infection for the past eight years, has placed the alpaca industry in a good position of biosecurity. The Australian Alpaca Association's *Q-Alpaca* program is based on individual animal identification, movement records and herd biosecurity. Any animal over 12 months of age that dies or is euthanased and any cria under 12 months of age with emaciation or diarrhoea that dies or is euthanased are examined post-mortem by an approved veterinarian with a standard range of tissues submitted for laboratory examination.

DISCUSSION

These assurance schemes have been developed to broaden the involvement of livestock owners in the assessment and management of the risk associated with Johne's disease, mainly in the infected regions of Australia. This is a complex task requiring widespread and effective communication. A prevalent perception is that Johne's disease is a problem only for owners of herds / flocks that are known to be infected and as a threat to owners of pedigree herds and flocks whose livelihoods depend on unrestricted access to markets for breeder replacements. Hence the majority of owners have avoided confronting their own situation and many have unwittingly continued to spread infection through selling animals from their non-assessed herds. Unfortunately, most animal buyers have not appreciated the risks associated with buying animals from non-assessed herds and flocks in infected regions. The emphasis on communicating risk of infection relies on everyone having a explicit infection status or score, and that doing nothing in infected regions is considered to be relatively "high risk".

Some States have declared that an assurance score or category is mandatory in order to sell sheep; South Australia mandates declaration of the Dairy BJD Score. While this approach increases awareness quickly, it relies on government commitment to audit compliance and on occasion prosecute recalcitrant offenders. Continued awareness and understanding of the control programs must be encouraged by industry leadership to create the environment wherein declarations are not exceptional but instead part of the normal spectrum of animal biosecurity and product assurance. Current advances in lifetime individual animal identification in Australia and in declaring animal health status favour this approach, as long as producers are not overburdened with complex paperwork.

Declaration of a single score that reflects risk removes some of the biological complexity inherent in the control programs. The other purpose of epidemiologically sound scoring systems is that they outline pathways along which producers can advance their score that are seen to be fair. This progress is achieved by preventing new infections and reducing disease (e.g. by vaccination or improved calf/lamb rearing methods) and by objectively assessing their herd or flock status. Because of the impact of a positive test on a herd's or flock's trading position in the past, most producers are still reluctant to test. This is being overcome by industry levies to subsidise testing as available and by the opportunity to gain a better infection risk score through infection control management which, in some cases, is also subsidised.

Although governments currently might mandate declaration and industries provide subsidies, in the end, the market must be the major driver seeking assurance from these schemes. This will require continued communication and auditing systems that not only detect problems but also educate users and confirm the market's confidence in the schemes.

CONCLUSION

Australia's recent development and ongoing implementation of risk-based assurance schemes is a bold step in changing the culture of detecting and controlling Johne's disease. The prior largely regulatory model has not been successful in infected regions and is no longer sustainable. The cultural shift requires recognition that the disease is a problem for the industry as a whole. Control can be successfully managed only by widespread application of biosecurity and risk assessment at the front gates of most livestock properties.

ACKNOWLEDGEMENTS

Many imaginative, dedicated and energetic people have worked collaboratively in developing and implementing fair and practical schemes to involve most producers in managing Johne's disease. The Australian livestock industries owe a great deal to these livestock producers and the staff of industry organisations and government animal health and production services.

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Further details of Australia's Johne's disease programs and links can be found at www.animalhealthaustralia.com.au.

Johne's disease control programs in the Israeli dairy herds

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ABSTRACT

The Israel control program for Johne's disease was initiated in 2003. This voluntary program aims to detect infected herds and provide management approaches for the reduction or prevention of herd infection.
During 2003 and 2004 88 dairy herds were tested (17400 cows). The mean withIn herd seroprevalence mean was 2.7% (min 0%, max 9%). Sixty-one percent of the herds were found to be infected with paratuberculosis (54.5% of these had clinical cases in the last 3 years and in 6.5% MAP was isolated from fecal cultures). In 20% of the herds, the infection could not be confirmed despite a more than 0 and up to 4% seropositivity. 19% didn't show any signs of infection (0% seroprevalence without clinical cases).
A seroprevalence cutoff of 4% had 95% positive predictive value for the prediction of true infection of the herd (at least one positive culture or clinical case). The relative risk for true infection was 1.84 higher in herds which numbered more than 80 cows (p=0.02), and 1.5 higher in open herds (which purchased cows in the last 5 years) (p=0.021).
Despite the high number of infected herds the in herd serological prevalence do not reach high. Implementing a proper management practices without introducing new cows into the herd and testing with smart selection may result reducing prevalence while maintaining economical and healthy herds.

Keywords: control program, Paratuberculosis, Johne's disease, Israel.

INTRODUCTION

The Israeli dairy industry consists of 1083 dairy herds with 100,000 dairy cows producing 1146 million liters of milk per year. Milk production is planned by quotas.
Johne's disease is a well-known and recognized disease in Israel. Its routine diagnosis is based on clinical signs and laboratory tests, primarily fecal smear (acid-fast stained), microscopically examined for mycobacteria and serological (ELISA) tests.
In the past, the primary way of dealing with the disease was culling the clinically affected cows. Little was done to prevent new infections by changing management practices. However, animal trade was controlled by requiring that all cattle moving from one herd to another must be tested for Johne's disease by ELISA in advance. Only seronegative cows are permitted to be moved. The herd prevalence was not taken in consideration when moving individual cows.

In 2003 a voluntary control program was established and dairy farms were asked to participate.
The objectives of the program were to evaluate the within and across herd prevalence of Johne's disease nationally. In addition, a risk assessment was completed to develop management practices that would improve biosecurity.

Control program design
The ensuing stages of the program are as follows:
1. Farm risk assessment focused on 5 management practices: maternity pen, colostrum and waste milk usage, suckling replacement calves, weaned replacement calves and replacement strategy (closed or open herd). A mark (0 to 5 scale) was given to each risk area and a total mark calculated to every

management practice. The evaluation of all 88 herds was done by the same person in order to reduce the subjectivity of the evaluation.
2. The herd owner was required to complete a management plan.
3. Whole adult herd testing by ELISA.
4. Fecal samples from ELISA positive cows using Harrold's media.
5. Herds were classified between 1 and 8 depending on clinical and serological prevalence and fecal cultures as follows (scheme 1)

NO		YES
No seropositive cows = **class 1, 2 or 3**		Herd seroprevalence is 5% or less = **class 7**
Seroprevalence 2%> without positive fecal culture = **class 4**		Herd seroprevalence is over 5% = class 8
Seroprevalence 2-5% or positive fecal culture) = **class 5**		
Seroprevalence 5%< with or without fecal culture = **class 6**		
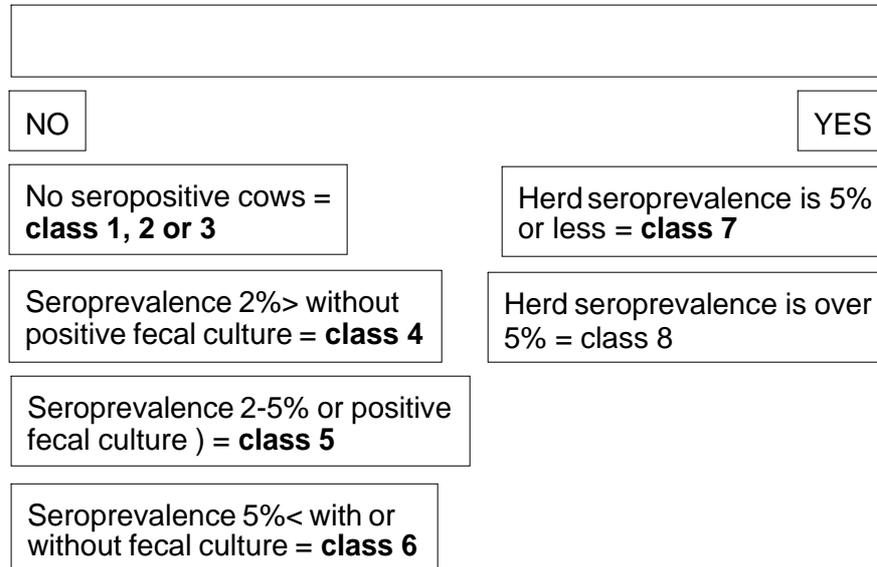
Scheme 1 Classification of dairy herds by clinical or serological prevalence or by fecal culture

6. A herd safety mark is calculated (0-100% scale) using the risk assessments results, diagnostic testing results and clinical prevalence.
7. A management program is devised to reduce the risk of infection in herd and from outside sources incorporating these recommendations:
    a.   Farms must use a clean dry maternity pen for calving of one cow only at a time.
    b. Colostrum or waste milk from seronegative cows only or milk must be pasteurized or milk replacer must be used.
    c. Different or cleaned tools in the young livestock pens.
    d. Fecal shedders are culled, and seropositive cows are marked and subsequent culling based on milking and other health factors.
    e. Herd replacemtents may be introduced into the herd only from herds classified at a lower risk of infection than the farm buying and after permission of the "program field coordinator".

STATISTICAL ANALYSES

The serologic cut-off point for prediction of true infection was analyzed using ROC curve. Management practices were analyzed by chi square.

RESULTS

The results shown here include 88 dairy herds with 17,400 cows evaluated in 2003 and 2004.
Herd size averaged 198 cows (minimum 28, maximum 890). Cows were Israeli Holstein in their first to seventh lactations and included both milking and dry cows.
"Truly infected herds" were herds defined as containing at least one cow with clinical signs of Johne's disease or one fecal culture positive cow within the last 5 years. Sixty-one percent of the herds were found

to be "truly infected" with MAP (54.5% with clinical cases and 6.5% with fecal culture (Figure 1). These herds showed positive serologic prevalences ranging from 0 to 8% (Figure 2). Thirty nine percent of the herds were "not confirmed as infected" (20% of these had positive serologic results ranging from 0.2% and 6% of the herd; 19% were seronegative).
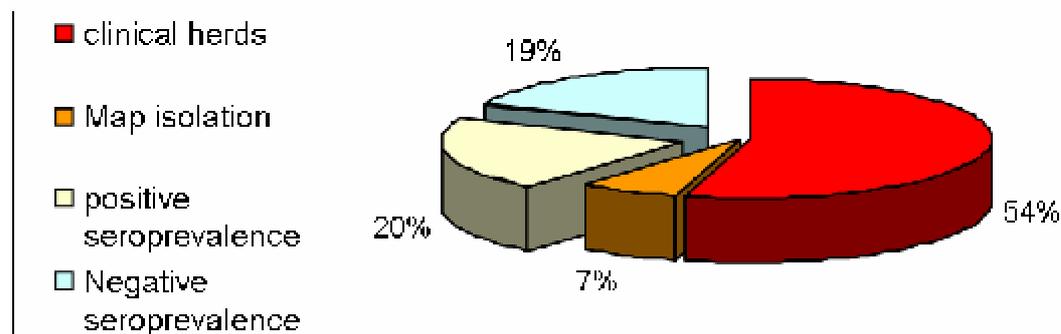

**Fig. 1**. Johne's disease prevalence by laboratory diagnosis
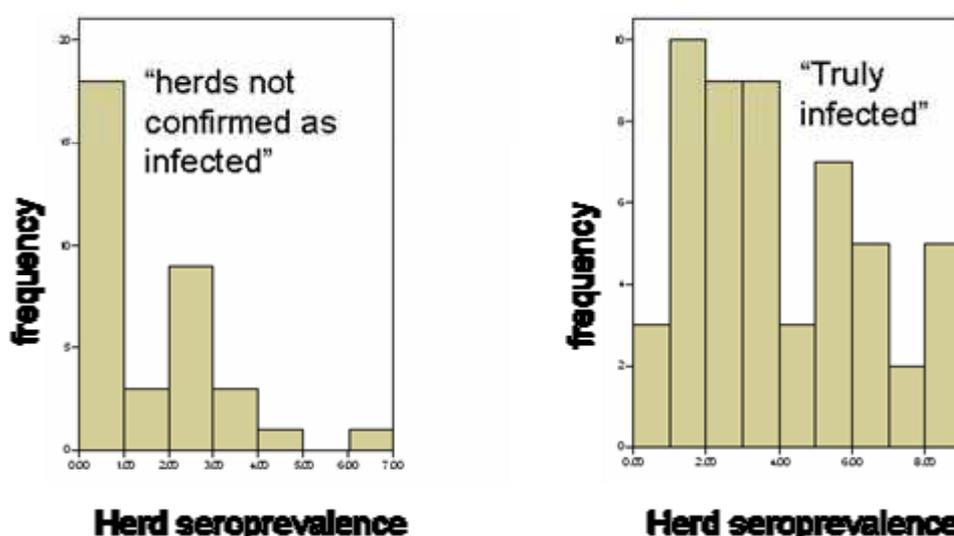
Fig. 2. Seroprevalence in "truly infected herds" with Clinical cases or MAP isoloation and "herds not confirmed as infected"

The ROC curve demonstrated a seroprevalence cutoff of 4% for prediction of true infection of the herd (PPV=95%, NPV=50%).

Three of the risk areas were statistically associated with herd prevalence: herd size, replacement purchase policy and maternity pen management. Herds milking 80 cows or more had a relative risk of being infected that was 1.84 times higher than herds milking fewer cows. (p=0.02, OR=1.84). Herds that purchased at least one cow in the last 5 years (open herds) had a relative risk 1.5 higher then those that were closed during the same time period (p=0.02, OR= 1.5).

The association between maternity pen practices and true infection of the herd with MAP was not significant statistically. However, herds that used a separate clean and dry maternity pen were more likely to seropositive rates less than 4% of the herd (p=0.02, OR= 0.7).

DISCUSSION

Classification of herds with a clinical history of Johne's disease or culture of MAP is a useful method with a minimal risk of misclassification. If serologic results are available, herds may be classified as positive with 95% confidence if the seroprevalence reaches 4% or higher (PPV=95%). Classification of herds with a lower prevalence as "negative" may lead to high proportion of false negative herds (NPV=50%).

The control program's management practices are being adopted by the farmers because they understand the disease and routes of transmission and because diagnostic testing costs have been subsidized.

Even though the connection between some of the management practices and infection with MAP could not be proved (i.e., the association between the practice and prevalence was not statistically significant) the farmers have continued with all aspects of their management programs.

CONCLUSIONS

A large proportion (61%) of Israeli dairy herds are infected with MAP but within herd prevalence remains below 8% even in herds considered to have suboptimal management practices. Implantation of a control program integrating education for management practices (mainly clean maternity pens, separation of adult cows from young livestock and maintaining closed herds), testing (ELISA and fecal culture) and a culling strategy that incorporates the risk of transmission may reduce the number of new MAP infections and preserve economic milk production for the future.

Application of a pilot paratuberculosis control program in an Alpine area in Northern Italy

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ABSTRACT

Paratuberculosis is a rapidly spreading, costly disease gaining an increasing importance for Italian dairy herds. An area in the Alpine part of Lombardia with an overall sero-prevalence among adult animals of 19% was selected. A pilot voluntary control program was developed to gradually decrease the prevalence of the disease in participating herds. The program was designed to be affordable both in cost and labour. The program starts with a risk assessment evaluation, and then farmers are invited to implement management strategies to prevent transmission of infection. A total of 1429 samples (1300 blood and 129 faeces) were obtained from 45 dairy herds in 2004-05. The pilot program was implemented in 37 herds. Animals were tested before parturition by a commercial ELISA and test-positives were scheduled for culling. Cows with suspect and weak-negative results were tested after parturition by real-time PCR. The preliminary results showed that after 6 months test-positive ELISAs decreased and the number of test-negatives increased. The real-time PCR positive samples were 28% of the faecal samples taken post-partum from ELISA weak-negative and suspect cows. The program was well accepted by the farmers. These preliminary data suggest that the program could be efficacious in controlling paratuberculosis.

Keywords: paratuberculosis, dairy herd, control program, diagnosis.

INTRODUCTION

Paratuberculosis is a chronic granulomatous enteritis which affects ruminants causing weight loss, emaciation and in some species untreatable diarrhoea. The causative agent of the disease is *M. avium* subsp. *paratuberculosis* (MAP) an acid-fast slow-growing bacillus. Paratuberculosis was first described in 1894 by Frothingham and Johne (cf. Harris and Barletta, 2001). As early as 1913 MAP was proposed as a possible causative agent in Crohn's disease (cf. Kennedy and Benedictus, 2001), an inflammatory bowel disease affecting humans but it was only in 1984 when Chiodini et al. (1984) first isolated MAP from a patient with Crohn's disease. The importance of MAP in public health needs further evaluation. On the other hand, the economic impact of paratuberculosis for domestic agriculture is a fact. The disease is present virtually worldwide. Herd prevalence in Europe has been estimated between 7% and 55% (Manning and Collins, 2001) and in Italy, regional data indicates a herd prevalence of 27% (Robbi, 2002).

Paratuberculosis costs dairy herds up to US$1.5 billion per year in the USA while costs for individual animals are estimated to be US$100 per year per animal in moderately infected herds and more than US$200 per year per animal in heavily infected herds (Kennedy and Benedictus, 2001). While farmers may measure loss only as culling of clinical animals, this direct cost represents just a small part of the problem. Direct costs are due to premature culling of clinical animals, reduction of milk production, 14.6%-19.5% in clinical animals and 6%-16% in subclinical animals, decline of body condition leading to lower slaughter value, poor feed conversion, higher rates of mastitis and infertility, longer calving intervals and greater susceptibility to other diseases (Benedictus et al., 1987; Wilson et al., 1996, Johnson-Ifearulundu et al., 1996). Indirect losses occur with an increase in infection control costs, veterinary services, diagnostic tests and management changes.

The high costs of the disease, its widespread occurrence and the possible zoonotic role of MAP justify measures to limit the spread of the infection within and among dairy herds. However, the development of control programs has to overcome two major problems. The first is related to technical aspects such as the

low diagnostic test sensitivity and long and invisible incubation period of the infection. The other problem is related to farmers' response and relatively poor understanding of the economic importance of the disease.

A preliminary field study in the Northern area of the Lombardia region showed that the seroprevalence of MAP was 19.5 % in multiple lactation cows, 8.4% in first lactation cows and 4.5% in heifers (Liandris et al., 2004). Therefore, a control program (Fig. 1) to eradicate the disease has been developed. The program was designed to be help farmers participate by permitting sampling throughout the year and by utilizing less expensive diagnostic tools such as ELISA vs. PCR and culture.

MATERIALS AND METHODS

Herd enrolment

Farmers wishing to enter the control program were contacted by a trained practitioner who assisted them to fill in a risk factor questionnaire for their herd. Four risk areas were reviewed: calving, pre-weaned calves, post-weaned calves/heifers and adult animals. In farms where data on the presence of infection was not available, sampling to identify sero-positive animals was completed.

Sampling procedure

This screening consisted of testing 15 randomly selected animals more than two years old by a commercial ELISA (Svanovir, Svanova, Sweden). Samples were obtained from animals at about 30 days before parturition, and the samples were tested by a commercial ELISA. Faeces of weak negative and suspect animals were tested at about 15 days after parturition using a commercial Real-Time PCR. For the real-time PCR, faeces were collected directly from the rectum and were transported to the laboratory under refrigeration temperature and conserved at 4°C till they were processed (no more than 4 days).

Diagnostic test

Animals were subdivided into five groups based on S/P values: negative, weak negative, suspect, weak positive and positive. Blood was collected in vacuum tubes left to coagulate at room temperature and then transported in laboratory under refrigeration where it was centrifuged and kept at 4°C until processed (no more than 3 days). For the ELISA, the manufacturer's instructions were followed.

For the extraction and purification of the DNA from the faeces the Adiapure® kit (Adiagene, France) was used. Briefly, 1 g of faeces was placed in a 50 ml tube containing 20 ml sterile deionised water and vortexed well to create a homogeneous suspension. The sample was left to settle for 20 minutes and 300 μl of the supernatant were placed in a 1.5 ml tube containing 300mg of glass beads and 300 μl of lysis buffer 1. The sample was treated in a mixer mill (MM301, Retsch, Germany) for 10 minutes at 30Hz and centrifuged at 7500 x g per 5 minutes. The supernatant (300 μl) was loaded into a filter on a vacuum manifold assembly to eliminate cellular fragments. The sample was loaded on a second filter to eliminate lipids, proteins and oligonucleotides. The purified DNA was stored at –20°C.

For the amplification of extracted DNA the Adiavet® paratb PCR Real Time kit (Adiagene, France) was used. The kit contains an internal control. The amplification reaction contained 25μl of master mix and 2μl of extracted DNA. Amplification took place in an Opticon 2 (Bio-Rad, USA) machine. Samples were heated at 52°C for 2 minutes, 95°C per 15 minutes and then followed 45 cycles at 95°C for 15 sec and 60°C for 60 sec.

RESULTS

A total of 1429 samples (1300 blood and 129 faeces) were obtained from 45 dairy herds in 2004-05. In 21 of the herds, no prior data on MAP presence were available and therefore the sampling procedure described above was completed. In the other 24 herds, previous testing showed that MAP infection had been detected and therefore they were enrolled in the program without further screening.

Among the 21 herds subjected to screening, only 8 were classified as test-negative for MAP and were not enrolled in the control program. Among the blood samples 590 were collected for herd-screening purposes, while the others were collected in herds following the control program (516 pre-calving, 149 post-calving and 45 on request).

The control program (Fig. 1) starts with ELISA testing of cows during drying-off period. If the ELISA test gives a positive or suspect result the farmer should discard the colostrum. If the result is positive, the cow should not be bred and the animal should be culled at the end of lactation. Colostrum from cows with negative or weak-negative ELISA results can be given to the calf. Cows with weak-negative and suspect ELISA results are tested by real-time PCR 2 weeks after calving and, based on the results, the described recommendation are applied.

The overall distribution of ELISA S/P values and the distribution of ELISA results by type of sampling are reported in Fig. 2 and Table 1, respectively. In screening and pre-calving sampling, about 70% of samples were negative or weak negative. The number of positive and weak positive samples was higher among the post-partum samples (25% of the results), while suspect results were 8% and 23% respectively in pre- and post- partum samples.

Table 1. Distribution of ELISA results classified by type of collection.

Collection type	Negative	Weak negative	Suspect	Weak positive	Positive
Screening (590 samples)	54.7	20.0	10.7	9.8	4.7
Pre-partum (513 samples)	57.3	15.4	8.12	13.6	5.5
Post-partum (86 samples)	24.4	26.7	23.3	15.1	10.5
Other (44 samples)	50.00	13.6	6.1	27.3	2.3
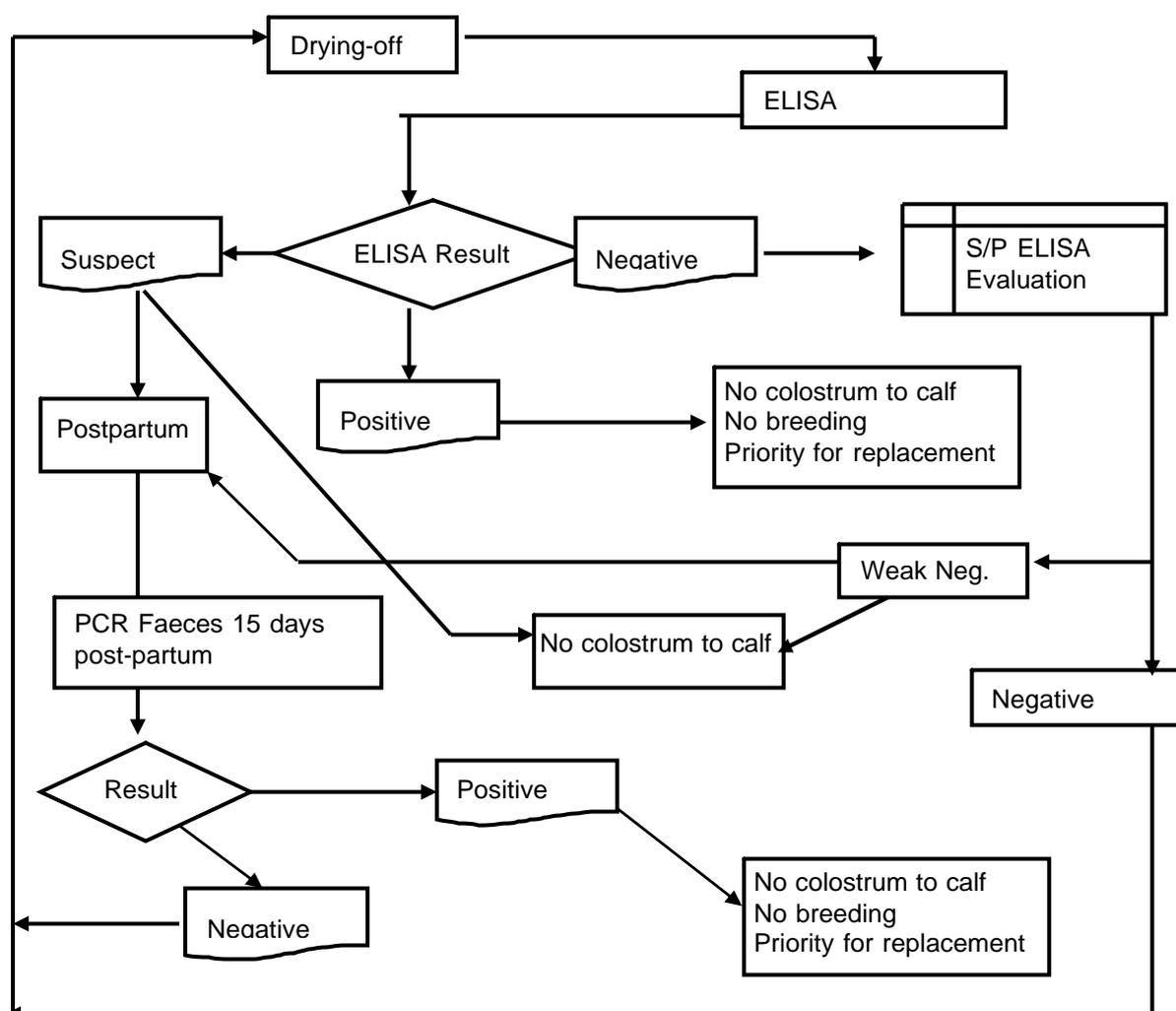
Fig. 1. Control program.
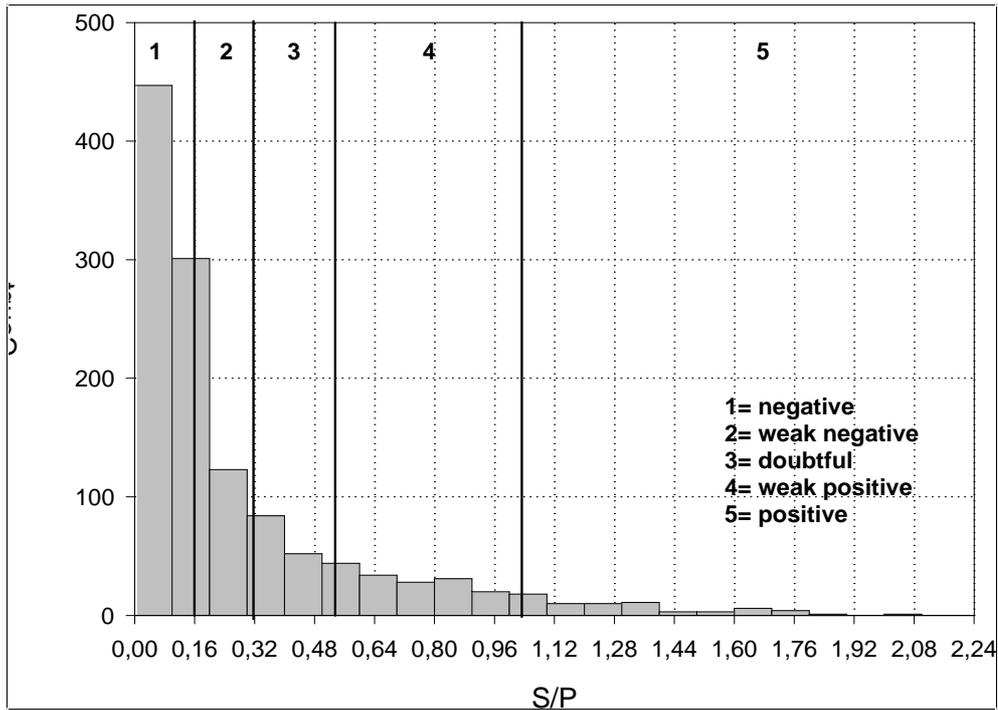
Fig. 2. Distribution of S/P values among 1300 blood samples taken in 45 dairy herds
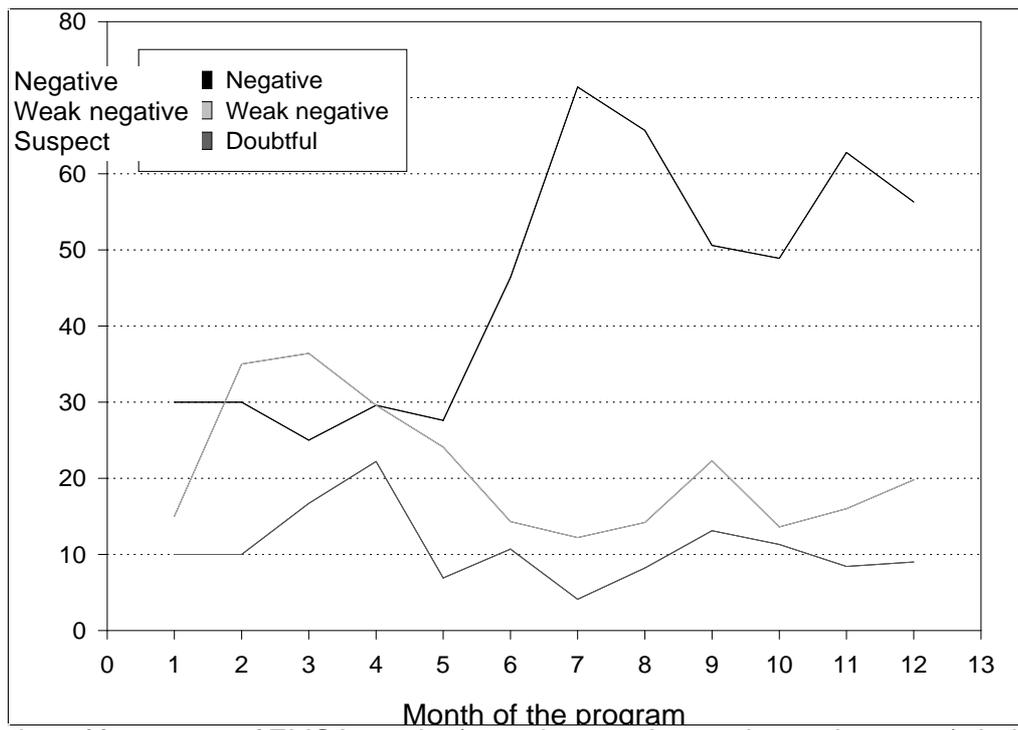
Fig. 3: Distribution of frequency of ELISA results (negative, weak negative and suspect) during the control program.
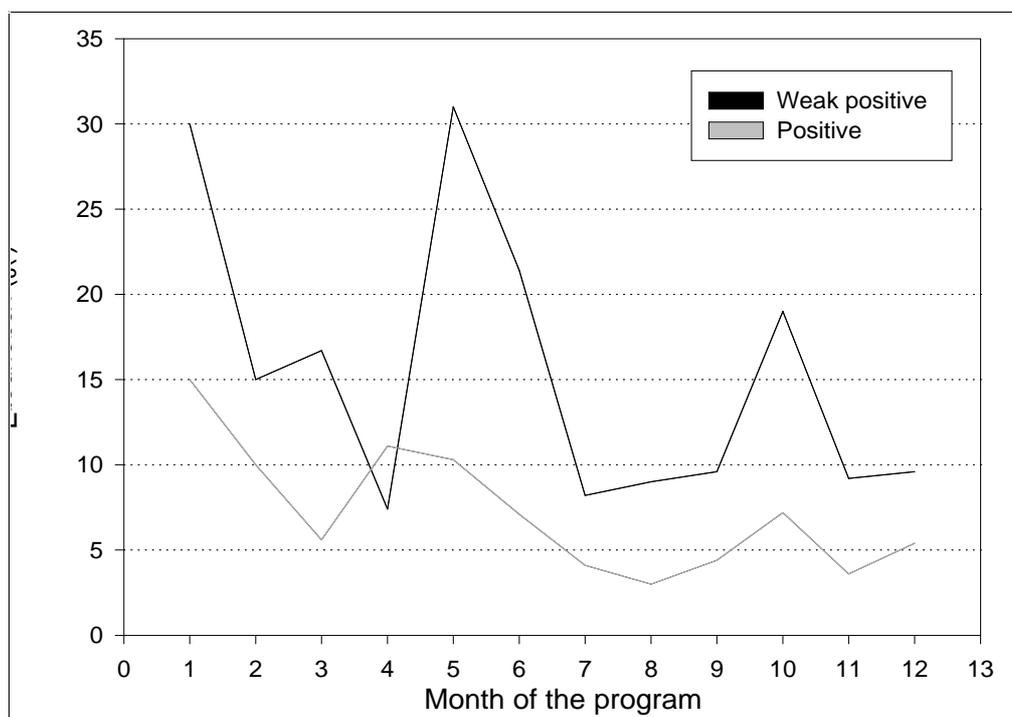
**Fig. 4:** Frequency of ELISA results (weak positive and positive) during the control program

A frequency trend in ELISA results was also observed by month in control program (Fig. 3). Negative samples increased greatly after the 6th month of the program, while weak negative and suspect results stayed more stable at 20% and 10% respectively. The frequency of positive samples (Fig. 4) decreased from 15% (1st month) to 5% (12th month). Weak positive samples ranged widely over the follow-up period, but some decrease could be observed. The values ranged from 15-30% at the beginning of the control program vs. 10-20% at the end of the follow-up period.

Table 2 reports the comparison of ELISA and real-time PCR. While the number of paired samples processed to date is relatively few, cows with negative and weak negative ELISAs were PCR-positive 11-14% of the time, suggesting that the number of ELISA false-negatives could be relatively low. Faeces from cows with ELISA results in the suspect range were PCR-positive in 44% of the cases.

Table 2. Correspondence between ELISA results and Real-Time PCR results

ELISA result category	Real-time PCR Result %	
	Negative	**Positive**
Negative (9 samples)	89.0	11.0
Weak negative (21 samples)	85.7	14.3
Suspect (18 samples)	55.6	44.4
Weak positive (11 samples)	81.8	18.2
Positive (5 samples)	60.0	40.0
No ELISA (65 samples)	75.4	24.6

Table 3. Correspondence between ELISA results and Real-Time PCR results by timing of sample collection

Sample type	Real-time PCR Result %	
	Negative	**Positive**
Pre-Partum (31 samples)	83.9	16.1
Post-partum (93 samples)	72.0	28.0
Other (5 samples)	66.7	33.3

When real-time PCR data were analyzed by timing of sample collection (Table 3), some differences were observed. Pre-partum samples were positive in 16% of the cases, while 28% of post-partum samples were positive.

DISCUSSION

The program was designed to be sustainable for farmers both from a practical and an economic point of view, and it should also be effective in reducing the spread of infection within the herd. These considerations were the rationale for selecting the program's particular diagnostic tests and protocols.

The serologic test used is characterized by a higher sensitivity and a lower specificity in comparison to other ELISA tests (Böttcher and Gangl, 2004). The approach and the diagnostic tests applied imply that colostrum (and milk) from test-negative cows only should be fed to calves. Even if some of the samples are false-positive, this approach could reduce the risk of spreading the infection through colostrum and milk.

A reduction in the frequency of ELISA positive tests over time and the relatively low number of real-time-PCR positive faecal samples in absence of clinical cases suggest that the program could be effective in reducing the risk of infection. The control program was well accepted by the farmers who had to take blood and faecal samples and to change the management of cows and calves as recommended. The most significant management changes were banning the use of colostrum and milk produced by "non-negative" cows and the priority given replacing test-positive cows. However, once explained, these changes too, were put in place by farmers.

Once this pilot study is completed, and if confirmed to be effective, it would be a useful test to enlarge this program to other herds in Italy.

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A national Johne's disease veterinary certificate program: key to success

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ABSTRACT

Critical to the implementation of the United States' Johne's disease control program is education and certification of regulatory and local veterinarians. A basic national curriculum was determined, but states were responsible for conducting their own training. The result was a variety of training programs, ranging from two hours to three days.

It became evident the US needed an educational program to provide a uniform base of knowledge and deliver a consistent message about Johne's disease diagnosis and control. Local action was also required to allow for state-specific practices, policies, and procedures. Proof of participation, verification of proficiency and records for the veterinarian and the state would be needed as well as the capability to educate unlimited numbers of veterinarians at their convenience. Web-based education could accomplish all of these objectives.

With Johne's experts across the country, we developed the certificate program in a modular format: four basic information modules and two modules with national and state program information for a total of six hours of continuing education. The last two modules could be customized for each state, if needed. Administratively, we adapted a "learning portal" to simplify management and access for program administrators and participating veterinarians.

This successful online program has, so far, been adopted by 35 states, and the US Department of Agriculture. Since it's inauguration in February 2004, the Online Johne's Disease Veterinary Certificate Program has trained over 450 veterinarians with over 2700 credit hours of continuing education. Development of virtual farm visits, a required refresher course, and virtual seminars are currently in progress. We are intrigued with the idea of expanding the program to other countries.

Key words: online education, continuing education, national education program

INTRODUCTION

In 2002, the US Department of Agriculture (USDA) created a national control program for Johne's disease. USDA recognized that veterinarians are the advisors most trusted by producers, and therefore the best candidates to deliver Johne's disease education. Therefore, in order to expand the veterinarians' knowledge of Johne's disease, and to ensure a consistent message, the USDA mandated that veterinarians in each state must be educated and certified to implement the control program. National standards and rules regulating the program were established, with each state able to implement the program as it wished, adding state-specific rules and policies more stringent than the national standards if desired. Each state was to hire a Designated Johne's Coordinator (DJC) in charge of that state's control program.

Funds were allocated to states in 2003 to train vets and enroll herds. In order to enroll in the program, the farm had to be evaluated by a Johne's-certified veterinarian who would conduct a risk assessment and develop a herd management plan for that farm. The number of enrolled farms is directly related to the number of veterinarians trained and certified to complete risk assessments.   Future funding for states was

to be contingent on the number of farms or ranches enrolled in the control program in the prior year. In October 2003, at the National Johne's Working Group meeting, a core curriculum was outlined for certifying veterinarians. How the veterinarians were to be trained and certified was a matter for each state to decide and accomplish. Therefore, fifty states and Puerto Rico were each faced with training and certifying local veterinarians to implement the national control program. What resulted was a variety of training programs that ranged from two hours to three days.

As a large dairy state, the Wisconsin Department of Agriculture, Trade, and Consumer Protection (DATCP) received a significant percentage of the funds dedicated for Johne's disease. It also had the needed to educate numerous veterinarians. DATCP turned to the University of Wisconsin, School of Veterinary Medicine, and UW-Madison's Wisconsin Technology Resource for Educating Care-providers (WisTREC). It was decided that web-based education could address the program's needs to:

- Provide a uniform base of knowledge and practice about Johne's disease to veterinarians;
- Deliver a consistent message about Johne's disease diagnosis and control;
- Allow customization of the content for state-specific information about the local program;
- Amplify a variety of experts' knowledge and expertise;
- Be easily updated and enhanced;
- Provide education that could be used and delivered in a variety of ways;
- Educate an unlimited number of veterinarians at their convenience;
- Verifiy proficiency and participation for the veterinarian and the state; and
- Grant continuing education credit as well as certification.

Although Wisconsin was using its funds to develop the program, the broader goal was to make a product that could be used by all states and easily customized for their use. Time was of the essence because the more veterinarians that were certified, the more farms that could be enrolled in the Johne's control program. Also, the more farms enrolled in the control program, the more USDA money dedicated to Johne's disease control the state would receive.

METHODS

Development
Fundamentally we had two tasks. One was to develop a high quality, standardized yet flexible certification program for individual states. The other was to create a structure that allowed states to administer their own programs using standardized courses.

First we considered our audience of large animal veterinarians. As evidenced by the dairy industry listserv, Dairy-L, and the listserve for the American Association of Bovine Practitioners (over 1600 subscribers), a large and increasing number of these veterinarians were using the Internet for professional purposes. However, we realized that many would not have high speed Internet access; therefore we needed to design for education delivery by modem. We needed a program that veterinarians could use at their own pace and at any location. Self-learning programs also avoided the high cost on instructor-led training.

We started with a certificate program for Wisconsin veterinarians, while keeping in mind its potential use as a national certificate program. Using the curriculum laid out by the National Johne's Working Group and approved by the US Animal Health Association, we outlined a certificate program consisting of 6 modules: four basic modules that would provide generic information needed by all veterinarians across the country and two modules with programmatic information ("Introduction" module and "Rules and Regulations") that could be customized for each state, if needed. Each module was developed as a narrated PowerPoint presentation with case examples, finished off with a quiz to verify completion of the module. We introduced the concept for the education plan at the National Johne's Working Group meeting in October 2003 with an anticipated delivery date of January 1, 2004.

We included experts from across the country to provide content, review, edit, add perspectives, and help narrate the modules. One of the editor/narrators we used was the National Johne's Disease Control Program Coordinator, Dr. Michael Carter. Wisconsin's Designated Johne's Coordinator (DJC), Dr. Beth

Patton, was the content expert for the "Introduction" and the "Rules and Regulations" modules for Wisconsin's program. Nebraska also developed its own state-specific certificate program. Using Wisconsin's two state-specific modules as a template, Nebraska's DJC edited the modules to include Nebraska information and then narrated the new presentation. Similarly, Colorado is creating its own state-specific program.

We used software from SoftTV (www.SoftTV.com) called ShowandTell. This program had the advantage of capturing audio while playing a PowerPoint presentation as well as allowing editing of individual slides. This made it easy to work with content experts across the country. They could each narrate their slides, send the digital audio files and then we could patch the segments into the PowerPoint show. ShowandTell also provided a way to present the slides, audio, and text as well as other resources such as handouts and links, thereby providing web accessibility. It is a relatively low-tech solution that the content experts were able to use on their own without training.

By the middle of December 2004 we had 3 modules ready for piloting by 12 Wisconsin state veterinarians. Once the modules were ready, all the state DJCs were asked for their review. The main request by other state DJCs was to develop generic modules for the "Introduction" and "Rules and Regulations" modules following the national guidelines, since many states were just adapting those modules and therefore wouldn't need state-specific modules. With funds from USDA, we subsequently developed two modules with national program information and added them to the four basic modules. This Standard Online Johne's Disease Veterinary Certificate Program may be used in states that adopt the national control rules and regulations as written. (http://vetmedce.vetmed.wisc.edu/JDVCP)   States could then decide if and how they wanted to use the online certification program, using all six generic modules or customizing some of them for their own state.

Implementation
The second task was an administrative and technological challenge. We needed a vehicle that would provide access to the veterinarians, allow them to pay either by credit card or by providing a pre-paid access code, test them and present them with completion certificates, be able to award continuing education credits, and keep records of the completed modules for the veterinarians and the state regulatory agency. We also wanted to allow states to administer their own programs. We adopted a continuing education portal offered by Netkeva (http://Netkeva.com) and created the online continuing veterinary medical education site, http://VetMedCE.org. Netkeva worked with us to incorporate the changes and additional functionalities to facilitate administration and access by states and their veterinarians. Figuring out what we needed the portal to accomplish and then getting it to work proved to be the biggest challenge. State veterinarians and Wisconsin veterinary practitioners provided valuable input that improved the educational content, delivery, and administration of the program. Important modifications included:
- Provision of access codes so states could buy registrations for their veterinarians;
- Clustering of modules into "programs" so the veterinarians could register just once for all modules of interest;
- Module hierarchies so the learners were forced to finish one module before proceeding to the next. (This was especially important when we wanted to assure veterinarians had completed the six modules before proceeding to on-farm risk assessment training);
- Randomization of test questions and the ability to retake tests if they didn't pass;
- Creation of "sub-portals" so each state could have its own identity and program ownership;
- Access for state officials to its own state registration and module completion data.

During testing of the modules we learned a number of lessons about the delivery of the content. Our biggest challenge stemmed from our commitment to making this program available to people on modem connections. Most, if not all, of our modifications had to do with this limitation. From our experiences we have the following insights to offer:
- While it is additional work, we ended up providing the content in three formats: RealPlayer, Windows Media Player, and a No-audio version. Evaluations show that 23% of users in the United States use RealPlayer, 35% use Windows Media Player, and 39% use the No-audio version.
- Large files and elements can be a problem. We compress the pictures as best we can and we also compress the PowerPoint presentation and audio as much as possible.

- We added a short "flash" presentation at the beginning of the program to teach learners how to navigate and manipulate the presentations. It is required viewing before taking the first module. This step has cut down considerably on the number of technical support requests. At some point, however, it may become voluntary.
- Even with all our efforts to provide low web bandwidth options, we have had a number of requests for a CD version or downloadable version. There are significant drawbacks to CDs: instant updating and improving the products is not possible and it is too easy for others to "borrow" the intellectual property and incorporate it into other, perhaps inaccurate, teaching resources.

Once the Standard program was ready, we gave states three options:

1. Use the Standard Johne's Disease Veterinary Certificate Program without modification. Veterinarians would simply go to the VetMedCE.org website, register for and take the standard Johne's disease program. They would receive 6 continuing education (CE) credits and a Standard Johne's Disease Veterinary Certificate that they would then need to submit to their state's DJC for verification.
2. Modify the generic modules to create their own state's certification program. Modifications would include:
   a. A customized web page with the state's logo and background information.
   b. Access to the state's certificate program from the list of choices, i.e. *"New Mexico Johne's Disease Veterinary Certificate Program"*.
   c. Receipt of 6 CE credits and a certificate from "New Mexico" with the agency's logo, signature, etc. to each veterinary participant.
   d. State official access to the VetMedCE database to verify activity in their program. These records can be downloaded to an Excel or Access program for analysis and can also be printed.
3. Customize the generic modules to include their own state-specific rules and regulations. In addition to all the features of Option 2 above, two of the six modules could be revised and re-produced specifically for the specific state. We would help the state's DJC edit, create, and narrate the PowerPoint presentations for the Introductory module and the Rules and Regulations module. They could also create a seventh module to explain how veterinarians should proceed to get the on-farm risk assessment training, if required.

CONCLUSION

The Online Johne's Disease Veterinary Certificate Program was inaugurated on February 6, 2004. To date, 20 states are using the Standard certificate program without modifications (Option 1), 12 states have their "own" program using the generic modules (Option 2), and 3 states have customized the modules with state-specific information (Option 3) for a total of 35 participating states. In addition, USDA has incorporated the Standard program in their annual training of the Designated Johne's Coordinators and State Veterinarians. As of August 1, 2005, 752 veterinarians have been trained and 3932 credit hours of continuing education have been provided. Development of virtual farm visits (as possible replacements to on-farm visits), a required refresher course, and virtual seminars are currently in progress.

This model for providing high quality, consistent education while providing flexibility and customization has proven to be useful and effective. We are currently pursuing the use of this model for nationalization in two other arenas: an aquatic veterinarian certification program and a national distribution of a women's health series for breast and cervical cancer screening.

ACKNOWLEDGEMENTS

LINKS

SoftTV (www.SoftTV.com)
Netkeva (http://Netkeva.com).
Standard Online Johne's Disease Veterinary Certificate Program (http://vetmedce.vetmed.wisc.edu/JDVCP)

The Victorian OJD Management and Control Program

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ABSTRACT

This paper documents the evolution of the OJD control and management program in Victoria, a state in south - eastern Australia. It follows on from previous papers delivered at the Sixth and Seventh International Colloquia on Paratburculosis in 1998 and 2001 respectively.

Progress since 2002 has demonstrated that it is possible to build industry ownership and engagement in an effective disease control program. Since its inception in 1996, the Victorian program has moved from a largely government sponsored effort heavily reliant on bureaucratic administrative processes to an industry sponsored program supported by government. The program's goal is now infection management, not eradication.

This paper outlines the progress made since 2002 and identifies the factors that contributed to a program that now enjoys a greater degree of confidence and support from the sheep industry. A key factor to the success of the ovine Johne's disease (OJD) management program in Victoria from 2002 to 2005 was the appointment of a representative industry ministerial advisory committee whose chairman enjoyed excellent credibility with sheep farmers in the management and control program. Subsequently, the industry advisory committee developed a financial assistance package to assist farmers whose properties were under trading restrictions for OJD. Underpinning committee's work was an extensive and ongoing communication process, a collaborative working relationship with the Department of Primary Industries' animal health staff, and the creation of a social support mechanism for affected producers.

Appointment of an independent and broadly based representative industry ministerial advisory committee
The report of the OJD ministerial advisory committee in 2001, headed by a former judge, made it clear that major changes were needed to the program for the effective management and control of ovine Johne's disease in Victoria.[1] In particular, the report identified the need for greater industry involvement in the decision making process. It also emphasised that if any future program was to enjoy support from the sheep industry, that program needed more effective communication and the building of relationships between key stakeholders from both industry and government.

With this in mind, a subsequent Minister for Agriculture appointed a second OJD advisory committee in 2002. Its role was to advise the Minister on developments in relation to OJD that might affect policies and procedures in Victoria, and the nation, for disease control, communication, financial assistance and social support.

In establishing this committee the Minister had called for expressions of interest from all interested and affected groups in Victoria.[2] Thus, when the committee was appointed it had representatives from all sectors of the sheep industry: breeders, veterinarians, stock agents, OJD lobby groups, the Victorian Farmers' Federation (VFF) and the Department of Natural Resources and Environment (NRE)[3]. The intention was for the committee to provide a forum for an open and transparent discussion of the issues so that decisions about the future management of the disease in Victoria would be made with the involvement of all industry sectors. This would increase the level of ownership and commitment to the implementation of any future program.

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[1] Fogarty, John, 'Advice to Minister for Agriculture, Hon, Keith Hamilton', July 2001
[2] Advertisement in 'The Weekly Times' newspaper, April, 2002
[3] NRE was disbanded in 2003 and two departments created - Dept of Primary Industries & Dept Sustainability & Environment.

The composition of the committee was as follows:
- a person representing the broader sheep industry;
- a person representing merino sheep breeders;
- a person representing meat sheep breeders;
- a person representing private veterinary practitioners with expertise in sheep;
- a person representing stock agents;
- a social scientist with rural experience;
- a person representing the Secretary of the Department
- the President of the VFF Pastoral Group or nominee;
- the President of the OJD Action Group or nominee.

The selection of the chairperson was a crucial decision. The committee voted to select its own chair and chose a sheep producer who had been directly affected by the previous program, Frank Tobin. After Tobin's property was destocked he initiated a campaign to stop the policy of eradication by forming a lobby group called the OJD Action Group. The efforts of this group met with success when a newly elected State government stopped the eradication scheme in 1999. Tobin now sat on the new advisory committee as the representative of the OJD Action Group who had also fought hard for the interests of sheep producers affected by the restrictions of the management program post 1999. Subsequently, the OJD Action Group lobbied for a new program that, whilst science-based, would be more sympathetic to the needs of farmers. In particular, they were keen to explore ways that would eventually allow owners of infected flocks access to sheep trading as a reward for any actions taken to control OJD on their properties. It was clear that engaging sheep producers who had the disease on their property was critical to controlling the spread of the disease. The appointment of Frank Tobin as Chairman of the new committee clearly signalled this new approach.

All industry representatives on the committee had one vote. The Department of Primary Industries' representation was limited to one vote to be exercised by the Secretary's representative, the Executive Director of Biosecurity Victoria. A number of other Departmental staff were to support the committee by providing expert technical advice, including the two most senior veterinarians, the Chief Veterinary Officer and the Manager of Animal Health Operations. The committee was also to be supported through the creation within the Department of a communications and support officer to assist with the writing and implementation of an effective communication strategy and also to create a support structure for producers in the control and management program.[4]

From the first meeting, it was evident that all representatives on the committee came with a willingness to work collectively for the interests of the sheep industry. Whereas the previous program had clearly been divisive, there was now a desire and expectation that a new approach could be devised.

The committee was appointed for a two-year term and was given sufficient resources to accomplish its tasks. The Minister later extended its term for an extra year[5].

Financial assistance package devised by industry advisory committee
One of the first actions of the new advisory committee was to devise a financial assistance package for properties that were infected with OJD. Previous financial assistance had taken the form of compensation for destocking infected farms. The new package provided incentives for owners of infected flocks to actively manage the disease.[6] It was also hoped that the package would encourage a number of producers whose flocks were suspected of being infected ('suspect' status) to undertake testing to determine whether the disease was present, and if so at what prevalence.[7]

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[4] This position formally titled OJD Support Program Coordinator was not filled until December, 2002
[5] The committee's term ran from August 2002 – October 2005
[6] These properties had an 'infected' status where it had been proven by laboratory testing that the flock had been exposed to OJD bacteria.
[7] Suspect flocks were assessed by departmental animal health staff as having significant risk   of exposure to OJD bacteria either through the purchase of sheep from an infected flock, or as neighbours to an infected flock. Both 'infected' and 'suspect' flocks were prevented from trading sheep. Sheep leaving these properties went directly to slaughter.

The assistance package encouraged producers to seek technical advice to enable them to make informed choices about the control of OJD on their farms. Either government animal health advisers or private consultants could provide this expert technical advice. Furthermore, the type of advice was not limited to veterinary advice, as producers were able to consult other professionals, such as an accountant or agricultural consultant with expertise in whole farm management.

The financial assistance measures were funded by the sheep industry through the Sheep and Goat Compensation Fund, raised by a levy of 12c per sheep sold in Victorian saleyards.

Specifically, the package involved:
- a subsidy of up to $3000 for professional advice to affected producers, to allow informed decisions about the management of OJD on their property;
- a subsidy of $50 per test-pool for undertaking an infected flock profile using pooled faecal testing (PFC)
- financial assistance of $20 per head to cull sheep considered to pose a high OJD risk (identified through flock profile testing), up to a maximum of $10,000;
- subsidisation of OJD vaccine (50% of purchase price) for vaccination of:
    - ewe lambs (other than slaughter lambs) each year for 3 years; or
    - 50% of the whole flock in the first year.

In an average flock of 4,000 sheep, the assistance measures were likely to amount to around A$20,000.

Initially, the response to the package was slow. However, over time it gained increasing favour, especially following:
- face-to-face communication by committee members at meetings held across the state[8]
- on-farm activities run by support groups formed under the communications and support project,
- a decrease in the stigma attached to the presence of the disease on a farm as more properties were confirmed as being infected and the impact of the disease on farm profitability became more clearly defined.[9]

The assistance package was reviewed at regular intervals in response to feedback from producers about the usefulness of each of its components. In particular, significant changes were made following a state - wide 'tour' by the advisory committee in mid-2003.
 These changes included:

- A subsidy of up to $500 provided to owners of 'Suspect' flocks for professional advice from an accredited veterinarian to allow for informed decision-making concerning testing and vaccination strategies.[10]

- Access to the vaccine subsidy for flocks considered 'at risk' of infection following the introduction of sheep from an infected flock, even if the at risk flock returned a negative test.

- Increasing the vaccine subsidy so that it provided 50% of the purchase price of vaccine for vaccinating all lambs (other than slaughter lambs) over a three-year period.

This rapid response to feedback from affected sheep producers continued to build the credibility of the advisory committee with the sheep industry.

Extensive and ongoing communication
In order to gain the support of the sheep producers for the financial assistance package, and later to seek input, plus gain support for a national risk-based sheep trading scheme (the Assurance Based Credit, or ABC scheme[11]), it was crucial to establish effective and ongoing communication with key stakeholders.

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[8] To support this communications effort one of the veterinarians from private practice sitting on the committee gave15 technical presentations in 2003 and 17 technical presentations in 2004.
[9] Number of infected properties rose from 45 in December 2002 to 337 in March 2005.
[10] If these suspect flocks tested positive then they were only eligible for $2500 of the available $3000 under the financial assistance package.

A flexible communication strategy was devised with the following characteristics:
EXTERNAL AUDIENCES
- Feedback was sought from all sectors of industry. This allowed modification of both the communication strategy and in the OJD control program. A good example of this flexibility was the adjustment to the financial assistance package described above. The main method of seeking feedback was through public meetings held across Victoria in 2003 and 2004.[12] An initial round of meetings was held with producers directly involved in the control program early in 2003. The main focus was to listen to their concerns. In mid-2003 a second round of meetings involving all sheep producers was conducted to seek input into a new national OJD control and management program. The final round of meetings conducted in 2004 was held to explain the ABC sheep trading scheme to industry[13]. All rounds of meetings allowed producers to meet with the advisory committee, regionally based animal health staff, and the support program coordinator. The meetings used a mix of formal presentations, small group discussions and a question and answer forum. Anonymous feedback forms were provided at every meeting so that those present could comment on any aspect of the meeting, the current OJD program, or the proposed future national program.
- Access to all committee members, key departmental animal health staff, and the support program coordinator was encouraged by circulating their names and phone numbers widely.

INTERNAL AUDIENCES
- A great deal of effort was made to include all departmental staff in the communications strategy by sharing information between key providers of the Victorian program - the representatives on the advisory committee, animal health staff and the support program coordinator. Discussion was encouraged within these circles and commitment sought on key decisions such as changes to tracing of infected and suspect properties, the setting up of the OJD Newsletter, adjustments to the financial assistance package, and Victorian responses to developments at an interstate level to name a few. The inclusive nature of these internal communications ensured ownership of, and commitment to, the Victorian program. In comparison with the pre-2002 program, a key feature was the integration of the key stakeholders in the decision making process and the relative harmonious nature of their relationships. As time went by and central features of the approach met with industry approval and acceptance, the level of trust and sense of team work grew thereby further enhancing the commitment of stakeholders to the new program.

Social support mechanism for producers in the control program
An essential recommendation of the former advisory committee was the creation of a support program coordinator to provide assistance to producers who were affected by the regulations of the OJD control program.[14] This position was filled in December 2002. The coordinator sat on the Advisory Committee as a non-voting member and was a departmental employee. Within the Department the position was put at a distance from the animal health operations branch so that the coordinator would be seen by producers as an independent figure able to advocate and work for their interests. The position was set up as a separate project with its own budget and time frame. It was expected however that the coordinator would work closely with the animal health staff and the advisory committee.

The selection criteria for the position had emphasized that the coordinator have experience and skills in communication. It was not seen as necessary for the coordinator to have any technical skills on OJD or experience and knowledge of the sheep industry. The appointee came with a background in education and communication and had been raised on a sheep property in Victoria.

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[11] The ABC scheme encourages producers to purchase and market sheep under a point score indicating the quality assurance of the sheep to be purchased. It caters for the needs of both affected producers (infected or suspect properties) and unaffected producers because the points system and its supporting Animal Health Statement (AHS) enable a purchaser to gauge the risk of introducing sheep with a lower score to their sheep flock. The seller of the sheep also benefits from the scheme as the AHS attests to the actions taken (e.g testing, vaccination, abattoir surveillance) to improve the quality of the sale sheep. The ABC scheme is a significant departure from the regulatory approach of the past where ownership and decision making about the disease was largely outside the control of producers.

[12] An effort was made to run a number of meetings in remote locations as well as more centralised regional town centres.

[13] See Appendix A

[14] Fogarty John, op cit, July 2001, pp19 - 22

The main elements of the support program were as follows:
- The formation of support groups across the state
- An OJD newsletter
- Delivery of a three day communication module for departmental animal health staff to enhance their skills in working with producers
- Communication advice and support to the advisory committee

Support groups
Support groups were set up across the state and were formed by producers who wanted to increase the level of knowledge of the disease within their communities. The philosophy behind the group concept was to give the control and ownership of the issue to the group. Each group was therefore provided with its own budget and given the freedom to choose a group coordinator from within their community. This person, paid from their group budget, became the organiser and with the group's input decided on what activities to run for the group's benefit. The backgrounds of the coordinators were varied,[15] as were the activities run by the groups.[16] The support groups took a leading role in educating their group members about the ABC scheme and worked closely with department animal health staff to ensure producers had relevant and accurate information that informed their decision making about the disease and the management program. This collaborative approach helped to take much of the stigma and negativity out of having the disease diagnosed on properties.

OJD Newsletter.
The objectives of the newsletter were:
- To increase the level of producer knowledge about OJD and the control and management program
- To promote success stories
- To minimise the fear and drama about the disease through the dissemination of information, and
- To enhance the communication networks between key stakeholders.

Six editions have been produced since March 2003. Contributors have been diverse with a significant number of articles written by producers, veterinarians and department animal health staff.

Communication module for animal health staff
The brief for the module was written by the support program coordinator and delivered by an external consultant. It was designed to enhance the communication competencies of staff to better equip them for their role in assisting farmers to make informed decisions for the management of OJD on their property. The module had a "people focus" component and "self management" focus component.[17]
Communication advice and support to the advisory committee.
The support program coordinator worked closely with the committee on all significant communications and was present at all rounds of meetings in 2003 and 2004. Organisation, collation and distribution of all feedback from all meetings were the responsibility of the coordinator.
The Department of Primary Industries has undertaken an independent evaluation of the support project to understand what worked and how well the project met the needs of affected producers.[18]

CONCLUSION

The successful engagement of the sheep industry in the decision making and implementation processes for the control and management of OJD has been a crucial factor in contributing to a more producer-oriented program in Victoria. A more representative sheep industry advisory committee has been important in bringing about significant change to the program, especially regarding sheep producers' attitudes towards working with government.

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[15] Examples: A veterinarian, sheep producers, an agricultural consultant, an assistant in a veterinary practice, a stock agent
[16] Examples: Post mortem of infected tissues, pathways to trade ABC scheme, vaccination demonstration
[17] "People focus" sought to increase knowledge of how people learn, assimilate and process information to understand their world. The self management focus sought to increase staff awareness of how to monitor and challenge their own negative emotions such as anxiety and to learn a more optimistic explanatory style.
[18] See Appendix B

The work of the advisory committee was enhanced through an extensive communications with the sheep industry, especially with affected producers in the management program.

REFERENCES

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VACCINATION

For the period 1 July 2004 to the present time:

Number of infected property requests	411
Number of Suspect property requests	82
Number of MAP requests	92
Number of Nil Assurance requests	470
Total doses	1,052,761

Number of application's for vaccine from flocks not known to be infected illustrates that more non affected flocks are now developing preventative management strategies.

Appendix A

Total numbers participating in public meetings to explain the Assurance Based Credit Scheme (producers only)

Benalla	66
Edenhope	22
Hamilton	36
Kyneton	46
Sale	26
Pyramid Hill	20
Seymour	46
Skipton	62
Swifts Creek	29
TOTAL	353

Information here does not include: a) meetings with stock agents, post July 2004; b) meetings with producers conducted by local Dept. Primary Industries animal health staff

Feedback sheet analysis

Location	Total no. of respondents	Outstanding rating	Very good rating	Good rating	Fair rating
Benalla	22	5	13	4	
Edenhope	16	3	11	2	
Hamilton	22	7	13	2	
Kyneton	26	7	14	5	
Sale	15	4	9	1	1
Skipton	42	7	28	6	1
Swifts Creek	15	4	10	1	
Pyramid Hill	No data				
Seymour	No data				
Total	158	37	98	21	2
%		23%	62%	13%	1.2%

Analysis of questions asked during and after the presentations

Tracing	8
Testing	15
Vaccine and vaccine safety	56
Animal Health Statement & scoring	45
Financial assistance	2
Terminal lambs	4
Tags	18
Abattoir surveillance	2
Saleyards	1
Other (e.g SheepMAP, prevalence levels, road movements, spread of the disease, policing of AHS)	17

Appendix B

Edited summary of the OJD support project evaluation conducted by Dr Lucia Boxelaar, University of Melbourne, August 2005.

The Communication and Support Project aimed to:
▪ Improve the information available to farmers
▪ Improve the capacity of animal health staff to support farmers
▪ Improve farmers' control over the decision making process with respect to OJD'

Evaluation approach
1. Semi-structured telephone interviews
A total of 18 semi-structured telephone interviews were conducted to explore the perspectives of a range of stakeholders in the project. These included:
- Interviews with 11 farmers involved with the support groups
- Interviews with three senior veterinary officers of the Department of Primary Industries
- Interviews with three of the five support group coordinators, to obtain their perspective on the role of the support groups in assisting farmers dealing with OJD
- One interview with a representative of the Victorian OJD Advisory Committee

2. Self-administered questionnaire mailed out to farmers

3. Email questionnaire with Department of Primary Industries animal health staff

4. Group interview with the Victorian OJD Advisory Committee

The outcomes achieved by the project
▪ Increased capacity of farmers to deal with OJD
▪ Increased capacity of AH staff to support farmers
▪ Farmers taking responsibility for OJD
▪ De-stigmatisation of the issue
▪ Improved uptake of the ABC Scheme
▪ Improved networks, relationships and trust between farmers, within communities and with DPI staff.

Five success factors were identified:
▪ The group approach
▪ The emphasis on communication
▪ The one-on-one support provided to farmers where necessary
▪ Farmer ownership of the groups
▪ The fact that the coordinator was an 'outsider' and 'newcomer' with strong communication skills, who was able to act effectively as a broker of relationships and information.

'It must be noted here that the evaluation has focused on identifying the logic of the project, i.e. *what* outcomes were achieved and *how.* While the data provides some insight into the extent to which these outcomes were achieved, definitive statements about this cannot be made without further empirical investigation.'

Increased capacity of farmers to deal with OJD

'Analysis of evaluation data suggests that the project helped farmers in managing the disease in a number of ways. Firstly, it raised their understanding of the disease itself, the way in which it spreads and how it manifests itself in sheep. It also made farmers aware of control strategies, particularly vaccination; and finally, it helped farmers to develop the necessary skills to manage OJD, including diagnosis of OJD and vaccination skills.'

Increased capacity of AH staff to support farmers

'Interviewees and email questionnaire respondents suggest that the Winning Through Woolly Thinking course increased staff's awareness of their communication style and staff report they have developed communication skills that allow them to interact with farmers more effectively'

Farmers taking responsibility for OJD

'Another outcome of the Communication and Support project that is reported by several interviewees is that the group approach facilitated a process whereby farmers were able to take responsibility for managing the OJD issue'

De-stigmatisation of the issue

'Several interviewees suggest that through the support groups, people started talking more openly about their issues in a supported environment. This openness assisted in taking away some of the fear and ignorance about the disease, which in turn helped the spread of accurate information about it'

Improved uptake of the ABC Scheme

'Interviewee responses suggest that another outcome of the support group has been that they have helped the introduction of the ABC Scheme and enhanced levels of acceptance of this scheme'

Improved networks, relationships and trust
'Interview data suggests that one very important outcome of the Communication and Support project is the improved relationships, networks and trust between farmers and DPI staff. This was an especially salient theme in the interviews, with 57 references made to this outcome in 18 interviews'.

Appendix C

Quotes from farmers involved in communications activities (from OJD support project evaluation conducted by Dr Lucia Boxelaar, University of Melbourne, August 2005.

'As far as I'm concerned it made me, by talking over things with people about how they cope and how they handled the whole thing, it encouraged me to test and find out my status and move on. I am still waiting on the results of that but I am quite confident, I am not at all worried about it if I do get it. Through being part of this group I am quite confident that I will handle the situation a lot better (Farmer).' p24

'I do know as a result of the group who has got it and where the hotspots are, and I know now … as a result of the group meetings that it is not as big an issue… When someone comes up and tells me he has OJD, I say, Oh, yes. What am I going to do about it now? I am not going to tear my hair out (Farmer)'p24

'It has taken away the alienation, the whispers that they have something terrible. It has been people stepping forward and supporting one another rather than pushing them to the periphery (Support Group coordinator).'p.27

'It was a great help for us all to meet, to discuss how we felt about it and to be able to talk to other farmers who had it, it relieved you of that stigma (Farmer).'p.27

Development of a milk quality assurance program for paratuberculosis: from within- and between herd dynamics to economic decision analysis

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ABSTRACT

A new surveillance program was modelled that focuses on limiting the concentration of *Mycobacterium avium* subsp. *paratuberculosis (*MAP) to a certain maximum number of bacteria per litre of bulk milk. In this new program dairy herds are distinguished in two categories, 'Green' and 'Red', where Green stands for the pool of certified herds that produce milk with a MAP concentration of <1,000 litre and Red herds with milk concentrations > 1,000 litre. The program is based on 3 parts: (1) an intake procedure (certification), (2) a surveillance procedure to monitor Green herds, and (3) infection control procedures for Red herds. Models were developed to predict the progress over time of Green and Red herds for certain herd factors (size, prevalence, MAP in bulk milk). Data from several test regimens based on blood or faecal tests were combined with information on herd sanitation measures and animal purchase policies. Results of epidemiological models were used in an economic decision analyses.

Keywords: Certification**,** surveillance, control, modelling, *Mycobacterium avium* subsp. *paratuberculosis.*

INTRODUCTION

In the Netherlands a certification and surveillance program for *Mycobacterium avium* subsp. *paratuberculosis (*MAP*)* has been developed, aiming at eradication of *MAP* at the herd level. In the program, herds can obtain a MAP 'free' status following five annual herd examinations, provided all faecal culture results are negative. The first herd examination is done by ELISA and faecal culture of ELISA-positive animals; the 2nd through 5th by pooled faecal culture (Benedictus et al., 1999). *A* program such as this that aims at eradication of *MAP* at the herd level is inherently expensive and there are no incentives for farmers to participate. Therefore, few farmers participate. However, the most important goal from a food safety point of view is to reduce the number of *MAP* bacteria per litre of bulk milk. Thus, an important research question is can we design a new program that limits the number of MAP bacteria per litre of bulk milk? Can the program be simple, cheap and give farmers enough incentives to join and stay with the program for many years?

In the present study a new certification and surveillance program was developed for farms with 'low-risk' or 'low-MAP' bulk milk. These farms will guarantee a certain component of their bulk milk, that is, to contain fewer than a preset number of MAP bacteria per litre. In the certification and surveillance program dairy herds are distinguished in two categories, 'Green' and 'Red', where Green stands for the certified herds that guarantee milk with MAP concentration below the preset quantity. An intake diagnostic test scheme determines which farms will receive a Green or Red status. Green herds are monitored regularly with a surveillance test scheme. Management improvements and trade restriction may help to improve the milk quality in the Green pool, and may help Green herds retain their Green status in the future (vs. moving to the pool of Red herds). A control program assists Red herds to progress to receive a Green status (perhaps again) after a certain time. The control program consists of test and cull of positive animals, whether or not combined with management improvements (step 1, 2 and 3 of PPN, the Paratuberculosis Program in the Netherlands, see Groenendaal et al., 2002 and 2003) and/or trade restrictions (here: purchase of live animals from Green farms only).

MATERIALS AND METHODS

Various alternative programs for certification-and-surveillance-and-control of MAP on low-prevalence Dutch dairy herds were evaluated in this study, assuming an initial herd-level prevalence in the country of 30%, i.e. at the start (before the intake procedure) we assume that 30% of the dairy herds is infected with MAP, and 70% is free from MAP. This prevalence was recently found in the Netherlands (van Weering et al., 2004).

Evaluated test schemes are based on sampling individual animals for blood or faeces (individual faeces test, pooled faeces test, serum ELISA). Testing of bulk milk samples for *MAP* on a large scale is not yet possible, so this test method was not evaluated. Through modeling we make the step from the number of test-positive animals in a herd to the number of MAP bacteria in milk. To evaluate the effectiveness of the various programs three models were used in this study:

(1) The simulation model JohneSSim for within-herd transmission of MAP in a closed herd. This is a stochastic and dynamic simulation model that simulates (a) the herd dynamics, (b) the disease dynamics within the herd, (c) the control of Johne's disease and (d) the economic consequences at the herd level. Details and input parameter values can be found in Groenendaal et al. (2002) and in Weber et al. (2005). With this model the effectiveness of the intake and surveillance procedures in closed herds were determined, as well as the infection control procedures in Red herds. The economic and epidemiologic output of this simulation model served as input for the other two models (see below).

Preventive management in the simulated herds was set to reflect management practices in the Dutch dairy industry (background management, see Groenendaal et al., 2002). An additional simulation assumed that *all* herds took the following preventive management measures: improved hygiene around birth (step 1 of PPN), colostrum from own dam only, and feeding of milk replacer only (step 2), and effective separation of young stock from adult cows from birth to the end of the first year (step 3). Because these measures also affect other animal diseases, only 50 % of all costs of these management measures were attributed to the control of paratuberculosis in this study. Some of these input parameters were updated in Febuary 2004 and are presented in van Roermund et al. (2004) and Weber et al. (2005).

The expected number of MAP bacteria per quantity of bulk milk was the sum of MAP shed directly into milk, and the numbers added through contamination of milk by faeces from faecal shedders. Faecal contamination of milk was estimated to amount on average to 40 mg per litre (Stadhouders and Jørgensen, 1990. For an extensive overview of MAP bacteria and/or CFU's in milk and in faeces, see van Roermund et al., 2004). Based on these data, assumptions were made on the on-farm MAP contamination of bulk milk (Table 1). Faecal contamination was considered the prime source of MAP in milk. The expected concentration C_{Map} of MAP bacteria in bulk milk was approximated by the average concentration of MAP in milk in all animals in the herd.

Table 1. Assumed concentration of MAP bacteria in milk for each stage of the infection-and-disease process in adult cattle.

Stage	Proportion of animals	Direct shedding of *MAP* in milk (bacteria per litre)	Faecal contamination of milk (g per litre)	*MAP* concentration in faeces (bacteria per gram)	*MAP* in milk through faecal contamination (bacteria per litre)	Total *MAP* in milk (bacteria per litre)
Latent infected		0	0.04	0	0	0
Lowly infectious	0.8	0	0.04	0	0	0
	0.2	0	0.04	10^2	4	4
Highly infectious	0.6	10^2	0.04	10^2	4	104
Highly infectious	0.24	10^2	0.04	10^4	400	500
Highly infectious	0.16	10^2	0.04	10^7	400	4.001×10^5
Clinical disease		10^4	0.04	10^9	4×10^6	4.001×10^7

What may be considered an acceptable concentration of MAP bacteria in on-farm bulk milk is not known. In the present study a maximum concentration of 1000 MAP bacteria per litre bulk milk was decided as acceptable by experts from Dutch dairy organisations (NZO/NIZO), based on their knowledge of the effect of pasteurisation.

Intake, surveillance and control schemes were first studied separately and then integrated in simulations with JohneSSim. Those selected can be found in Table 2. In the integrated simulations, herds were permitted to migrate between the statuses Green and Red. At intake, test-negative herds were designated as Green while test-positive herds were designated as Red. Thereafter, Green herds were re-classified as Red if a test-positive animal was found in the herd during surveillance. Red herds were re-classified as Green following the required number of annual test-negative herd examinations, as determined in the separate analyses of the intake and control schemes.

Table 2. Intake and surveillance of Green herds and control in Red herds simulated with the JohneSSim model. A positive result of the ELISA or pooled faecal culture (PFC) during intake and surveillance was confirmed by individual faecal culture (IFC). During Control in Red herds IFC-positive animals and their lastborn calf were culled. However, results of ELISA during control in Red herds were not confirmed by another test; ELISA-positive individuals and their lastborn calf in Red herds were culled.

Scheme	Intake			Surveillance				Control		
	Test	Animals		Test	Interval	Animals		Test	Interval	Animals
i1-s1-c1	i1	ELISA	All, ≥3yr	s1	ELISA	1 yr	All, ≥3 yr	c1	ELISA	1 yr	All, ≥3 yr
i1-s1-c7	i1	ELISA	All, ≥3yr	s1	ELISA	1 yr	All, ≥3 yr	c7	IFC	2 yr	All, ≥2 yr
i1-s2-c1	i1	ELISA	All, ≥3yr	s2	ELISA	2 yr	All, ≥3 yr	c1	ELISA	1 yr	All, ≥3 yr
i1-s2-c7	i1	ELISA	All, ≥3yr	s2	ELISA	2 yr	All, ≥3 yr	c7	IFC	2 yr	All, ≥2 yr
i5-s1-c1	i5	PFC	All, ≥2yr	s1	ELISA	1 yr	All, ≥3 yr	c1	ELISA	1 yr	All, ≥3 yr
i5-s1-c7	i5	PFC	All, ≥2yr	s1	ELISA	1 yr	All, ≥3 yr	c7	IFC	2 yr	All, ≥2 yr
i5-s2-c1	i5	PFC	All, ≥2yr	s2	ELISA	2 yr	All, ≥3 yr	c1	ELISA	1 yr	All, ≥3 yr
i5-s2-c7	i5	PFC	All, ≥2yr	s2	ELISA	2 yr	All, ≥3 yr	c7	IFC	2 yr	All, ≥2 yr
i5-s5-c7	i5	PFC	All, ≥2yr	s5	PFC	2 yr	All, ≥2 yr	c7	IFC	2 yr	All, ≥2 yr

(2) The analytical model. For the total population of dairy herds that interact with each other by trade of living animals, a new model was developed. This mathematical model describes a large group of herds (divided in Green herds and Red herds). The model is deterministic, and variation among herds is modelled by statistical distributions. Input parameters of this model were aligned with those used in JohneSSim, such as the distribution of initial infection prevalences within herds, life expectancy of animals (infected or not), relative infectiousness in various stages of infection, test sensitivity in various stages of infection, and the within-herd transmission rate of MAP. For the within-herd dynamics of paratuberculosis in each herd, the transmission is described by one parameter (beta), with a default value based on simulation results with

JohneSSim. This mathematical model was used to study the progress in time of the group of Green herds and of the group of Red herds (pool size, prevalences in the two pools, MAP in bulk milk etc).

Animal trade, i.e. here purchase of live animals from Green herds, is based on actual data of the Netherlands of the year 2000. In that year 37% of all dairy herds purchased cattle, and 63% did not. The average number of purchased life animals by open cattle herds was 7 per herd per year (Velthuis, 2004). In the model for the 'open herds' scenario (animal trade allowed), 63 % of the herds was treated as closed, and 37 % of the herds purchased animals (7 per herd per year). Of course for the 'closed herds' scenario (no animal trade), no animals were purchased by any herds.

(3) The economic decision analysis determines the preferred decision for a farmer: should I join the new program or not? The decision of an individual farmer whether to join the program or not will be based on many different aspects (e.g. former experiences with other programs, the amount of labour, the time it will take, the yearly costs, the investments, the benefits and the chances of receiving these benefits, beliefs, etc.). A way to determine the economically preferred decision of a farmer, given the set of alternatives he has, is by analysing a decision tree. A decision tree includes three aspects of the decision making process, namely the costs, the benefits and the risks. In a decision tree all alternative actions available for the decision maker and the outcomes determined by chance are structured in a chronological order. The producer's goal that drives a decision is the highest Expected Monetary Value (EMV). More background information on decision analysis and decision trees can be found in Hardaker et al (2004), Clemen (1991) and TreeAge (1999).

The decision tree analysis models the costs, losses and the probabilities to change status: Green to Red or Red to Green. The decision tree weighs the economic elements with the risks and shows the preferred decision based on this. When the preferred decision is not to join the program the decision tree estimates the milk price differentiation for Green farms that is needed to change the preferred decision to joining the program. This milk price differentiation serves as an incentive for farmers to join the program.

A decision tree has three elements: 1) decisions to make; 2) outcomes based on probabilities, and 3) the value of the specific outcomes. Each element is explained below. A farmer has to make several decisions in time considering a new MAP program: (1) Should I join the program at the start? (2) Should I continue the program given the test results of the intake procedure?, (3) Should I continue the program during surveillance given the test results of the jth test round ? (This decision can be repeated for each test round). It is assumed that a farmer has no prior information on the true infection status of his herd. After the intake procedure a farmer knows more about the infection status of the herd and uses this information when the next decision is made. This new information is also considered in the decision tree. Another scenario that has been modelled is that once he has decided to join, it is not possible for a farmer to leave the program before the sixth test round,
The uncertain events within the MAP program included in the decision analysis as probabilities are: (1) the chance that the farm is infected with MAP at the start of the program, (2) the chance of being classified as Green at the intake given that the farm is infected with MAP, (3) the chance of being classified as Green at the intake given that the farm is free of MAP, (4) the chance of becoming free of MAP within the period between two tests given that the farm is infected at the start of this period, and (5) the chance of becoming infected with MAP within the period between two tests given that the farm is free at the start of the period. The input for the decision analysis (probabilities) is the output of JohneSSim and of the analytical model.

These two elements, that is decisions and chances, influence the value of the specific outcome (pay-off function): profit, costs and losses. The values of the specific outcomes are calculated in a profit function that includes the net present value (i.e. the value in today's prices) of the following costs and benefits: (1) yearly program costs, (2) test costs, (3) costs for management improvements, (4) losses due to MAP, and (5) milk price differential between Green farms and all other farms. The higher price per litre of milk produced by Green farms compared to other farms is a premium that serves as an incentive to encourage farmers to comply with the program and to compensate them for the costs incurred to earn a "Green" status.

The 36 alternative scenarios for paratuberculosis that have been evaluated in this study are the 9 test schemes of Table 2, each with and without management measures and with and without animal trade (9x2x2).

RESULTS

Epidemiology. We assumed an initial overall prevalence of paratuberculosis in participating herds of 30%, i.e. 30% of the herds were considered infected and 70% were considered to be free. As a result, 90% and 83% of the herds receive a Green status after intake I1 (ELISA) and I5 (faecal culture) respectively. After that, the number of Green herds drops during the first 10 years. This is due to the Green status but truly infected herds that are detected later and reclassified as Red herds. Only with management measures can increase in number of Green herds can be seen, as control methods (C1=ELISA or C7=faecal culture) permit Red herds to reach Green status. As an example the percentage of Green herds after 8 years is given in Table 3 (recall that there were 90% at the start in Year 0 after Intake I1). (For codes I, S and C, see Table 2.)

As noted, the pool of Green herds decreases during the first years. As Green herds found to be infected are removed from the pool, the prevalence of infected animals and the MAP bacteria per litre bulk milk in the pool of Green herds falls, showing an improvement in Green herds' milk quality in time (not shown here; see van Roermund et al, 2004).

Immediately after the intake procedure, the PVN (predictive value negative) is very high: about 97.8% and 99.7% of the Green herds produces milk with MAP<1000 bacteria/litre after intake I1 (ELISA) and I5 (faecal culture) respectively. However, the small fraction of Green herds producing milk with MAP>1000 bacteria/litre have a significant effect on the *average* MAP content of milk of all Green herds combined, keeping the overall concentration above the limit. The MAP concentration is above the limit for the first 5 years when ELISA is used as the intake assay (I1). This is due to the very skewed distribution of MAP bacteria in milk per herd. After intake procedure I5 (faecal culture) however, the average MAP content of milk in the pool of Green herds drops immediately to the level of 1000 bacteria/litre (see van Roermund et al, 2004).
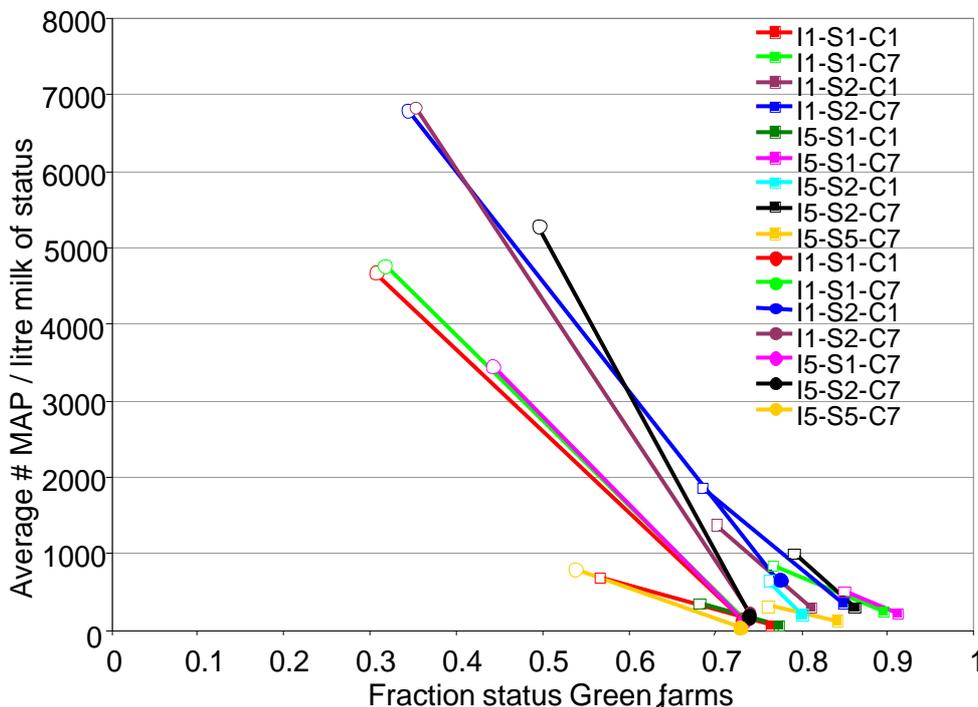
Figure 1. MAP bacteria per litre bulk milk in the pool of Green herds versus fraction of farms in the Green pool, 8 years after the start of the program. Open dots or squares represent open farms, closed dots or squares represent closed farms. Squares represent farms with management measures (=step123), dots represent farms without management measures.

Figure 1 shows MAP bacteria in bulk milk for Green herds in year 8. The best programs are in the lower-right corner of this figure: these programs result in a higher fraction of participating herds receiving a Green designation, and a low average bulk milk MAP content (after 8 years). From this figure it becomes clear that

acquiring replacements outside of the home herd (purchase from Green herds; open farms: open dots) has a strong negative effect on both outputs. Programs with better outcomes are in the lower-right corner (I5-S1-C7, I1-S1-C7, I5-S2-C7, I1-S2-C7, and I5-S5-C7); they all require management measures and a closed herd. Just one test scheme permits an open herd and does not require management measures yet still keeps the average Green herd MAP concentration below the limit. This scheme is I5-S5-C7, the only one using only faecal culture for all three program components: intake, surveillance and control.

Tabel 3. Percentage Green herds after 8 years.

Test scheme Intake-Surva.-Control	Management: yes Herd: closed	Management: no Herd: closed	Management: yes Herd: open	Management: no Herd: open
I1-S1-C1	77	73	57	31
I1-S2-C1	81	77	70	35
I1-S2-C7	85	74	69	34

The intake procedure (I) and the surveillance procedures (S) appear to have less effect than the type of control procedure on Red herds (all faecal culture C7), and the presence of management measures plus the absence of animal trade. With the fecal culture control scheme C7 Red herds go back to the pool of Green herds sooner (after two negative test rounds) than C1 (ELISA control), explaining the larger size of that pool. In the absence of animal trade, the effect of management measures seems relatively minor (compare closed squares with closed circles), but this may be due to the short analysis period of 8 years.

When found test-positive, a Green herd shifts to the pool of Red herds and is treated as if it had never been classified otherwise (i.e. status is based on the PVN value discussed above). For the pool of Red herds it was found that 6 ELISA or 2 faecal culture test-negative herd examinations are needed to reach a PVN value of 97.8-99.7% (depending on intake I1 or I5).

For the Red herds management measures are very important (see van Roermund et al., 2004) in controlling the percent of infected animals and MAP content of milk. Animal trade is less important for a Red herd, since purchase of an infected animal has a marginal effect on the prevalence of an already infected herd. Except for a short time immediately after culling faecal culture-positive animals (control C7) when management measures are applied on the farm, the average MAP content of milk in the group of Red herds is always above 1000 bacteria/litre. In fact within one year of culling the MAP content rises to at least 7000 bacteria/litre (see van Roermund et al., 2004).

Decision analysis. The model predicts that without the milk price incentive, farmers will not join the program. A farmer will drop out if classified as a Red herd (at the intake procedure or after a test round during the surveillance procedure) even if the milk price differentiation is €0.01 per litre milk. Higher milk price differentiations were not studied here. The one exception is program I1-S1-C7 (without management measures). For this program the optimal decision for a Red farm after testing positive at intake procedure is to join the control procedure for another test round (at a milk price differentiation of at least €0.003). If the herd tested positive again, the optimal economic decision is to quit the program.

The minimal milk price differentiation needed to change the decision from 'no' to 'yes' to join the intake-procedure is between €0.0005 and €0.0051. If a program is designed in such a way that a farmer cannot stop joining the program before the 6th test round of the surveillance procedure, the milk price differentiation is higher and should be between €0.0009 and €0.0080, depending the program. The higher milk price differentiation is needed to balance the higher program costs during 6 test periods. The programs I1-S2-C1, I1-S1-C1 and I1-S2-C7 (without management measures) have the lowest costs for participants. The average yearly costs for Green and for Red farms are €388 and €1065 for program I1-S2-C1, €609 and €1085 for program I1-S1-C1 and €386 and €1647 for program I1-S2-C7. With management measures these costs are much higher: €2110 and €2538 for program I1-S2-C1, €2332 and €2529 for program I1-S1-C1 and €2007 and €3183 for program I1-S2-C7. For a more extensive overview and for benefits, see van Roermund et al. (2004).

A cost-effectiveness analysis is presented in Figure 2. In this figure the milk price differentiation between Green and Red farms (needed to give a farmer enough incentive to join a quality assurance program voluntarily) is set out against the fraction of participating farmers that will have a Green status after 8 years.

Two clusters can be distinguished in this figure: the programs with management measures (step123, right upper corner) and the programs without management measures (left). This shows that management measures results in a higher milk price differentiation and in a more effective program (defined here as the percent of herds classified as Green). The difference in the upper and lower end of each line indicates the effect of animal trade. All programs perform better when no trade is allowed. However, the ban on trade has a greater impact when no management measures are included. The 'cheapest' programs that are most effective (where the fraction is higher than 70%) require closed herds but do not require management measures.
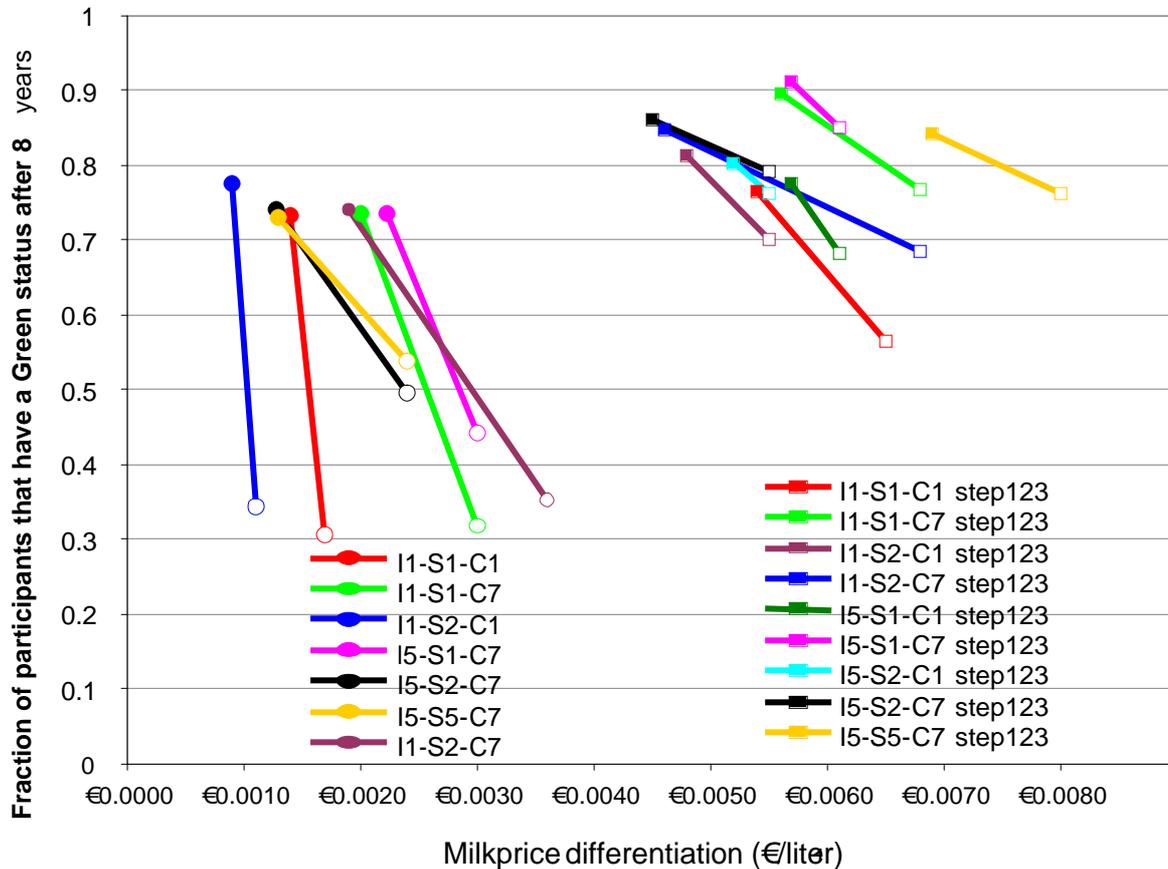
Figure 2. Milk price differentiation needed to give a farmer enough incentive to join a quality assurance program voluntarily vs. the fraction of participating herds that will have a Green status after 8 years. The milk price differentiation is based on the assumption that a farmer joins the program for the intake procedure and remains for at least 6 infection control. The upper end of each line (closed dots or squares) represents closed farms in a program where no trade is allowed, whereas the lower end (open dots or squares) represents open farms where trading is allowed.

The average concentration of MAP bacteria per litre of Green herd milk is given in Figure 3 in relation to the milk price differentiation. As in Figure 2 two clusters can be seen: the programs with management measures (step123, to the right) and the programs without management measures (to the left).

In setting a target concentration level of less than 1000 MAP bacteria per litre for Green farms on average, the models show that open herds and no management measures are not optimal with one exception (as mentioned above for the scenario that a farmer remains in the program before the sixth test round of the surveillance and control procedures). When management measures are applied almost all programs (with or without animal trade) are provide milk that meets or is below the MAP target concentration.
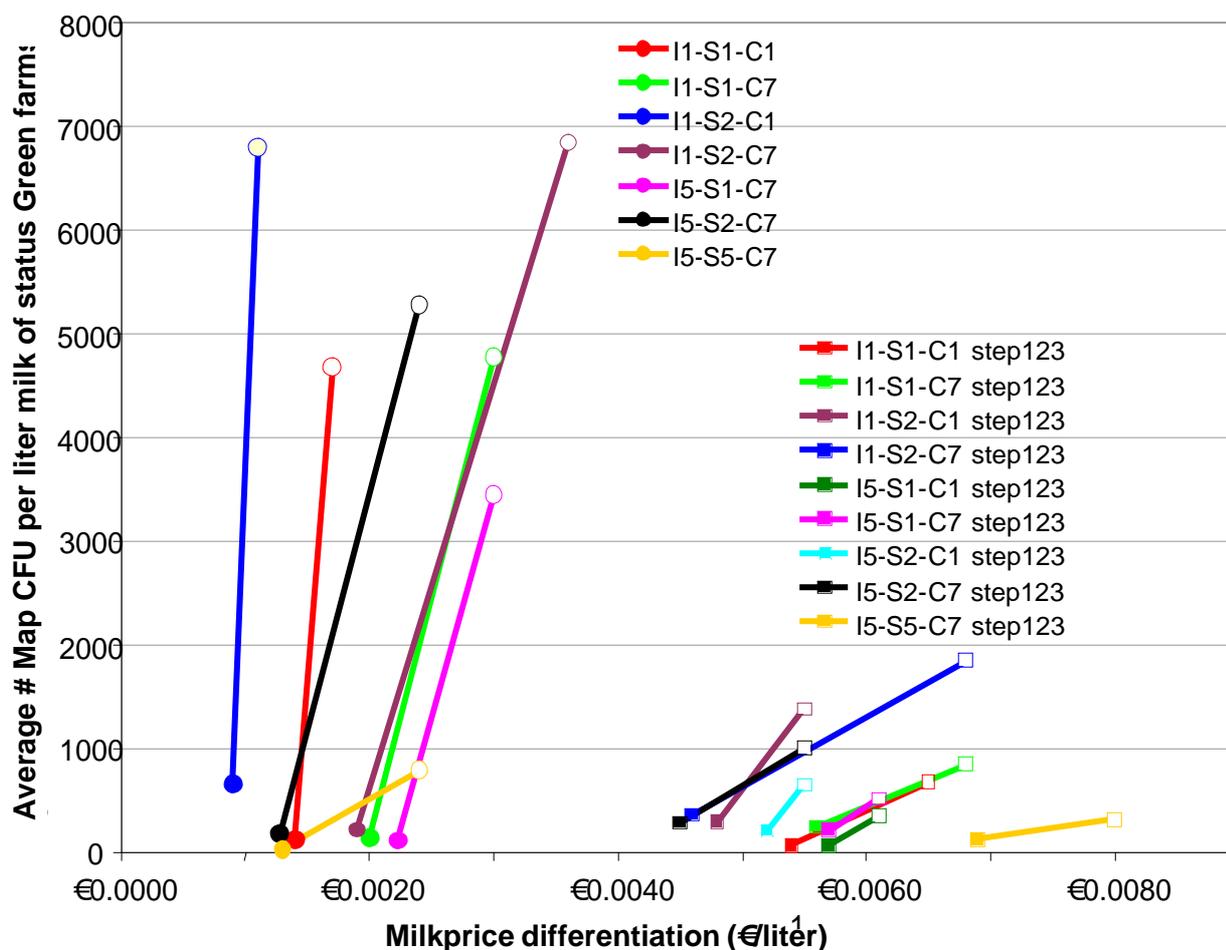
Figure 3. Milk price differentiation needed to give a farmer enough incentive to join a quality assurance program voluntarily for MAP vs. the average number of MAP per litre of milk of Green farms per year. The milk price differentiation is based on the assumption that a farmer joins the program for the intake procedure and remains for at least 6 test rounds during the control procedure. The lower end of each line (closed dots or squares) represents closed farms in a program where no trade is allowed, whereas the upper end (open dots or squares) represents open farms where trading is allowed.

Management improvements on farms can be costly, and based on this study are less important than reducing animal trade (see Figure 2). However, the positive effect of management measures increases if animal trade is allowed. Furthermore, management measures are important on Red farms, and if they are not taken, the pool of Green farms will never increase in size.

DISCUSSION AND CONCLUSIONS

According to the models, Green herds must be closed to animal trade. The choice of intake and surveillance policies themselves is less important than the effect of animal trade and of management measures. Management measures have less effect when animal trade is restricted (during the first 8 years), but are always very important on Red herds. The control component C7 (individual faecal culture) for Red herds increases the number of herds in Green herd pool due to the shorter lag time of becoming Green again (2 negative test rounds), and C7 is effective at lowering the concentration of MAP in Red herd milk. If there is no milk price differentiation for milk produced by Green farms the preferred decision for a farmer is not to join any program. If a milk price differentiatial is introduced, the Green farms will join. The majority of all dairy farms in the Netherlands will be certified as Green. When a farmer receives a Red status (at the intake procedure or after a test round during the surveillance procedure) the preferred decision is to stop the program immediately even if the milk price differentiation is € 0.01 per litre milk. This will happen with 10-14% of initially participating farms.

As for any modelling study, the reliability of results depends on the accuracy of the model's assumptions. The most uncertainty in these models concerns the lack of data on the amount of MAP bacteria in milk and the effect of management measures on the within-herd transmission of the infection. Both factors' assumptions were tested in a sensitivity analysis (see van Roermund et al., 2004 and Weber et al., 2005). In the models MAP in milk is contributed by a small fraction of highly infectious animals plus by the clinical animals (see Table 1). This skewed distribution shown by the model should be verified in the field. It is also important to realise that prevalence impacts of management measures in the program are still based on expert opinions (see Groenendaal et al., 2002) that have yet to be proven. They are now being studied on 17 heavily infected farms in the Netherlands during 2001-2005.

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Milk quality assurance programmes for paratuberculosis: stochastic simulation of within-herd infection dynamics and economics

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ABSTRACT

A bulk milk quality assurance programme for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in dairy herds was simulated with a stochastic simulation model (JohneSSim). Herds were certified as 'low-MAP bulk milk' if, with a certain probability, the concentration of MAP in bulk milk did not exceed a maximum acceptable concentration (MAC). The MAC, less than 10^3 MAP per litre, was based on pasteurisation studies. The programme starts with an intake procedure; test-negative herds enter a surveillance procedure and test-positive herds enter a control procedure. The aim of this study was to simulate combinations of various intake, surveillance and control procedures to evaluate their epidemiological and economic effects in a population of closed Dutch dairy herds.

The results showed that herd examinations by ELISA for intake and surveillance effectively ensure the quality of 'low-MAP bulk milk': >96% of simulated certified herds were below the MAC. Preventive management measures had little influence on the MAP bulk milk concentration in herds certified as 'low-MAP bulk milk', but had an important positive effect on the number of certified herds. Culling of test-positive animals based on biennial faecal culture was more effective than culling based on annual ELISA. Average total discounted costs for 20-year participation in a programme consisting of intake by ELISA, surveillance by biennial ELISA and control by biennial faecal culture were €6·10^3 per herd. On average, additional preventive measures increased these costs to €40·10^3 per herd.

This study showed that a bulk milk quality assurance programme based on MAP concentrations for closed Dutch dairy herds is feasible and provided decision-makers with information on the cost-effectiveness of different programmes.

Keywords: cattle, paratuberculosis, stochastic simulation model, certification, surveillance.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) infections in cattle are of concern to the dairy industry in part due to the as-yet-unresolved issue of its potential role in Crohn's disease in humans (Anon. 2000, Chacon et al. 2004, Herrewegh et al 2004). If MAP is causally implicated, then milk is a possible vehicle by which humans may acquire the infection, because MAP has been detected in raw milk and may not be effectively inactivated by pasteurisation (Sweeney et al., 1992b, Streeter et al. 1995, Grant et al. 1996, Millar 1996, Sung and Collins 1998, Grant et al. 1999, Giese and Ahrens 2000, Corti and Stephan 2002, Gao et al. 2002, Grant et al. 2002a & b, MacDonald et al. 2002, Pillai and Jayaro 2002, Sevilla et al. 2002, Rademaker (NIZO food research, the Netherlands) personal communication 2004). A milk quality assurance programme for paratuberculosis in dairy herds may reduce the potential risk of MAP transmission to humans through consumption of milk and milk products.

Certification-and-surveillance programmes for MAP-free herds have been developed in several countries (Kennedy et al. 2001). These programmes generally aim at providing buyers with a low risk of acquiring the infection through trade of cattle. In the Netherlands, a certification-and-surveillance programme has been

developed in which herds can obtain 'MAP-free' status following five negative annual herd examinations (the first herd examination by ELISA and faecal culture of ELISA-positive animals, the 2nd through 5th examination by pooled faecal culture; Benedictus et al., 1999). Control programmes for MAP-infected herds generally aim at its elimination. These certification, surveillance and control programmes are inherently expensive and participation is often restricted to a minority of herds. By July 1st, 2005, only 473 of approximately 23.000 Dutch dairy herds had obtained 'MAP-free' status. An alternative control approach is to develop a milk quality assurance programme that focuses on reducing the concentration of MAP in bulk milk rather than eradication of MAP infection from cattle. Herds in a milk quality assurance programme can be certified as 'low-MAP bulk milk' if, with a certain probability, the concentration of MAP in bulk milk does not exceed a pre-set maximum acceptable concentration. This does not necessarily mean that the herd is free of MAP infection. Thus, such a milk quality assurance programme might possibly be run at considerably lower costs than the current Dutch certification, surveillance and control programme. Therefore, the aim of this study was to simulate different milk quality assurance programmes to evaluate their epidemiological effects and economic consequences for a population of closed Dutch dairy herds.

A milk quality assurance programme was simulated, starting with an intake testing procedure. Test-negative herds enter a surveillance program and test-positive herds enter a control program. Herds that are found to be test positive during surveillance shift to the control program. The control program aims to suppress the infection in the herds, such that the milk quality can be guaranteed and the herd can move to the surveillance program. Different milk quality assurance programmes were simulated with a stochastic model JohneSSim (Groenendaal et al. 2002). Varied alternative test schemes based on herd examinations by serology (ELISA) or individual faecal culture (IFC) were simulated. All programmes were simulated with and without preventive management measures taken by all participating herds. The simulation assumed all herds were closed.

MATERIALS AND METHODS

The JohneSSim model. The JohneSSim model is a stochastic and dynamic simulation model that simulates (a) herd dynamics, (b) disease dynamics within the herd, (c) control of Johne's disease and (d) economic consequences at the herd level. The herd dynamics of a typical Dutch dairy herd and the infection and disease process in a 20-year period are simulated. The model and its use to study certification and surveillance programmes have been described in detail (Groenendaal et al. 2002, Weber et al. 2004). Repeated runs of the model provide insight into the variation in outcome at the farm level. Results at a higher aggregation level (e.g. national level) are obtained by simulating different types of dairy herds and aggregating the results according to their relative abundance. Both infected and non-infected herds are simulated.

Assumptions in JohneSSim model for present study. All herds were assumed to be closed (i.e. no purchase of animals and no new introductions of MAP). Herd size was assumed to be 65 adults (≥ 2 yr.) initially, and to increase by 5% per annum. Eighty to 100% of heifer calves were raised in the herd, while a surplus of heifers was sold shortly before 1st calving. Mean annual milk production was 8000 kg. Initial herd-level true prevalence was assumed to be 0.30, based on a recent study in the Netherlands (van Weering (AHS, the Netherlands), personal communication, 2004). The assumed distribution of the initial within-herd true prevalence in infected herds is shown in Fig 1. Economic assumptions on losses caused by infection with MAP, costs of participation in the quality assurance programme and costs of preventive management measures were updated (Tables 1-3). All costs were discounted at a real interest rate (approximated by interest rate minus inflation rate) of 5% per year. Assumptions on test characteristics are shown in Table 4. Preventive management in the simulated herds was set to reflect the distribution of management practices in the Dutch dairy industry ('background' management; Groenendaal et al. 2002). Assumptions on effectiveness of additional preventive management measures, imposed on the 'background' management, have been described in detail previously (Groenendaal et al. 2002). By default, effective separation of young stock from adult cattle was assumed to reduce incidence through faecal contamination of the environment by 90%.
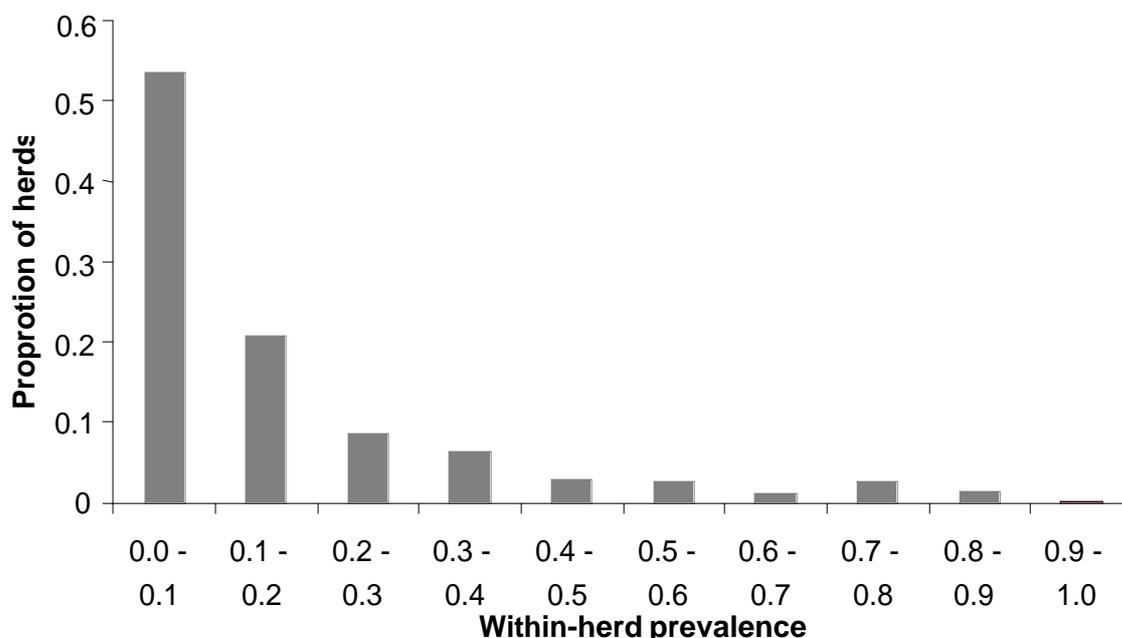
Fig. 1. Assumed starting distribution of within-herd true prevalence in infected herds.

Table 1. Assumptions on losses caused by infection with MAP. Losses did not include effects of a potential reduction in milk consumption due to consumer concerns.

Category		Costs (Euro)
Milk production	Reduction depends on infection state: 5% (early) to 20% (clinical)	0.08 / kg
Treatment	Treatment of clinical case	30
Reduced slaughter value	Standard slaughter value (per cow)	448.75
	Reduction depends on infection state (early 5%, infectious 10%, clinical 100%)	
Missed future income	Retention Pay Off (depending on parity, month in lactation and production level assuming no alternative use of production factors)	- 111.63 to 1431.23

Table 2. Variable costs (Euro) of participation in the bulk milk quality assurance programme. Subscription costs were 90 Euro per year. Costs do not include Value Added Tax (VAT for subscription and laboratory tests = 6%; VAT on other costs 19%).

Test / action	Costs : veterinarian	Transport	Lab / submission	Lab / test
Veterinarians' visit	22 per visit			
IFC	2.75 per animal	10	7.80	30.00 per animal
ELISA	2.75 per animal	10	7.80	6.15 per animal

Table 3. Assumed costs (Euro) of preventive management measures (including labour at 18.21 Euro per hour). Fifty percent of the costs of additional preventive management measures imposed on the 'background' management (Groenendaal et al. 2002) were attributed to the control of paratuberculosis.

Category		Loss or costs
Calving	Costs of cleaning per year	€ 100 per year
	Extra labour (hygiene, milking own dam) per calving	Own dam colostrum of € 9.11
Milk replacer	280 litres of artificial milk, 8 litre of milk replacer per kg milkpowder, costs of milkpowder € 1.30 per kg, value of bulkmilk €0.20 per litre.	42 litre vs. rest milk = € 6.83 238 litre vs. bulkmilk = - € 9.11 Total = - € 2.28
Hygiene barrier	Between adult stock and young stock	€ 726.71 per year (including labour)
Roughage	Better quality roughage, straw during housing in summer season only.	€ 39.03 for calves 0 – 6 months
Housing	Separate housing of animals 0 – 70 days (initially 5 animals)	€ 487.5 per year; 5% increment per year
	Separate housing of animals 70 – 180 days (initially 7 animals)	€ 682.5 per year; 5% increment per year
	Separate housing of animals 180 – 360 days (initially 9 animals)	€ 877.5 per year; 5% increment per year

Table 4. Assumptions on sensitivity (Se) and specificity (Sp) of individual faecal culture (IFC) and serum ELISA.

	Stage of infection	IFC	ELISA
Se	Early, minimal shedding	0	0.01
	Low level shedding	0.40	0.10
	Heavy shedding	0.95	0.60
	Clinical disease	0.90	0.80
Sp	Not infected	1 [a]	0.997 [b]

[a] Reinders (1963); [b] van Maanen et al. (2002).

Shedding of MAP in milk. The assumptions made on shedding of MAP in milk depended on the stage of infection are shown in Table 5. These assumptions were based on the available quantitative data on direct shedding of MAP in milk, faecal contamination of milk and shedding of MAP in faeces (Chiodini et al 1984, Stadhouders and Jørgensen 1990, van der Giessen et al. 1992, Sweeney et al. 1992a & b, Streeter et al. 1995, Millar 1996, Rossiter and Burhans 1996, Nauta and van der Giessen 1998, Giese and Ahrens 2000, Pearce et al. 2001, Corti and Stephan 2002, MacDonald et al. 2002, Grant et al 2002a, Grant et al. 2002b, Pillai and Jayaro 2002, Sevilla et al 2002, Rademaker (NIZO food research, the Netherlands), personal communication 2004, Stehman (Cornell University, USA), personal communication, 2004).

Acceptable concentration of MAP-organisms in milk. The concentration of MAP organisms in on-farm bulk milk that can be considered acceptable for eventual human consumption after pasteurization is unknown. Firstly, no quantitative data on exposure to MAP (either alive or dead organisms) are available and the probability of human disease is unknown. In the present study, we assumed that no viable MAP organisms should be present after commercial pasteurisation. Secondly, the results of pasteurisation studies seem to be inconsistent. Stabel et al. (1997) found pasteurisation in a test tube to be ineffective, but found complete killing using spiked milk samples with a lab-scale pasteuriser with a turbulent flow of milk during pasteurisation. Similarly, MAP cells could not be recovered in milk samples that were spiked with approximately 10^6 MAP cells, after disruption of cell clumps, if high-temperature short-time (HTST) pasteurisation was performed at 72°C with holding times 10 s (Rademaker et al, 2002). HTST pasteurisation is expected to give a 5 to 6 $\log_{10}$ reduction in numbers of viable MAP in milk (Grant et al. 1996, Grant et al. 1999, Gao et al. 2002). However, surviving MAP cells were detected in 19 of 33 (55%) spiked milk samples pasteurised by HTST when MAP was initially present in a concentration of 10^4 CFU per ml (Grant et al 1996). Furthermore, MAP was found to survive HTST pasteurisation in milk from 2 infected herds (Grant et al 2002a). Sung and Collins (1998) concluded that MAP may survive HTST pasteurisation when the initial organism concentration is greater than 10^3 cells per litre. Therefore, in the present study, we considered a concentration of MAP organisms in milk less than 10^3 per litre acceptable.

Table 5. Assumed concentration of MAP in milk for each stage of the infection in adult cattle (Total MAP in milk = direct shedding + faecal contamination $*$ MAP in faeces).

Stage	Proportion of animals by stage	Direct shedding of MAP in milk (organisms/L)	Faecal contamination of milk (g/L)	MAP in faeces (organisms per gram)	Total MAP in milk (organisms per litre)
Early stage infection	0	0	0.04	0	0
Low shedder	0.8	0	0.04	0	0
	0.2	0	0.04	10^2	4
Heavy shedder	0.6	10^2	0.04	10^2	10^2
	0.24	10^2	0.04	10^4	$5 \cdot 10^2$
	0.16	10^2	0.04	10^7	$4 \cdot 10^5$
Clinical disease	0	10^4	0.04	10^9	$4 \cdot 10^7$

Bulk milk quality assurance programmes. In our simulations, certified 'low-MAP bulk milk' dairy herds were assigned a status 'green', while other dairy herds were assigned a status 'red'. Thus, 'green' herds were herds with a high probability that the concentration of MAP in bulk milk was <10^3 per litre. The intake procedure was done two years after the start of the simulations. Initial assignment of a status to a herd was based on the results of the intake: test negative herds were classified as 'green' and test-positive herds as 'red'. Thereafter, 'green' herds were regularly monitored in a surveillance scheme; if a herd converted to a

test-positive status, it was moved to the pool of 'red' herds. An infection control scheme was applied to 'red' herds. Test-positive cattle and their last-born offspring were culled.

Various alternative test schemes for intake (i), surveillance (s) and control (c) were simulated (Table 6). The number of negative herd examinations required for a 'red' herd to move to the pool of 'green' herds was determined by the probability that the concentration of MAP in bulk milk was $<10^3$ per litre. A test-negative 'red' herd became 'green' if this probability was equal to, or higher than, the probability for a 'green' herd to have $<10^3$ MAP per litre immediately after the intake procedure.

All programmes were simulated with and without additional preventive management measures imposed by all participating herds on their 'background' management. When applied, the preventive measures were: improved hygiene around birth, colostrum from own dam only, feeding of artificial milk replacer only, and effective separation of young stock from adult cows from birth to the end of the first year.

Model output. In the present study, relevant herd-level outcomes over time were the within-herd true prevalence, test prevalence, concentration of MAP in bulk milk, and costs spent on the quality assurance programme. Relevant aggregate level outcomes over time included the proportion of herds initially categorized as 'green', the average concentration of MAP in bulk milk from 'green' herds, the proportion of 'green' herds with $<10^3$ MAP organisms per litre of bulk milk and costs spent on the bulk milk quality assurance programme (including herd examinations, subscription costs, preventive measures and cull of infected animals).

Table 6. Simulated test schemes for intake (i), surveillance (s) and control (c) for cattle. In the intake and surveillance procedure, a positive ELISA result was confirmed by individual faecal culture (IFC); IFC positive cattle and their lastborn calf were culled. In the control procedure, all ELISA or IFC positive cattle were culled.

Scheme	Intake		Surveillance			Control		
	Test (once)	Animals	Test	Interval	Animals	Test	Interval	Animals
i1-s1-c1	ELISA	All, $\geq$3 yr	ELISA	1 yr	All, $\geq$3 yr	ELISA	1 yr	All, $\geq$3 yr
i1-s1-c7	ELISA	All, $\geq$3 yr	ELISA	1 yr	All, $\geq$3 yr	IFC	2 yr	All, $\geq$2 yr
i1-s2-c1	ELISA	All, $\geq$3 yr	ELISA	2 yr	All, $\geq$3 yr	ELISA	1 yr	All, $\geq$3 yr
i1-s2-c7	ELISA	All, $\geq$3 yr	ELISA	2 yr	All, $\geq$3 yr	IFC	2 yr	All, $\geq$2 yr

Sensitivity analyses. The influence of various input parameters on the study results was analysed by changing one parameter at the time. These sensitivity analyses were performed with test scheme i1_s1_c1, with or without additional preventive management measures taken in the herds. Two possible values were assessed for each of four scenarios: (1) The default numbers of MAP bacteria in milk (Table 5, last column) were multiplied by 10^4 and 10^6 respectively to study the effect of altering the value of this uncertain parameter. (2) The model's default effect of preventive management measures was to reduce the probability of infection through the environment by 90%. An alternative assumption was tested whereby the reduction was only 50%. (3) By default, the initial herd-level true prevalence was 0.30. Alternatively, a prevalence of 0.56 was simulated. (4) By default, multiple negative herd-examinations were required for a 'red' herd to become 'green' (based on the probability for such herd to have $<10^3$ MAP per litre of bulk milk). The effects on the model of requiring only one negative herd-examination to receive a 'green' status was tested.

RESULTS

Simulated bulk milk quality assurance programmes. At intake (scheme i1: ELISA, all cattle $\geq$3 yr, ELISA positive confirmed by IFC), 90% of all herds were test-negative and classified as 'green'. The remaining 10% of herds were test-positive (i.e. ~35% of the infected herds at that time, and none of the non-infected herds) and therefore classified as 'red'. The assumed within-herd prevalence of adult cattle in 'green' and 'red' herds at the intake is shown in Fig. 2A. The concomitant distribution of the concentration of MAP in bulk milk is shown in Fig. 2B. Immediately after the intake procedure (with scheme i1), 98% of 'green' herds had a concentration of MAP in bulk milk $<10^3$ per litre. During control in 'red' herds, two consecutive negative herd-examinations by IFC or six consecutive negative herd-examinations by ELISA were required to reach the same probability of having $<10^3$ MAP per litre milk. Therefore, by default, 'red' herds were re-

classified as 'green' only after two consecutive negative herd-examinations by IFC, or six consecutive negative herd-examinations by ELISA.
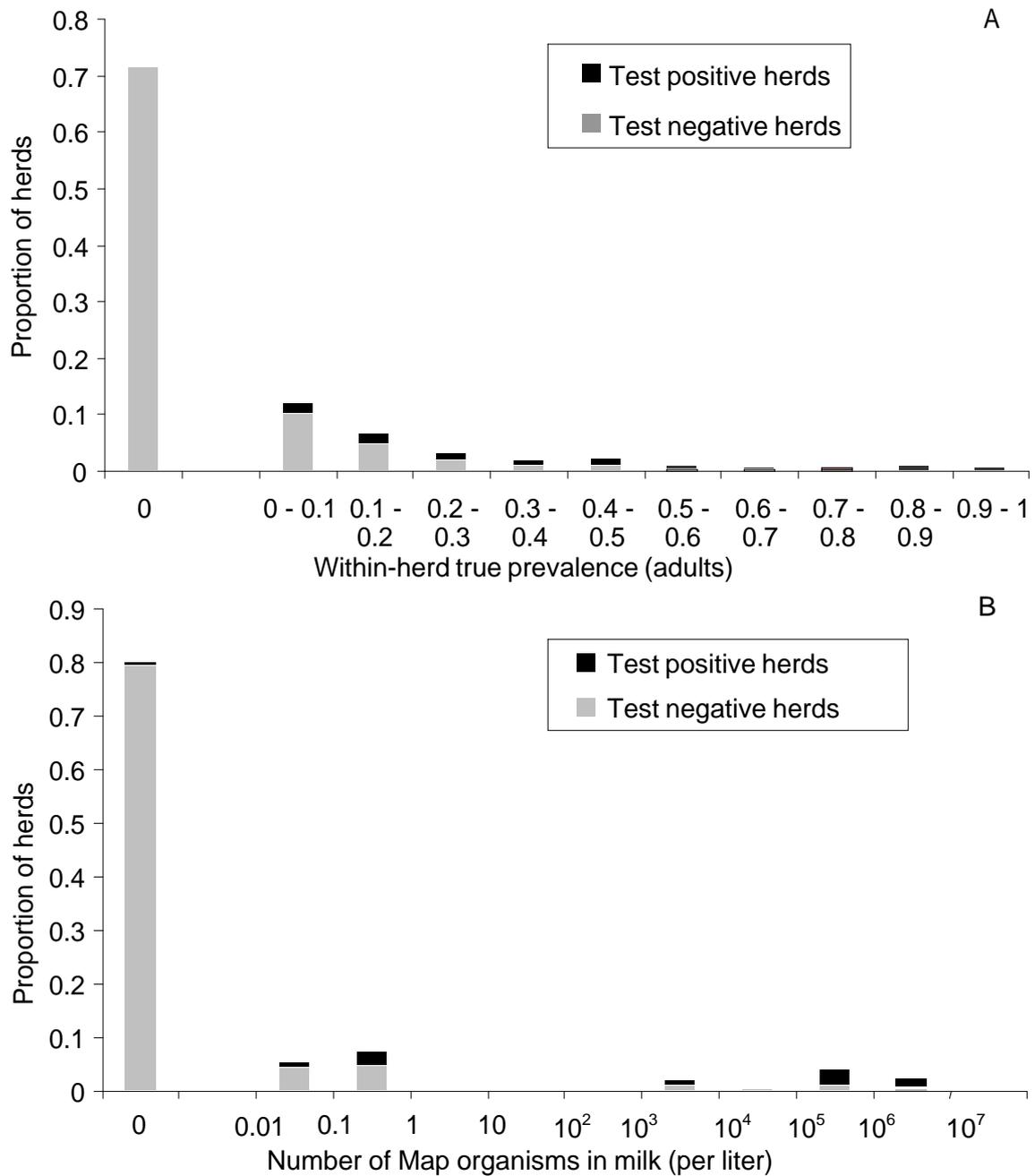

Fig. 2. Estimated within-herd prevalence in adult cattle (A) and estimated number of MAP organisms per litre of bulk milk (B) immediately after intake in simulated herds that were test-positive ('red') and test-negative ('green') at intake, using intake scheme i1 (ELISA, all cattle ≥3 yr).

The proportion of herds classified as 'green' decreased over time if no preventive measures were taken (Fig. 3A). However, if preventive management measures were taken, this proportion first decreased, but, thereafter, increased towards 86% - 99%, depending on the test scheme used (Fig. 3B). Preventive measures were pivotal for 'red' herds to become 'green'. Furthermore, these measures reduced the proportion of 'green' herds that lost their status. If preventive measures were taken, culling based on IFC was more effective than culling based on ELISA.
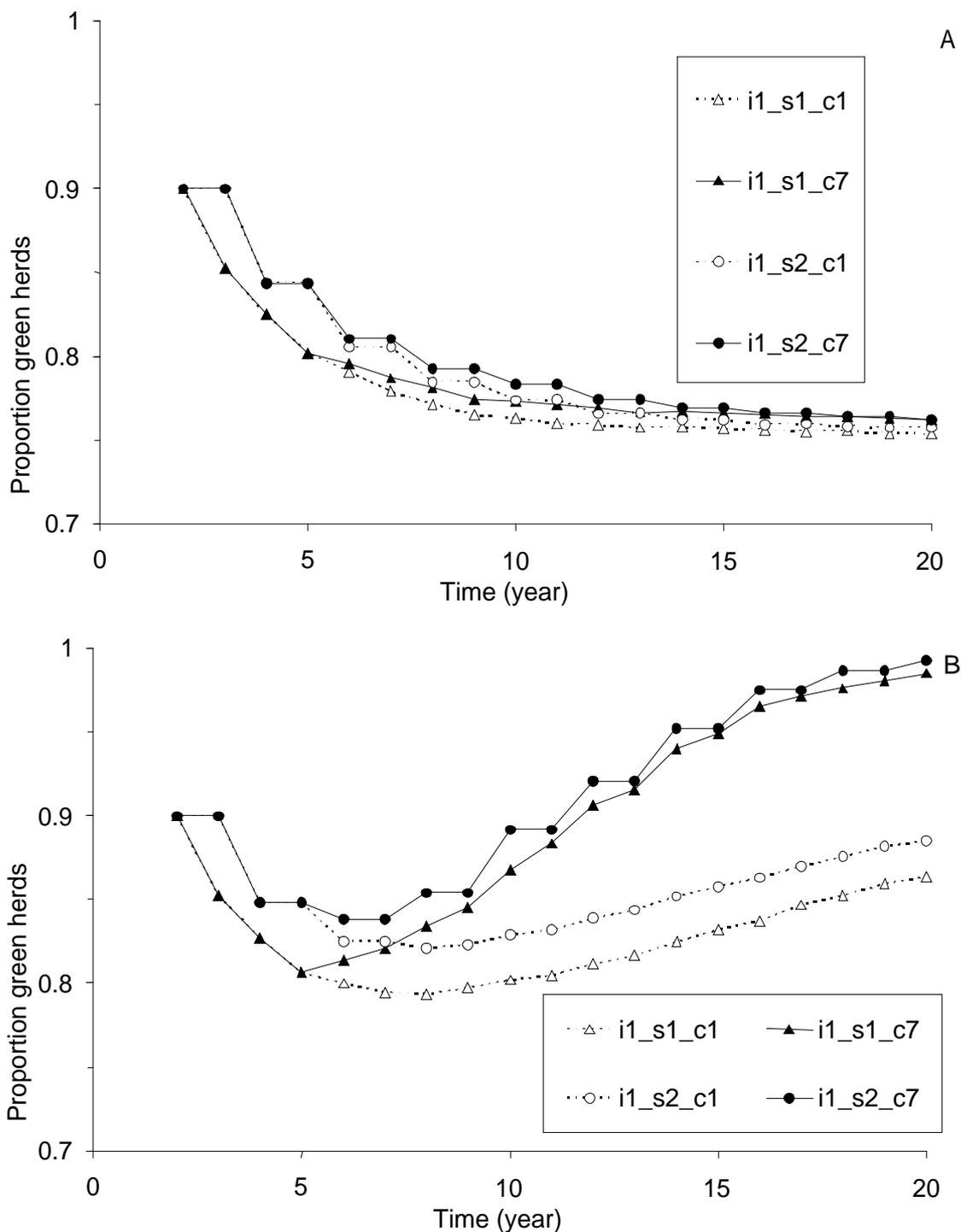
Fig. 3. Proportion of herds that are classified as 'green' over time, assuming a population of closed herds with an initial herd-level true prevalence of 30%. (A) Without additional preventive measures, (B) with additional preventive measures. Test schemes are defined in Table 4.
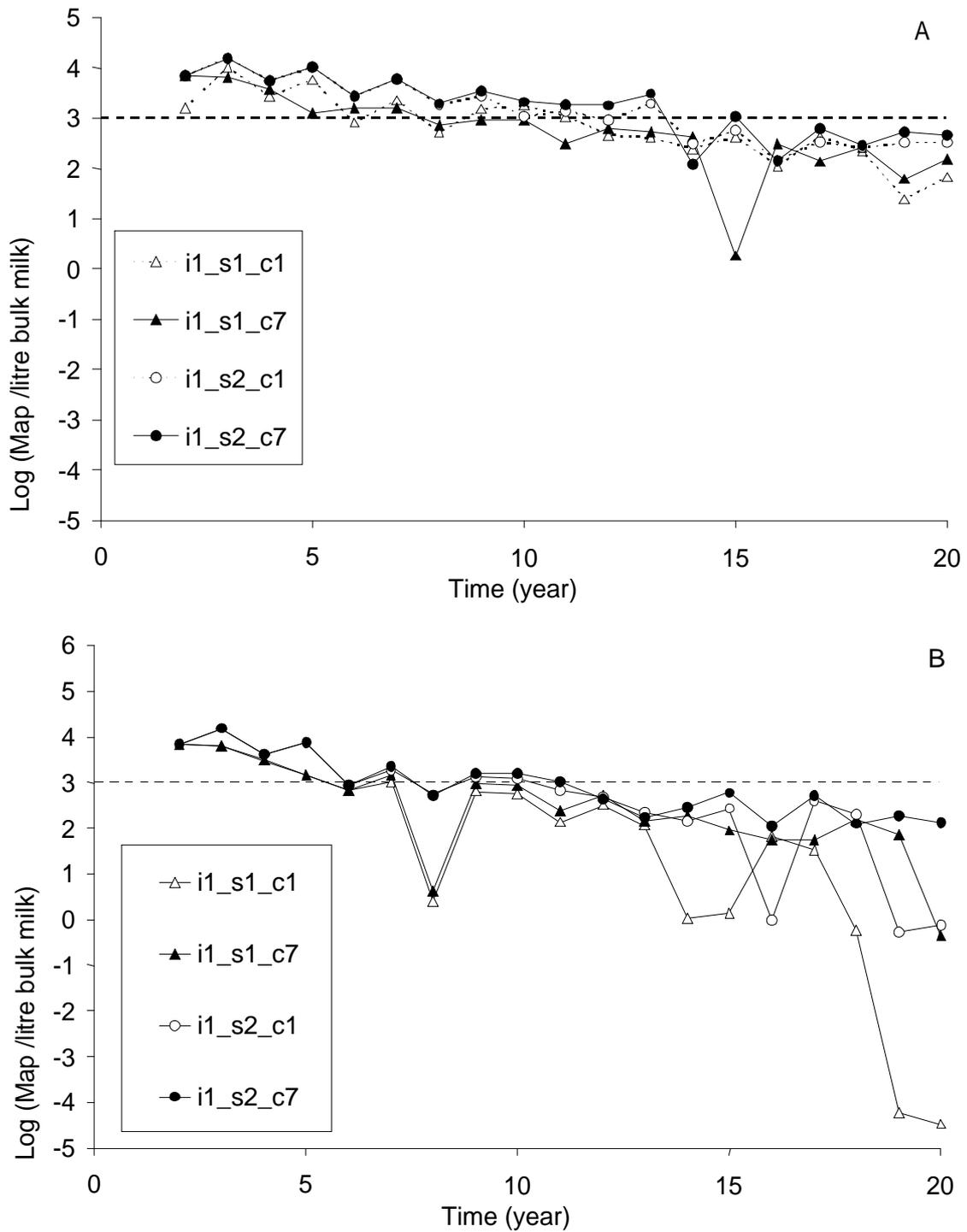
Figure 4. Logarithm ($\log_{10}$) of the average number of MAP bacteria per litre of bulk milk from 'green' herds (A) without preventive management measures, (B) with preventive measures. Test schemes are defined in table 4. The dashed horizontal line indicates the threshold of 10^3 MAP bacteria per litre of bulk milk. Test schemes are defined in Table 4.
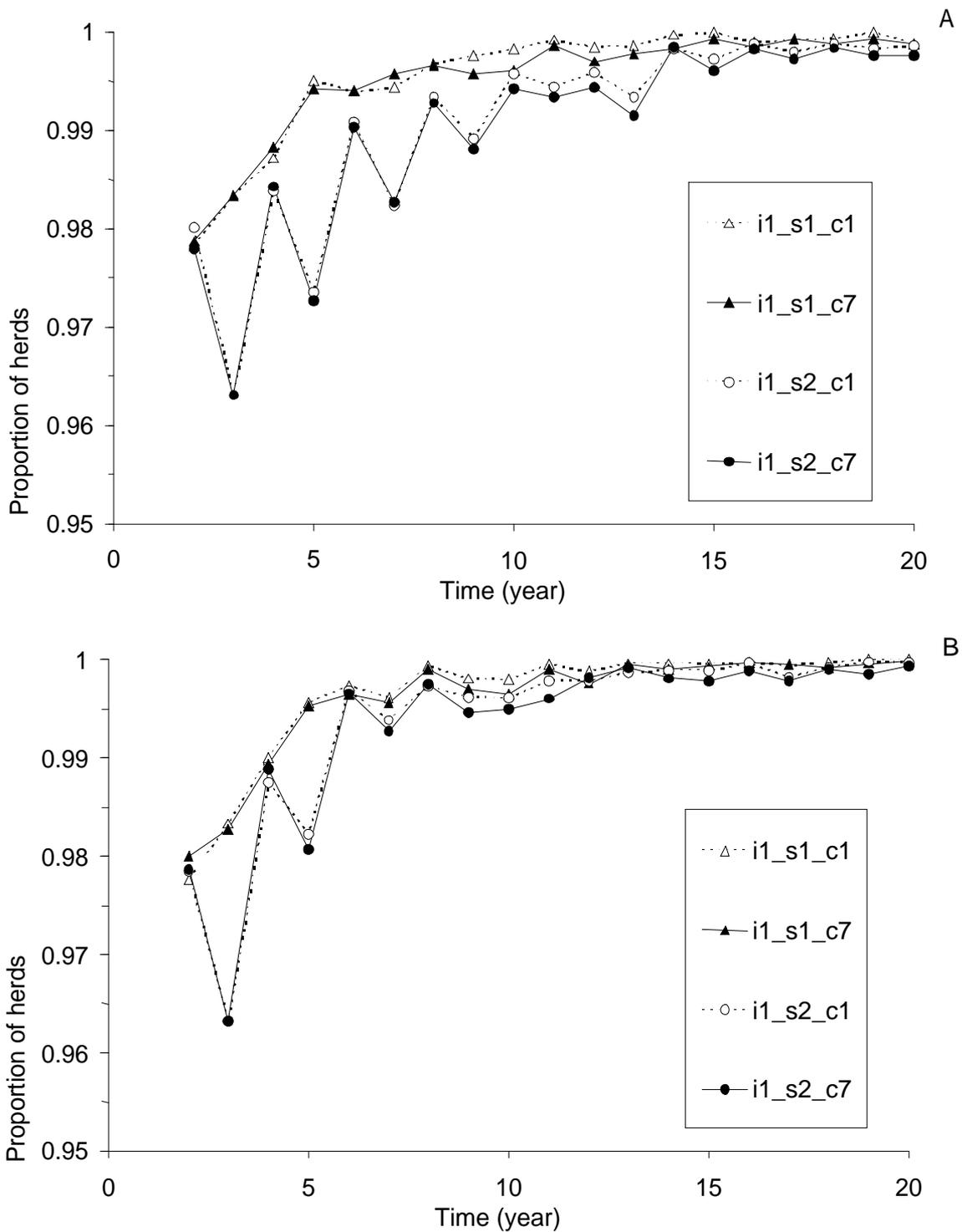
Fig. 5. Proportion of 'green' herds with <10^3 MAP per litre of bulk milk (A) without additional preventive measures. (B) with additional preventive measures. Test schemes are defined in Table 4.

Depending on the scheme used and whether or not additional preventive measures were taken, the estimated average concentration of MAP bacteria per litre of bulk milk in 'green' herds did not decrease below 10^3 before year 8 to 15 (Fig. 4). The proportion of 'green' herds with <10^3 MAP per litre of bulk milk increased towards 100% in year 20 (Fig. 5).

The median cumulative discounted costs during the 20 simulated years for schemes without additional preventive measures ranged from 6,000 to 10,000 Euro (Fig 6). For schemes with additional preventive measures these costs were higher, ranging from 40,000 to 44,000 Euro. However, the 90% range of costs

was much broader if no preventive measures were taken; therefore, for some schemes, the 95% percentile of costs were higher if no preventive measures were taken than if preventive measures were taken.
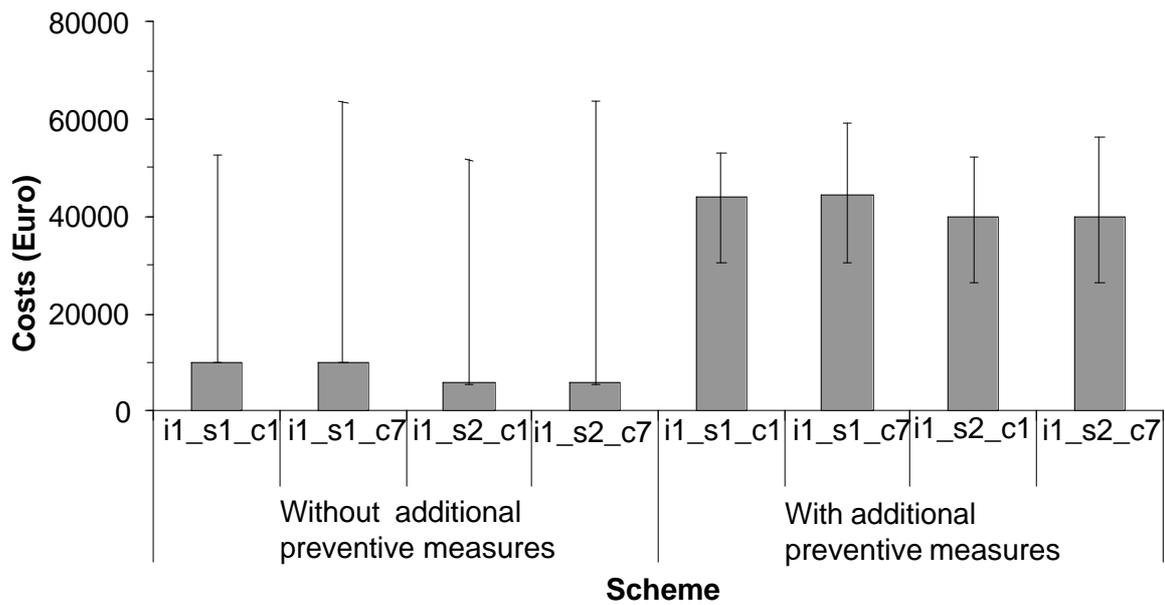

Fig. 6. Median cumulative discounted costs per herd up to year 20 (averaged over all 'green' and 'red' herds). Error bars indicate the 5% to 95% range. Test schemes are defined in Table 4.

Sensitivity analyses. When control of environmental transmission through preventive measures was assumed to be 50% vs. 90% effective, the number of "green" herds after 8 years was increased by only 5% (81% vs. 86%). If no preventive measures were taken, the number of herds certified as "green" dropped to 75%. Changing the value of the environmental contamination control variable in the model had no effect on the proportion of 'green' herds with $<10^3$ MAP per litre bulk milk.

If the default level of contamination of milk with MAP was multiplied by 10^4 or 10^6, the proportion of 'green' herds with $<10^3$ MAP per litre bulk milk was reduced by up to 10% during the first years after intake. However, beyond approximately year 10 (i.e. 8 years after intake), this effect was very small (Fig. 7).

If a higher initial herd-level prevalence was assumed (0.56 instead of 0.30), the proportion of 'green' herds in year 20 was reduced by 21% (54% instead of 75% without additional preventive management measures; 75% instead of 86% with additional preventive measures). The proportion of 'green' herds with $<10^3$ MAP per litre of bulk milk during the first years of the simulations was decreased by up to 2%, but this decrease was small beyond year 10. Effects on the cumulative discounted costs up to year 20 were negligible.

Changing the assumption about the number of negative herd examinations by ELISA that were required for a 'red' herd to be re-classified as 'green' has a strong effect. When the model assumed that only one, vs. six, negative tests were required, over 99% of herds were classified as 'green' in year 20 (instead of 86%), if additional preventive management measures were taken. The reason is, of course, that 'red' herds move to the pool of 'green' herds sooner. If no additional preventive measures were taken, there was only a minor effect on the proportion of 'green' herds. However, the bulk milk 'quality' of these 'green' herds was lower: the proportion of 'green' herds with $<10^3$ MAP/l was reduced by up to 2% if only one negative herd examination by ELISA was required instead of six.
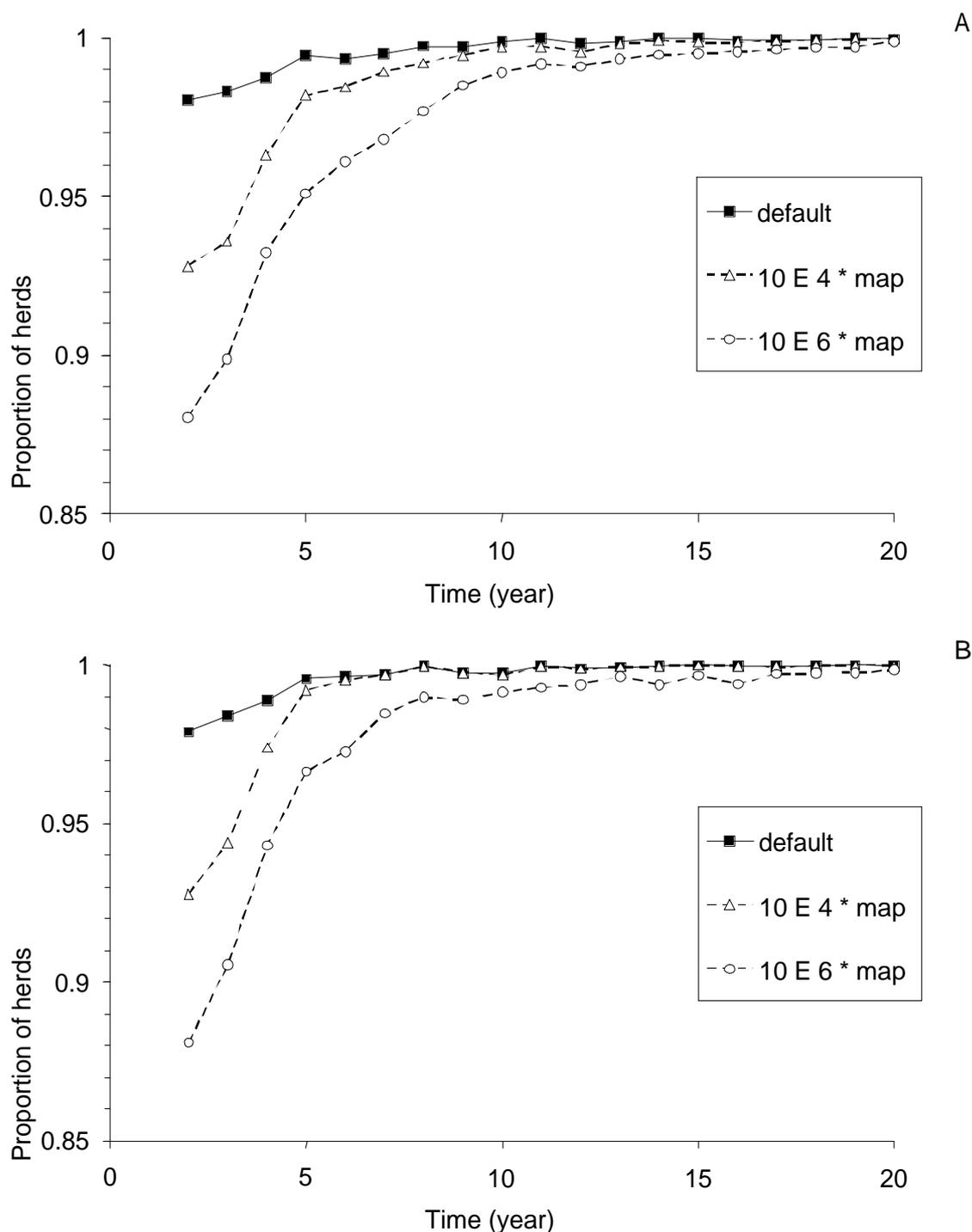
Fig. 7. Sensitivity analysis for the effect of contamination of milk with MAP, using scheme i1_s1_c1. Default concentrations of MAP bacteria in milk are given in Table 5. Alternatively, these concentrations were multiplied by 10^4 and 10^6. Proportion of 'green' herds with <10^3 MAP per litre of bulkmilk, (A) without additional preventive measures, (B) with additional preventive measures being taken.

DISCUSSION

To our knowledge, this is the first modelling study of a bulk milk quality assurance programme for paratuberculosis in dairy herds. By aiming to reduce MAP concentration in the bulk tank, a milk quality assurance programme can be run at considerably lower costs than certification, surveillance and control programmes that attempt to establish low infection risk trade of cattle and eliminate MAP at the herd-level.

Key elements in a successful bulk milk MAP quality assurance programme are (1) preventive measures to reduce the risk of introduction of MAP in participating herds (including trade restrictions), (2) preventive management measures to reduce the risk of within-herd spread of MAP, and (3) effective intake, surveillance, and control procedures. The present study was restricted to closed herds. Effects of animal trade were analysed separately using a mathematical model (van Roermund et al. 2005). In the present study, additional preventive management measures beyond test-and-cull to reduce within-herd spread of MAP were found to have a major effect on the proportion of herds that can be certified as 'low-MAP bulk milk' (i.e. 'green' in this study). These management measures were pivotal for test-positive ('red') herds to move to certification as 'low-map bulk milk' ('green'). However, these measures only had a minor effect on the actual bulk milk quality of 'low-MAP bulk Milk' herds ('green').

It would be optimal to base the intake, surveillance and control procedures on accurate measurement of the concentration of MAP organisms in bulk milk. However, to our knowledge, techniques to routinely quantify MAP in large numbers of bulk milk samples are not yet available. Therefore, we simulated intake, surveillance and control procedures based on tests at the animal-level (ELISA, faecal culture). The results showed that herd examinations by ELISA for intake and surveillance effectively ensure the quality of 'low-MAP bulk milk': >96% of simulated certified herds (increasing to >99% after 10 years) were below the $<10^3$ MAP/l. However, culling of test-positive animals and their last-born offspring based on biennial faecal culture was more effective than culling based on annual ELISA.

While fundamental assumptions were made in the present study, the impact of those considered to be most critical were evaluated through our sensitivity analyses. Due to deficiencies in the current methodology, it has so far been impossible to accurately quantify MAP organisms in milk from a dairy herd with paratuberculosis (Dundee et al, 2001; Grant et al, 2002a). For instance, colony forming units can not simply be translated to concentrations of MAP organisms, because of clumping of MAP in specimens and insensitivity of culture, while quantitative PCR results are estimates only of the total number of both viable and dead MAP organisms. Our sensitivity analyses showed that a 10^6 fold increase in the assumed concentration of MAP in milk from infected animals would initially decrease by 10% the number of certified 'low-MAP bulk milk' ('green') herds with $<10^3$ MAP per litre. However, such high concentrations of MAP in milk are probably not biologically plausible (for example, a clinical animal would have to be shedding $4 \cdot 10^{12}$ MAP/litre of milk). Even if this occurred, the effects of such an increase in MAP in milk from infected animals on the bulk milk quality of 'green' herds were very small beyond year 10 (i.e. 8 years after the intake procedure).

It is concluded that a bulk milk quality assurance programme for paratuberculosis in closed dairy herds is feasible. Preventive management measures should be recommended to participants given that they considerably increase the probability of obtaining and keeping a 'low-MAP bulk milk' status in the long term. Serology is sufficient for intake and surveillance in the programme. However, for control in test-positive herds, culling based on faecal culture results is more effective than culling based on ELISA results. The present study provided decision-makers with information on the cost-effectiveness of different programmes.

ACKNOWLEDGEMENTS

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Control of paratuberculosis in live cattle and semen imported to Sweden 1995-2004

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Abstract

Objective: This presentation describes measures taken to prevent paratuberculosis from entering into and spreading in Sweden. Materials and Methods: Sweden has a favourable situation with a very low prevalence of paratuberculosis. Therefore, when Sweden joined the EU in 1995 the Ministry of Agriculture applied for additional guarantees regarding paratuberculosis. While this application is under consideration the Swedish Board of Agriculture (SBA) has decided that all imported animals must be tested in quarantine for paratuberculosis. A control programme in beef herds, financed by the SBA, has been in place since 1998. This programme prohibits purchase of animals from non-tested herds. A voluntary import control, the Swedish Farmers´ Disease Control Programme, which is run by the Swedish Animal Health Service, advises farmers to import semen or embryos instead of live animals and recommends that imported animals come from tested herds. In addition, a policy in the dairy industry prohibits purchase of imported animals to dairy herds. Results: The import of live animals to Sweden is low. Between 1995 and 2004, 278 animals were imported, the majority being pedigree beef animals. Since 1998 there has been no import of dairy cows. All imported animals have been tested with negative results by faecal culture as well as serology, and in most cases serological testing has also been performed in the exporting herd. The import of semen has steadily increased since 1995, from approximately 130 000 to 215 000 doses/year. The majority of semen donors have been tested serologically. Swedens low prevalence of paratuberculosis has been maintained during the period described. Conclusions: Import of live animals constitutes a high risk of introducing paratuberculosis. Regulations of the authorities (SBA) together with measures initiated by a voluntary import control run by the Swedish Animal Health Service have successfully limited the numbers and thereby minimized the risks.

Laboratory proficiency in testing for antibodies to Mycobacterium avium subspecies paratuberculosis: A new approach for the United States

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Abstract

The goal of proficiency testing for antibodies to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is to assure users that laboratories are able to conduct serology testing in a way that will provide quality results to be used in the Voluntary Bovine Johne's Disease Control Program. Historically, the panel of 25 sera, including some duplicates, was graded based on qualitative (positive/negative) results. Qualitative scoring of test results will often lead to selection of a panel of sera that give extreme values (positive or negative) to avoid challenges of the interpretation around the cutpoint of the test. The Z-scoring system, which is currently used in Australia, can be used to score quantitative outcomes of tests and has the potential to be modified and adopted for use with many laboratory tests. A combination of positive/negative and Z-scoring has been adopted by the United States for MAP ELISA antibody testing. The Z-scoring system uses the median and inter-quartile range values from the participating labs' results in the analysis. This Z-score can then used to evaluate inter- and intra-laboratory variation in ELISA readings. Graphical representation of summed Z-scores may assist labs in identifying and resolving laboratory errors. The combination of scoring methods should provide more assurance in the quality of MAP antibody results for the US and has the potential to improve laboratory proficiency.

Paratuberculosis in cattle in the Czech Republic: current situation results of national control program and the main risk factors

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Abstract

Paratuberculosis (PTB) in cattle was diagnosed for the first time in one imported Danish Red Pied cow (1962), subsequently in one herd (1967) and in 16 herds (1984-1988) of grazing cattle originating from two areas where heifers were reared in the pastures in contact with imported cattle and sheep. However, cattle import into the family farms formed in those years (1990-1992) and subsequently into large farms (1993-1996) introduced PTB. According to OIE, 1.7 million head of cattle were kept in 5,405 farms in the year 1998. Analysis of data showed that 29,234 head of cattle in 428 groups were imported (1992-1998) and PTB was successively diagnosed in 84 (19.6%) groups before 2004. Since 1998, a national subsidized control programme based on the faecal-culture of all animals older than 18 months twice a year has been adopted. In the year 2003, 3,500 of 28,000 cattle herds could be considered as economically significant from the aspect of milk production. PTB is annually controlled in 25 to 30 herds, particularly of dairy cattle. The primary risk factor for the spread of PTB through the infected dairy cattle herds is represented by the widely used feeding of calves with mixed colostrum and milk, and the use of the progeny of infected dams in breeding. PTB is transmitted through cattle herds by cows and heifers purchased from cancelled herds and also by animals and bulls imported for natural breeding. The risk of spread of PTB to free living animals is above all represented by the introduced extensive grazing system of cattle. The introduction of the certificate programme should better motivate the farmers to both sanitation of infected herds and purchase of animals from non-infected herds.

Screening the Swedish sheep population for paratuberculosis

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Abstract

In Sweden, paratuberculosis is compulsorily notifiable in all animals and a stamping out policy is applied in infected herds. A total of 53 infected cattle herds have been identified since 1993, all linked to animal imports. Screening activities, based on faecal culture, have been undertaken in dairy cattle in 2001 and 2004, and there is a voluntary control programme for beef cattle, also based on faecal culture. Paratuberculosis has never been detected in Swedish sheep, but an infected ram was identified in quarantine in 1999. Since 1993, yearly screening of the sheep population has been undertaken. For 10 years serology (AGID) was used, but in 2004 this was replaced by faecal culture (modified Löwenstein-Jensen medium with mycobactin and modified Middlebrook 7H10 with mycobactin for 6 months). An average of one sero-positive sample was found every year, but further investigations into these herds, including slaughtering of the positive animal and testing of all other animals in the herd, revealed no paratuberculosis. No positive faecal samples have been found. Apart from the screening activities, suspect clinical cases and suspect cases found at post mortem or slaughter are further investigated. Although it isn't safe to say that Swedish sheep are free from paratuberculosis, it can be concluded that the infection, if present, remains at a very low level.

Immunology of bovine paratuberculosis: the hosts' perspective

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ABSTRACT

Mycobacterial infections constitute a major thread to cattle populations worldwide. The major mycobacterial infections are tuberculosis, caused by infection with *M. bovis* (MB), and paratuberculosis, caused by infection with *M. avium ssp. paratuberculosis* (MAP). The interaction between the hosts immune system and MAP is a complex interplay reflecting the intricate ways in which this successful pathogen manipulates the host. The completion of the prototype genome of MAP has lead to the identification of species specific as well as conserved antigens. These antigens can be used in functional assays to study host pathogen interactions in detail. Mycobacterial heat shock proteins are potent antigens for many lymphocytes, and are used widely as defined antigens to study interactions between mycobacteria and the host. This review provides an overview of immune interactions between MAP and the bovine immune system with special focus on reactivity of $\alpha\beta$ T cells, $\gamma\delta$ T cells and B cells towards mycobacterial heat shock proteins in paratuberculosis.

Key words: cattle, heat shock protein, immunology, *Mycobacterium paratuberculosis*, pathogenesis

INTRODUCTION

Mycobacterial infections constitute a major threat to cattle populations worldwide. The major mycobacterial infections are tuberculosis, caused by infection with *M. bovis* (MB), and paratuberculosis, caused by infection with *M. avium ssp. paratuberculosis* (MAP). The description of a chronic granulomatous infection of the small intestine in a cow, by Johne and Frottingham in 1895, was the first report on paratuberculosis; although at the time they considered it to be an unusual case of bovine tuberculosis. (Johne and Frothingham, 1895) Paratuberculosis is a slowly developing disease, with a long asymptomatic period during which the infection is spread. It eventually causes a wasting syndrome in animals progressing to the clinical stage of the disease months or more likely years after infection. Both diagnosis, especially in the early asymptomatic stages of the disease, as well as protective vaccination are notoriously difficult.

Another major point of interest lies in the physiology of the bovine immune system in which $\gamma\delta$ T cells are the major T cell population in the circulation during the first 6-12 months of life. (Hein and Mackay, 1991) As opposed to $\alpha\beta$ T cells, many questions on the function of the $\gamma\delta$ T cell populations during infection and inflammation are still unanswered. Heat shock proteins (Hsp) have been shown to be immunodominant antigens for B and $\alpha\beta$ T cells, and they also have been shown to be antigens for the $\gamma\delta$ T cells. (Boismenu and Havran, 1997; Born et al., 1999) This review provides an overview of interactions between MAP and the bovine immune system with special focus on reactivity towards mycobacterial Hsps in paratuberculosis.

Innate immunity to MAP
Young calves, especially in the first six months of life, are most susceptible to MAP infection which is transmitted via milk and environmental contamination. (Sweeney, 1996) The nature of this apparent age resistance is poorly understood, however it may be related to the extensive lymphoid areas, Peyers patches (PP), in the jejunum and ileum of calves that gradually disappear during the first year of life. (Liebler, 1985) MAP is taken up by M-cells in the mucosa of the PP of the small intestine. (Momotani et al., 1988) With the disappearance of the M-cell rich PP a porte-d'entrée for the bacteria disappears as well, although recent evidence obtained in goats and calves point to uptake of MAP via enterocytes as well. (Schleig et al., 2005; Sigurardottir et al., 2005) Major membrane protein and fibronectin attachment proteins have been shown to

be involved in the attachment of MAP to intestinal mucosa. (Bannantine et al., 2003; Secott et al., 2002, 2004) Following expulsion at the basal side of the M-cell the bacteria are taken up by macrophages underlying the domes of the PP. (Momotani et al., 1988; Sigurdardottir et al., 2001) The bacteria persist inside the macrophages, surviving its microbicidal mechanisms by altering the normal phagosomal maturation pathways and preventing apoptosis. (Kuehnel et al., 2001; Pieters, 2001; Weiss et al., 2004) In addition the initiation of the acquired immune response may be hampered by impaired production of pro-inflammatory cytokines such as TNF , and increased expression of IL-10 (Coussens et al., 2004; Weiss et al., 2002). Heat shock proteins may provide an activation signal to antigen-presenting cells (Wallin et al., 2002) although a specific induction of regulatory T cells limiting tissue damage has also been described (van Eden et al., 2005). Mycobacterial heat shock protein of 70kD is specifically recognized by bovine antigen presenting cells, like macrophages and dendritic cells, and may also lead to increased production of IL-10. (Langelaar et al., 2005b; Langelaar et al., 2005c) Ultimately the infected macrophages, restricted in their capacity to migrate, accumulate at the site thus forming poorly organized lepromatous granulomatous lesions. (Clarke, 1997) NK cells, unexplored until recently, may bridge innate and specific immunity, through production of IFN in response to soluble factors released by adherent cells following antigenic stimulation.(Olsen et al., 2005; Storset et al., 2004)

Acquired immunity to MAP
Infection is followed by a prolonged asymptomatic phase, two years or more, in which neither immune responses (humoral and cell mediated), nor excretion of bacteria in the feces can be measured. (Clarke, 1997) Most notably the first signs of infection are IFNγ production and lymphoproliferation in response to challenge with mycobacterial antigens such as purified protein derivative (PPD), indicating activation of cell mediated immunity (CMI), and intermittent shedding of bacteria in the feces. During the years to follow bacterial excretion will increase, as will CMI, and antimycobacterial antibody responses may become detectable. Cows can remain in this stage of the disease for years, an apparent equilibrium between host and pathogen. Different host phenotypes can be distinguished by shedding frequency and quantity. (Koets et al., 2005b; Whitlock et al., 2005)

A limited number of animals rapidly progress to the clinical stage of the disease characterized by incurable diarrhea due to the expanding granulomatous lesions in the intestinal wall. Ultimately these animals die of emaciation caused by the protein losing enteropathy. In the animals that develop clinical disease, antigen specific CMI wanes while excretion of the pathogen increases exponentially, thus contributing correspondingly to spread of the disease. (Chiodini, 1996) It has been hypothesized that the decrease in CMI and the increased antibody responses reflects a switch in immune reactivity from type 1 to type 2 responses (Chiodini, 1996; Clarke, 1997) based on the T helper cell dichotomy first described by Mosmann and co-workers (Mosmann et al., 1986). Although this dichotomy is not as clear-cut in outbred species as it is in different murine strains, studies regarding bovine type 1 and type 2 immune responses have confirmed the crucial role of IL-4 and IFN-γ as driving cytokines. (Brown and Estes, 1997) Protective immunity against paratuberculosis is considered to be essentially cell mediated. As such, protection depends on the interactions between T cells and infected antigen presenting cells. (Kaufmann, 2001)

Recent advances such as the completion of the sequencing of the prototype genome of MAP lead to a rapid expansion of knowledge on sequences with potential diagnostic value and individual protein antigens. The main focus of these antigen discovery approaches focuses on finding antigens specific for MAP for diagnostic purposes. (Bannantine et al., 2002; Paustian et al., 2005) These antigens are subsequently tested for immunogenicity in model systems and MAP infections revealing interesting new antigens that warrant further testing. (Bannantine et al., 2004; Nagata et al., 2005) Although species-specific antigens may prove useful in diagnostic approaches (Stratmann et al., 2004) it remains to be seen whether this type of antigen is a major target for the immune system. From an evolutionary viewpoint it may be more efficient for the immune system to target conserved essential targets in pathogens as these are less likely to change rapidly, and cross-protective effects may be beneficial.

Despite their high degree of conservation Hsps are essentially very immunogenic proteins. In mycobacterial diseases the Hsp have been found to induce both T and B cell activity (Ivanyi et al., 1996). As reviewed by Matzinger (Matzinger, 1994) the Hsp recognition by immune cells could deliver an efficient danger signal to activate immune responses. They are challenging antigens for the immune system as they may provide a universal signal for infection, based on epitopes unique for or shared by pathogens. Furthermore,

expression of self-Hsp derived epitopes by infected cells may also facilitate immune recognition by shared mechanisms (Kaufmann, 1990). The sharing of epitopes between host and pathogen may potentially lead to autoimmunity. However, pre-immunisation with Hsp often leads to (partial) protection against, rather than induction of, autoimmunity in a number of experimental disease models, probably via induction of regulatory self-Hsp-reactive T cells.(van Eden et al., 2005) Our research focuses on the unravelling of immune responses to MAP Hsp60 and Hsp70, as these constitute an evolutionary and functionally important strategy to deal with mycobacterial infections.

B lymphocytes

As is seen with other mycobacterial diseases, e.g. tuberculosis, antibodies contribute little to protective immunity against those intracellular bacteria. Currently there is little insight into B cell responses in early stages of disease. Based on the available assays, it appears very little if any antibody production is evident in early stages post infection. Recent data using new recombinant antigens and an intra-tonsillar route of infection do show early antibody responses, in fact these antibody responses precede cell mediated responses in this model. (Waters et al., 2003) The serological response to crude mycobacterial antigens during paratuberculosis has been a subject of many studies aiming to improve diagnosis of this infection (Abbas and Riemann, 1988; Collins et al., 1993; Hilbink et al., 1994; Jark et al., 1997; Sockett et al., 1992; Spangler et al., 1988; Vannuffel et al., 1994). The results of serological studies have been generalized to argue in favor of a Th2 driven antibody response in later stages of the disease.

A distinction can be made between IFN-γ dependent (Th1) antibody isotypes and IL-4 dependant Th2 related isotypes (Abbas et al., 1996). Likewise for cattle, it has been shown that IgG1 and IgA as opposed to IgG2 and IgM isotypes can be classified respectively as type 2 vs. type 1 associated isotypes (Brown and Estes, 1997; Brown et al., 1999; Estes et al., 1994; Estes et al., 1995). Studies with isotype specific ELISAs and different antigens showed this to be an oversimplification. They indicated that the change in type 1 and type 2 responses appears to be driven by a loss of type 1 reactivity, however loss of type 2 reactivity to certain antigens also occurs. In these studies recombinant heat shock protein 60 (Hsp60) appears to be a dominant B cell antigen while recombinant Hsp70 does not. (Koets et al., 2002; Koets et al., 2001)

CD4$^+$ T lymphocytes

Most of the work on CD4$^+$ T cell reactivity in paratuberculosis has focussed on protein antigens of MAP, mostly derived from crude preparations and to a lesser extent using recombinant protein antigens. Despite work in the human TB field (Moody et al., 2000) studies regarding T cell reactivity to non-protein antigens of MAP are in their early days, although the first indications of CD1 restricted anti-MAP lipid responses have been found. (Van Rhijn et al., 2005) The putative switch from protective Th1 to permissive Th2 responses has been studied with respect to T cell reactivity from paratuberculosis infected animals however these studies were unable to clearly show the hypothesized Th1 to Th2 switch. While the decrease in IFN production is evident, indications for increased Th2 are less clear. Upregulation of IL-10 and TGF expression appears to be more consistent. (Coussens et al., 2005; Lee et al., 2001; Stabel and Ackermann, 2002)

Recombinant Hsp70 is a dominant T cell antigen in paratuberculosis as indicated by high proliferative responses in vaccinated animals and in asymptomatic shedders compared to cows with clinical signs of paratuberculosis. (Koets et al., 1999) A similar low level of cell mediated immunity (CMI) was observed in focal tissue lesions of animals with clinical signs of disease. Further analysis by immunohistochemistry and flow cytometry indicated a loss of CD4+ Th cells from those lesions. (Koets et al., 2002)

Collectively these data on CD4$^+$ Th cell driven CMI suggest that the decrease in reactivity during progressive bovine paratuberculosis is more likely due to a loss of mycobacterial antigen specific CD4$^+$ Th cells rather then redistribution of T helper cells. The loss of CD4$^+$ T cells in mycobacterial diseases has previously been linked to activation induced apoptosis through cell-cell contact and soluble mediators (Coussens et al., 2005; Dalton et al., 2000; Das et al., 1999; Florido et al., 2005; Grell et al., 2005). Another mechanism for the loss of CMI may involve activation of regulatory T cells during the course of disease. These regulatory T cells, producing anti-inflammatory cytokines IL-10 and TGF- , have a role in the maintenance of self-tolerance. However recent studies suggest that regulatory T cells can be induced against bacterial, viral and parasite antigens in vivo. They might prevent infection-induced immunopathology resulting in prolonged pathogen persistence by suppression of protective Th1 responses.

(McGuirk and Mills, 2002) Indications have been found that these mechanisms are active in advanced cases of paratuberculosis. (Coussens et al., 2002; Coussens et al., 2004; Khalifeh and Stabel, 2004) It remains to be elucidated whether the host employs those mechanisms attempting to reduce inflammatory damage or whether the ability to induce host regulatory T cells should be considered a pathogenic trait of MAP, possibly mediated by interaction with DC-SIGN and TLR on dendritic cells similar to tuberculosis.(van Kooyk and Geijtenbeek, 2003)

CD8$^+$ T lymphocytes

The role of the CD8$^+$ T cells in protective immunity has been subject of debate, however cytotoxic T cells do contribute to mycobacterial infection resistance. (Cho et al., 2000; Stenger and Modlin, 1998) It has been shown that CD8$^+$ cytotoxic T cells are induced during bovine tuberculosis (Skinner et al., 2003), however data on the induction of CD8$^+$ T cells during paratuberculosis either using crude or recombinant antigens is limited. Recent data using flowcytometric approaches to study antigen specific activation of subsets does indicate some early activation of CD8$^+$ T cells. (Koo et al., 2004)

No specific CD8$^+$ CTLs have been described for mycobacterial Hsp70, however this molecule has gained recent attention as it was shown to possess so-called cross-priming capabilities. Hsp70 belongs to a group of molecules that are able to translocate protein epitopes, complexed to them or linked as a fusion protein, into the cellular MHC class I presentation pathway when administered extracellularly, instead of being processed and presented via the MHC class II pathway (Li et al., 2002). This process is receptor mediated and to date several receptors have been described to be involved such as the α2 macroglobuline receptor (Basu et al., 2001; Binder et al., 2000) and LOX (Delneste et al., 2002). Several studies have indicated that these properties can be used to generate CTLs that recognise intracellular pathogen related antigens in the context of MHC class I. Such protein based, adjuvant free, vaccine systems may prove to have substantial benefits over using e.g. attenuated pathogens to induce CTL (Srivastava and Amato, 2001) We however thusfar have been unable induce CTL using this system (Langelaar et al., 2005a).

$\gamma\delta$ T lymphocytes

The immune system of ruminants has a number of characteristics unique among mammals such as the abundance of T lymphocytes with a $\gamma\delta$ T cell receptor present in blood of young animals. The $\gamma\delta$ T cells can represent up to 75% of blood T lymphocytes during the first 6-9 months of life. (Davis et al., 1996; Hein and Mackay, 1991; Wyatt et al., 1996) The majority of these $\gamma\delta$ T cells express the workshop cluster 1 (WC1) molecule. Based on the expression of the WC1 molecule, unique to ruminants and belonging to the scavenger receptor cysteine-rich domain family, the $\gamma\delta$ T cells can be divided into two subpopulations, WC1+ and WC1-. (Clevers et al., 1990; Takamatsu et al., 1997; Wijngaard et al., 1994) Furthermore, within these subsets additional markers show differentiation into at least 4 functionally different   T cell populations. (Park et al., 2000) The recognition of antigen by $\gamma\delta$ T cells is incompletely understood, although several restriction elements have been described. While a minority of the $\gamma\delta$ T cells recognizes antigen in the context of MHC I or II, the majority of $\gamma\delta$ T cells see their antigen differently. Antigens may be recognized via non-classical MHC I (MHC Ib) molecules (Soloski et al., 2000), lipid based antigens by presentation in CD1 molecules (Porcelli et al., 1998), or direct interaction with pathogen associated molecular patterns (Hedges et al., 2005). Ruminant $\gamma\delta$ T cells express a very diverse repertoire of T cell receptors in contrast to mice and humans. Hence the ruminant $\gamma\delta$ T cells may have a better developed capacity to recognize diverse ligands. (Hein and Dudler, 1997; Hein and Mackay, 1991)

In the course of intracellular mycobacterial infections two, apparently opposite, functions of the $\gamma\delta$ T cell population have been described. Evidence has been presented that the $\gamma\delta$ T cell population contributes to clearance of infection by cytotoxicity (Olin et al., 2005), or the secretion of IFN-γ via a positive feedback loop sustained by activated antigen specific CD4$^+$ T cells and IL-12, possibly enhancing the conditions for a microenvironment that favors cell mediated immune responses in early stages of infection. (Garcia et al., 1997) Recent studies on cattle experimentally infected with *M. bovis* show that there is early activation of WC1$^+$ $\gamma\delta$ T cells with a strong proliferative response to *M. bovis* antigen in vitro, and relative low production of IFN-γ.(Smyth et al., 2001) However, in models for intra-cellular infection, it has been shown that $\gamma\delta$ T cells may predominantly have an immunoregulatory function via IL-10 which downregulates Th1 and NK-based IFN-γ production, thus controlling Th1 responses (Flesch and Kaufmann, 1994; Hsieh et al., 1996). Similar results have been obtained with regard to the role of $\gamma\delta$ T cells in models for bovine mycobacterial diseases,

indicating that the $\gamma\delta$ T cell population does not restrict the growth of the mycobacteria but allows for formation of epitheloid granulomata as observed in progressive bovine paratuberculosis. (Smith et al., 1999; Tanaka et al., 2000)

In earlier studies T cells have been described displaying cytotoxic activity towards CD4 T cells thus contributing to the loss of cell mediated immunity.(Chiodini and Davis, 1992) In support of this theory, the loss of CD4 T cells during the final progressive stages of paratuberculosis has been shown to be associated with an increase in WC1$^+$ $\gamma\delta$ T cells. (Koets et al., 2002) A third functional role of T cells may be that they act as antigen presenting cells (APC) which has been shown to be the case in cattle (Collins et al., 1998) and recently also in humans (Brandes et al., 2005). In the latter case it was shown that they could function as professional APC. This in turn would also tie in with data on bovine T cells being able to directly respond to pathogen associated molecular patterns (PAMP).(Hedges et al., 2005) Although the overall role of $\gamma\delta$ T cells remains elusively complex, linking innate and adaptive immunity, their regulatory functions may under certain conditions prevail over those inducing protective immunity.

CONCLUSIONS

In the course of bovine paratuberculosis dramatic changes in the immune reactivity to the mycobacterial pathogen occur that are related to the outcome of the infection. This review has focused on some of the major players affecting this outcome. Current evidence points to an antigen specific mechanism that causes the demise of CD4$^+$ helper T cells that recognize mycobacterial antigens. As a consequence of the progressive loss of these CD4$^+$ T cells the infection progresses beyond control leading to extensive intestinal pathology eventually leading to the death of the cow. Major questions at this point relate to the mechanism of this loss of CD4$^+$ T cells. The direct interaction between infected macrophages and dendritic cells on the one hand and CD4$^+$ T cells on the other hand may lead to the induction of apoptosis in the CD4$^+$ T cell population. This could occur via signalling mechanisms through e.g. Fas – FasL interactions or via cytokines. (Dockrell, 2001; Gao and Kwaik, 2000; Mustafa et al., 2001) It may also be the result of a regulatory function of $\gamma\delta$ T or Treg cells, which may start a prolonged down regulation of continuous activation of the CD4$^+$ T cells during these chronic infections in a misdirected attempt to limit tissue damage. This eventually leads to uncontrolled mycobacterial replication. (Coussens, 2004)

It is important to note that a substantial amount of data has been gathered using peripheral blood lymphocytes. As the infection, and therefore the early stage of host-pathogen interaction, is confined to the mucosal immune system of the small intestine it is important to use techniques such as intestinal lymphatic cannulation (Hein et al., 2004) to study whether these mechanisms also play a role locally, and at early stages of infection.

The Hsps have been shown to be dominant antigens in paratuberculosis. In comparison with Hsp60, the Hsp70 induces predominantly cell mediated responses. When using it as a vaccine we were recently successful in showing a protective effect of Hsp70 immunisation in a MAP challenge experiment. In contrast to expectations a prominent antibody response, absent during natural infection, preceded relatively low CMI responses. (Koets et al., 2005a)

The influence of host genetics on resistance to paratuberculosis is yet another field which will develop in the coming years. Some studies have provided indications of genetic factors involved in resistance to paratuberculosis.(Estonba et al., 2005; Koets et al., 2000; Mortensen et al., 2004) The completion of the bovine genome and technological advances in SNP discovery and typing will support expansion of this field. Further studies on this complex mycobacterial disease in natural hosts are indispensable to better understand the pathogenesis as well as aid the rational design of eradication strategies both on the individual animal as well as the population level.

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Studying the pathogenesis of paratuberculosis: the enduring challenge

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INTRODUCTION

Pathogenesis is defined as the origination and development of disease, more specifically the cellular events and reactions and other pathologic mechanisms occurring in the development of disease. It is the product of the confrontation between a pathogen and its host. The pathogen strives to enter the host to replicate and then be released for dissemination whilst the host fights to reduce the damage and eliminate the pathogen. The host response and hence the outcome of infection is determined by a complex interplay of factors including the genetic background and immune status of the host. Animals have evolved highly efficient recognition systems and effective inflammatory and immune responses to restrain, contain and eliminate microbial pathogens. However, microbial pathogens continuously adapt and evolve sophisticated ways of circumventing the host immune defences and so the host-pathogen battle continues. Therefore, to study and understand pathogenesis we need to consider both the pathogen and the host.

Studying the pathogenesis of paratuberculosis is an enduring challenge. The causative organism, *Mycobacterium avium* subspecies *paratuberculosis* (MAP), replicates extremely slowly and many isolates are refractory to culture *in vitro*. The disease has a prolonged incubation period of two to five years during which time it is difficult to detect the pathogen and study host-pathogen interactions. Although MAP is a successful pathogen in nature it is difficult to reproduce the characteristic features of the disease by experimental infection. These factors have severely hampered studies on the pathogenesis of paratuberculosis. Consequently, we have little knowledge of the underlying mechanisms of pathogenesis from the perspective of both the pathogen and the host. A clearer understanding of pathogenesis is of paramount importance if we are to be successful in intervening in the process to either prevent or treat infected animals.

This paper will review the progress that has been made during the past five years in understanding the pathogenesis of paratuberculosis from the perspective of how we study pathogenesis, with particular emphasis on the use of animal models. During this time, novel approaches employing new technologies have been applied. In some cases, this has been possible as a result of the availability of the complete MAP genome sequence and advances with respect to the genetic manipulation of MAP. These technologies will be reviewed by John Bannantine (Bannantine, 2005) and will not be discussed in detail here. Pathogenesis is a huge topic and it is not possible to cover everything in one short paper. Therefore, I have selected those studies which I think have yielded important and interesting information or have used novel approaches. The immunological aspects of pathogenesis will be covered by Ad Koets in his paper entitled 'Acquired immunity to bovine paratuberculosis: the host's perspective' (Koets, 2005) and similarly will not be covered in detail in this paper.

PATHOGENESIS

Identification of MAP virulence factors
An obvious approach to studying pathogenesis is to identify virulence factors produced by the pathogen that have a role in pathogenesis. A virulence factor is broadly defined as "a component that results in a pathogen's being able to survive or exploit the special environment represented by the host, or which

results in damage to the host". As such, virulence factors encompass diverse molecules and are involved in all stages of infection and pathogenesis including adherence to, ingestion by and survival in macrophages, modulation of the host immune response, persistence and dormancy. There have been a number of studies undertaken to identify MAP virulence factors and the molecular mechanisms underlying pathogenesis. Studies have tended to focus on the identification of antigenic proteins as these are likely to be key factors in pathogenesis, could be MAP-specific and potentially could be exploited for diagnostics or vaccine development. Immunogenic determinants were initially identified by screening MAP expression libraries with antisera from experimentally infected animals (Stevenson *et al.,* 1991; De Kesel *et al.,* 1993; Inglis *et al.,* 1994; El-Zaatari *et al.,* 1994; Bannantine and Stabel, 2001;) or from naturally infected clinical cases of paratuberculosis (Cameron *et al.,* 1994). The latter's advantage is that it is possible to detect immunogenic antigens expressed *in vivo* that may be absent from or poorly expressed in bacteria grown *in vitro*. This approach is now referred to as *in vivo* induced antigen technology or IVIAT. Some of the proteins identified using this approach have been further characterised and utilised in serological tests (Vannuffel *et al.,* 1994; El-Zaatari *et al.,* 1997; Shafran *et al.,* 2002) but their precise role in pathogenesis has not been elucidated. The 70KDa heat shock protein has been extensively studied with respect to its immunogenicity and more recently, as presented in this Colloquium, with respect to protection and potential as a subunit vaccine against bovine paratuberculosis (Koets *et al.,* 2005). The 34kDa putative serine protease identified by Cameron *et al.* (1994) has since been demonstrated to enhance the survival of MAP in macrophages (Heaslip, 2002). The 35KDa major membrane protein (Banasure *et al.,* 2001; Bannantine and Stabel, 2001) has been shown to have a role in the invasion of bovine epithelial cells (Bannantine *et al.,* 2003). More recently, Stratmann *et al.* (2004) identified MAP-specific ABC transporter proteins expressed on the mycobacterial cell surface *in vitro* and *in vivo* and are recognised by the immune response of infected animals. The ABC transporter operon was found to be located within a 38Kb pathogenicity island specific for MAP that also encompassed several iron uptake-related gene clusters potentially involved in pathogenesis.

The availability of the complete genome sequence of MAP has permitted the construction of a MAP partial protein array providing an alternative approach for identifying novel immunogenic antigens (Bannantine *et al.,* 2005). Sixty four MAP proteins are represented on the array including unknown hypothetical proteins and previously characterised antigens. The array has been screened with antisera from immunised rabbits and mice and from non- and MAP–infected cattle. Ten proteins were recognised by infected cattle of which seven represent novel antigens that have not been described previously.

Identification of antigens that elicit a Th1 type response has been more difficult. Nagata *et al.* (2005) recently employed expression library technology to identify and isolate interferon-gamma (IFN-) –inducing antigens by screening a MAP expression library using the IFN-   assay and peripheral blood mononuclear cells (PBMCs) from infected cattle. Three recombinant proteins were identified, two of which were members of the PPE protein family.

Progress also has been made regarding the identification of other MAP virulence factors. Hughes *et al.* (2005) have used a proteomics approach employing 2-D electrophoresis and Matrix Assisted Laser Desorption Ionisation time of flight (MALDI-tof) to identify potential virulence factors. They compared the proteomes of MAP grown *in vitro* and MAP isolated from the ilea of clinical cases of ovine paratuberculosis and identified a set of ten proteins whose expression is upregulated during natural infection. These proteins appear to be involved in metabolism and adaptation to physiological change expressed in the host environment and further studies will determine their precise role in the pathogenesis of paratuberculosis. No doubt with the availability of the complete MAP genome sequence, further virulence factors will be identified by searching for homologues of genes and proteins that have been found to be involved in the pathogenesis of other related organisms.

In order to demonstrate that a particular gene is important for pathogenesis it is necessary to fulfil molecular Koch's postulates (Falkow, 2004). This requires the construction of mutant strains that lack the gene of interest. Such studies had been hindered by the inability to genetically manipulate MAP. Two groups have succeeded recently in producing mutant libraries by transposon mutagenesis (Cavaignac *et al.,* 2000; Livneh *et al.,* 2005). These libraries have been screened to identify additional genes that may be involved in pathogenesis and also could be used to isolate mutants of putative virulence genes identified in the studies outlined previously.

Studying host-pathogen interactions
Studying host-pathogen interactions in paratuberculosis is particularly difficult due to the protracted period over which the disease develops. Subclinically infected animals are difficult to diagnose so investigation of the early events in pathogenesis and the mechanisms of progression from subclinical to clinical disease requires experimental models. The use of *in vitro* tissue culture systems has permitted elucidation of some important host-pathogen interactions but they can never reproduce the dynamic and complex interactions that occur within the whole animal. The ultimate model system therefore is infection of the intact animal host.

In vitro systems
In vitro systems have proved extremely useful for studying specific events in the early stages of pathogenesis, particularly entry and intracellular survival of the pathogen. The primary entry point for MAP infection is believed to be the M cells, specialised cells within the follicle associated epithelium (FAE) lining the Peyer's patches in the intestine. After entering the M cells, MAP is probably transported to the subepithelial lymphoid tissue where it is phagocytosed by subepithelial macrophages. M cells are very difficult to maintain *in vitro* and therefore most *in vitro* studies investigating attachment and uptake have been conducted using a variety of cell lines including Caco-2 cells that can be induced to develop an M cell like phenotype *in vitro*, T-24, and HT-29 intestinal epithelial cells. The mechanisms of attachment and uptake of MAP are poorly understood but progress has been made in this field in recent years. Two mycobacterial invasion proteins have been identified recently (Secott *et al.,* 2002; Bannantine *et al.,* 2003; Secott *et al.,* 2004). Secott *et al.* (2002) showed that MAP expresses a fibronectin attachment protein (FAP) that binds soluble fibronectin, which facilitates attachment and uptake in T-24 and Caco-2 cells. Their results suggested also that $\alpha 5 \beta 1$ integrin may serve as the host cell receptor for fibronectin bound FAP. As the distribution of receptors on cells in culture can often be different to that in intact tissue, it was necessary to conduct further investigations in an animal model. The researchers injected wildtype and antisense FAP mutant MAP strains independently or together with blocking peptides or antibodies into murine gut loops and monitored invasion of M cells by immunofluorescence microscopy (Secott *et al.,* 2004). These studies showed that MAP targeting and invasion of M cells is via formation of a fibronectin bridge between FAP and integrins on M cells. Bannantine *et al.* (2003) conducted experiments in cultured Madin-Darby bovine kidney cells and showed that the MAP 35KDa major membrane protein is a surface exposed protein that may play a role in the invasion of bovine epithelial cells. Schleig *et al.* (2005) developed an *in vitro* model for investigating attachment of MAP to bovine intestinal organ cultures. They found significant differences in the ability of different MAP strains to attach but could demonstrate no differences in attachment among different regions of the intestinal tract. Coating of the organisms with fibronectin affected attachment but appeared to be strain related. Therefore it is likely that there will be other factors governing the preferential targeting of M cells and that these will be revealed in future studies. Likewise, as will be described later, M cells may not be the only cells through which uptake of MAP occurs.

There have been a considerable number of *in vitro* studies investigating the entry and survival of MAP within macrophages. These studies make use of bovine monocyte- derived macrophages or murine bone marrow-derived macrophages and cell lines including J774 derived from a mouse tumour that has characteristics typical of macrophages. The molecular mechanisms by which MAP enters macrophages are unknown. Cheville *et al.* (2001) investigated the binding of MAP to murine macrophages by incubating with monoclonal antibodies to complement receptor CR3 and adding the tripeptide sequence RGD which is the binding site on bacteria for integrins. The results suggested that CR3 appears to be one receptor for the attachment of MAP to macrophages facilitating phagocytosis.

More progress has been made in characterising host macrophage responses to MAP internalisation and how MAP resists killing. Studies using J774 murine macrophage cell cultures have shown that phagosomes containing live MAP fail to mature and acidify whereas killed MAP were identified in acidified phagosomes and phagolysosomes (Cheville *et al.,* 2001;Hostetter *et al.,* 2003;Kuehnel *et al.,* 2001). Phagosomal maturation can be evaluated using stage specific markers detected by immunofluorescent labelling and confocal microscopy. Phagosomes containing live MAP exhibited increased levels of the early endosomal marker transferrin receptor and decreased levels of the late endosomal marker lysosome associated membrane protein 1 relative to those containing killed MAP (Hostetter *et al.,* 2003). Kuehnel *et al.* (2001) reported that phagosomes containing live MAP were reduced in their ability to acquire internalised calcein,

BSA-gold or lysosome associated membrane protein 2. The effects of cytokine activation on the ability of monocytes and macrophages to phagocytose and kill MAP have also been studied. Cytokine activation of J774 cells prior to infection appears to restore the ability of the cells containing MAP to proceed to the late phagosomal stages (Hostetter *et al.,* 2002).

Coussens and coworkers (2002, 2003, 2004b) have pioneered an alternative approach and applied microarray and quantitative real-time PCR (Q-RT-PCR) technology to investigating the host-pathogen interactions in naturally MAP infected cattle (Coussens *et al.,* 2002;Coussens *et al.,* 2003;Coussens *et al.,* 2004b). Microarray technology can provide information on the molecular basis for MAP pathogenesis and has the advantage of providing a global picture of gene expression. EST and cDNA microarrays were developed for studying bovine immunobiology (Yao *et al.,* 2001). Coussens *et al.* (2002) used a bovine specific total leukocyte (BOTL) cDNA microarray to evaluate the response PBMCs from subclinically and clinically MAP infected cattle. This study demonstrated predominant repression of immune cell gene expression in PMBCs from clinical MAP infected cattle and that this was not due to a general loss of immune cell function. A total of 83 genes showed decreased expression including those encoding microspherule protein 1, fibroblast growth factor and Lyn B protein kinase. Only eight genes exhibited up regulation including those encoding bovine CD40L, IFN- , interleukin 10 and tissue inhibitor of matrix metalloproteinases. In contrast, MAP stimulation of PBMCs from subclinical cattle revealed a predominate trend to activate immune cell gene expression. A total of 71 genes showed increased expression including those encoding bovine CD40L, several matrix metalloproteinases and SPARC (secreted protein, acidic and rich in cystine) whereas only 16 genes were down regulated including those encoding the bovine orthologues of cytochrome oxidase subunit III, interleukin 1 (IL-1) receptor type I and fibrinogen-like 2 protein. Further experiments using an expanded BOTL cDNA microarray demonstrated that there were major differences in the gene expression patterns between PBMCs isolated from infected and uninfected cattle regardless of *in vitro* stimulation with MAP (Coussens *et al.,* 2003). The observed differential gene expression could not be explained by gross differences in the relative immune cell populations in the two groups of animals. Aho *et al.* (2003) extended the microarray studies to compare the gene expression profiles of ileal tissues from MAP infected and uninfected control cattle (Aho *et al.,* 2003). These studies demonstrated upregulation of IL-1 and tumour necrosis factor receptor-associated protein 1 (TRAF1) mRNA and protein expression in ileal tissues of MAP infected cattle. IL-1 is a proinflammatory cytokine associated with granuloma formation and maintenance and TRAF1 is a proapoptotic intracellular signalling molecule. Both proteins may have an important role in MAP pathogenesis and have been the subject of further investigation, the results of which have been presented at this Colloquium (Coussens *et al.,* 2005). High levels of TRAF1 are located primarily within infiltrating macrophages and IL-1 appears to be secreted from cells within lesions in MAP infected animals. In addition, Q-RT-PCR has been used to study the expression of selected proinflammatory cytokines in PBMCs from MAP infected cows (Coussens *et al.,* 2004a). The microarray approach has been used also by Weiss and coworkers to characterise the response of bovine monocyte-derived macrophages to MAP infection and to investigate sequential gene expression patterns by bovine monocyte-derived macrophages associated with ingestion of MAP (Weiss *et al.,* 2004a; Weiss *et al.,* 2004b). The results of these studies revealed discrepancies with those of Coussens *et al.* (2002) possibly due to the different microarray platforms used and the use of a mixture of lymphocytes and monocytes rather than monocyte-derived macrophages. This highlights the importance of defining the experimental conditions when using microarray technology and verifying the results with additional assays measuring function or protein quantification.

Animal models
Small animal models for paratuberculosis offer a number of advantages. Small animals are easy to house, maintain and handle and generally the pathogenic features can be measured after a much shorter post infection incubation period than in ruminants. The disadvantage is that they do not always mirror the features of ruminant paratuberculosis. The murine model is particularly useful for studying host-pathogen interactions involving the immune response. Mouse strains have been produced with mutations that render them deficient in specific components of the immune response and there is a host of immunological reagents and assays available for studies. A number of small animals have been investigated as models for paratuberculosis including chickens (Larsen and Moon, 1972; Valente *et al.,* 1997), guinea pigs (Francis, 1943), hamsters (Hirch, 1956; Beran *et al.,* 2005) and rabbits (Mokresh and Butler, 1990). There has been more interest in developing the rabbit model in recent years following reports of paratuberculosis in wild rabbits (Greig *et al.,* 1997; Greig *et al.,* 1999; Raizman *et al.,* 2005) and the ability of rabbit isolates of MAP

to produce paratuberculosis in experimentally infected calves (Beard *et al.,* 2001). A recent study by Vaughan *et al.* (2005) orally challenged adult and juvenile rabbits with a wildtype bovine MAP strain and followed disease progression by bacterial and histopathological examination over 36 months. Unfortunately, as with previous studies, it was not possible to consistently reproduce the disease as bacteria were cultured from the tissues of only two of four adult and three of 16 juvenile rabbits and microscopic lesions were detected in four of the diseased rabbits. All studies so far have employed New Zealand White rabbits and the outcome of experimental infection may be influenced by the genetic background of the host or host preference of the MAP strains selected.

In contrast, experiments using mice have made a valuable contribution to our understanding of the pathogenesis of paratuberculosis. These have been reviewed elsewhere (Harris and Barletta, 2001) and only recent experiments will be highlighted here. The mouse model is very versatile and can be used to study different aspects of MAP pathogenesis by selecting the appropriate strain of mouse, age at time of, dose and route of inoculation. Knockout mice have been exploited during the past five years to investigate the interaction between MAP and the host immune system. Tanaka *et al.* (2000) used BALB/c T-cell receptor (TCR)   knockout mice to study the role of   T cells in MAP infection. The results of the study suggested that   T cells may be important for the development of epithelioid granulomata during MAP infection but are probably not involved with the elimination of the mycobacteria from the host. Stabel and Ackermann (2002) also utilised TCR knockout mice to investigate the role of   and   T cells in resistance to MAP infection. They demonstrated that irrespective of bacterial strain or infection period, TCR- - knockout mice had higher levels of colonisation of MAP in their tissues compared to TCR- -knockout mice or control mice suggesting that   T cells are critical for bacterial containment. Another interesting study was conducted by Mutwiri *et al.* (2001) who investigated the intestinal pathophysiological changes induced by MAP infection in beige/scid mice. This research group used Ussing chambers to show that MAP infection produced significant abnormalities in intestinal transport parameters. The results suggested that intestinal tissue from infected mice was less permeable to ions but that there was increased ion secretion. There was also evidence that the inflamed intestinal tissue had neural and/or epithelial damage. The model shows that mucosal pathophysiologic changes consistent with chronic inflammation following MAP infection can occur via T cell independent mechanisms.

Although mouse models offer unique opportunities to study interactions of MAP with the host immune system, mice are not a natural host for MAP infections and they cannot accurately mimic all aspects of MAP pathogenesis in ruminants. Ultimately, pathogenesis has to be studied in the natural host. The calf model has been used extensively for studying the interaction of MAP with the bovine immune system and has been reviewed recently (Coussens, 2004). As with other animal models, the parameters used for MAP infection can influence the pathogenic features observed and a cautionary approach should be taken when interpreting results. Waters *et al.* (2003) investigated the usefulness of the intratonsillar inoculation route as an experimental model for MAP infection. These experiments challenged the dogma of a Th2 response occurring late in pathogenesis as mycobacteria-specific antibody was detected as early as 134 days post challenge and concurrently with a cell mediated response. Koo *et al.* (2004) demonstrated that high-dose exposure via the oral route lead to a uniform and reproducible infection consistent with that described in animals directly inoculated with MAP. A proliferative immune response to MAP was not readily detected until five months post inoculation suggesting that MAP interferes with pathways of activation associated with antigen-presenting cells. This study also highlighted the advantages of using multicolour flow cytometry for monitoring immune responses during disease progression.

The calf model has not been widely used to study uptake of MAP or the histopathological features of MAP pathogenesis. The most notable progress in the past five years in this field has come from studies in goats. Subclinical paratuberculosis in goats following experimental infection has been characterised using immunological assays and microbiological culture (Storset *et al.,* 2001). Sigurdardottir and co-workers have described some very elegant experiments to study the uptake of MAP through the distal small intestinal mucosa (Sigurdardottir *et al.,* 2001;Sigurdardottir *et al.,* 2005). MAP was injected into isolated loops of ileum and after one hour the segments were excised and processed for light and electron microscopic analyses (Sigurdardottir *et al.,* 2001). MAP was observed in the FAE both in the cytoplasm of M cells and in the cytoplasm of intraepithelial leukocytes in M-cell pockets and occasionally between M cells in the ileal dome. MAP was not found in association with the absorptive epithelium. Uptake of MAP was found to be rapid, cells being detectable after just 30 minutes. More recently, the research group has used the everted sleeve method initially developed for the *in vitro* measurement of intestinal absorption to look at the uptake

of MAP in more detail (Sigurdardottir *et al.,* 2005). This method is particularly useful in that it provides a quantitative as well as a qualitative method of studying bacterial uptake although it does have limitations relating to how long mucosal integrity can be preserved. Small everted sleeves derived from areas of the intestine with and without Peyer's patches were incubated for one hour in a suspension of radiolabelled MAP. The study showed that MAP could enter the intestine through areas with and without Peyer's patches and therefore that uptake is not restricted to M cells and can occur via enterocytes. Valheim *et al.* (2002) undertook studies to investigate the association between the morphology of the Peyer's patches and the lesions of paratuberculosis in goats. The observed morphological similarities between the ilealcaecal-valve Peyer's patch and the jejunal Peyer's patches but a different morphology for the ileal Peyer's patch which undergoes involution during the first 12-18 months of life. The persistent organised lymphoid tissue of the ilealcaecal valve and jejunal Peyer's patches but not the involuted ileal Peyer's patch could sustain the development of granulomatous lesions during subclinical paratuberculosis. The characterisation of macrophages and occurrence of T cells in the intestinal lesions of subclinically infected goats has also been reported by Valheim *et al.* (Valheim *et al.,* 2004).

Sheep models also have been used to study ovine paratuberculosis, particularly the early immunopathological events in infection (Begara-McGorum *et al.,* 1998) but it is difficult to reproduce early histological lesions between experiments. This could be due to a number of reasons including differences in the breed of sheep or the viability, dose, or MAP strain used. It has been reported that infections are more easily established using MAP prepared directly from mucosal tissue rather than from *in vitro* cultures (Stewart *et al.,* 2004). Whether this is a dosage effect (it is very difficult to count MAP in intestinal tissue) or due to upregulation of virulence factors in *in vivo* grown cells is yet to be established. Previous experimental infections have employed cattle-type strains that are easier to propagate in the laboratory but it has recently been demonstrated that sheep can be experimentally infected with ovine-type strains (Reddacliff and Whittington, 2003; Stewart *et al.,* 2004). Hein *et al.* (2004) have adopted a novel approach for studying regional immune responses in naturally MAP infected sheep with clinical signs of paratuberculosis (Hein *et al.,* 2004). They cannulated the afferent and efferent lymphatic vessels draining the jejunum enabling continuous sampling of lymph up to a period of 107 days. This enabled phenotypic analysis and cytokine gene expression profiling of the intestinal lymph cells. There is no doubt that this approach will prove extremely useful for gaining real-time insights into host-pathogen interactions as they occur *in vivo* within the gut associated lymphoid tissue.

CONCLUSION

Our knowledge and understanding of the pathogenesis of paratuberculosis has improved over the past five years but there are still many unanswered questions and much more to learn. The availability of the MAP genome sequence and novel molecular technologies including those for the genetic manipulation of MAP will accelerate further studies but will need to be combined with appropriate *in vitro* and *in vivo* models to evaluate pathogenesis. Although current animal models offer a range of approaches for studying different aspects of the disease, further refinement and development of novel models is a future area for research. An approach that has not yet been adopted for paratuberculosis is the application of non-invasive techniques for studying pathogenic bacteria in the whole animal (Camilli, 1996). Bioluminescence monitoring has been used successfully for monitoring *Salmonella* and *Citrobacter rodentium* infection in mice (Contag *et al.,* 1995; Wiles *et al.,* 2004; Burns-Guydish *et al.,* 2005) and for detecting *Yersinia pseudotuberculosis* in dissected Peyer's patches and spleen (Forsberg and Rosqvist, 1993). The technique has limitations imposed by its requirement for oxygen but could nevertheless be extremely useful. Once reliable and reproducible systems for genetically manipulating MAP are in place, it should be possible to construct reporter strains of MAP for bioluminescence studies. There is a promising and exciting future for paratuberculosis research.

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Flow cytometric analysis of the immune response to *M. avium* subsp. *paratuberculosis* in experimentally infected calves.

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ABSTRACT

Multi-parameter flow cytometry was used to analyze the immune response to *M. avium* ssp. *paratuberculosis* (MAP) in experimentally infected neonatal calves. A persistent proliferative response to MAP purified protein derivative and soluble MAP antigens was detected 3 weeks post infection (PI). CD4+ with a memory phenotype (CD45R0+) was the predominant cell type responding to antigens. Few CD8+ T cells proliferated in response to antigens until 16 months PI. T cells proliferated in culture also, but there was little difference between the response observed with cells from controls and experimentally infected animals cultured with antigens. WC1+ CD2- γδ T cells were the predominant type of T cells in all cultures. WC1- CD2+ T cells were present but always comprised a lower proportion of T cells in cultures with and without antigen. CD2+ and CD2- NK cells proliferated extensively in some cultures of cells from control and infected animals in the presence and absence of antigen. Although it is not clear what modulatory effect, if any, T, NK, and CD8+ T cells had on the observed proliferative response to antigens, they were always present.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Ptb). The disease has been difficult to control because of the lack of diagnostic assays that can detect infected animals before they begin shedding bacteria into the environment (Barrington et al., 2003; Rideout et al., 2003) and the lack of an efficacious vaccine (Kalis et al., 2001; Harris and Barletta, 2001; Barletta et al., 2000). At this juncture, information remains limited on the factors affecting the immune response to MAP. Studies to date have shown the time course of disease is characterized by a long latency period with no clinical signs. No defined time point has been identified during the latency period when immune control of infection begins to wane and animals begin shedding bacteria. It is thought that loss of immune control is associated with a depression or loss of cell mediated immunity (CMI) and an increase in the antibody response to MAP antigens. The underlying defect that could be associated with an alteration in CMI is dysregulation of dendritic cell and macrophage function mediated by MAP (Martino et al., 2004; Zur Lage et al., 2003; Tooker et al., 2002; Weiss et al., 2002; Mariotti et al., 2002). Disruption of gene activation and signaling through genes encoding of TNF-α, IL-1, IL-12, and IFN-γ and their receptors could lead to a breakdown in the immune response controlling infection (Koets et al., 2002; Ottenhoff et al., 2000; Dorman and Holland, 2000; Jouanguy et al., 1999; Bloom et al., 1992). The effector mechanisms used by CD4+ T cells include secretion of IFN-γ, which activates bactericidal activity in macrophages, and secretion of lymphotoxin, perforin, and granulysin (Canaday et al., 2001; Roach et al., 2001). Disruption could also affect CD8+ T cells (Canaday et al., 2001). The present study was conducted to gain further information on

the immune response to MAP infection and determine the earliest time point a response can be detected using multicolor flow cytometry (FC).

MATERIALS AND METHODS

Animals
Ten Holstein newborn calves, obtained from the Washington State University MAP-free dairy herd, were used in the present study. The first eight calves were used in the primary study. They were housed and maintained according to the Institutional Animal Care and Use committee guidelines and AAALAC. Five animals were randomly selected and given daily oral doses of MAP (10^7 bacteria) for seven days. The three remaining animals were housed separately and served as controls. Two calves were obtained later and maintained for 6 months before being infected with MAP. They were inoculated with a single oral dose of 10^8 bacteria.

Blood processing for tissue culture and flow cytometry
PBMC used in cultures were obtained from buffy coat fractions of blood collected in acid citrate dextrose (ACD) separated by density gradient centrifugation with Accu-Paque (density 1.086) (Accurate Chemical & Scientific Corp., Westbury, NY). Residual erythrocytes were lysed with H_2O. Dead cells and debris were removed from cultured cells by density gradient centrifugation before use in flow cytometry (FC).

For analysis of the composition of peripheral blood at the time of initiation of culture, 10 ml of blood was collected in ACD and lysed in Tris-buffered NH_4Cl to remove erythrocytes. The resultant cells were washed several times in phosphate buffered saline containing 20% ACD (PBS/ACD) to remove excess platelets and then used in flow cytometry as described (Davis et al., 1995).

Antigens
MAP purified protein derivative (PPD), Johnin, was obtained from the National Veterinary Service Laboratory. Soluble antigen (SAg) was prepared from an isolate (8544) used in previous studies.(Eriks et al., 1996; Eriks et al., 1994) MAP was grown at 37 °C in Bacto-Middlebrook 7H9 broth enriched with 7 % (v/v) ESP-para-JEM™GS (OADC, Trek Diagnostic Systems, Cleveland, OH, USA), 4 % (v/v) ESP-para-JEM™AS (antibiotics which selectively grow MAP cells, Trek Diagnostic Systems), 0.1 % (v/v) mycobactin J (*Allied Monitor*, Fayette, MO, USA), and 0.05 % Tween 80 in a shaking incubator. MAP was harvested by centrifugation at 1500× g for 10 min at 4, washed three times in PBS, suspended in PBS, pH 7.4 containing 0.01% Tween 80. The mycobacterial concentration was determined spectrophotometrically. The bacteria were killed by irradiation. They were exposed to a ^{60}Co source for 26 hrs (2.5 Mrad). Soluble MAP antigen was prepared by vortexing MAP cells mixed with the same amount (v/v) of zirconia/silica beads (Biospec Products, Inc., Bartlesville, OK, USA). Phenylmethylsulfonyl fluoride (PMSF) was added to soluble MAP antigen at a final concentration of 1 mM to prevent protein degradation. The protein concentration of soluble antigen was determined by measuring O.D. at 280 nm.

Cell culture
PBMCs were resuspended in RPMI 1640 medium supplemented with 13% bovine calf serum (BCS), 2ME, and antibiotics and then distributed in T 75 tissue culture flasks (3×10^6 cells/ml). Two flasks each were stimulated with MAP PPD (20 µg/ml), soluble antigen (SAg, 4 µg/ml) or RPMI 1640 complete medium alone. At 6 or 7 days post stimulation, the cultures were collected and subjected to density centrifugation to remove dead cells and then labeled for flow cytometry as described (Davis et al., 1995).

Flow cytometry
The monoclonal antibodies (mAb) used in the present study are shown in Table 1. The combinations of mAbs used in the analysis are shown in Fig. 1. In some combinations, two mAbs of the same specificity and isotype were used to increase the intensity of the fluorescent signal. The mAbs specific for the chain of the TCR were used in combination with mAbs to CD3 and CD2 to distinguish T cells from the CD2$^+$, WC1$^-$ and CD2$^-$ WC1$^+$ T cell subsets. WC1 (workshop cluster 1) is an unique molecule expressed on a subset of T cells only found in Artiodactyla (Davis et al., 1998; Davis et al., 1996a). Previous studies have shown the WC1$^-$ subset is the same as the chain$^+$ CD2$^+$ WC1$^-$ subset and that the WC1$^+$ subset is

the same as the   chain$^+$ CD2$^-$ subset (Davis et al., 1996a). WC1 is used in the text with CD2 when describing results obtained on the CD2$^+$, WC1$^-$ and CD2$^-$ WC1$^+$ populations of   T cells. All mAbs were used at 15 µg/ml. In brief, 10^6 cells were incubated for 15 minutes in 96 well conical bottom assay plates, in the respective cocktails of mAbs (50 µl of each mAb) at a final volume of 250 µl of PBS/ACD, containing 0.5% horse serum, and .09% azide), washed 3✕ and then incubated for an additional 15 minutes in cocktails of isotype specific second step reagents conjugated with fluorescein (FL), phycoerythrin (PE), PE-Cy5 (TRI-COLOR) or Cy5 (Caltag, Burlingame, CA, Southern Biotechnology Associates, Birmingham, AL). Following incubation, the cells were washed 2✕ in PBS/ACD and fixed in 2% PBS buffered formaldehyde and refrigerated until examined.

Table 1. Monoclonal antibodies used to determine the composition of peripheral blood mononuclear cells before and after culture with antigens

mAb	Ig isotype	Specificity
AKS1	IgG1	NKp46 (Storset et al., 2004)
MM1A	IgG$_1$	CD3 (MacHugh et al., 1998; Davis et al., 1993; Howard et al., 1991)
MUC2A	IgG$_{2a}$	CD2 (Howard and Naessens, 1993; Larsen et al., 1990)
BAT76A	IgG$_{2a}$	CD2 (Davis and Splitter, 1991; Larsen et al., 1990)
ILA-11A	IgG$_{2a}$	CD4 (Baldwin et al., 1986)
CACT138A	IgG$_1$	CD4 (Bensaid and Hadam, 1991)
CACT187A	IgG$_1$	CD4 (Koo et al., 2004)
B29A	IgG$_{2a}$	CD5 (Davis et al., 1990)
BAQ83A	IgG$_{2b}$	CD6 (Koo et al., 2004)
7C2B	IgG$_{2a}$	CD8α? (Koo et al., 2004)
CACT80C	IgG$_1$	CD8α (MacHugh and Sopp, 1991)
BAT82A	IgG$_1$	CD8β (MacHugh and Sopp, 1991)
GB21A	IgG$_{2b}$	$\gamma\delta$ T δ chain specific (Davis et al., 1996a)
CACT61A	IgM	$\gamma\delta$ T δ chain specific (Davis et al., 1996a; Parsons et al., 1993)
CACT148A	IgM	$\gamma\delta$ T δ chain specific (Davis et al., 1996a)
GC44A1	IgG$_3$	CD45R0 (Koo et al., 2004)
BAQ44A	IgM	B cells (Davis et al., 1990; Hein et al., 1989)
DH59B	IgG$_1$	CD172a (Alvarez et al., 2000; Davis et al., 1987)
LCTB2A	IgG$_3$	CD25 (Davis et al., 1996b)
CACT116A	IgG$_1$	CD25 (Naessens et al., 1992)
CACT114A	IgG$_{2b}$	CD26 (Lee et al., 2002)
CACT200A	IgG$_1$	ACT1 (Koo et al., 2004)
CACT26A	IgG$_1$	ACT2 (Davis et al., 1996b)
LCTB6A	IgG$_1$	ACT9 (Davis et al., 1996b)
GB110A	IgM	ACT16 (Davis et al., 1996b)
CACT195A	IgM	ACT27 (Koo et al., 2004)
CACT216A	IgM	ACT28 (Koo et al., 2004)
CACT225A	IgG$_1$	ACT29 (Koo et al., 2004)
CACT185A	IgG$_1$	ACT30 (Koo et al., 2004)
CACT152A	IgM	ACT31 (Koo et al., 2004)
CACT191A	IgM	ACT32 (Koo et al., 2004)
H34A	IgG2b	MHC II(Davis et al., 1987)

A FACSort flow cytometer equipped with argon and red lasers, a Macintosh Quadra computer, and Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) were used to collect data. FCS Express software (De Novo software, Thornton, Ontario, Ca) was used to analyze the data.

Analysis of the proliferative response to MAP antigens
In the first study, blood was collected and processed for cell culture every two weeks for two months, once a month to 14 months and every 2 months to 2 years and every 3 months to 3 years when the study was completed. FC was used to collect data, using combinations of mAbs for 3 or 4 color analyses (Fig. 1). Selective gating was used to obtain results on specific subpopulations of lymphocytes. Granulocytes and nonspecific signals were excluded at the time of data acquisition. Gates were placed on CD4$^+$ and CD8$^+$ T cells at the time of data acquisition because of the low frequency of these subpopulations in unstimulated cultures. In the first study, NK cells were detected indirectly using a combination of anti-CD3, -CD2, -CD25, and -   T to identify CD2$^+$ and CD2$^-$ null cell populations. For analysis of   and   T cell subsets, a selective gate was placed on CD3$^+$ cells. Exclusion gates were placed on CD3$^+$ and CD5$^+$ T cells to identify

CD3$^-$ CD5$^-$ null cells. mAbs specific for CD25 and CD26 were used to monitor the activation status of and   T cells and other cells in the cultures (Lee et al., 2001; Davis et al., 1996b). An additional mAb was used to monitor the upregulation of a molecule, ACT2, uniquely expressed on activated   T cells (Table 1) (Davis et al., 1996b).

	1	2	3	4	5	6	7
Control	M1A MUC2A GB21A LCTB2A	AKS1 MUC2A GB21A	ILA11A CACT116A CACT114A GC44A1	7C2B CACT116A CACT114A GC44A1	ILA11A CACT200A GC44A1	LCTB6A ILA-11A GC44A1	CACT138A CACT187A GB110A GC44A1
Specificity	CD3 CD2 $\gamma\delta$ chain CD25	NKp46 CD2 $\gamma\delta$ chain	CD4 CD25 CD26 CD45R0	CD8 CD25 CD26 CD45R0	CD4 ACT1 CD45R0	ACT9 CD4 CD45R0	CD4 ACT16 CD45R0
	8	9	10	11	12	13	14
Control	CACT138A CACT187A CACT195A GC44A1	CACT138A CACT187A CACT216A GC44A1	CACT225A ILA-11A GC44A1	CACT185A ILA-11A GC44A1	CACT138A CACT187A CACT152A GC44A1	CACT138A CACT187A CACT191A GC44A1	CACT26A CACT114A CACT61A CACT148A LCTB2A
Specificity	CD4 ACT27 CD45R0	CD4 ACT28 CD45R0	CD4 ACT29 CD45R0	CD4 ACT30 CD45R0	CD4 ACT31 CD45R0	CD4 ACT32 CD45R0	ACT2 CD26 $\gamma\delta$ δ chain $\gamma\delta$ δ chain CD25

Fig. 1. Combinations of mAbs used in 3 and 4 color flow cytometry. Specificities of mAbs correspond from top to bottom as shown.

Statistics
Data were analyzed by either repeated measures, one-way analysis of variance followed by the Tukey-Kramer multiple comparison test, or Student's *t* test.

RESULTS

Flow cytometric analysis of the proliferative response to MAP antigens
At the initiation of culture, CD4$^+$ and CD8$^+$ T cells comprised, on average, ~16% and 10%, of the ungated cells respectively. CD2$^+$ $^-$ T cells comprised ~ 42% of the CD3 gated cells. CD2$^+$ $^+$ and CD2$^-$ $^+$ T cells comprised ~ 4% and 47% of the CD3$^+$ gated cells respectively. CD3$^-$ CD2$^+$ cells comprised ~1 – 3% of the cells. The CD3$^-$ CD2$^-$ cells i.e., B cells, monocytes, dendritic, and null cells comprised ~ 38% of the cells. B cells, both CD5$^+$
and CD5$^-$ populations, comprised a variable proportion of cells at the start of culture (Koo et al., 2004). The composition of cultured cells changed with time following infection with MAP. Comparison of the percentage of cells in electronic gates placed on small (gate R1) and large (gate R2) lymphocytes showed there was a clear increase in the proportion of large proliferating cells in gate 2 (Fig. 2). However, up to 5 to 6 months, an unexplained proliferation of lymphocytes in some cultures of cells from infected and control animals made it difficult to detect a specific immune response to MAP. Examination of the cultures by FC revealed that the proliferation was primarily attributable to the non-specific proliferation of CD3$^-$ CD2$^-$ and CD3$^-$ CD2$^+$ null cells. These cells proliferated both in the presence and absence of antigen. Analysis of the CD172A, CD5, CD6, B cell combination showed that a subset of these cells expressed CD6 (Koo et al., 2004). Because some of the null cells expressed CD8, it was difficult to exclude them with an electronic gate at the time of data acquisition and also when attempting of analyze the   CD8 T cell response to MAP.
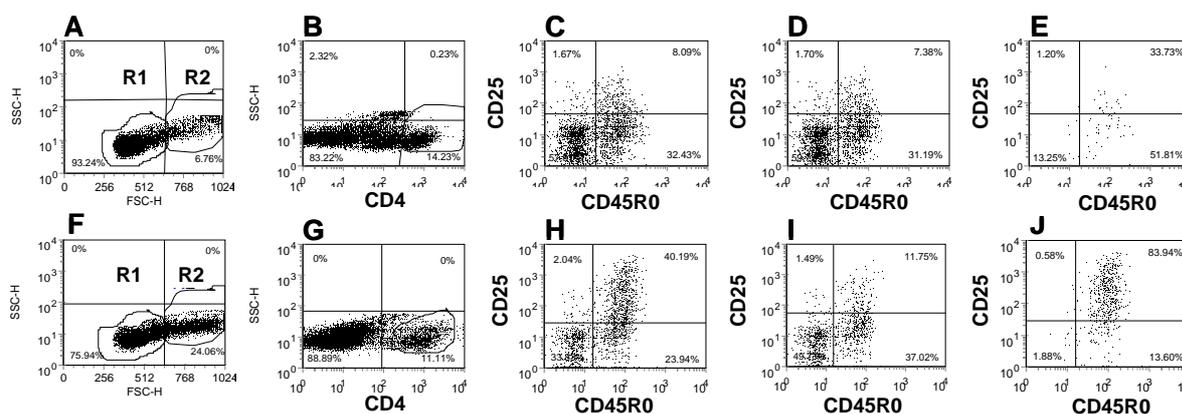
Fig. 2 Representative dot plot profiles showing the use of selective electronic gating to analyze the composition of lymphocytes following culture with Map antigens. A – E, cells cultured without antigen. C and H: selective gates placed on R1 (small cells), R2 (large cells), and CD4 to show pattern of expression of CD25 on CD4 naïve (CD45R0-) and memory (CD45R0+) T cells. D and E: selective gates placed on R1 (small cells) and CD4 to show pattern of expression of CD25 on CD4 naïve (CD45R0-) and memory (CD45R0+) T cells. E and J: selective gates placed on R1 (small cells) and CD4 to show pattern of expression of CD25 on CD4 naïve (CD45R0-) and memory (CD45R0+) T cells. The gating shows CD25 is only expressed on large proliferating CD4 memory T cells.

By 6 months, a consistent proliferative response was observed in the presence of PPD and SAg. After 6 to 7 days of culture, 70 – 80% of the T cells were present in the large lymphocyte gate. Analysis of gated CD4$^+$ T cells showed that the majority of CD4$^+$ T cells fell in the large lymphocyte gate and that they were CD45R0$^+$, CD25$^+$, and CD26$^+$. Further examination at 1 to 3 years showed additional activation molecules were also upregulated (Fig. 3).

The proliferative response of CD8$^+$ T cells appeared minimal during the first year. They comprised less than 10 % of cells in stimulated and unstimulated cultures from both control and infected animals. At 1 year, some CD8$^+$ T cells from infected animals were beginning to respond to antigen but there were still background problems in some cultures because of the presence of CD8$^+$ null cells. By 16 months a consistent response was more evident in antigen stimulated cultures where null cells were low in frequency. Selective gating showed the responding cells were increased in size and CD45R0$^+$, CD25$^+$, CD26$^+$.

    γδ T cells comprised a large proportion of cells present at the initiation and after 6 to 7 days of culture. The majority of the γδ T cells were CD2$^-$ WC1$^+$. The frequency of this subset varied from 40 – 50% of the total T cell population in both control and infected animals. The CD2$^+$ WC1$^-$ population varied from 5 – 10%. The relative proportions of these subsets did not change appreciably in unstimulated cultures from control and infected animals. The frequencies of both populations were lower in antigen stimulated cultures from infected animals containing proliferating CD4$^+$ T cells. γδ T cells expressed CD25 and ACT2 at low levels at the beginning of culture. CD25 was expressed predominantly on WC1$^+$ CD2$^-$ γδ T cells. They did not express CD26. All γδ T cells expressed CD45R0. γδ T cells in cultures from control and infected animals exhibited an activated phenotype in the presence of PPD and SAg. Cells with the activated phenotype were present in the large lymphocyte gate.

In this set of animals, the proliferative response to PPD and SAg remained the same until the experiment was completed at 3 years PI. CD4$^+$ CD45R0$^+$ memory T cells were the predominant population in all antigen stimulated cultures. Proliferating CD8$^+$ CD45R0$^+$ memory T comprised less that 10% of the cells in the cultures. Activated γδ T cells and null cells comprised a variable proportion of cells in all cultures.

% Positive cells
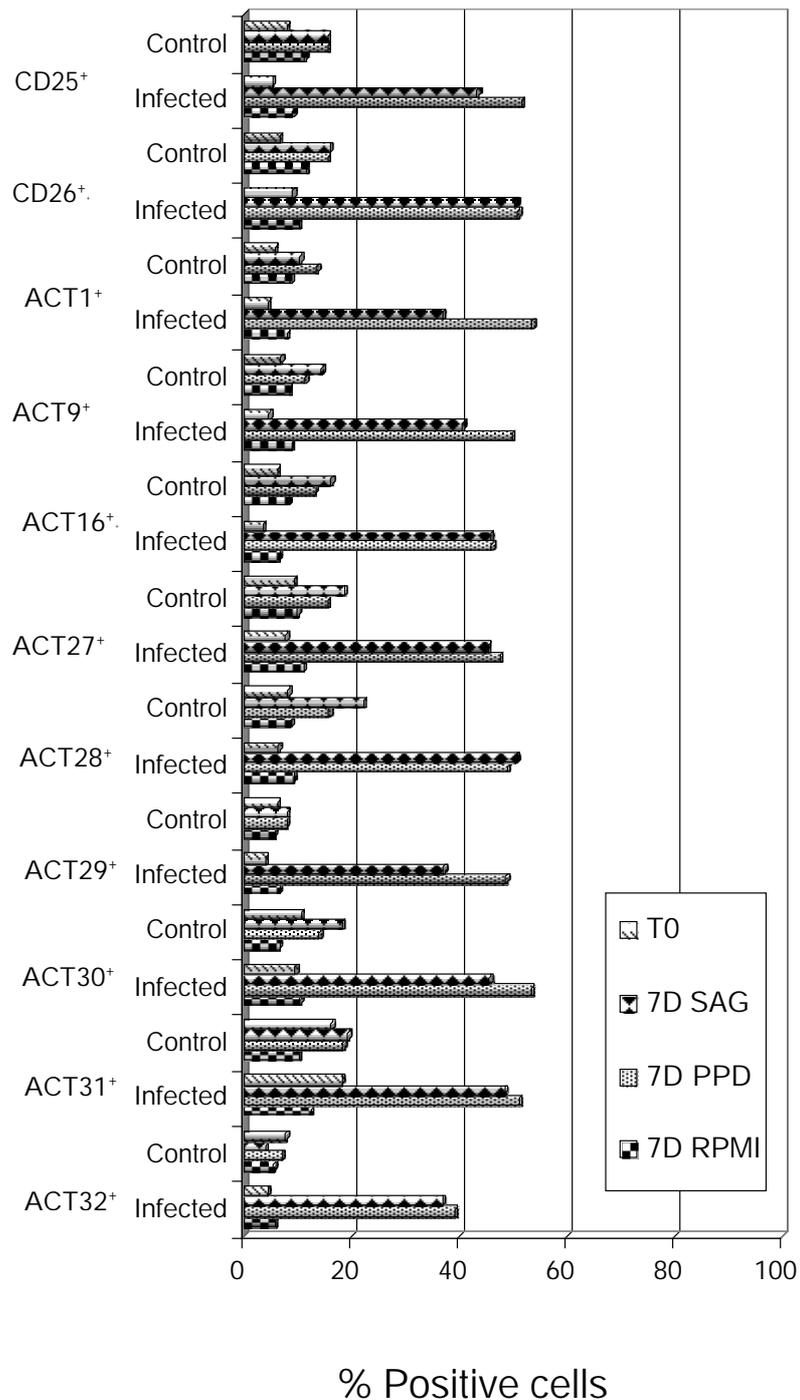
Fig. 3. Summary of data showing the upregulation of molecules expressed on CD4 memory T cells following culture with SAg. Electronic gates were placed on cells in R1, R2, and CD4 (see Fig. 2).

A second study was conducted to determine the specificity of null cells and also determine if a CMI response to MAP antigens could be detected earlier than 6 months. Two calves were maintained for 6 months and then infected with 10^8 bacteria by the oral route. FC analysis showed there was no response to PPD before infection. At 3 weeks PI the calves were killed to examine tissues for the presence of bacteria. Blood was collected for FC analysis. As shown in Fig. 2, a CD4 memory T cell was detected against PPD. Multiple activation molecules were upregulated on CD4+ CD45R0+ T cells (Fig. 4). Because of background and the low frequency of CD8 T cells in cultures, a specific CD8 response could not be clearly demonstrated.
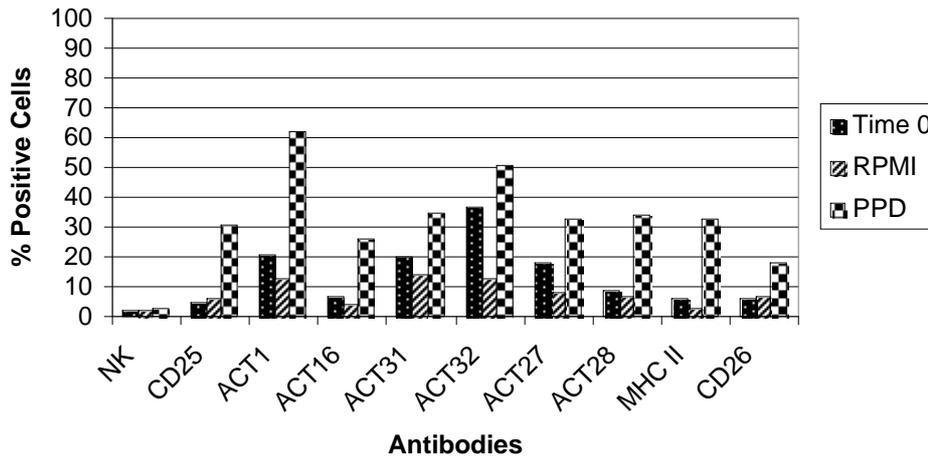
Fig. 4. Summary of data showing the upregulation of molecules expressed on CD4 memory T cells following culture with PPD. Electronic gates were placed on cells in R1, R2, and CD4 (see Fig. 2).
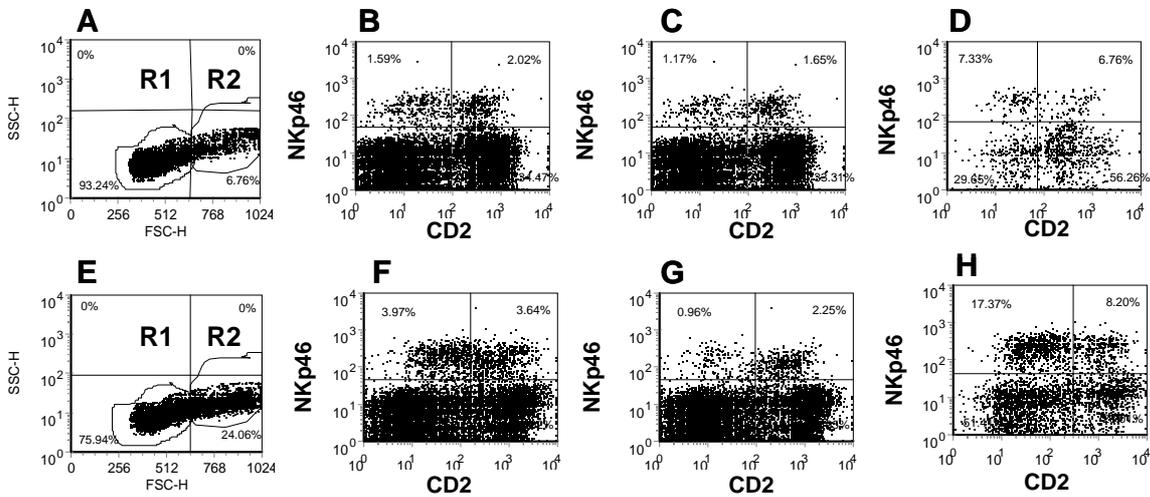
Fig. 5. Representative dot plot profiles showing the expression of NKp46 on CD2- and CD2+ NK cells in cultures of cells in the presence absence of PPD. A – D, cells cultured without antigen. B and F: selective gates placed on R1 (small cells), R2 (large cells). C and G: selective gates placed on R1 to show expression of NKp46 on CD2- and CD2+ NK cells. D and H: selective gates placed on R2 to show expression of NKp46 on CD2- and CD2+ NK cells. Gating shows NK cells have been activated in antigen stimulated cultures.

To establish whether the null cells detected in the present studies were NK cells, the NKp46 specific mAb, AKS1 was used in 3 color FC with anti-CD2 (MUC2A) and anti- chain (GB21A) mAbs. Analysis verified the null cells were actually NK cells (data not shown). As shown in Fig. 5, NK cells were activated and proliferated in cultures of cells from the two calves. The proportion of CD2$^-$ NK cells increased in relative proportion to the CD2$^+$ population. Activated NK cell expressed both CD25 and CD26 (data not shown).

DISCUSSION

The findings presented here summarize and extend ongoing studies on the immune response to MAP. Until now, it has been difficult to characterize the response using in vitro assays involving the use of tritiated thymidine. Multi-parameter flow cytometry has provided a way to circumvent this problem. With this method, all populations of cells in the culture can be phenotyped and monitored for changes in activation status

during culture. Electronic gating on side and forward light scatter affords a way to correlate changes in cell size with activation and proliferation of cells in the absence and presence of antigen. The frequency of each population can be assessed using side scatter versus a given fluorescence channel. Selective gating on specific subsets of cells permits analysis of the expression of molecules upregulated on activated cells. In combination with gating on small and large cells, it is possible distinguish memory T cells from naïve T cells and characterize the activation status of memory T cells. Using multicolor FC, we have been able to examine the complexity of cultures of cells and monitor changes in the composition in the presence and absence of antigen. Analysis has shown that NK and   T cells can comprise a high proportion of activated proliferating cells in both unstimulated and antigen stimulated cultures. An unexpected finding is that NK cells are activated and proliferate in both the presence and absence of antigen. In some unstimulated cultures of cells from control and infected animals, NK cells comprised 40% or more of the cells in culture at 6 days. Where antigen specific proliferation occurred, NK cells usually comprised 10% or less of the cells in the culture. Both $CD2^+$ and $CD2^-$ NK cells were present in the first and second study (Fig. 6). Selective gating on CD4 T cells showed CD4 memory T cells were the only cells that proliferated in the presence of antigens. Multiple membrane expressed molecules were identified that are upregulated only on CD4 memory T cells. In contrast to the first study, it was possible to detect a memory T cell response at 3 weeks PI. Selective gating on small and large lymphocytes showed activation molecules were expressed on large proliferating cells. In both the first and second study, it was difficult to detect a $CD8^+$ T cell response to MAP antigens. The frequency of $CD8^+$ T cells was always low.

The studies show that oral exposure to MAP leads to uniform infection as assessed by the appearance of a specific immune response to PPD and SAg, findings similar to those obtained by intratonsillar exposure to MAP.(Waters et al., 2003) By 6 months, a persistent proliferative response dominated by $CD4^+$ T was observed to both antigens. Although still present, NK cell proliferation appeared to diminish with increase in age of the animals. This made it possible to detect an antigen specific CD8 memory T cell response.

In conclusion, multicolor flow cytometry has allowed us to analyze the composition of cell cultures from uninfected animals and animals infected with MAP and detail the events that occur following antigen stimulation. Importantly, we have established that it is possible to detect a memory T cell response to antigens as early as 3 weeks following oral exposure to MAP. Further studies are now in progress to characterize memory T cells during the early phases of infection and identify factors associated with the development of protective immunity that is present in the silent phase of infection.

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In vivo and in vitro characterization of *Mycobacterium avium* subsp. *paratuberculosis* mutants

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ABSTRACT

Identification of genes responsible for the pathogenesis of *Mycobacterium avium* subsp *paratuberculosis* (MAP) is important in understanding how this bacterium causes disease. A transposon mutagenesis system was developed to randomly deliver a transposable element (Tn*5367*) into the genome of the virulent wild type strain K-10. We obtained 13,500 independent mutants necessary for a 95% theoretical coverage of the genome. In this study, we focused on further *in vitro* and *in vivo* characterization of mutants 1F3, 40A9 and 4H2. We used these mutants in baby-goat and rodent infection experiments and *in vitro* cell culture assays. We focused on the initial critical steps in the pathogenesis: gut invasion and colonization *in vivo*, and phagocytosis and survival of MAP in macrophages. *In vivo*, no reduction in colonization was found in either goats or mice regardless of bacterial strain used for inoculation, samples were IS900 positive by PCR and acid-fast organisms were seen in samples of ileum, jejunum and mesenteric lymph nodes six months after inoculation. All three mutants maintained their ability to colonize the small intestine in both goats and mice. *In vitro*, we showed that MAP induces NO production using monocyte-derived bovine macrophages, BoMac macrophage cell line, and in the mouse macrophage-like cell line RAW264.7. Initial findings showed that all mutant strains induced less NO production relative to K-10. Macrophage infection and intracellular survival were also reduced for the mutant strains in comparison to K-10. Experiments to assess the overall virulence of MAP strains in goats and mice are in progress.

Key words: *Mycobacterium avium paratuberculosis*, transposon mutants, virulence, macrophages, mice, goats.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of a severe gastroenteritis in ruminants, known as Johne's disease. Johne's disease is prevalent in domestic ruminants worldwide, and has significant impact on the global economy. Natural infection by MAP is usually acquired in the first months of life. The main symptoms of the disease are diarrhea of varying degrees and weight loss secondary to granulomatous enteritis (Harris and Barletta, 2001). Infection seems to occur through the ingestion of MAP through contaminated milk or other food products by young animals. The MAP organisms target the lymphoid tissue of the intestinal mucosa, and past evidence has indicated that the bacterium enters the host by the M cells at the Peyer's patches (Momotani et al., 1988). Once in the submucosa, MAP infects macrophages and replicates intracellularly within granulomatous structures. Colonization and growth of pathogenic bacteria within the host tissue is a complex process involving recognition of the host by the bacteria, rapid reprogramming of the pathogen gene-expression pattern and timely expression of components that carry out specific interaction with host cells. These components may include adhesins, invasins, toxic proteins and virulence factors. Identification of genes responsible for the pathogenesis of MAP is important in understanding how this bacterium causes disease. Very little is known about the molecular basis of the infection, nor about the genes that allow MAP to target and survive in the lymphoid tissue of the intestinal mucosa and to replicate intracellularly within macrophages.

The development of genetic systems including transposon mutagenesis and reporter genes (Foley-Thomas et al., 1995; Harris et al., 1999; Williams et al., 1999; Harris et al., 2002) makes the application of the molecular version of Koch's postulates feasible. Up to date, few candidate virulence determinants have been described (Secott et al., 2002; Bannantine et al., 2003; Paustian et al., 2005). The role of these genes and others in the pathogenesis of this microorganism requires functional analysis of mutant strains. In vitro assays contribute greatly to our understanding of bacterial pathogenesis, but they frequently cannot replicate the complex environment encountered by pathogens during infection.

In our previous studies, we used phAE94 as a vector to deliver a transposable element (Tn*5367*) randomly into the bacterial genome (Bardarov et al., 1997; Pelicic et al., 1997; Harris et al., 1999). The *aph* gene present in Tn*5367* provides a selectable marker to distinguish mutants carrying a transposon insertion. For this study, strain K-10, a virulent bovine MAP isolate with low number of *in vitro* passages (Foley-Thomas et al., 1995) was used to generate a transposon mutant bank with phAE94, and yielded a collection of approximately 5,000 mutants. Theoretically, mutants attenuated in virulence from this bank can be isolated for further testing in animal or cell culture model systems (Harris et al., 1999). Recently, the usefulness of the phAE94 delivery vector system was confirmed using the MAP strains 989 and ATCC 19698 (TMC 1613), in which approximately 2,000 mutants were generated (Cavaignac et al., 2000). The MAP strain K-10 mutant collection has been recently expanded by the Barletta laboratory to a representative bank of the 13,500 independent mutants necessary for a 95% theoretical coverage of the genome with transposon insertions in all non-essential genes (Barletta et al., 2003).

This paper describes the in vivo and in vitro characterization of mutants 1F3, 40A9 and 4H2 identified from this pool based on various screening strategies.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Mycobacterium avium paratuberculosis (MAP) strain K-10 and K-10 transposon mutant strains (Harris et al., 1999) were grown at 37^0C in Middlebrook 7H9 (Difco) supplemented with 0.2% glycerol, 10% OADC, 2 mg/L Mycobactin J (Allied Monitor, Lexana, KS), 0.05% Tween 80 and 1% cyclohexamide (M-OADC-TW broth). Strain K-10 (pWES4) expresses the green fluorescence protein (GFP) gene and the aminoglycoside phosphotransferase (*aph*) gene, which confers kanamycin resistance, as previously described (Harris et al., 2002). This strain was grown in M-OADC-TW broth; 50 µg/ml kanamycin was added. For K-10 mutant strains, 50 µg/ml kanamycin, 40 µg/ml L-tryptophan and vitamins were added. When grown on plates, agar was used to solidify the medium. Organisms were passed into fresh broth weekly to maintain log phase growth. Prior to macrophage infection, all MAP preparations were pelleted by centrifugation at 5,000g for 15 min, washed in phosphate-buffered saline (PBS) with 0.05% Tween 80, and declumped by at least 25 passages through a 25-gauge needle, and centrifuged at 250 *g* for 3 min to remove clumps. MAP organisms were serially diluted and counted on a bacterial hemocytometer. When required, organisms were heat-killed at exposure to 65^0C for at least 30 minutes. MAP strains and mutants and animal samples were tested for the presence of *IS900* and *hsp65* gene by PCR. For further confirmation of MAP identity, restriction fragment analysis (RFLP) using restriction enzyme *Pst*I was performed on PCR products of the *hsp*65 gene (Eriks et al., 1996).

Animal models

Eight male Saanen goat kids aged 7 to 14 days were obtained from a commercial herd with no history of paratuberculosis and CAE (caprine arthritis encephalitis). Their dams were tested and found serologically negative for paratuberculosis and CAE. Each pair was kept in a separate pen and the daily cleaning and handling procedures were designed to minimize cross contamination among the four groups. Each goat was given 1×10^9 bacteria mixed with milk-replacement 3 times a week for 9 weeks, a total of 27 times (Storset et al., 2001). Each pair was given a different strain of bacteria: K-10(pWES4), or one of the transposon mutants. Six months after the beginning of inoculations the goats were euthanatized, complete post mortem examination was done on all goats, and feces and tissue were collected for bacterial culture, for IS*900* PCR and histology. For mice experiments, BALB/C mice 2-3 days old were inoculated with K10 (pWES4), transposon mutants or PBS. 1×10^6 bacteria were given orally.

Delayed-type hypersensitivity test.
A delayed-type hypersensitivity (DTH) test was performed by an intradermal injection of the left flank of each goat with 0.1 ml of standardized avian tuberculin (PPDa), 0.1 ml of PPD from MAP (PPDp, Veterinærinstituttet Oslo, Norway) and 0.1 ml saline. Skin fold thickness at time of injection, measured with slide calipers, varied from 2.7 to 5.5 mm. The reaction was read after 72 hours. An increase of 3.0 mm or more was considered a positive reaction.

Bacteriological examination
Clinical samples (2 g) were mixed with 8ml 4% sterile NaOH and incubated at room temp for 15 min. After centrifugation 2500*g* for 15 min. the debris were mixed with 5ml 5% oxalic acid (Storset et al., 2001; Kruip et al., 2003). Following incubation and centrifugation as before, the debris were mixed with 4ml physiological saline. Three drops of the suspension were plated on 7H9 agar plates supplemented as described above. Visible colonies were tested for acid-fastness, and AF positive colonies were plated with and without mycobactin J. Acid-fast colonies with mycobactin J dependency were tested by *IS900* PCR to confirm identification as MAP.

Cells, infection of macrophages and phagocytosis assays.
A bovine macrophage cell line (BoMac), peripheral blood bovine monocytes, monocyte derived bovine macrophages, and RAW 264.7 murine macrophage-like cell line were used in this study. All cells were cultured and maintained in complete media; RPMI-1640 medium supplemented with 4 mM L-glutamine, 1% penicillin/streptomycin, 10% heat inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), and 25 mM HEPES (Gibco) in a 5% CO_2 humidified incubator at 37°C (Stabel and Stabel, 1995). Peripheral blood bovine monocytes were isolated from peripheral heparin-anticoagulated blood samples collected from normal paratuberculosis-free adult Holstein cows. Blood was placed over Ficoll-Hypaque (Sigma), centrifuged 3500 rpm for 30 min, and peripheral mononuclear cells were separated and placed in complete media. For preparation of monocyte-derived macrpohages, the mononuclear cells were allowed to adhere to a 75-mm^2 plastic tissue flask for 90 min, and nonadherent cells were washed off. Adherent cells were left to mature undisturbed for 6 days (Weiss et al., 2001). Cells were harvested by scraping or by incubating with 0.05% trypsin/EDTA (1:5000) solution (Biological Industries, Kibbutz Beit Haemek, Israel) after which macrophages were resuspended in complete media without penicillin/streptomycin (AB-free medium) and enumerated with hemocytometer. Viability was assessed by trypan blue exclusion. Macrophages were seeded at 1.5 X 10^5 cells/ml onto 24 well tissue culture plates, sometimes containing 16 mm round microscope coverslips to form a non-confluent monolayer, or 1.0 X 10^6 cells/ml onto 96 well flat bottom tissue culture plates, unless indicated otherwise below.

Measurement of NO.
The production of NO was determined by assaying culture supernatants for nitrite accumulation by the Griess reaction (Zhao et al., 1997). Briefly, aliquots (50 µl) of the supernatants were harvested and distributed into 96-well microtiter plates. Equal volumes of the Griess reaction solutions (1% sulfanilamide in 2.5% phosphoric acid proceeded by 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) were then added. The plates were centrifugated (400*g*) for 15 min, and the reaction was allowed to proceed for another 15 min at 37^{0}C. The absorbance at 540 nm was measured with a model 808 BioTek enzyme-linked immunosorbence assay reader. The amounts of NO in the samples were then calculated from a sodium nitrite standard curve prepared for each experiment.

Statistical analyses.
Each experiment was performed in duplicate or triplicate and repeated 3 times or more.

Differences within experimental groups were analyzed by Student t-test and differences between experimental groups were analyzed by one-way analysis of variance (comparison of means). Statistical analyses were performed using the SPSS 10.0.1 (SPSS Inc., Chicago IL, USA) and *P*<0.05 was considered significant.

RESULTS

Animal models

Two of the baby goats used in this experiment were inoculated with one of 4 strains of MAP: K-10(WES4), 1F3, 40A9, and 4H2. Strain 1F3 displays a halo-less phenotype on siderophore-detection medium; strain 40A9 is a random transposon mutant that grows faster on D-cycloserine; and strain 4H2 is a colony morphology mutant (Barletta lab). Six months after the beginning of inoculations a DTH test was performed. In all pairs except the one inoculated with 1F3 at least one goat had a positive reaction to PPDa and PPDp (Table 1). In the pair inoculated with 4H2 both goats reacted strongly. DNA extracted from all tissues for the presence of *IS900*. In all goats, at least one tissue sample was positive by *IS900* PCR (Table 1). Acid-fast organisms were found in formalin fixed sections collected from all goats (Table 1).

Newborn mice were orally inoculated with K-10 (pWES4) and 4H2. 11 weeks after inoculation only one of the 4 mice tested was IS*900* positive by PCR. 3 weeks later all mice tested were found positive by PCR but not histologically. IS*900* positive bacteria were cultured from intestinal tissue samples of mice inoculated with 4H2. No pathological changes could be detected on histological examination of the gut 9 months after inoculation.

Table 1. Results of diagnostic tests and pathological findings for goats 6 months after infection with MAP K-10 strain and transposon mutants of K-10

MAP strain	Goat no.	Intestinal tissue			DTH		Serology ELISA	Fecal culture
		ZN staining	IS900 PCR	Histopathology	PPDa	PPDp		
K-10	198	+	+	-	-	-	-	-
K-10	189	+	+	-	+	+	-	-
1F3	178	+	+	-	-	-	-	-
1F3	129	+	+	-	-	-	-	-
40A9	200	+	+	-	-	-	-	-
40A9	163	+	+	-	+	+	-	-
4H2	191	+	+	-	+	+	-	-
4H2	168	+	+	-	+	+	-	+

ZN = Ziehl-Neelsen staining; DTH = delayed-type hypersensitivity test; PPDa = purified protein derivative avium; PPDp = purified protein derivative paratuberculosis.
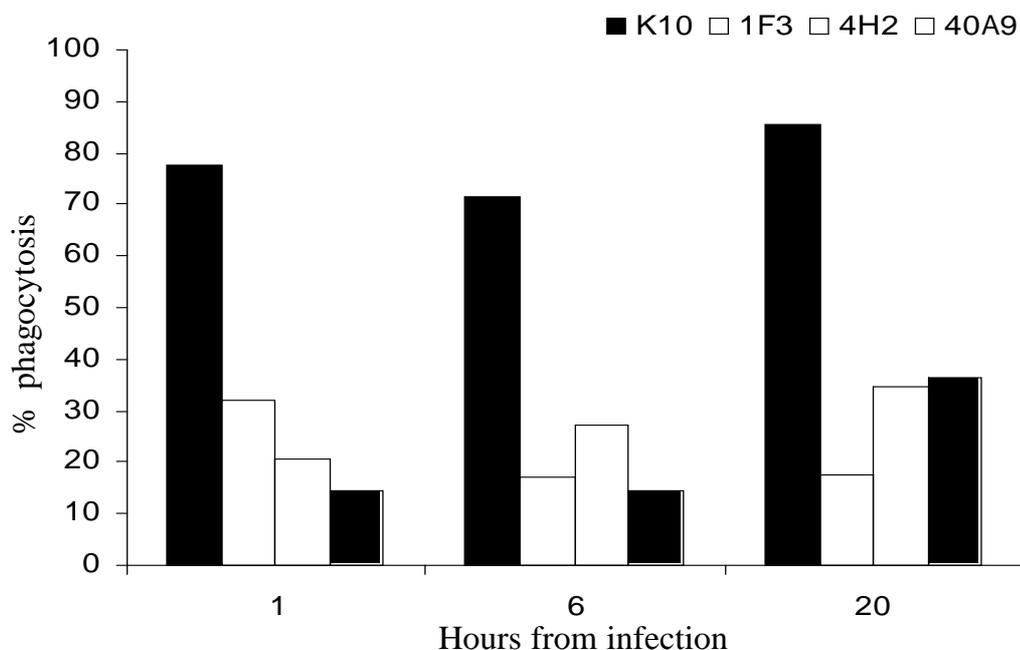
Fig. 1. Percent of cells infected with MAP K-10 strain and transposon mutants 1F3, 4H2 and 40A9 at different times after infection. 10^5 cells of the BoMac cell line were infected with 10^6 FITC-labelled bacteria (MOI 1:10). At 1, 6, 20 hours after infection cells containing fluorescent organisms were counted via fluorescence microscopy. At least 100 cells at 4 different fields were counted for each trial.

Macrophage phagocytosis assays
The rate of phagocytosis by BoMac cells was considerably different between MAP K-10 and the transposon mutants 1F3, 40A9 and 4H2 (Fig 1). Phagocytosis of the transposon mutants was significantly lower when compared to the wild type K-10 strain over all time points. Similar results were obtained using the RAW 264.7 murine cell line (data not shown). This effect was not a non-specific transposon effect since many other mutants were similar to K-10 (Harris NB and Barletta RG, unpublished results).

Induction of NO system in macrophages by MAP
Several MAP strains and mutants were tested for dose dependent induction of NO synthesis in bovine and murine monocytes and macrophages. A dose dependent synthesis of NO was observed in all cells infected by MAP organisms, although considerable differences were observed across MAP strains and cells. MAP infected monocyte-derived bovine macrophages released significantly higher levels of NO than bovine monocytes. RAW 264.7 cells produced very high levels of NO in response to MAP while lower levels were measured in monocyte-derived bovine macrophages (data not shown). Significant differences were observed between K-10 and the mutant organisms which elicited lower levels of NO. Although K-10 consistently induced higher levels of NO, induction of NO by the transposon mutants was inconsistent between the two cell types. Mutant 1F3 induced lower levels of NO in the murine cell line while mutant 40A9 induced lower levels of NO in the bovine primary macrophages.

DISCUSSION

We have tested the capacity of three MAP K-10 transposon mutants (altered colony morphology, defective siderophore production and D-cycloserine resistant) and have found that these mutants as well as GFP-expression MAP K-10 (pWES4) were able to infect and colonize the gut of baby goats. Due to the long incubation time of the infection in goats we were unable to determine if these strains are also equally virulent. The colony morphology mutant, 4H2, was the only strain we were able to culture from a fecal sample of an inoculated goat and the gut of inoculated baby mice. When compared to K-10, less phagocytosis and less NO production occurred for all 3 mutants. Infection of macrophages and survival in macrophages are the hallmarks of mycobacterial pathogenesis (Honer zu Bentrup and Russell, 2001; Hope et al., 2004). Numerous studies have demonstrated the important role of the NO system in the pathogenesis of mycobacterial diseases (Cooper et al., 2002). This system probably plays a dual role: limiting the growth of the organism and controlling the host's immune response. Although previous studies demonstrated the ability of MAP to induce NO production by macrophages, this effect was considered insignificant (Zhao et al., 1997; Jungi et al., 1999; Hostetter et al., 2005). Induction of the NO system by MAP seems to differ considerably among strains and species. Our preliminary data indicate that the NO system does not affect survival of MAP in macrophages; however, it still unclear if this system plays any role in the regulation of the immune response to the organism. The exochelin deficient mutant 1F3, an extremely slow growing organism in vivo, was able to infect and colonize the gut of baby goats. The reason for the significant of lack of intradermal DTH response to PPDp and PPDa in the 1F3 inoculated animals is still unknown, although it might indicate a defective cell-mediated immune response to this organism. Work to sequence the transposon insertion sites in these mutants is currently underway to enable us to explain these mechanisms further.

ACKNOWLEDGEMENTS

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Experimental challenge model for paratuberculosis in red deer

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ABSTRACT

Paratuberculosis is an emerging problem in farmed red deer (*Cervus elaphus*) in New Zealand. Red deer may develop clinical disease as young as 8 months of age and herds may suffer outbreaks involving up to 25% of groups of young animals.

In order to study the pathogenesis, epidemiology and immunology of this disease in deer, we have developed an experimental infection model that closely mimics natural disease. It utilises oral challenge of 3 month old deer with approximately 10^9 live "deer/bovine" strain *M. paratuberculosis* recovered from the mesenteric lymph nodes of clinically affected yearling deer. In two studies involving a total of 59 young deer, clinical Johne's disease developed in 26% and 31% of deer, 5 to 11 months after challenge. The gross and microscopic appearance of the lesions and the immunological responses were indistinguishable from natural Johne's disease in red deer. This model has subsequently been used in vaccine and epidemiology studies.

Key words: deer, Johne's disease, *M. paratuberculosis*, experimental challenge model.

INTRODUCTION

Johne's disease (paratuberculosis) has emerged as a significant problem on deer farms in New Zealand and overseas (Mackintosh et al 2004). At least 6% of ~5000 deer farms in New Zealand are infected (de Lisle et al 2003). The disease causes sporadic losses in adult deer, as occur with Johne's disease in sheep and cattle, but a major difference in this species is the outbreaks of clinical disease in young animals (8-15 months old). These can involve 10-25% of animals on some farms. Current research is aimed at understanding the epidemiology of the disease to try to prevent these outbreaks. This paper describes the development of an experimental infection model intended to be used for efficient research into the epidemiology, immunology and control of Johne's disease in deer.

MATERIALS AND METHODS

At the end of March 2002, a group of 43 newly weaned 4 month old red deer fawns were moved onto an isolated part of the deer farm at AgResearch Invermay. The deer were challenged daily, for 4 consecutive days, with a dose of 10^9 cfu *M. paratuberculosis* organisms. The inoculum was prepared from live organisms recovered from lymph nodes of a clinically affected, yearling red deer. The number of bacteria was estimated using phase contrast microscopy and the confirmed retrospectively by plate culture. The isolate was confirmed as *M. paratuberculosis* and strain-typed as the "Bovine" using IS*900* and IS*1311* PCR techniques.

All animals were examined and weighed monthly for the first 3 months, then fortnightly for one month and weekly for the last 7 months of the trial. Blood samples were taken monthly throughout the trial. Lymphocyte transformation assays were carried out from March to November 2002; IgG1-specific antibody ELISA assays were carried out from May to March 2003. Two trials were completed using the oral inoculation model.

Deer that developed a clinical infection were euthanased early in the course of disease since Johne's disease is more acute in young deer than in adult livestock.

A presumptive diagnosis of Johne's disease was made when individual animals showed a loss of condition, especially over the loins and there was a loss of >10% liveweight over 2 weeks. This weight loss was usually preceded by a week or two of when no weight was gained at a time when their herd mates were gaining 1-1.5 kg per week. Affected animals developed soft semi-liquid faeces or diarrhoea. The remaining unaffected deer were killed 10-12 months post challenge. All animals were subjected to post mortem examination and tissue samples were taken for BACTEC culture and histopathology to confirm the diagnosis and to gauge the severity of the disease.

RESULTS

Trial 1
One deer died of malignant catarrhal fever and was excluded from the trial. The first clinical case of Johne's disease was diagnosed 5 months post challenge. A total of 11 clinical cases (26%) were diagnosed over the period 5-11 months post challenge (deer aged 9-15 months). All 11 clinically affected deer had gross lesions typical of Johne's disease, including enlarged ileo-caecal and jejunal lymph nodes and prominent cord-like lacteal ducts between the small intestine and the draining lymph nodes. Three animals had multiple small caseous lesions in the jejunal lymph nodes. Some, but not all, had obvious thickening of the jejunum and ileum. Eight had severe histopathological lesions, while the microscopic lesions in three deer were relatively mild.

Four of the 31 clinically unaffected deer had small caseous lesions in their jejunal lymph nodes. Five had relatively severe histopathological lesions, while 26 had mild histopathological lesions or were unaffected.

The immunological responses are shown in Fig.1 and 2 and Tables 1 and 2. It appears that the group mean IgG1 antibody responses peaked in October, and declined slightly after November due to the removal of the clinically affected animals that had the highest antibody levels.
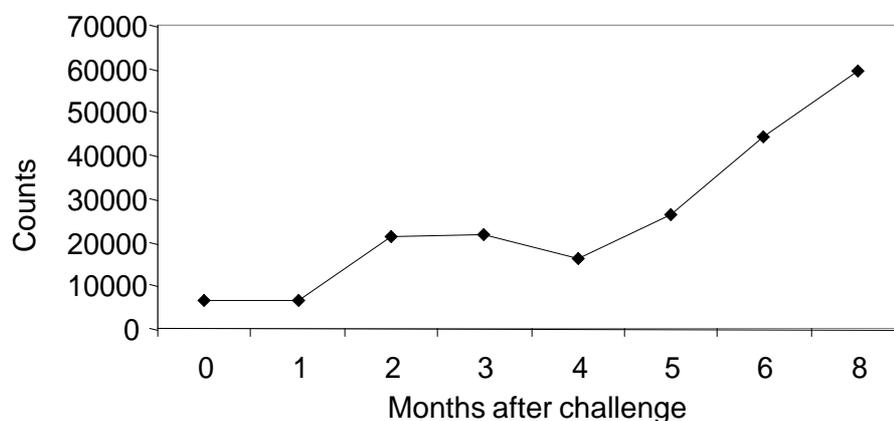
Fig. 1. Mean Johne's lymphocyte transformation assay responses

Table 1. Mean Johne's lymphocyte transformation assay responses (± standard error of the mean) by months after challenge and the number of deer remaining in the group.

Months after challenge	0	1	2	3	4	5	6	8
Mean LT response (counts)	6318	6255	21037	21679	16305	26310	43981	59478
No. deer	43	43	43	43	43	43	41	31
± SEM	853	1011	3058	3580	3684	4061	4279	6546
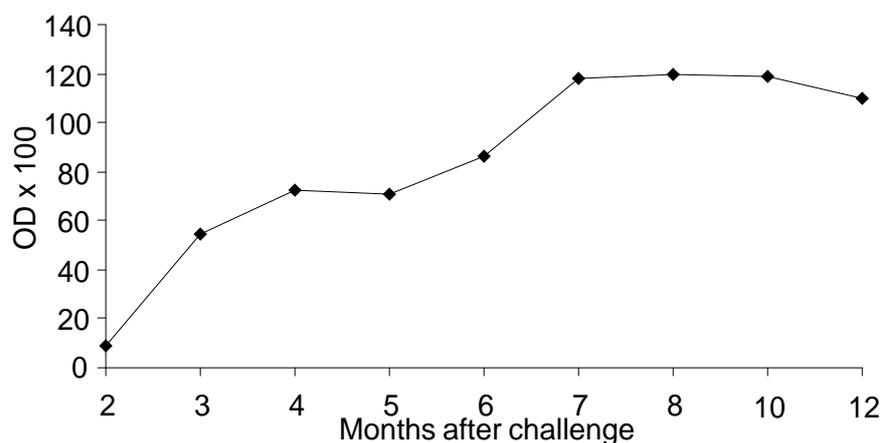
Fig. 2. Mean Johne's IgG1 ELISA responses

Table 2. Mean Johne's IgG1 ELISA assay responses (± standard error of the mean) by months after challenge and the number of deer remaining in the group.

Months after challenge	2	3	4	5	6	7	8	10	12
Mean IgG1 response (OD)	9	55	73	71	86	118	119	119	110
No. deer	43	43	43	43	41	37	31	20	11
± SEM	2.1	9.1	9.1	8.8	9.8	9.0	11.5	16.5	23.5

Trial 2
After the initial trial, the challenge model was used to investigate the effects of the size of the challenge and the strain of _M. paratuberculosis_. One of the dose rates was identical to that used in the first trial and 5/16 (31%) young red deer developed clinical Johne's disease, 6-9 months post challenge (see paper in this proceedings: Mackintosh et al "_M. paratuberculosis_ strain and dose response trial in red deer")

CONCLUSION

Natural outbreaks of Johne's disease may involve 10-25% of groups of 8-15 month old farmed red deer. This experimental infection model caused similar attack rates in this age group and produced disease and immunological responses indistinguishable from natural Johne's disease in red deer

This model should provide an efficient "natural" model to investigate epidemiology, immunology and vaccine efficacy. Because this model takes less than 12 months to complete, it is significantly more efficient than sheep or cattle models that take 2-3 years to complete.

ACKNOWLEDGEMENTS

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IFN- production from NK cells: implications for diagnostic testing

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Key words: NK cells, secreted mycobacterial antigens, IFN-

INTRODUCTION

The IFN-γ test is used to detect bovine tuberculosis and paratuberculosis at an early stage of the infection before the development of clinical signs. The rationale for measuring IFN-γ production as a diagnostic parameter is that T-cells, previously sensitised through the T-cell receptor/MHC complex, will recognise antigens specific for a particular infection. It has been demonstrated however that young calves especially often respond to both avian and bovine PPD in the IFN-γ test without having any evidence of infection (McDonald et al. 1999, Jungersen et al. 2002). An explanation for these observations can be that the exposure of the newborn animal to a variety of new antigens stimulate the immune system and may result in a higher degree of activated cells in peripheral blood. These activated cells could subsequently be more easily triggered to produce IFN-γ by innate mechanisms. The fact that young animals are more prone than adult cattle to elicit this non-specific IFN-γ production in response to PPD makes it unlikely that it is caused by environmental mycobacteria sensitisation. We have previously shown that neither CD4+, nor CD8+ nor δT-cells were responsible for IFN- production in non-infected cattle (Olsen and Storset 2001). A possible source is thus the NK cells that are involved in the early immune responses against intracellular pathogens and are known to secrete large amounts of IFN- . Bovine NK cells have recently been characterised and a monoclonal antibody against the NK cell specific receptor, NKp46, is available (Storset et al. 2003, Storset et al. 2004). The aim of the present work was to study the influence of NK cells on the IFN-γ production in young calves.

MATERIALS AND METHODS

Antigens.

A 14 kDa secreted antigen (MPP14) was purified from *M. avium* subsp. *paratuberculosis* as described previously (Olsen et al. 2000) Purified Protein Derivative (PPD) from *M. tuberculosis* was obtained from the National Veterinary Institute, Oslo, Norway. Recombinant ESAT-6 was kindly provided by Dr. John Pollock Veterinary Science Division, Stormont, Belfast and Dr. Peter Andersen, Statens Serum Institute, Copenhagen. Native MPB70 was provided from Prof. Morten Harboe, The National Hospital, Oslo. MPP14, ESAT-6 and MPB70 were used at a concentration of 2 μg/ml and PPD at 10μg/ml.

Animals.
Clinically healthy Norwegian Red dairy cattle in areas free from paratuberculosis and bovine tuberculosis were used in the present study.

IFN-γ production in whole blood.
Heparinised whole blood was dispensed into 24-well tissue culture trays (1 ml/well) and stimulated with purified MPP14, ESAT-6, MPB70 and PPD and incubated at 37°C in 5% CO_2 in air for 24 hours. The plasma was removed and assayed for IFN-γ using the bovine IFN-γ ELISA (Bovine IFN-γ EASIA, Biosource, Nivelles, Belgium) according to the manufacturer's instructions.

Depletion of NK cells.
Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by density gradient centrifugation (1150 x g, 20 min) on Lymphoprep (Nycomed Pharma, Oslo, Norway). NK cells were removed using MACS system with an LD column (Miltenyi Biotec Ltd., Bisley, UK). The cells were stimulated with MPP14, ESAT-6, PPD or medium alone and incubated at 37°C in 5% CO_2 in air for 24 hours. The supernatant was assayed for IFN- .

Intracellular staining for IFN-γ
Performed as described previously (Olsen and Storset 2001)

## RESULTS

Altogether 31% of the animals had an IFN- response to ESAT-6, 50% to MPP14 while 37% responded to PPD. In contrast, no IFN- response was detected in response to MPB70. In general, the animals responding to MPP14 also responded to ESAT-6 and PPD. ESAT-6 is present in the *M. tuberculosis* complex and has also been detected in *M. kansasii* and *M. flavens* (Harboe et al. 1996) while MPP14 was detected by Western blotting in the *M. avium* complex and *M. scrofulaceum* (Olsen et al. 2000).

To identify the cells producing IFN-γ, intracellular staining for IFN- together with labelling for surface markers and analysis by flow cytometry was used (Olsen et al. 2005). The method identified NK cells as the main IFN- producing cell-type while little IFN- was produced by CD4+ cells (Fig. 1). Furthermore PBMC from responding animals were depleted of NK cells using a monoclonal antibody directed against NKp46, followed by secondary antibodies coupled to magnetic beads. The removal of NK cells completely abolished the IFN- production in response to MPP14, ESAT-6 and PPD confirming that NK cells are a source for IFN- production.

DISCUSSION

The IFN- test has been used in several countries to diagnose early stages of bovine tuberculosis. The use of specific proteins like ESAT-6 has clearly increased the specificity of this test (Pollock and Andersen 1997, Buddle et al. 2003). However, it is widely recognised that the test cannot be used in young animals due to non-specific IFN- production (McDonald et al. 1999, Jungersen et al. 2002). This was confirmed in the present study where one third of young cattle from areas free from both bovine tuberculosis and paratuberculosis responded with IFN- production to various antigens was tested. It is unlikely that such a large percentage of study animals were sensitised to mycobacteria harbouring both MPP14 and ESAT-6, and this suggested that other mechanisms for IFN-γ production were present
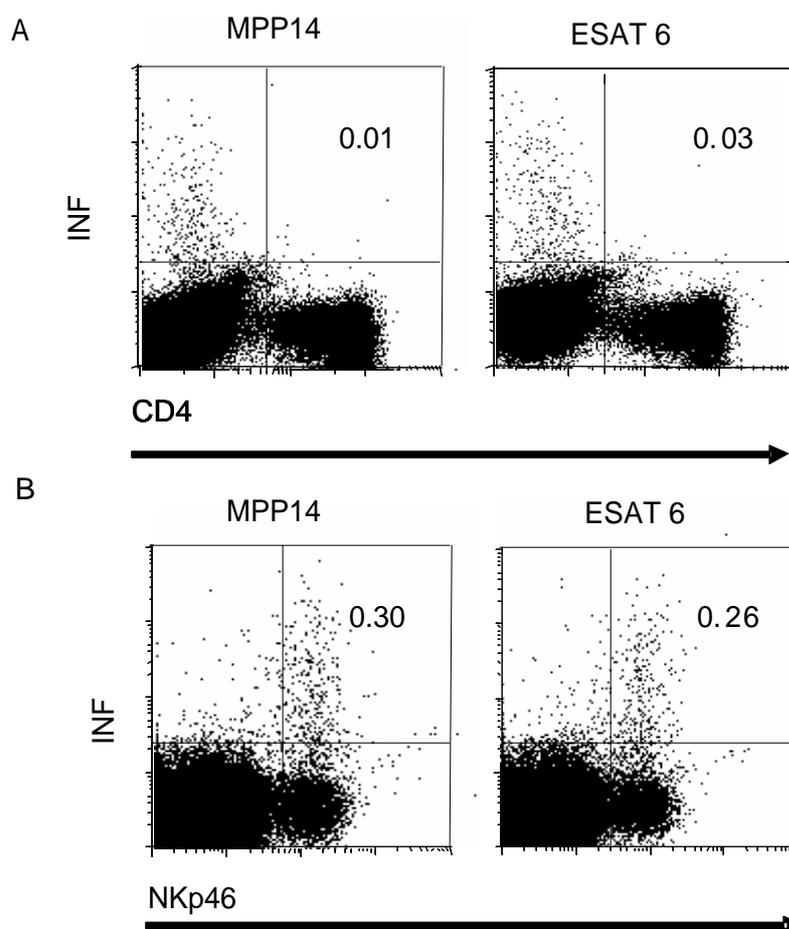
Fig. 1. Flow cytometry plots showing IFN- production by NK cells. Whole blood was stimulated with MPP14, ESAT-6 or left unstimulated for 6 hours. The cells were labelled with mAbs against; A) CD4 and B) the NK-cell marker NKp46 followed by permabilisation and labelling for intracellular IFN- . Numbers in upper right quadrant indicate the percentages of total lymphocytes that are IFN- producing CD4+ (A), IFN-γ producing NK cells (B).

In conclusion, our results demonstrated that NK cells are a source of innate IFN- production, and this provides an explanation for the problems encountered in the young calves. This is also in agreement with the finding that young calves had the highest proportion of NK cells in peripheral blood (Kulberg et al. 2004). Consequently, it is likely that NK cells are responsible for the majority of the non-specific IFN-production observed in young cattle. Methods that inhibit IFN- production from NK cells would therefore lead to an improvement of the IFN- test for detection of bovine mycobacterial diseases. This should be a focus of further research in addition to the identification of specific antigens from *M. a. paratuberculosis.*

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Immunohistochemical detection of TGF-β in paratuberculosis granulomatous lesions

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ABSTRACT

"In situ" detection of TGF-β in different types of lesion associated with MAP infection was completed using histochemical methods. Samples of intestine (ileocaecal valve, ileum and jejunum) with and without lymphoid tissue and mesenteric lymph nodes were obtained from lambs and calves experimentally infected with MAP. The Avidin-Biotin Peroxidase technique was employed using a polyclonal antibody against human TGF-β. Lesions present in the samples were classified as: focal (formed by small granulomas, with no or very low number of mycobacteria located in the lymphoid tissue and seen in the initial or latent stages of the infection); and diffuse forms (composed of an extensive granulomatous infiltrate located in the lymph nodes and intestinal lamina propria, showing moderate to large amounts of bacilli and seen in the advanced stages of MAP infection). In all the cases, positive immunolabelling was absent or seen sporadically in macrophages forming focal granulomas, both located in the lymph node or intestinal lymphoid tissue. However, in diffuse granulomatous lesions present in the intestinal mucosa, the majority of macrophages were TGF-β positive and showed a high intensity of staining. A clear relationship was seen between the presence of high numbers of bacteria in the cytoplasm and the intensity of the staining. These results suggest that high levels of TGF-β could be related to the inability of macrophages to kill MAP and thus the progression of the infection. Low levels of TGF-β, seen in focal lesions, could be related to the initial phases or forms of resistance of the infection.

Key words: Pathology, granulomas. immunohistochemistry. TGF-β.

INTRODUCTION

Paratuberculosis is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp *paratuberculosis* (MAP). Following oral infection, MAP invades intestinal macrophages and are capable of resisting host defences and multiplying intracellularly to reach very high numbers. Such events may take years to develop and are usually accompanied by marked inflammatory changes in the intestine including a macrophage infiltration. Many of these recruited cells subsequently become infected (Chiodini et al., 1984).

A wide variability in histological lesions has been recognized in paratuberculosis-infected animals (Clarke, 1997). Lesions associated with natural paratuberculosis have been divided into different categories based on the location of lesions in relation to intestinal lymphoid tissue, severity, inflammatory cell types and numbers of mycobacteria present (Pérez et al., 1996; González et al., 2005) and related to the clinical status of the animals. In those showing clinical signs, diffuse lesions with three different pathological forms were described, the "paucibacillary" form, in which the inflammatory infiltrate is composed of lymphocytes with some macrophages but few, if any, mycobacteria, the "multibacillary" form, in which macrophages, filled with numerous mycobacteria are the main inflammatory cell, and the "intermediate", showing features of both types (Pérez et al., 1996; Clarke, 1997; González et al., 2005). Besides this, in animals with no clinical signs or gross lesions, "focal" lesions, formed by small, well-demarcated granulomas located exclusively in the intestinal lymphoid tissue or mesenteric lymph nodes and with no or scant acid-fast bacteria (AFB) were also described (Pérez et al., 1996; González et al., 2005).

The host immune response has been thought to be an important factor in the development of the different lesional forms seen in MAP infected animals (Clarke, 1997). The macrophage has a central role in mycobacterial killing, antigen processing and presentation, and directing inflammatory and immune processes (Alzuherri et al., 1996). These mechanisms are influenced strongly by macrophage cytokines, such as transforming growth factor β (TGF-β). This is an immunomodulatory cytokine that has a suppressor effect on the cellular immune response at different levels (Letterio and Roberts, 1998). A decrease in IFN-γ production, an essential factor for containment of mycobacterial infection (Othieno et al. 1999), has been associated with an increase in the expression of TGF-β.

The main goals of this work were the evaluation of the "in situ" expression of TGF-β assessed by immunohistochemical methods in granulomatous lesions associated with MAP infection, and to study the relationship between lesion type and the amount of AFB present in the lesions.

MATERIALS AND METHODS

Animals and tissue samples
Tissue samples were obtained from 4 calves and 5 lambs experimentally infected with MAP together with 8 adult cows and 7 sheep with natural paratuberculosis infection. All of them had been previously confirmed as infected by MAP by bacteriological isolation from feces and PCR.

For this study, a total of 40 samples from the ileocaecal valve and ileum (15 ovine and 25 bovine) and 29 from the caudal jejunal mesenteric lymph node (13 ovine and 16 bovine) were employed. They had been fixed in 10% buffered formalin and embedded in paraffin wax and 4μ sections stained with haematoxylin-eosin (HE) and Ziehl-Neelsen method for acid-fast bacteria (AFB).

The amount of AFB present in the lesions were subjectively scored from + (solitary or very few bacilli present in the granuloma) up to +++ (the majority of the cytoplasm of the macrophages filled with AFB).

Immunohistochemical studies (IHC)
Selected sections were examined by the avidin-biotin complex (ABC-P) method, with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) following the instructions of the manufacturer. The following reagent was used as primary antibody: chicken IgG anti-human TGFβ serum (R&D Systems, Minneapolis, USA) at a dilution of 1 in 150. The specificity of the technique was controlled by (1) omission of the first specific antibody and replacement by normal chicken serum, (2) omission of the second biotinylated antibody, (3) omission of the ABC complex, and (4) omission of diaminobenzidine. All controls gave negative results.
Positivity was subjectively scored from + (few cells immunolabelled showing a weak reaction) up to +++ (strong postivity seen in a large number of cells).

RESULTS

Lesions associated with MAP infection were detected in all the samples examined (Tables 1 to 4). They were classified as _focal_, formed by small groups of macrophages and giant cells, located exclusively in the intestinal lymphoid tissue or mesenteric lymph nodes; _multifocal_, with granulomas spreading to the lamina propria, without disrupting the normal architecture of the area and showing a variable number of AFB, or _diffuse,_ characterized by a severe and diffuse granulomatous infiltrate in the intestinal wall that caused a marked thickening of the mucosa in several parts of the intestine. According to the nature of the inflammatory infiltrate and the amount of AFB present, diffuse lesions were divided in "_multibacillary_", formed mainly by macrophages and large numbers of AFB,"_paucibacillary_" showing large numbers of lymphocytes together with some macrophages and few or none AFB, and "_intermediate_", seen as mixed forms between the two diffuse types.

As it can be seen in tables 1 to 4, the antibody against TGF-β employed was able to stain macrophages and giant cells both in bovine and ovine tissues. A close relationship has been observed between the amount of AFB and the intensity of the staining. In focal lesions (Fig 1 to 3), located both in the intestinal Peyer´s patches or mesenteric lymph nodes, the immunolabelling of macrophages or giant cells forming the lesion was absent or scant (in these cases, they were one or few AFB). A similar result was found in isolated granulomas found in the lamina propria, forming multifocal lesions. Diffuse multibacillary lesions, with high amount of AFB, showed an intense immunorreaction (Fig 4), whereas in diffuse paucibacillary forms, with none or few bacilli, immunostaining was absent or sporadic (Fig 5). In the diffuse intermediate forms, the intensity of the staining varied according to the amount of AFB present (Fig 6).

Table 1. Evaluation of the AFB amount and TGF-β immuno- staining according to the lesional type, in the bovine lymph node sections. In brackets, number of sections showing the indicated scoring.

Type of lesion Lymph node	No of examined sections	Amount of AFB by ZN	Intensity of IHC. TGFβ
Focal	6	+ (1) 0 (5)	+ (1) 0 (5)
Multifocal	5	++ (2) + (3)	++ (2) + (3)
Diffuse multibacillary	1	+++ (1)	+++ (1)
Diffuse paucibacillary	3	+++ (1) + (2)	+++ (1) + (2)
Diffuse intermediate	1	+ (1)	+ (1)

Table 2. Evaluation of the AFB amount and TGF-β immuno- staining according to the lesional type, in the bovine intestinal lamina propria sections. In brackets, number of sections showing the indicated scoring.

Type of lesion Lymph node	No of examined sections	Amount of AFB by ZN	Intensity of IHC. TGFβ
Focal	4	0 (5)	0 (5)
Multifocal	4	+ (2) 0 (2)	+ (2) 0 (2)
Diffuse multibacillary	2	+++ (2)	+++ (2)
Diffuse paucibacillary	2	++ (2)	++ (2)
Diffuse intermediate	13	+++ (2) ++ (8) + (3)	+++ (3) ++ (7) + (3)

Table 3. Evaluation of the AFB amount and TGF-β immuno- staining according to the lesional type, in the ovine lymph node sections. In brackets, number of sections showing the indicated scoring.

Type of lesion Lymph node	No of examined sections	Amount of AFB by ZN	Intensity of IHC. TGFβ
Focal	5	+ (2) 0 (3)	+ (1) 0 (4)
Multifocal	2	0 (2)	0 (2)
Diffuse multibacillary	4	++ (3) + (1)	++ (3) + (1)
Diffuse paucibacillary	2	0 (2)	0 (2)

Table 4. Evaluation of the AFB amount and TGF-β immuno- staining according to the lesional type, in the ovine intestinal lamina propria sections. In brackets, number of sections showing the indicated scoring.

Type of lesion Lymph node	No of examined sections	Amount of AFB by ZN	Intensity of IHC. TGFβ
Focal	4	0 (4)	0 (4)
Multifocal	2	0 (2)	0 (2)
Diffuse multibacillary	7	+++ (7)	+++ (7)
Diffuse paucibacillary	2	0 (2)	0 (2)

DISCUSSION

The close relationship encountered between the amount of AFB and the intensity of the immunostaining by IHC for TGF-β suggest that a higher "in situ" expression of this cytokine is related to the inability of macrophages for limiting the multiplication of MAP. Other studies have found a higher expression of TGF-β, measured by real-time PCR, in peripheral blood mononuclear cells and ileum tissue samples from cattle showing the clinical stage of MAP infection, with respect to uninfected controls and subclinically affected animals (Coussens et al, 2004; Khalifeh and Stabel, 2004). This is in agreement with our results, since clinically affected animals usually show diffuse multibacillary or intermediate forms (Pérez et al., 1996; González et al., 2005), that we found to be related with marked immunostainings .

In paratuberculosis, as in other mycobacterial infections, a cell-mediated immune response is vigorously active during the early stages of disease. This type of immunity limits disease progression and is characterized by elevated antigen-specific IFN-γ production (Stabel, 2000). Animals in subclinical stages of disease shed low numbers of AFB in their faeces and, as infection progresses to a more clinical state, bacterial shedding is increased and IFN-γ production declines (Stabel, 1996, 2000). The decrease of IFN-γ has been associated with an increase in the expression of TGF-β (Letterio and Roberts, 1998; Khalifeh and Stabel, 2004). Animals having focal lesions without TGF-β expression mount strong cell-mediated immune responses, showing high levels of IFN-γ in the peripheral blood after the proper stimulus. Conversely, diffuse multibacillary forms are associated with low circulating levels of this cytokine (Pérez et al., 1999). The increase of the regulatory cytokine TGF-β seen "in situ" in our study in animals harbouring high levels of AFB suggests that a change in the balance of cytokines at the site of inflammation would contribute to the inability of the host to control MAP multiplication and infection progression.

In clinical cases of human Crohn´s disease, where MAP has been described by some as playing a role as aetiologic agent, an over-expression of TGF-β has been also observed (Friess et al., 1998). In other mycobacterial diseases such as human leprosy, macrophages forming lepromatous forms show stronger immunohistochemical labelling than those present in the tuberculoid forms, where immunostaining is usually absent (Goulart et al., 1996). These results support our findings, bearing in mind that focal and diffuse multibacillary forms have been compared to tuberculoid and borderline-lepromatous forms of human leprosy (Pérez et al., 1996; González et al., 2005).

CONCLUSION

In paratuberculosis granulomatous lesions, the expression of high levels of TGF-β in the macrophages assessed by IHC is closely associated with the numbers of AFB present in the cells. Thus, the over-expression of this cytokine would be related to the inability of the macrophages to limit the multiplication of MAP. These results confirm the latent character of the focal lesions, in which the host controls the progression of the infection, as opposed to diffuse forms that are associated with clinical signs and an increased expression of TGF-β.

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Ag85 homologues from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens in experimentally infected cattle

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ABSTRACT

The characterization of protective antigens is essential for the development of an effective, sub-unit based vaccine against paratuberculosis. Surface-exposed and secreted antigens, present abundantly in mycobacterial culture filtrate (CF), are among the well-known protective antigens for *M. tuberculosis* and *M. bovis*. In order to study the vaccine potential of CF in Johne's disease, *M. avium* subsp. *paratuberculosis* (MAP) ATCC 19698 was first adapted to grow as a surface pellicle on synthetic, protein-free Sauton medium and CF was prepared from 4 week old cultures. Strong proliferative and IFN-γ responses against MAP CF could be detected as early as 4 weeks post infection (p.i.) in PBMC cultures from a bull infected by the intravenous route with 10^8 CFU of MAP ATCC 19698. Analysis of the immune response against MAP CF separated by electro-elution demonstrated that these early bovine T cell responses were mostly directed against fractions in the 30 kDa region, containing homologues of the Ag85 complex of BCG with well-documented vaccine potential. We conclude that homologues of the antigen 85 family are immunodominant antigens of MAP in experimentally and naturally infected cattle, inducing strong proliferative and IFN-γ T cell responses. Their potential as possible vaccine candidates remains to be determined.

Key words: culture filtrate, mycolyl-transferase, Ag85, antigen

INTRODUCTION

M. avium subsp. *paratuberculosis* (MAP) causes Johne's disease, a severe gastroenteritis in ruminants, with a significant impact on the agricultural economy, particularly the dairy industry (Harris and Barletta, 2001). In the Belgian cattle population, paratuberculosis prevalence was determined by a serologic survey conducted from December 1997 to March 1998. This approach resulted in an estimated true herd prevalence of MAP infection of 6% (Boelaert et al., 2000). Cell-mediated immune responses seem to control the initial infection for a sustained period of time and clinical symptoms appear in cows only after a number of years, often after the first or second calving. This may be due to the enhanced intracellular multiplication of MAP caused by alterations in the hormonal milieu (Feola et al., 1999).

Vaccines consisting of whole killed or attenuated live MAP bacilli can provide partial protection by delaying fecal shedding and reducing the number of clinically affected animals, but they do not protect against infection. In the context of bovine tuberculosis (*M. bovis*) control and eradication programs, it is worth mentioning that animals immunized with these paratuberculosis vaccines develop positive reactions in the tuberculin skin test and therefore paratuberculosis vaccination is subject to approval by the veterinary services. Moreover, because of the impact on trading of living animals, farmers are reluctant to use paratuberculosis vaccination. It is clear that the development of an efficient paratuberculosis sub-unit vaccine that would not interfere with tuberculosis detection may be useful.

The precise MAP antigens that induce a protective immune response are poorly defined. Secreted and surface-exposed cell wall proteins are major antigens recognized by the protective immune response

against *M. tuberculosis* and *M. bovis*. Immunization with whole culture filtrate, a rich source of extracellular proteins, can protect mice and guinea pigs to some extent against subsequent challenge with the tubercle bacillus (Andersen, 1994; Pal and Horwitz, 1992). A key fraction of the secreted proteins in culture filtrates of *M. tuberculosis* and *M. bovis* BCG is the Ag85 complex. A 30-32 kD family of three proteins (Ag85A, Ag85B and Ag85C) (Wiker and Harboe, 1992), each of which possesses an enzymatic mycolyl-transferase activity required for the biogenesis of cord factor (trehalose-dimycolate). The proteins are encoded by three paralogous genes (*fbpA, fbpB* and *fbpC*) located in distinct regions of the bacterial genome (Content et al., 1991). Both Ag85A and Ag85B are promising candidates for future tuberculosis vaccines (Baldwin et al., 1998; Huygen et al., 1996; Kamath et al., 1999; McShane et al., 2004).

The genes encoding the three Ag85 components from MAP have been sequenced; at the protein level a 99% sequence identity with *M. avium* was found (Dheenadhayalan et al., 2002). Comparisons have also been made between the mature protein sequences of MAP Ag85 and *M. bovis* Ag85 (note that the latter differs from those of *M. tuberculosis* by only one residue in position 100 of the mature Ag85B protein). For the 85A protein (Map 0216) 82% of sequences are identical, for the 85B protein (Map 1609c) 86% and for the 85C protein (Map 3531c) 87% of amino acid sequences are identical between MAP and *M. bovis* (Dheenadhaylan et al., 2002).

Little is known about far on the immune recognition of culture filtrate (CF) antigens and of Ag85 homologues in particular, during MAP infection. Here, we report on CF and Ag85-specific T cell responses in cattle experimentally or naturally infected with MAP. Our results indicate that Ag85 homologues are immunodominant T cell antigens in MAP infection, inducing strong proliferative responses. Their vaccine potential for bovine paratuberculosis remains to be determined.

MATERIALS AND METHODS

Bacteria and Antigens.
MAP ATCC 19698 was purchased from the American Tissue Culture Collection (ATCC) and grown on solid Löwenstein-Jensen medium. Subsequently, cultures were maintained in liquid 7H9 medium supplemented with 10% OADC and mycobactin J (Allied Laboratories Inc, Synbiotics Europe) (2 μg/ml). After an intermediate passage for 6 months on solid potato soaked in synthetic, protein-free Sauton medium supplemented with mycobactin J, bacteria were grown as a surface pellicle on Sauton medium. MAP was grown for 4 weeks at 39°C, the culture filtrate was separated from the bacteria and CF proteins were precipitated by 80% saturated ammonium sulfate. Precipitate was extensively dialyzed against PBS. Bacteria were stored as concentrated pellets in Sauton with 20% glycerol at -70°C. *M. bovis* BCG and *M. tuberculosis* H37Rv culture filtrates were prepared from 2 week old cultures on synthetic Sauton medium as described previously. Native 30-32 kDa Ag85 was purified from BCG CF using sequential chromatography on Phenyl-Sepharose, DEAE Sephacel and Sephadex G75 (de Bruyn et al., 1987). PPD-B from *M. bovis* Vallée and PPD-A from *M. avium* (strain 42*)* were kindly given to us by Dr. J. Nyabenda from the Pasteur Institute Brussels.

Electro-elution and SDS-PAGE.
 Ammonium sulfate-concentrated CF from MAP was further concentrated by dialysis against poly-ethylene-glycol and 10 mg of CF was separated by electrophoresis on a 12 % SDS-PAGE. The acrylamide gel was electro-eluted into thirty 3 ml fractions using a Whole Gel Eluter (Biorad) according to manufacturer's instructions. Fractions were stored in PBS (1x)-0.5% glycerol. Whole CF and the electro-eluted fractions were analyzed on a 12% SDS-PAGE stained with silver nitrate. Ag85 homologues were visualized in Western blot analysis, using Ag85 specific Moab TD17.4 (Huygen et al., 1993).

Peptide synthesis.
Peptides spanning the entire sequence of the mature BCG Ag85A sequence (295 aa) were synthesized as 20-mers overlapping by ten residues, with the exception of the 19-mer 35-53 and the carboxy-terminal peptide spanning aa 275-295. Peptides spanning the entire mature BCG Ag85B

sequence (285 aa) were synthesized as 18-mers overlapping by 9 residues, with the exception of a 21-mer spanning aa 240-260 and the carboxy-terminal 15-mer 271-285. Peptides spanning the entire mature BCG Ag85C sequence (294 aa) were synthesized as 20-mers overlapping by ten residues, with the exception of two 18-mers (aa 31-50 and 41-60, residues 33 and 34 lacking in Ag85C) and the carboxy-terminal 14-mer 281-294 (Rosseels et al., 2005).

Experimental cattle infection.
One bull from the Friesian-Holstein breed originating from a herd officially free of bovine tuberculosis was infected at the age of 11 months by the intravenous route with 10^8 CFU of MAP (ATCC 19698) grown as a surface pellicle on synthetic mycobactin J supplemented Sauton medium for 4 weeks. This animal was persistently infected by MAP as indicated by the presence of viable MAP in the retropharyngeal lymph nodes at the day of culling, one year after infection. At no time was the bull sero-positive.

Proliferation assays in infected cattle.
Blood was collected in heparin tubes by venipuncture and rested for 24 hr at room temperature to decrease background proliferation of unstimulated cells. Proliferative responses were analyzed using a whole blood assay in 10% autologous plasma. Briefly, heparinized blood was diluted 1:10 in RMPI-1640 medium supplemented with 5.10^{-5} M 2-mercapto-ethanol. A volume of 180 μl of cells was mixed with 20 μl of antigen (final concentrations of CF and PPD: 25 μg/ml, purified Ag85: 5 μg/ml) in 96 well round–bottom microwell plates, and cultures were incubated in a humidified CO_2 incubator for 7 days. After 6 days, cells were pulsed overnight with tritiated thymidine (0.4 μCi/well) and collected on a Titertek Cell Harvester. Radioactivity recovered on the filters was counted in a Betaplate Liquid Scintillation Counter and results expressed as mean cpm ± SD of triplicate cultures.

Cultured (in vitro) interferon-γ production in infected cattle.
Cells were rested for 24 hr at room temperature as described for proliferation assays. Heparinized blood was centrifuged for 10 minutes at 1500 rpm, and plasma replaced by the same volume of RPMI-1640 supplemented with 10% FCS, penicillin, streptomycin and 5.10^{-5} M 2-mercapto-ethanol in order to reduce background IFN-γ levels in unstimulated cells. Leukocytes were counted in a Coulter Counter and whole blood cell cultures were adjusted to 10^6 WBC/ml in complete RPMI-1640 medium. A volume of 180 μl of cells was mixed with 20 μl of antigen in 96 well round–bottomed microwell plates and cultures were incubated in a humidified CO_2 incubator. PPD-A and PPD-B (Pasteur Institute Brussels) and CF were used at final concentrations of 25 μg/ml, synthetic peptides at 10 μg/ml, native Ag85 at 5 μg/ml and electro-elution fractions at a 1:10 dilution. After 6 days of culture, supernatants from at least three wells were pooled for each antigen and stored at -20°F until testing.

Bovine Interferon-gamma assay.
Bovine IFN-γ was determined using the Bovine IFN-γ EASIA cat.nr. KBC1232 (BioSource Europe S.A., Nivelles, Belgium). O.D. values were converted to pg/ml, using a standard curve of recombinant bovine IFN-γ (Serotec) at an initial concentration of 20 ng/ml.

RESULTS

Preparation of culture filtrate from MAP ATCC 19698 grown as a surface pellicle on synthetic Sauton medium.
MAP ATCC 19698 was adapted to grow as a surface pellicle on synthetic, protein free Sauton medium supplemented with mycobactin J. Comparison of the 4 week old MAP CF with the protein profile of a 2 week old *M. tuberculosis* H37Rv CF in SDS-PAGE, indicated that in the region of the 30-32 kD mycolyl-transferase (Ag85), one protein of approximately 30 kD was strongly expressed in the paratuberculosis CF. In tuberculosis CF, three protein bands were detected in this region, corresponding to Ag85B, Ag85A and Ag85C respectively (Pal and Horwitz, 1992) (Fig. 1A). Subsequently, proteins from the MAP CF were separated on the basis of decreasing molecular weight by SDS-PAGE and electro-eluted in 30 fractions. Fig. 1B shows the Sauton culture filtrate from MAP and its electro-elution profile (fractions 3 to 27). Western blot analysis of the electro-eluted fractions

from MAP CF with monoclonal antibody TD17.4 directed against Ag85 from *M. bovis* BCG, showed that Ag85 homologues were present in fractions 14 to 17 (Fig. 1C).
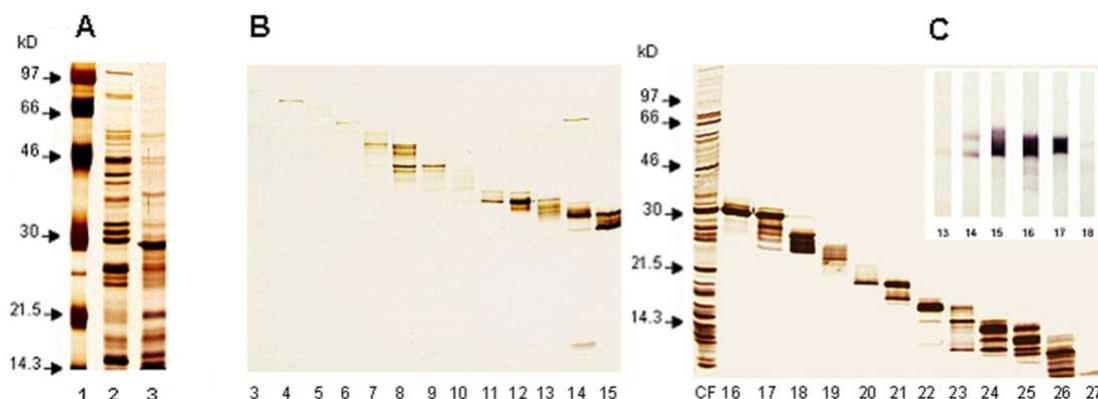
Fig. 1. A. Comparative SDS-PAGE (12%) of culture filtrate from *M.avium* subsp. *paratuberculosis* ATCC 19698 (lane 3) and *M. tuberculosis* H37Rv (lane 2) grown as surface pellicles on synthetic Sauton medium. Proteins were visualized by silver staining. Molecular weight markers are shown in lane 1. B. Electro-elution profile of MAP CF separated in 27 fractions. C. Western blot analysis of fractions 13-18 using Ag85 specific Moab TD 17-4.

Proliferative and IFN-γ responses against culture filtrate from M. avium subsp. paratuberculosis ATCC 19698 following intravenous infection with MAP ATCC 19698 in cattle.

As shown in Table 1 and Fig. 2, strong proliferative and IFN-γ responses against MAP CF could be detected as early as 4 weeks post infection (p.i.) in PBMC cultures from a bull infected by the intravenous route with 10^8 CFU of MAP ATCC 19698. Proliferative and IFN-γ responses against PPD-A and PPD-B were of comparable magnitude. Very strong proliferative and IFN-γ responses were also observed in response to native Ag85 purified from BCG CF. Responses to all these antigens were low to undetectable prior to infection. On the other hand, proliferative responses to *M. bovis* BCG CF were significantly lower than to MAP CF.

Table 1. Proliferative T cell responses in a 11 month old bull infected by the intravenous route with 10^8 CFU of *M.paratuberculosis* ATCC19698

Antigen	Pre-infection[a]	4 weeks p.i.
Control	495 ± 162	793 ± 305
CF *M. ptb*	529 ± 119	53,190 ± 8,670
CF *M. bovis* BCG	2,006 ± 849	14,060 ± 4,473
PPD-A	1,966 ± 679	112,540 ± 27,273
PPD-B	1,798 ± 677	89,818 ± 8,690
Ag85 BCG	721 ± 155	12,356 ± 1,269

[a]: mean cpm ± SD of proliferative responses of whole blood diluted 1:10 and cultured in triplicate for 7 days in the absence (control) or presence of the various antigens. ^{3}H thymidine was added to the cultures on the evening of day 6 and cells were harvested after overnight culture.
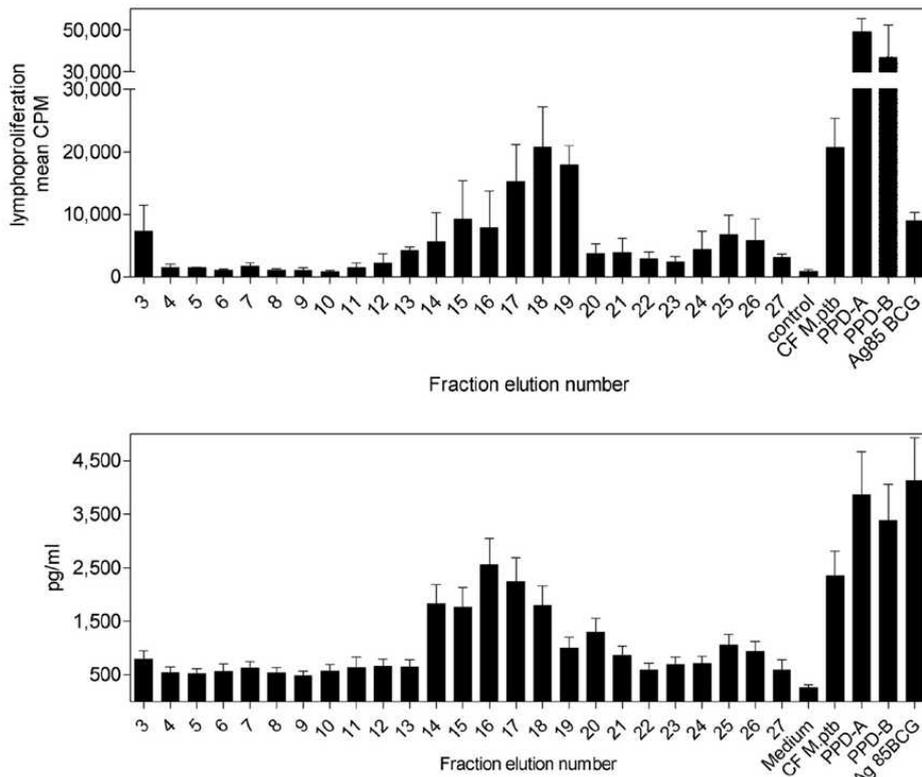
Fig. 2. Proliferative (mean cpm ± SD of 7 day cultures) and (*in vitro* day 6) IFN- (pg/ml) responses against electro-eluted fractions of *M. ptb* culture filtrate of PBMC (whole blood diluted 1:10) of a young bull infected with 10^8 CFU of *M. ptb* ATCC 19698 by the intravenous route 6 weeks previously.

Analysis of the immune response against MAP CF separated by electro-elution demonstrated that early bovine T cell responses (6 weeks p.i.) were mostly directed against fractions 15-19 containing the Ag85 homologues (Fig. 2). (Fractions from a different electro-elution that that shown in Fig. 1 were used, with an electro-elution profile slightly shifted to the right). At 4 months post infection, T cell repertoire broadened and positive responses were found against a wider range of fractions, throughout the electro-elution profile (data not shown).

Epitope mapping using overlapping synthetic peptides spanning Ag85A (20-mer peptides), Ag85B (18-mer peptides) and Ag85C (20-mer peptides) mature protein sequences from BCG indicated that Ag85B peptide 17, spanning aa 145-162, was strongly recognized by this animal (Fig. 3). Somewhat lower proliferative responses against the corresponding Ag85A peptide 15 spanning aa 141-160, were also detected, whereas responses to Ag85C peptides were very low. Lymphoproliferative responses to Ag85B$_{145-162}$ peptide in this intravenously infected animal were found consistently throughout the 12 month follow-up period (data not shown). Comparison of the sequence showed that this Ag85B$_{145-162}$ peptide sequence is identical in *M. bovis* BCG and MAP (YAGSLSALLD PSQGMGPS).
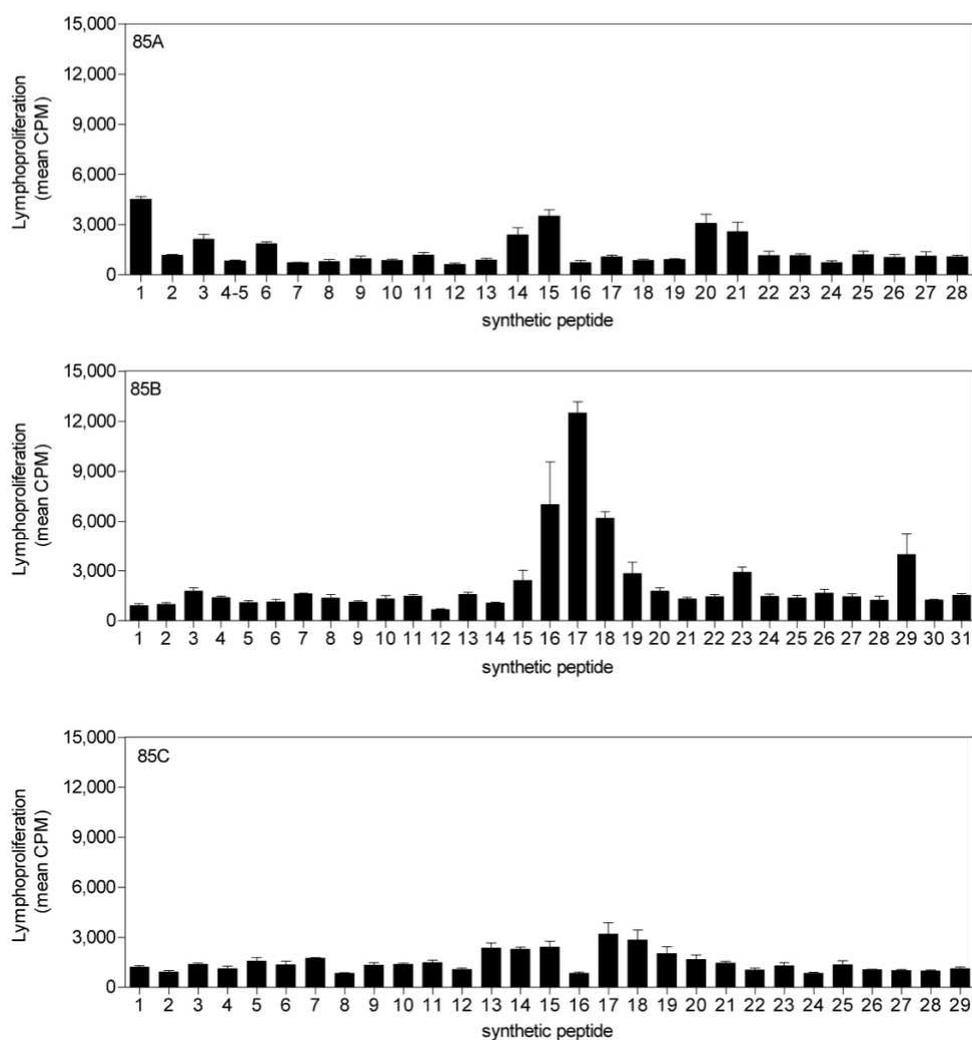
Fig. 3. Characterization of cross-reactive T cell epitopes of Ag85A, Ag85B and Ag85C in a young bull infected with 10^8 CFU of *M. ptb* by the intravenous route, using lymphoproliferation assays on quadruplicate cultures from PBMC (whole blood diluted 1:10) stimulated with overlapping synthetic peptides (10 g/ml) spanning the mature sequences of Ag85A, Ag85B and Ag85C from *M. bovis*.

DISCUSSION

Currently available vaccines against MAP are based on killed or live attenuated whole bacteria preparations. Animals vaccinated with these whole cell vaccines develop positive tuberculin skin reactions and are of lesser economic value, because trading of PPD positive animals is not permitted (EU Council Directive 64/432/EEC). Moreover, such animals may develop observable granulomas at the injection site, which can be a problem at inspection. An efficient subunit vaccine that would not interfere with bovine tuberculosis diagnosis would be very valuable in the management of paratuberculosis, but requires the characterization of immunodominant and protective antigens. A number of immunogenic proteins of MAP have been described (Harris and Barletta, 2001, but they have mostly been analyzed for diagnostic purposes (Bannantine et al., 2002) and little is known on their vaccine potential.

Here we have analyzed the immunogenicity of culture filtrate derived from MAP ATCC 19698. Very strong proliferative and IFN-γ responses could be detected in an experimentally infected bull as early as one month after infection against the MAP CF and against native Ag85, purified from BCG CF. Electro-eluted fractions from MAP CF containing the Ag85 homologues were also recognized very strongly early upon infection. Culture filtrate proteins such as Ag85 are produced by actively replicating,

live mycobacteria. In the phagosome of antigen presenting cells, these proteins can be secreted and processed very early upon infection, before actual destruction of the bacteria releases antigenic components from the cytoplasm (Harth et al., 1996). This may explain the very early recognition of Ag85 components by the immune system and also the confirmed vaccine potential of this protein family.

CONCLUSION

In conclusion, we show that experimental MAP infection of cattle elicits a rapid and strong T cell response against MAP culture filtrate proteins in general and against the Ag85 complex in particular. Analysis of the T cell response in cattle from a herd with reported cases of clinical Johne's disease has indicated that about 50% of the presumed naturally infected animals also show strong proliferative responses to the Ag85A and Ag85B homologues of this antigenic complex (Data not shown). Homologues of the antigen 85 family are immunodominant antigens of MAP in experimentally and naturally infected cattle, inducing strong proliferative T cell responses. Their potential as possible vaccine candidates remains to be determined.

ACKNOWLEDGEMENTS

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Sensitization with johnin purified protein derivative augments interferon- production in cows infected with *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

Measurement of secreted IFN- has proven to be a valuable tool for the detection of animals infected with mycobacterial pathogens, including *Mycobacterium avium* subsp. *paratuberculosis*. Previous reports have suggested that tuberculin skin testing can influence the performance of the IFN- assay. In the present study, healthy noninfected cows, and cows subclinically and clinically infected with MAP were administered an intradermal injection of johnin purified protein derivative and effects on secreted and intracellular IFN- were observed. Significant increases in secreted IFN- were noted in antigen-stimulated PBMC isolated from subclinically infected cows within 2 days of the skin test. Results for intracellular IFN- were more variable than those noted for secreted IFN- . Stimulation of peripheral blood mononuclear cells with medium only (nonstimulated), pokeweed mitogen, and JPPD resulted in transient elevations in intracellular IFN- , depending upon infection status of the animals and in vitro stimulant. When averaged over each bleeding day of the study, intracellular IFN- was higher within CD4+ and CD8+ subpopulations for infected cows compared to healthy controls throughout the study. When T cell populations were further defined by CD45RO expression, intracellular IFN- was higher for CD8+/CD45RO+ lymphocytes compared to CD4+/CD45RO+ cells. These results indicate that intradermal sensitization of cows in the early stage of infection with antigen will enhance the sensitivity of detection by the IFN- assay. In addition, CD8+ lymphocytes appear to play an important role as a mediator of MAP infection in naturally exposed cattle.

Key words: interferon-g, *Mycobacterium avium* subsp. *paratuberculosis*, skin test, cattle.

INTRODUCTION

Johne's disease (paratuberculosis) is a chronic progressive enteric disease of domestic and wild ruminants caused by the intracellular pathogen, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Chiodini et al., 1984). Clinical disease in adult cattle is characterized by intermittent diarrhea, weight loss, and inappetence. However, clinical signs are generally preceded by a long period of subclinical infection in which the infected animal is asymptomatic. It is not known what precipitates the progression from subclinical to clinical paratuberculosis in cattle but it is believed that a deviation in the host immune response occurs, causing a disruption in the ability of the host to contain the infection (Stabel, 2000). A reciprocal relationship has been documented between the host T cell responses and the extent of disease during mycobacterial infections (Orme, 1993; Koets et al., 2002; Welsh et al., 2005). Studies of human patients infected with M. tuberculosis suggest a predominance of Th1-mediated immunity in the early stages of disease, with a shift to Th2-mediated immunity in the more advanced stages (Orme et al., 1993). This shift in host immunity has also been observed for cows infected with MAP with strong IFN- responses noted in the early stages of disease, followed by an upregulation of IL-10 in the latter stages (Khalifeh and Stabel, 2004; Sweeney et al., 1998). Although secretion of other Th1-driven cytokines has been studied, the enhanced expression and secretion of IFN- in the early stages of infection suggest that this cytokine is critical for controlling mycobacterial infections. The upregulation of IFN- also makes it an attractive tool for the detection of subclinical infection in cattle with paratuberculosis (Huda et al., 2003; Stabel, 1996; Stabel and Whitlock, 2001). Previously, it has been shown that antigen-specific IFN- responses are enhanced after sensitization with mycobacteria (Barbosa et al., 2003; Gormley et al., 2004; Whipple et al., 2001), an effect that could be capitalized on to improve the sensitivity of the assay for the detection of infected

animals. The present study was designed to evaluate the effects on secreted and intracellular IFN-production after intradermal injection with johnin purified protein derivative (JPPD) in healthy noninfected cows and cows with subclinical and clinical paratuberculosis. In addition, the effects of in vivo sensitization on cell phenotypes present in the dermal induration and in the peripheral blood of healthy and infected cattle were determined.

MATERIALS AND METHODS

Animals

Three cows from each of the following infection groups were utilized in this study: noninfected healthy cows, cows naturally infected with MAP but asymptomatic (i.e., subclinical), and naturally infected cows with the clinical form of Johne's disease. Infection was monitored by bacteriologic culture of MAP from fecal samples using a standard method previously described (20). By definition, clinical animals were shedding more than 100 colony forming units (CFU) per gram of feces and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/g of feces. The non-infected control cows were purchased from herds with no history of paratuberculosis and were characterized by repeated negative fecal cultures. In addition, these animals were negative on all serologic assays (i.e., production of antibody specific for MAP and IFN-) performed during the period. All animal-related procedures were approved by the Institution Animal Care and Use Committee (National Animal Disease Center, Ames, Iowa).

Experimental protocol

Cows were bled on days –7 and day 0 (just prior to the intradermal injection), and on days 2, 4, 7, 10, 14, and 21 after intradermal injection. Blood was collected into vacutainer tubes containing sodium heparin for IFN- analysis and acid-citrate-dextrose for flow cytometric phenotype analyses. Pre-injection skin thickness measurements were taken with calipers and cows were given intradermal injections of johnin purified protein derivative (JPPD; National Veterinary Service Laboratories, Ames, IA) in 0.1ml (100 μg protein) volumes in the cervical region. After 72 h, changes in skin thickness (induration) at the site of injection were recorded. Fine needle aspiration of the cells at the site of the injection was performed at the same time with a 26 g needle and 3 cc syringe containing 1 ml of phosphate-buffered saline (0.15 M, pH 7.4) with 1 mM dithiothreotol (DTT; Sigma Chemical Co., St. Louis, MO). Flow cytometric phenotype analyses were then performed on the cell aspirates.

IFN- ELISA

Samples of whole blood (1 ml) were added to 24-well tissue culture plates and incubated either alone (no stimulation), or with concanavalin A (ConA; 10 μg/ml; Sigma), pokeweed mitogen (PWM; 10 μg/ml; Sigma), JPPD (10 μg/ml; NVSL), or a whole cell sonicate of MAP (MPS; 10 μg/ml; NADC) for 18 hr at 39°C in 5% CO_2. Plates were centrifuged at 500 x g for 15 min and plasma was harvested from each well. Plasma samples were frozen at –20°C until analyzed for IFN- concentrations by ELISA using a commercial kit (Bovigam, BioCor, Omaha, NE). Results were expressed as optical density units at 450 nm.

Flow cytometric analysis of cell surface antigens on leukocytes.

In order to differentiate immune cells, blood samples were collected into vacutainer tubes containing acid-citrate-dextrose (ACD) as the anticoagulant (BD Vacutainer Systems, Franklin Lakes, NJ) and processed as follows for 4-color flow cytometric analysis. Whole blood (75 μl) was dispensed into 96-well U-bottom microtiter plates. Red blood cells were lysed with distilled water for 10 seconds followed by restoring isotonicity with 10X PBS. After centrifugation and decantation 50 μl each of primary antibodies (CD4, GC50A1; CD8, BAQ111A; N12, CACT61A; CD25, CACT108A; CD45RO, GC44A; VMRD) were added into appropriate wells and incubated at room temperature for 15 minutes in the dark. Following centrifugation and decantation, 100 μl per well of secondary antibody cocktail (FITC, PE, PerCP, APC dyes) was added, and incubated at room temperature for 15 minutes in the dark. After centrifugation and decantation, cells were suspended with 200 μl per well of FacsLyse (BD Biosciences, San Diego, CA) and kept at 4°C until analysis. Data were acquired as 5000 events/sample using LSR flow cytometer and CellQuest software (both from BD Biosciences, San Diego, CA).

For the fine needle aspirates, cells were processed in the same manner as above without hemolysis. Analysis was limited to the expression of CD4, CD8, and N12 (only for infected cow samples) due to the limited number of cells.

Flow cytometric analysis of cell surface antigens and intracellular cytokines.
Blood samples were collected into vacutainer tubes containing sodium heparin as the anticoagulant (BD Vacutainer Systems). Each sample was divided into three aliquots of 1 ml and incubated for 20 hours at 39°C in a capped polystyrene tube alone (no stimulation), PWM (10 µg/ml in final), or JPPD (10 µg/ml). Brefeldin A (Sigma, Saint Louis, MO, final conc. 10 µg/ml) was added 4 hours prior to the termination of stimulation. Stimulated whole blood (100 µl) was incubated with 50 µl each of primary mAb for phenotype analysis (CD4, CD8, and CD45RO) for 15 minutes at room temperature in a microtiter plate (U-bottom, 96-well). After centrifugation the supernatant was decanted, red blood cells were hemolyzed with hypotonic saline followed by restoration with hypertonic saline. The peripheral blood mononuclear cells were then washed once with 200 µl/well of PBS. Cells were then incubated at room temperature in the dark for 10 min with 50 µl each of the secondary mAbs. After incubation and one wash with PBS, the cells were incubated with 50 µl/well of Fixation Medium (Caltag Laboratory, Burlingame, CA) for 15 minutes at room temperature in the dark. After one wash with PBS, 100 µl of permeabilization medium (Caltag Laboratory) and 10 µl each of PE-conjugated mAb for cytokine or isotype control were added. Cells were incubated for 15 minutes at room temperature in the dark. After two washes with PBS, cells were resuspended with 200 µl of PBS. Data were acquired for 20,000 events/sample immediately using LSR flow cytometer and Cell Quest software. Results are presented as the percentage of the cell population (either total PBMC or cell subset) that stained positive for each marker or cytokine.

Statistical analyses.
Data were analyzed by repeated measures using PROC MIXED procedure of the Statistical Analysis System (SAS, Cary, NC). Means and SEM are reported for all data. When significant effects ($P < 0.05$) due to treatment, day or treatment x day interactions were detected, means separation was conducted by the Tukey-Kramer option in SAS.

RESULTS

The results of the intradermal injection with JPPD are presented in Table 1. The three healthy noninfected control cows and the three cows with clinical MAP infection demonstrated only negligible increases in skin thickness (0.3 to 0.5 mm) after the intradermal skin test. In contrast, significant ($P < 0.05$) increases in swelling (9.3 to 15.6 mm) were noted for the three subclinically infected cows in this study.

Table 1. Effect of infection status of cows on induration size 72 hr after intradermal injection with johnin purified protein derivative

Infection status group	Induration (mm)	
(N = 3 for each)	**Pre-injection**	**Post-injection (72 h)**
Control	9.0 ± 0.5	9.5 ± 0.8
Subclinical	9.3 ± 1.2	15.6 ± 0.4[b,c]
Clinical	6.7 ± 0.7	7.2 ± 0.4

[a]Values are means ± SEM; n = 3. [b]Significantly different from pre-injection value, $P < 0.05$.
[c]Significantly different from other infection groups post-injection, $P < 0.05$.

In non-stimulated PBMC cultures neither the infection status (healthy, subclinical or clinical) nor in vivo sensitization via the intradermal injection was associated with the level of IFN- secretion (data not shown). For PWM-stimulated PBMCs, again differences among infection groups were not observed in IFN- secretion from after injection although IFN- production increased significantly ($P < 0.05$) between day 0 and day 2 for subclinical and clinical cows after the injection was performed (Figure1A). Interferon- was higher ($P < 0.05$) for control cows on day 0 compared to infected cows with no further increases noted for the through day 10 and then began to decline. In contrast, subclinically infected cows demonstrated the most noticeable increase in ConA-mediated IFN- production by PBMC during the study with significant ($P < 0.05$) increases observed on days 7, 10, and 21 as compared to day 0 (Fig. 1B). The rise in IFN- secretion after ConA stimulation of cells was much more protracted than that noted for PWM.
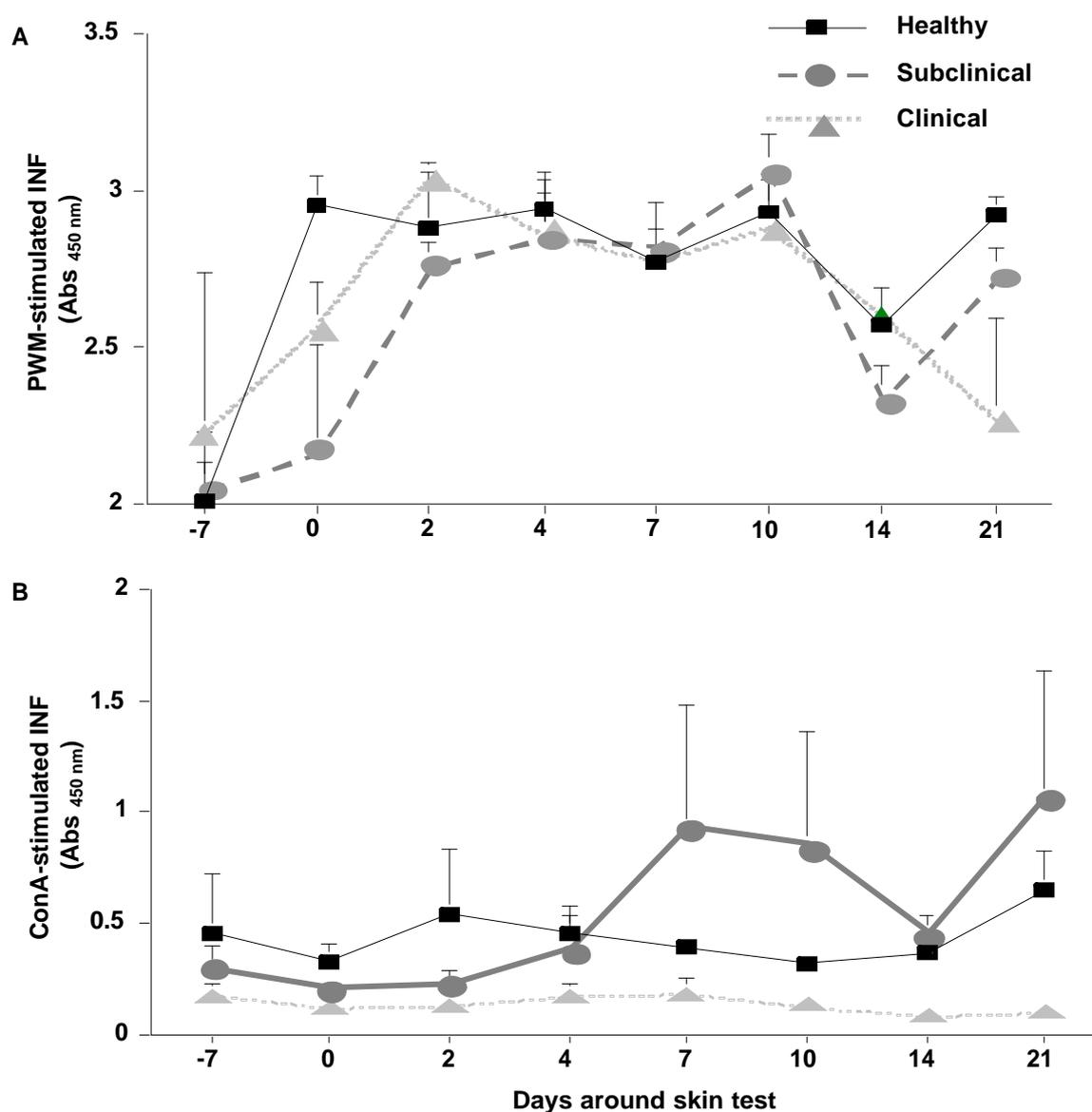
Fig.1. Effects of intradermal sensitization with JPPD on IFN- production (Abs450nm) after in vitro stimulation with PWM (A) and ConA (B) (N.B. different y-axis scales for graphs A vs. B)

Differences in antigen-specific IFN- production were more marked than mitogen-mediated IFN- , with higher (P < 0.05) IFN- production noted for the subclinically infected cows throughout the study when compared to the healthy and clinical cows (Fig. 2A/B). Although IFN- levels began to increase for subclinical cows within 2 days of intradermal injection, significant (P < 0.05) differences between pre- and post-injection IFN- were noted on days 4, 7,10, and 14.

Subpopulations of CD3+, CD4+, and CD8+ lymphocytes within whole blood were significantly (P < 0.05) higher for infected cows when compared with healthy, uninfected controls (Fig. 3A/B/C). In addition, a significant (P < 0.05) increase in CD3+ cells was observed after the intradermal injection with JPPD for all infection groups. Healthy control cows also demonstrated a significant (P < 0.05) increase in CD8+ cells post-injection that was not noted in the infected cows. The   TCR+ population did not differ among infection groups but did decline by day 10 after intradermal injection regardless of infection status (Fig. 3D).
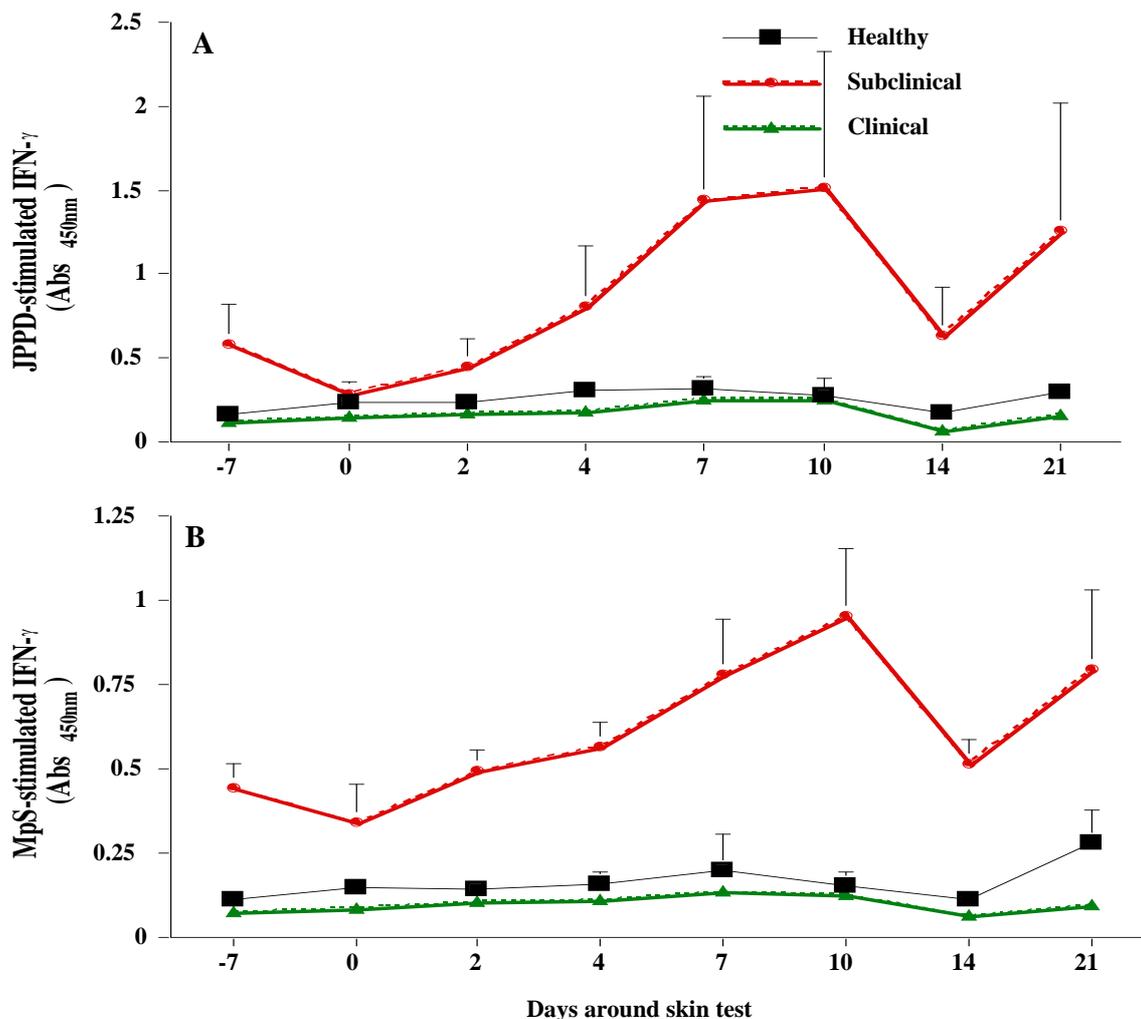
Fig. 2. Effects of intradermal sensitization with JPPD on IFN- production (Abs450nm) after in vitro stimulation with JPPD (A) and MpS (B)
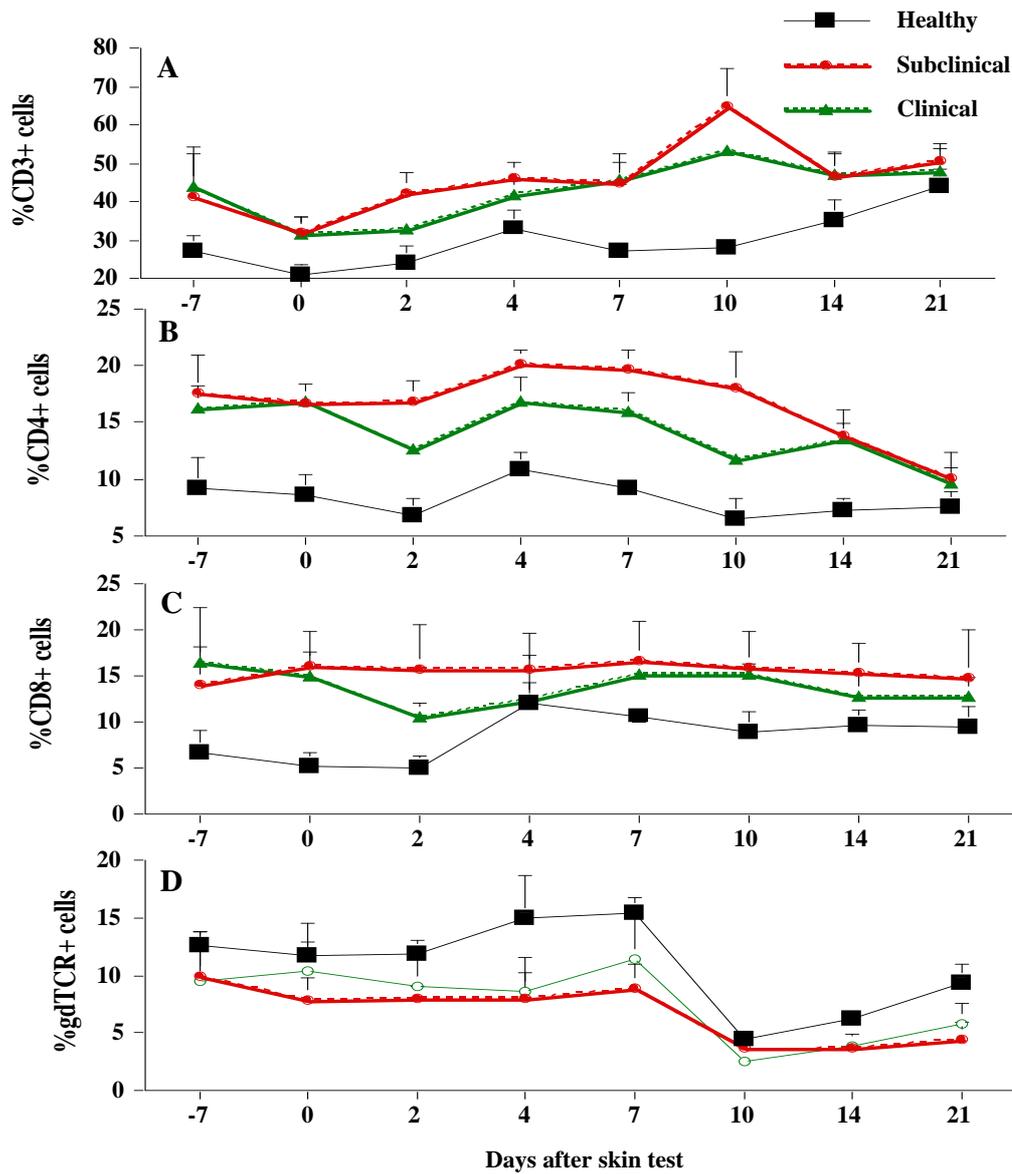
Fig. 3. Changes in T cell phenotypes (% of PBMC) after intradermal sensitization with JPPD; CD3 (A); CD4 (B); CD8 (C);   T cells (D).

The percentage of CD4+ and CD8+ T cells within the induration 72 hrs after intradermal injection with JPPD was significantly ($P < 0.05$) lower for the subclinically infected cows compared to healthy uninfected and clinical infected cows (Fig. 4). In addition, the CD8+ subpopulation was significantly ($P < 0.05$) lower than the CD4+ subpopulation for subclinically infected cows, whereas control and clinical infected cows had similar numbers of CD4+ and CD8+ cells. Due to the low cell recovery from the indurations of healthy and clinical infected cows,   T cell subpopulations were not adequately represented across treatments. Cell staining data however did reflect a decrease in expression of the cell surface markers for subclinical infected cows compared to the cows with clinical disease.
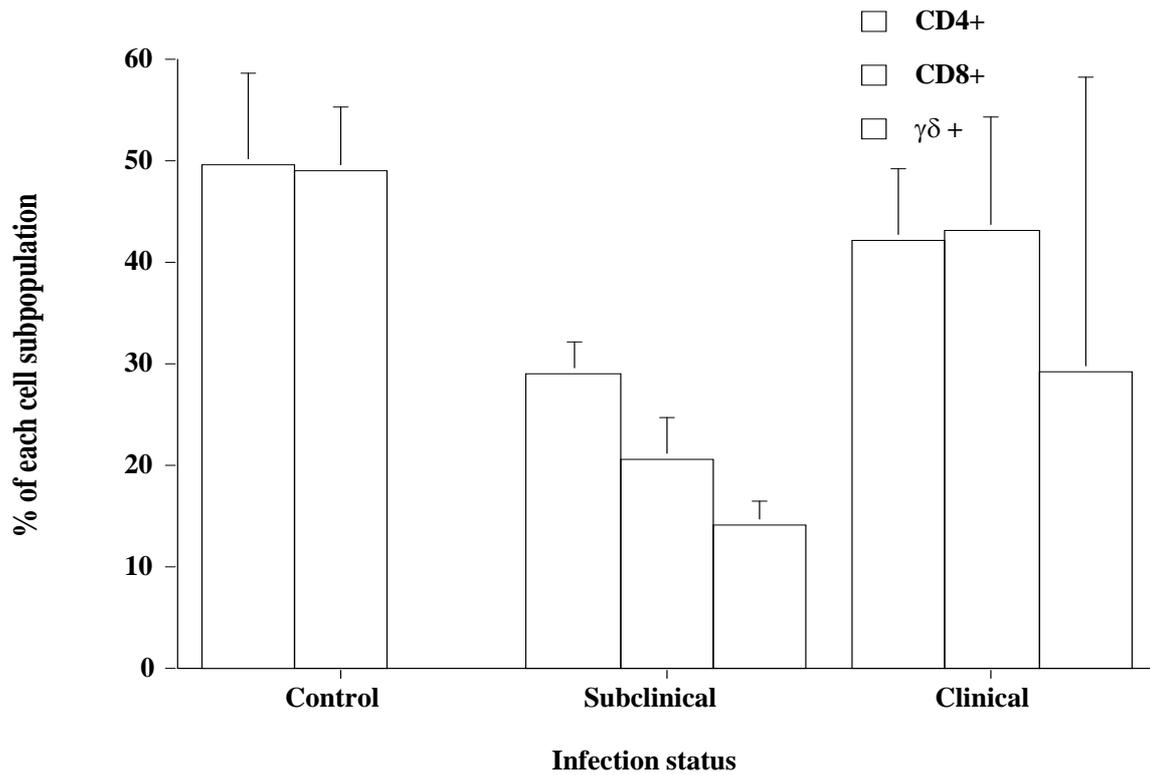
Fig. 4. Percentage of T cell subpopulations present in the induration 72 hrs after intradermal sensitization with JPPD.

Effects of intradermal sensitization on intracellular IFN- in PBMC in nonstimulated cultures and after stimulation of cells with PWM and JPPD are shown in Fig. 5. A significant (P < 0.05) rise in intracellular IFN- for nonstimulated cells was noted for all 3 infection groups by day 4 after the skin test and remained elevated only for the clinically infected cows (Fig. 5A). In contrast, stimulation of cells with PWM resulted in higher (P < 0.05) intracellular IFN- levels for clinical vs. subclinical infected cows throughout the study (Fig. 5B). In addition, a significant (P < 0.05) increase in PWM-stimulated intracellular IFN- was noted in cells from healthy cows on days 4, 7 and 10 after intradermal sensitization with JPPD (Fig. 9B). In contrast, a trend towards higher (P < 0.06) levels of intracellular IFN- was observed between subclinical infected cows and healthy controls after stimulation of PBMC with JPPD, when averaged throughout the study (Figure 5C).
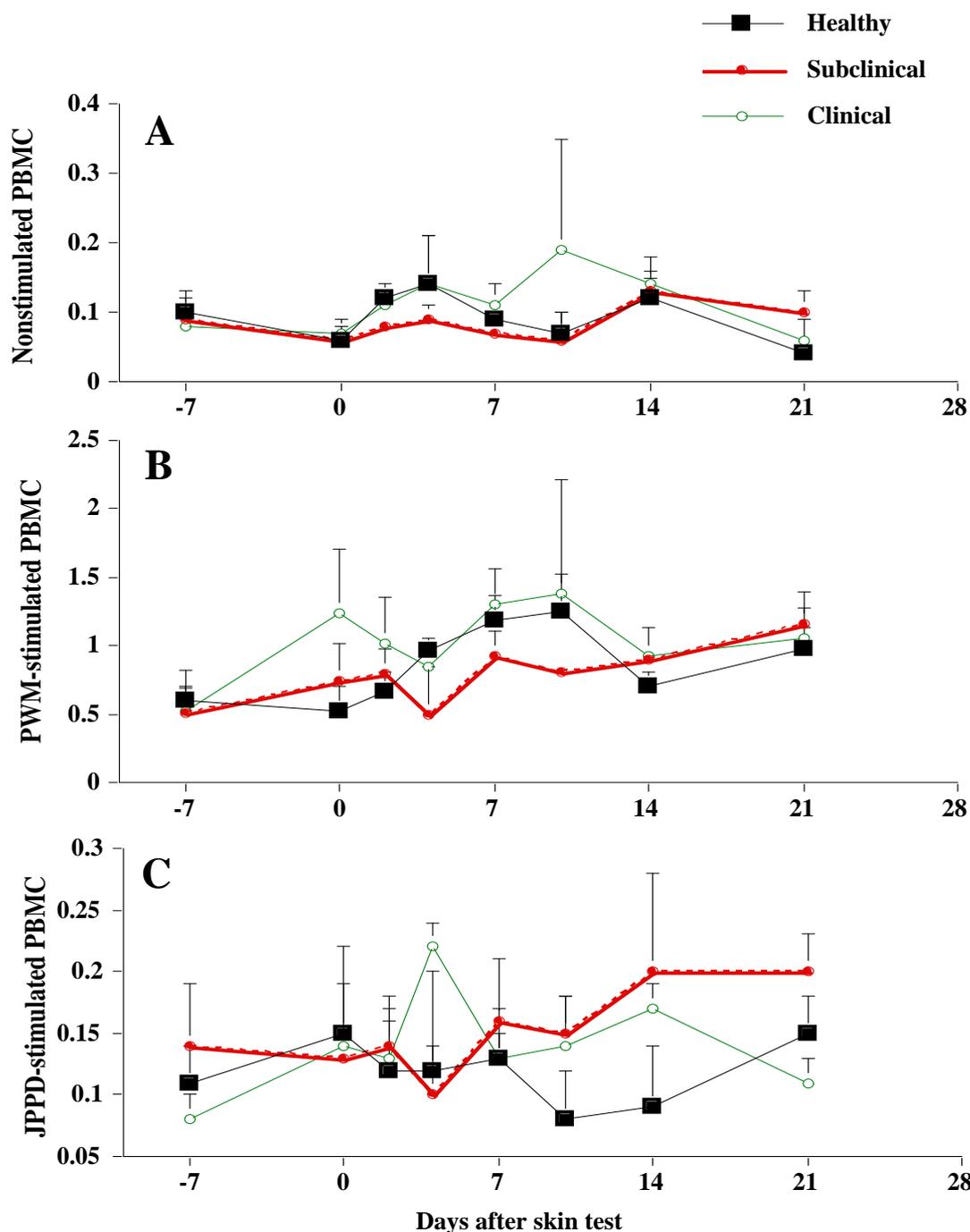
Fig.5. Intracellular IFN- in PBMC after in vitro stimulation with medium control (A), PWM (B); and JPPD (C). Results are expressed as the percentage of total PBMC that stained positive with the bovine anti-IFN- antibody.

When averaged over the study, intracellular IFN- within CD4+ and CD8+ subpopulations was significantly affected by the infection status of the cows but only in the nonstimulated cells. Higher (P < 0.05) levels of IFN- were noted for infected cows compared to the healthy controls in both T cell subsets (data not shown). When further delineated by CD45RO expression within T cell subsets, intracellular IFN- was predominantly expressed within the CD8+ subset (Fig. 6). Clinical infected cows had higher (P < 0.05) expression in CD4+ whereas subclinical infected cows had higher (P < 0.05) expression in CD8+ cells within nonstimulated PBMC compared to healthy control cows (Fig. 6A). Intracellular IFN- was significantly lower (P < 0.05) in CD4+/CD45RO+ cells from clinical infected cows after in vitro stimulation with JPPD (Figure 6B).
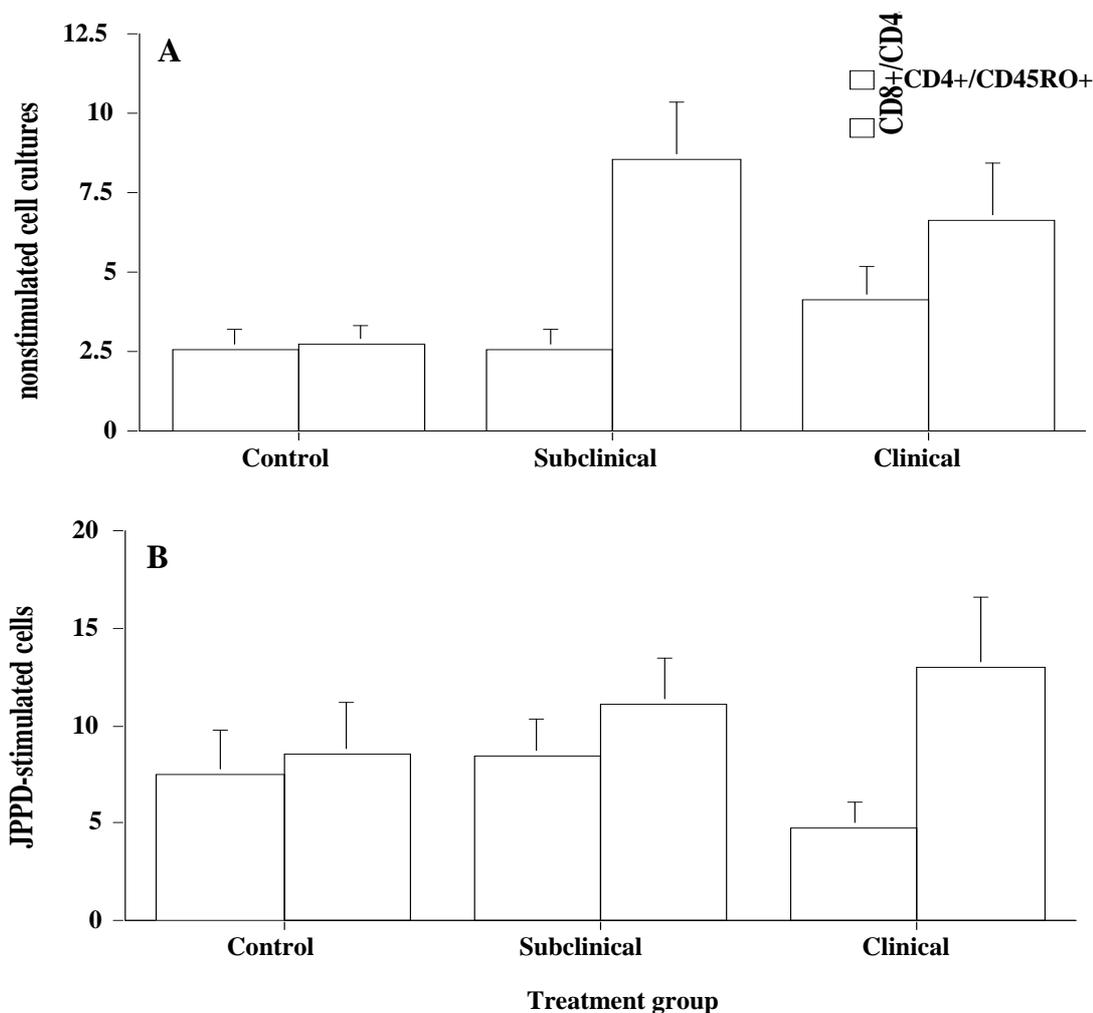
Fig. 6. Intracellular IFN- in CD4+/CD45RO+ and CD8+/CD45RO+ subsets after in vitro stimulation with medium control A) or JPPD (B). Results are expressed as a percentage of the cell subset that stained positive with the anti-bovine IFN- antibody and are presented as an average of values obtained from each bleeding date from the study within an infection group.

DISCUSSION

The effectiveness of diagnostic tools for the detection of animals infected with MAP is greatly affected by the biology of the disease. The progression from asymptomatic subclinical infection to a clinical disease state characterized by a classical protein-losing enteropathy corresponds with a concomitant shift from Th1-driven cellular immunity to Th2-mediated humoral immunity in the host. This means that a serologic diagnostic tool such as the ELISA for antibody cannot identify animals in both disease states. Yet the ability to detect MAP infection in the early stages of infection is paramount to controlling the spread of this disease. Measurement of IFN- production in response to in vitro mycobacterial antigen stimulation has been used successfully as an indicator of infection with M. tuberculosis, M. bovis, and MAP (Huda et al., 2003; Rothel et al., 1992; Stabel, 1996). In addition, studies with M. tuberculosis and M. bovis demonstrated that prior sensitization to the mycobacterial antigen by skin test or BCG vaccination enhanced the sensitivity of detection with the IFN- assay (Barbosa et al., 2003; Whipple et al., 2001).

In the present study, intradermal injection with JPPD resulted in a significant increase in secreted IFN- by PBMCs isolated from subclinical infected cows after in vitro stimulation with either MpS or JPPD antigen preparations. Little or no effect of the sensitization was observed in healthy cows or cows in the clinical stage of disease. The lack of response in control animals would indicate that they did not have appropriate antigenic recall mechanisms since they had not been previously exposed to MAP. In infected cows with clinical disease, IFN- secretion was likely attenuated through increased expression of IL-10 and resultant

downregulation of the IFN- responses in cows infected with MAP as previously been described (Khalifeh and Stabel, 2004). Interestingly, while sensitization with JPPD did not markedly affect the amount of PWM-mediated IFN- production for the 3 infection groups during the study, it did increase IFN- secretion in response to ConA in the subclinical infected cows. This would suggest that the T-cell population responding to JPPD antigen preparations corresponded more closely with the T-cell population that reacts to ConA stimulation. Unfortunately, further characterization of this population was not performed in the present study so it was not possible to distinguish whether CD4+, CD8+, subpopulations, or a combination of them, were responsible for the increased IFN- production after the skin test was performed. It is highly likely that each of these subpopulations contributed to the secretion of IFN- however it has been documented that CD4+ cells are the major T cell population responsible for IFN- secretion in cattle infected with MAP (Bassey and Collins, 1997).

Although variable throughout the time course of the study, detection of IFN- through intracellular staining within total PBMC populations was enhanced by intradermal injection and associated with the infection group status and the in-vitro stimulant. A weakly positive correlation between secreted IFN- and intracellular IFN- was observed after PWM stimulation of PBMC isolated from cows in all infection groups and for JPPD-stimulated PBMC isolated from subclinical infected cows. When segregated into CD4+ and CD8+ subsets neither subpopulation was consistently dominant in expression of intracellular IFN- . These data are supported by a study that evaluated patterns of intracellular cytokine expression in CD4 and CD8 cells from patients infected with M. leprae (1) but contrasted with data from two recent studies reporting that the CD4+ subset was responsible for the majority of intracellular IFN- noted in antigen-stimulated PBMC obtained from either vaccinated goats or calves experimentally infected with MAP (Hasvold et al., 2002; Waters et al., 2003). In fact, in the present study separation of the CD4+ and CD8+ subsets by CD45RO expression demonstrated that CD8+/CD45RO+ lymphocytes had higher expression of intracellular IFN- than CD4+/CD45RO+ cells. These data are supported by a study that evaluated IFN- production by both CD4+ and CD8+ lymphocytes in cattle following vaccination with BCG (Hope et al., 2000). Stimulation of PBMC with BCG-infected dendritic cells induced higher levels of secreted and intracellular IFN- for CD8+ cells than CD4+ cells. In addition, human subjects immunized with BCG had high numbers of CD8+ cells that produced IFN- in response to BCG-infected dendritic cells (Kawashima et al., 2003). These data clearly indicate that CD8+ lymphocytes play a critical yet uncharacterized role in host immune responses to MAP, perhaps equivalent to that of CD4+-mediated protection.

CONCLUSIONS

Intradermal sensitization cows with infections dramatically increased IFN- secretion by cultured PBMC after antigen stimulation, suggesting that the sensitivity of this assay can be increased for diagnostic purposes. Based upon the measurement of intracellular IFN- , it is also apparent that CD8+ T cells mediate host responses after exposure to MAP antigens but it is unclear as to their specific role. Further studies need to be conducted to elucidate the roles of T cell subsets during the progression of natural infection and their contribution to protection from or exacerbation of disease.

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Development and use of a M. avium subsp. paratuberculosis partial protein array for discovery of novel antigens

John Bannantine, W R Waters, M V Palmer, Vivek Kapur, Judy R. Stabel, M L Paustian

Abstract

With the genome sequence available for *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the technology is now developed for protein arrays to detect the presence of antibodies directed against MAP in host serum. The power of this approach is that it enables a direct comparison of MAP proteins to each other in relation to their immunostimulatory capabilities. In this study, over 60 MAP coding sequences were heterologously expressed and purified for use in a partial protein array. MAP proteins represented on this array include unknown hypothetical, cell surface, and MAP-specific proteins as well as previously characterized or known MAP antigens. The array was exposed to sera from MAP immunized rabbits and mice to identify immunodominant antigens in those artificial conditions. Furthermore, sera from non- and MAP-infected cattle were used to probe the array to identify antigens in the context of disease. Distinct sets of antigens emerged when data were compared between Johne's disease animals versus that of immunized mice and rabbits, indicating that immunodominant antigens in those artificial hosts may not represent antigens detected in the context of disease. Ten of the 64 proteins bound significant levels of antibody from clinical cattle. Of these ten proteins, three were previously identified as MAP antigens and the remaining seven represent novel antigens. Sera from three experimentally infected cattle identified at least one putative surface antigen detected early in infection. These data suggest this antigen may be useful in the early diagnosis of MAP infections. This powerful combination of genomic information, molecular tools, and immunological assays has enabled the identification of previously unknown antigens of MAP.

Relationship between Mycobacterium avium subspecies paratuberculosis, IL-1a, and TRAF1 in primary bovine macrophages

Paul M. Coussens, S K Chiang, A Aho, M Kuipel

Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a facultative intracellular pathogen that resides in host macrophage cells. Presently, little is known about how MAP is able to subvert the normal bacteriocidal functions of infected macrophages. Previously, our group identified a dramatic up-regulation of interelukin-1alpha (IL-1a) and tumor necrosis factor receptor-associated protein 1 (TRAF1) gene and protein expression within ileal tissues from MAP infected dairy cattle. IL-1a is a proinflammatory cytokine secreted by macrophages and other cells. TRAF1 protein is expressed by many cell types and has been demonstrated to negatively regulate apoptosis and other cell signaling functions. High-level expression of these two proteins could therefore have profound effects on macrophage function and apoptosis. We now present immunohistochemical evidence that high levels of TRAF1 protein are located primarily within macrophage cells infiltrating areas of active MAP infection, while IL-1a displays a diffuse staining pattern, consistent with secretion from cells within these lesions. We also report on experiments where cultured bovine monocyte derived macrophage cells (MDM) were challenged with live MAP and stimulated with recombinant IL-1a. Western blot analysis identified a dose dependent increase in TRAF1 protein levels in bovine MDM in response to infection with live MAP and to treatment with recombinant IL-1a. Using IL-1 receptor antagonist to block IL-1 receptors of bovine MDM, we also demonstrate that TRAF1 protein expression in MAP infected bovine MDM is dependent upon production of IL-1a following infection. Our studies further suggest that primary bovine macrophages cells constitutively express IL-1a and that TRAF1 protein increases with time in culture. MAP apparently uses the IL-1a – TRAF1 system to enhance TRAF1 protein expression in infected bovine MDM. These novel results provide evidence for a new avenue of research on the effect of MAP and other intracellular pathogens on macrophage signaling and apoptosis.

Proteomic comparison of Mycobacterium avium subspecies paratuberculosis grown in vitro and isolated from clinical cases of ovine paratuberculosis

Valerie Hughes, S Smith, A Garcia-Sanchez, Karen Stevenson

Abstract

Paratuberculosis (Johne's disease) poses a significant economic problem to beef, dairy and sheep industries in the United Kingdom and worldwide and is caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map). Understanding the interaction between host and Map at the molecular level will identify mechanisms of pathogenesis that promote bacterial survival in host tissue. We have used 2-D PAGE as a tool to investigate the virulent state of Map, incorporating the technique of beating the organism with zirconium/silicon beads to provide a comprehensive representation of the proteome of the organism. A direct comparison of the proteomes of Map scraped from the terminal ileum of ovine paratuberculosis cases and the identical strain grown *in vitro* is presented. These analyses have identified a set of ten proteins whose expression is up-regulated during natural infection: 1-Pyrroline-5-carboxylate Dehydrogenase (RocA), a putative Acyl-CoA Dehydrogenase (FAD E14), 2-Methylcitrate Dehydratase, Arginosuccinate Synthase (Arg G), Universal Stress Protein, 30S Ribosomal Protein S2 (RpsB), Peptidyl-prolyl cis trans isomerase (PpiA), Luciferase-like mono oxygenase, Thiosulphate sulphurtransferase (SseA) and Adenosine-tri-phosphate(ATP)-dependent Clp Protease (ClpB). The relevance of these proteins to the *in vivo*, virulent state of Map is discussed.

Use of Mycobacterium avium ssp. paratuberculosis 70 kD heat shock protein as a subunit vaccine against bovine paratuberculosis

Adriana P. Koets, A Hoek, M Langelaar, P Franken, W van Eden, Victor PMG Rutten

Abstract

Introduction: Paratuberculosis, caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP), constitutes a global threat to ruminant health. Eradication of paratuberculosis could be aided with a vaccine. The use of current whole bacterin vaccines is limited due to lack of efficacy and interference with tuberculosis eradication strategies. In the current study we evaluated recombinant MAP heat shock protein 70kD (Hsp70) as a subunit vaccine candidate. Materials & Methods: In total 40 female calves were randomly assigned to one of 4 experimental groups (n=10 each) which were orally infected (groups G3 and G4), or left uninfected (groups G1 and G2). Calves of G2 and G4 were vaccinated with 100 ug of Hsp70 in DDA adjuvant at day 0 and 365 of the experiment. Blood samples were taken every 2 weeks, and used for isolation of lymphocytes, and serological analysis. Antigen specific activation of lymphocytes was evaluated in flowcytometry and IFN-γ Elispot. Fecal samples were taken for culture of MAP 7 times during the first 2 years of the experiment, in which the calves were raised and fed conventionally. Results: The animals in G1 and G2 remained culture negative throughout the experiment. From the calves in G3, infection with MAP only, in total 8 out of 10 animals were tested positive for MAP at least once. In G4 calves, which were both infected and immunized, 4 out of 10 animals tested positive once at day 126 of the experiment. In the 2 years since, only 1 G4 animal of the 9 remaining animals tested positive for MAP while the other calves remained culture negative. Conclusions: Seen the strongly reduced shedding in G4 as compared to G3 we conclude that use of MAP Hsp70 as a subunit vaccine can aid in the eradication of bovine paratuberculosis.

Development of a calf ileal cannulation model to facilitate testing of existing and candidate vaccines for M. avium subsp. paratuberculosis

A J Allen, G M Barrington, Mary Jo Hamilton, Judy R. Stabel, S Robbe-Austerman, William C. Davis

Abstract

M. avium subsp. *paratuberculosis* (Map) is the causative agent of Johne's Disease, a chronic granulomatous enteritis that affects adult cattle worldwide. It is one of the most prevalent and costly diseases of dairy cattle. Control methods to date have been limited due to the lack of sensitive diagnostic tests and methods to assess the potential efficacy of candidate vaccines. To facilitate analysis of the immune response to Map and candidate vaccines we developed a calf ileal cannulation model. An indwelling "T"-shaped, plastic cannula (0.5-1.0 inch ID), is surgically placed in the ileum with the sampling portal exiting the body wall at the right paralumbar fossa. Inflatable catheters are inserted into the intestinal lumen through the portal and run both oral and aboral to allow flushing, sampling and direct infection of an isolated segment of bowel. The model augments the establishment of experimental infections, monitoring the development of local and systemic immune responses, and the evaluation of disease progression in a step by step manor. Biopsies can be obtained for culture, RT-PCR, and histopathology. Importantly, the model provides a way to assess the capacity of candidate vaccines to elicit a protective immune response over a short time course. If protective immunity is elicited, impaired survival of challenge bacteria introduced into the ileum should be demonstrable. The ileal cannulation model augments *in vitro* methods of testing the immune response to candidate vaccines.

Immune parameters and vaccine efficacy in Mycobacterium avium subspecies paratuberculosis experimentally infected sheep

D Begg, J Frank Griffin

Abstract

Johne's disease in ruminants is caused by the pathogenic bacterium *Mycobacterium avium* subsp. *paratuberculosis* (Map). Currently available Map commercial vaccines protect against clinical disease but not infection. Using an experimental model of ovine Johne's disease this study examined the proprietary Johne's vaccine Neoparasecä and an Aqueous formulation of Map 316F (AquaVax). Detailed immunological examinations including lymphocyte transformation assays, FACS analysis, IFN-γ and antibody detection from blood and gut associated lymphoid tissues was carried out on animals after vaccination and challenge with virulent Map to identify markers of protective immunity. Neoparasecävaccination provided significant protection against disease while AquaVax did not. Diseased animals had different immune responses from different gut lymphatic tissues which correlated with the histopathology observed. Immune animals had stronger cell-mediated responses and altered proportions of CD4+, CD8+, CD25+ and B cells in blood, spleen and the gut lymphatics, than diseased animals.

The study of Mycobacterium avium subsp. paratuberculosis virulence in golden hamsters

Vlademir Beran, Z Rozsypalova, Wuhib Y. Ayele, T Amemori, M Bartos, M Moravkova, A Horvathova, I Pavlik

Abstract

Virulence of five isolates was investigated in golden hamsters (Mesocricetus auratus) line HAN:AURA. In the first experiment, 24 hamsters were infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cattle isolate of RFLP type B-C1 from a herd with a rapid spread of infection. Hamsters were infected per os (po), subcutaneously (sc), and intraperitoneally (ip), per eight animals in each group. Shedding of the causative agent through faeces was monitored for 7 months; after euthanasia the gastrointestinal tract was examined. Excreting of MAP of identical RFLP type was induced by all routes of infection and was observed during the whole period of the experiment. In the following experiment performed for 32 months, 96 hamsters were infected with four MAP isolates of three RFLP types: B-C1 from larvae of syrphid flies and from a patient with Crohn's disease (CD), A-C10 from cattle with a slow spread of infection, and D-C12 from a CD patient. Faeces were cultured on solid Herrold's egg yolk medium and liquid Long medium with Mycobactin J. The hamsters survived in average for 20.5 months (range 5-32 months). No signs of chronic diarrhoea or emaciation were recorded. Culture examination of organs revealed the highest percentage of mycobacteria in hamsters infected with the isolate D-C12 (75% positive hamsters) and the lowest percentage in hamsters infected with the isolate of RFLP type A-C10 (29.2%). Twenty-four months later, a change of RFLP type D-C12 to RFLP type D-C18 was recorded; that was likely caused by a transposition of one IS*900* copy. After ip, sc, and po infections MAP was detected in the organs from 71.9%, 59.4%, and 37.5% hamsters, respectively. The culture on liquid Long medium did not increase sensitivity of culture examination. *Partially supported by the MinAgr of the CR (grant No. 0002716201) and EC: No. QLK2-CT-2000-00928 and SSPE-CT-2004-501903 (supports the attendance to this Congress).*

Ovine apoptotic responses to Mycobacterium avium paratuberculosis

S Browne, K de Silva, Richard Whittington, D Emery

Abstract

Johne's disease is a chronic wasting condition of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. ptb.*). A proportion of infected adult animals develop clinical disease more than 12 months after infection. More knowledge regarding the pathogenesis of this disease, including apoptotic responses during the course of infection, could allow advances towards earlier detection and control of the disease and identification of resistant sheep. Merino sheep aged 7 months were orally dosed with three doses of 0, 4×10^4, 4×10^5 or 4×10^7 *M. ptb.*/dose (2 animals per group) and samples were taken from ileal (ILN), jejunal (JLN) and prescapular (PLN) lymph nodes 15.5 weeks later. Isolated lymph node cells (LNC) were incubated with medium alone, *M. ptb.* antigen (10 µg/ml) or Con A (10 µg/ml) for up to 6 days. Caspase activity, a marker of apoptosis, was determined by flow cytometry on days 0, 2, 4 and 6. Regardless of disease status similar levels of caspase activity were observed in LNC immediately after isolation. At this very early stage in the development of Johne's disease, *in vitro* incubation with *M. ptb.* antigen did not induce apoptosis. There were no significant differences in the percentage of caspase positive cells when LNC were incubated in the presence of medium alone or *M. ptb.* antigen. These results suggest that *M. ptb.* antigen responses *in vitro* in ILN and JLN cells may not prove useful in identifying very early exposure to *M. ptb.* in sheep. Further studies are being undertaken to assess apoptotic contributions in ovine peripheral blood mononuclear cells during the course of *M. ptb.* infection.

Proliferative responses in peripheral blood and lymph node cells in the early stages of ovine Johne's disease

K de Silva, N Carter, D Taylor, L Di Fiore, Richard Whittington, D Emery

Abstract

Early detection is vital in controlling the spread of Johne's disease. Cell-mediated immune responses are predominant during the subclinical phase of disease when clinical signs are not evident. The aim of this study was to identify lymphoproliferative responses which may allow the detection of *Mycobacterium paratuberculosis* (*M. ptb.*) infection in subclinical stages in sheep. Merino sheep aged 7 months were orally challenged with three doses of 0, 4×10^4, 4×10^5 or 4×10^7 *M. ptb.*/dose (3 animals per group) and samples were taken from peripheral blood and ileal, jejunal and prescapular lymph nodes 15.5 weeks later. Proliferation of peripheral blood mononuclear cells (PBMC) and lymph node cells (LNC) in response to *in vitro* incubation with medium alone, *M. ptb.* antigen (10 µg/ml) or Con A (10 µg/ml) for 4 days was determined by flow cytometry. PBMC and LNC were also phenotyped using a panel of antibodies. *M. ptb.* was not detected by faecal culture in any of the animals and only one microscopic lesion, which had no acid-fast bacilli, was detected in one animal. At this very early stage of disease, proliferation of PBMC increased in response to *M. ptb.* antigen in two of three animals exposed to 4×10^5 or 4×10^7 *M. ptb.*/dose (6 fold and 4.5 fold respectively when compared to the medium only control), but not in the other two groups. Proliferation of LNC in response to *M. ptb.* antigen was seen in all animals, irrespective of experimental exposure to *M. ptb.* There was no difference in PBMC phenotype among the different groups. Further studies are being carried out to determine if PBMC proliferation assays can be used to detect Johne's disease at later, pre-clinical stages of disease.

Bovine 3'-UTR (GT11) variant of the NRAMP1 gene under-represented among paratuberculosis seropositive cattle

A Estonba, M Iriondo, O Oyanguren, Joseba Garrido, Marivi Geijo, I Sevilla, Ramon A. Juste

Abstract

Recent works have shown some coding and non-coding polymorphisms of the NRAMP1 gene altering susceptibility to M. tuberculosis and other intracellular pathogens in mice and humans. In cattle, NRAMP1 gene is a major candidate gene controlling natural resistance to brucellosis, but it has been shown recently that under field conditions it does not determine resistance or susceptibility to M. bovis. However, no data is available regarding paratuberculosis. Here, we present a case-control preliminary study in a naturally infected herd where the potential correlation of 3 polymorphic markers for the NRAMP1 gene with susceptibility or resistance to Johne's disease were investigated by comparing the distribution of frequencies of several polymorphims in a sample of 44 ELISA positive and 100 ELISA negative cattle. Only one polymorphism at the 3′-UTR microsatellite showed a different frequency between the two groups. The GT_{11} allele had a frequency significantly higher in the seronegative (0.22) than in the seropositive (0.07) group. Since ELISA has a low sensitivity and a detectable humoral immune response is closely related to the development of clinical disease, these results suggest that NRAMP1 is involved in lesion progression. Further research is needed to evaluate the effects of this genetic condition on the pathogenesis of paratuberculosis and, therefore, to determine whether genetic selection can be of practical value for control of paratuberculosis.

Vaccination in a paratuberculosis affected herd: some immunological and bacteriological indicators

Marivi Geijo, Joseba Garrido, I Sevilla, N Elguezabal, G Aduriz, Ramon A. Juste

Abstract

Control of paratuberculosis by fecal culture and culling is expensive and has not fully answered to its theoretical potential. Although vaccination is not expected to lead to rapid eradication of the infection, it might be the most practical approach to stop losses and to decrease transmission in the short term. In this study, we have analyzed some indicators of the effects of vaccination in a field trial where an experimental vaccine was applied in a heavily infected herd. Indirect ELISA, IFN-γ, and blood PCR were carried out every six months during one and a half year in a cohort of 21 dairy cattle in a heavily affected herd. Fecal culture and PCR were additionally used at the beginning and the end of the follow-up. Finally, 12 vaccinated and 9 non-vaccinated animals were killed and samples from the intestine and enteric lymph nodes were cultured for isolation of Map. Only two fecal cultures from the non-vaccinated group were positive at the end of the follow-up. Fecal PCR was positive in 3 vaccinated and in 3 non-vaccinated animals at the last control. Blood PCR showed a similar proportion of positive results among vaccinated and non-vaccinated cattle. All vaccinated and non-vaccinated cattle had high humoral and cellular immune responses that were probably related to limited differences in the response associated to vaccination. However, there was a significantly lower proportion of tissue culture positive animals in the vaccinated group. Infected animals, including a vaccinated one, showed a decreased immune response compared with their non-infected mates. These results suggest there was a strong background of immune stimulation in this affected herd which did not allow to clearly see the immune effects of vaccination. However, the vaccine used showed a strong effect in terms of infection protection.

Is antigen specific apoptosis of blood lymphocytes predictive of progressive paratuberculosis?

S N Grell, K Skovgaard, A Clemensen, Gregers Jungersen

Abstract

The immunologic mechanisms determining the progression of paratuberculosis from subclinical to clinical disease have not been finally determined. Induction of immunological T cell unresponsiveness has been suggested to play a role in progression of paratuberculosis. Here we focus on the T-cell immune responses in the different stages of the infection, with special focus on the possible loss of T cell activity caused by antigen-induced apoptosis . Cattle from two paratuberculosis infected herds were categorized into 3 progressive disease stages according to their cell mediated and humoral immune responses. Stages were defined as stage 0 (IFN-γ ÷, Ab ELISA ÷), stage 1 (IFN-γ +, Ab ELISA ÷) and stage 2 (IFN-γ +, Ab ELISA +). Whole blood samples were stimulated *in vitro* with a positive control (superantigen), *M. paratuberculosis* PPD (PPDj) and with PBS as a negative control. PBMC were purified and tested for induction of antigen-induced proliferation and apoptosis. By flow cytometry, a progressive antigen specific induced apoptosis (caspase-3 induction) was demonstrated in paratuberculosis infected animals in the different subclinical disease-stages but not in uninfected. Further studies showed that this antigen induced apoptosis were transferable, i.e. supernatant from apoptosis induced cultures could induce apoptosis in cultures with ParaTB naïve animals. Subsequent RT-PCR studies on an array of apoptosis genes show that Caspase 8 and FasL are upregulated in the apoptosis indicating apoptosis by Activation Induced Cell Death (AICD). <u>Conclusions:</u> Altogether these results indicate that the development of clinical paratuberculosis involves progressive loss of immune responsiveness and that T-cell death by AICD may represent one of the important components leading to an ineffective immune response against *M. paratuberculosis*. Furthermore, it can be speculated that a yet unknown apoptosis factor in plasma may be a future diagnostic tool to predict the transition from appropriate cell mediated immune responses to inappropriate humoral responses.

Comparative pathology of Johne's disease in deer and sheep

J Frank Griffin, Colin Mackintosh, R O'Brien, D J Turner

Abstract

The aim of this study was to characterise typical Jd lesions in farmed red deer in NZ, by comparing them with well-characterised descriptions of ovine Jd. A secondary objective was to determine whether the histopathology found following experimental infection of both sheep and deer was comparable to that seen in naturally infected animals. Twenty-six orally infected sheep, 5 naturally infected sheep, 8 experimentally infected deer and 8 naturally infected deer were used. Tissue was sampled from the gut mucosae, adjoining mesentery and lymph nodes to track progression of disease from the primary site of infection to the lymph node. Deer and sheep were experimentally infected by oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* (Map) isolated from the gut lymphatics of clinically affected deer and sheep, respectively. Independent studies have examined the strain type of Map isolated from naturally infected deer and sheep by IS*1311* typing. Using this system deer isolates of Map from deer were 'bovine' strain while sheep isolates typed as 'ovine' Map. There were no differences in histopathology or disease severity seen in either species following natural and experimental challenge. The histopathology seen in deer was comparable to that seen in sheep but some unique characteristics were evident. Deer exhibited foci of caseous necrosis within severe lesions. Macrophages seen in deer lesions often displayed unique ultrastructural features not seen in sheep. These findings highlight the need for increased awareness among pathologists for the unique histopathological characteristics of cervine Johne's disease. The findings show that in red deer (*Cervus elaphus*) caseated lesions can occur within the intestinal lymph nodes and need to be differentiated from caseated lesions caused by M. bovis infection. While grossly visible caseous lesions occur relatively rarely histopathological evidence of caseation is seen more commonly in deer than sheep.

Pathobiology of spontaneous and experimental paratuberculosis (S-5 strain) in goats with special reference to early lesions

S Hajra, Shri N. Singh, A K Srivastava

Abstract

Present study was aimed to diagnose early cases of paratuberculosis by demonstration and isolation of the *Mycobacterium avium* subsp. *paratuberculosis* (Bison type) in faecal and tissue samples. ELISA test and pathomorphological lesions were studied in experimentally produced paratuberculosis using MAP (Bison type) S-5 strain. Faecal sample from 142 goats from various organized herds of North India were subjected to smear (using centrifugation and decontamination method) and cultural examinations. Isolation of the mycobacteria was performed in all faecal samples and 74 tissue samples on HEY medium with or without Mycobactin-J after decontamination with 0.9% HPC. Experimental study was conducted on 13 goats (10 infected and 3 controls) where pathogenicity of S-5 strain was tested positively by gross and histopathological lesions (using routine H& E and ZN as special stain) and plate-ELISA test. Pathogenicity of the S-5 strain was proved and characteristics gross and microscopic lesions were observed on 90 DPI and onwards. Lesions showing infiltration of macrophages with AFB without granuloma formation simulating lepromatous form of human leprosy and typical granuloma as in tuberculoid form were found. Positive humoral immune response by using plate ELISA was observed on 90 DPI onwards showing antibody titer above the cut off value. There was an apparent linear correlation between the antibody level and days post infection. The performance of the different diagnostics test like examination of faecal smear by direct microscopy, faecal culture, scraping smear examination of organism from tissue pathomorphology and plate ELISA test had a linear relationship among them.

Microenvironment of intestinal granulomatous lesions from naturally occuring bovine Johne's Disease

Jesse M. Hostetter, E Huffman, A Dorn, M Wannemeuhler

Abstract

The intestinal lesions of *Mycobacterium avium* subspecies *paratuberculosis* (M. a. ptb) infection are characterized by extensive granulomatous lesions with high bacterial burdens. The objective of this study was to examine macrophage activation phenotype and pro-inflammatory cytokine expression specifically within intestinal granulomatous lesions from animals naturally infected with M. a. ptb. To test the hypothesis that granuloma macrophages have a non-activated phenotype, we examined granulomatous lesions from the ileocecal valve of cattle in the clinical stages of Johne's disease. We determined protein expression by immunohistochemistry and compared this to gene expression determined by quantitative reverse transcriptase real time polymerase chain reaction (Q-RT-PCR). Laser capture microdissection was used to recover granuloma cells from intestinal lesions, thereby allowing for direct sampling from the granuloma microenvironment. Our immunohistochemical studies included stains to detect mycobacterial antigen, iNOS as a marker of macrophage activation, and IFN-γ. Using laser capture microdissection, granuloma cells were collected onto a thermoplastic film held on a collection cap, (CapSure® HS LCM Caps). We collected roughly one 80% confluent cap per experiment. We measured gene expression of iNOS and IFN-γ in the captured cells by Q-RT-PCR using the Syber green system. The results of this study demonstrate that most granulomatous lesions had high bacterial burdens. Protein expression of iNOS was low within the granulomatous foci. Q-RT-PCR demonstrated variable IFN-γ gene expression within granulomas and surrounding lamina propria. Gene expression for iNOS within granuloma cells was low. These data suggest that macrophages in intestinal granulomatous lesions of bovine Johne's disease do not have strong iNOS expression, suggesting a non-activated phenotype.

Detection of B cell epitopes of M. avium subspecies paratuberculosis 70 kD heat shock protein using monoclonal antibodies

Adriana P. Koets, P van Kooten, T van den Ingh, W van Eden, Victor PMG Rutten

Abstract

Introduction: Bovine paratuberculosis is caused by infection of young calves with *Mycobacterium avium* ssp. *paratuberculosis* (MAP), and results in chronic granulomatous infection of the ileum. Relatively little is known about immunogenic properties of individual antigens of MAP. The aim of the present study was to define antibody epitopes of the 70 kD heat shock protein of MAP through the generation of monoclonal antibodies. Materials & methods: Mouse monoclonal antibodies were generated against recombinant MAP 70 kD heat shock protein (Hsp70) using conventional hybridoma technology. Antibodies produced by different hybridomas were primarily screened using Hsp70 as antigen in ELISA. Subsequent epitope mapping was performed using synthetic peptides representing different parts of the Hsp70. Monoclonal antibodies recognizing linear epitopes were tested for use in western blot, immunohistochemistry and electronmicroscopy. Results: In total, 8 hybridomas which recognized MAP Hsp70 were generated. Five hybridomas produced antibodies recognizing conformational epitopes on the Hsp70. Three of these hybridomas produced monoclonal antibodies recognizing 2 different linear epitopes of Hsp70. The 3 antibodies could successfully be used in peptide specific ELISA, western blots, immunohistochemistry and electronmicroscopy of tissues of animals infected with paratuberculosis. One epitope was conserved in multiple mycobacterial species, with the other epitope differentiation between MAP and M. tuberculosis / M. bovis was possible, however this epitope appeared to be present in at least some

Evidence for distinct host response patterns in cows experimentally infected with M. avium subspecies paratuberculosis

Adriana P. Koets, M Langelaar, A Hoek, Douwe Bakker, Peter Willemsen, W van Eden, Victor PMG Rutten

Abstract

Introduction: Bovine paratuberculosis is caused by infection of young calves with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and results in chronic granulomatous infection of the ileum. The aim of the present study was to perform a longitudinal follow up of immunological and microbiological parameters of calves experimentally infected in the first month of life. Materials & methods: Twenty experimentally infected calves were sampled monthly during 4.5 years. The PBMC were isolated, and phenotyped by flow cytometry using a panel of 8 monoclonal antibodies. Lymphocyte proliferation assays, with a panel of 6 mycobacterial antigens were used for evaluating T cell function. Antibody responses were measured using ELISA coated with MAP PPD antigen, recombinant MAP heat shock protein of 65 kD, and 70 kD. Fecal culture was performed monthly to acquire data on the mycobacterial shedding. Results: The results showed that based on fecal excretion patterns animals could be divided in three groups, one which is characterized by high frequent shedding (HFS), a group characterized by low frequent shedding (LFS) and an intermediate group (IM). Animals in the HFS group typically had lower cell mediated responses, especially during early stages of infection, when compared to animals from the LFS group. Conversely the HFS animals had early onset and higher antibody responses when compared to LFS animals. Conclusion: Based on fecal shedding patterns we identified different host response patterns to paratuberculosis infection. Whether these different response patterns reflect successful or failing immune responses remains to be established as neither LFS nor HFS animals in the study developed overt signs of clinical paratuberculosis in the 4.5 years of the study. The HFS animals likely contribute significantly more to spread of the infection in the population. Host genetic make up could explain the observed response patterns and as such is subject of ongoing follow-up studies.

Profile of certain cytokines in the experimentally infected paratuberculous sheep

A A Kumar, Bhupendra Nath Tripathi, B Sharma

Abstract

The immunopathology of paratuberculosis involving both humoral and cellular elements is mediated through several cytokines at the sites of lesions. The systemic immune response may not reflect the events taking place at the gut levels. The objectives of this study was to evaluate the expression of mRNA for cytokines IFN-γ, TNF-α, IL-2, IL-4, IL-6 in tissues of small intestine, mesenteric lymph nodes (MLN) and blood in 8 sheep experimentally infected with a caprine strain of *Mycobacterium avium* subspecies *paratuberculosis* and 3 uninfected control sheep. Histologically, 4 sheep had mild lesions and 2 each had moderate and severe lesions. Expression of cytokines were detected by RT-PCR using specific primers and expressed in relative units against GAPDH gene serving as control. Cytokines were expressed both in the tissues and blood of all infected and control sheep. In the intestinal tissues, mRNA for these cytokines was non-significantly increased as compared with the control sheep. In MLN of infected sheep these cytokines were better expressed 1.5 to 3 times with significantly increased levels of IL-4 as compared with the control sheep. On comparison of cytokine profiles in the small intestinal and mesenteric lymph node tissues and blood, TNF- α and IL-2 were significantly increased in the MLN, IL-4 in the blood and IL-6 in the small intestinal tissues. Increased levels of IL-4 in the blood might have inhibitory effect on the expression of IL-2. In general it was observed that levels of cytokines in the blood were more related with the profile in the MLN than those in the small intestine. Increase in the levels of Th1 and Th2 cytokine types suggests that both the cell mediated and humoral immune responses are triggered simultaneously with lot of individual variation.

Immunisation with a rHsp70-GFP fusion protein elicits cellular and humoral immune responses but no cytotoxic T cell reactivity in cattle

M Langelaar, A Hoek, W van Eden, Victor PMG Rutten, Adriana P. Koets

Abstract

Introduction: Bovine paratuberculosis is caused by infection of young calves with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). MAP causes intracellular infection in macrophages and results in chronic granulomatous enteritis. The aim of the present study was to investigate whether a heat shock protein (Hsp) based delivery system could be used to generate cytotoxic T cells in cattle as a tool to aid the elimination of MAP infected macrophages, as described in murine model systems. Materials & methods: A recombinant fusion protein, rHsp70-GFP, was produced according to previously published methods (Huang et al, 2000, J. Exp. Med. 191, 403) with some modifications. In the current study, the N-terminal receptor binding part of recombinant MAP Hsp70 was fused to enhanced green fluorescent protein (GFP), the latter serving as a model antigen. Five cows were immunized 4 times with the rHsp70-GFP fusion protein, an additional cow was immunized with GFP only. Peripheral blood mononuclear cells were isolated using density gradient centrifugation and used in lymphocyte stimulation assays. Target cells were pulsed with the rHsp70-GFP, GFP protein or GFP peptides. Cytotoxicity was measured using chromium release assays and a flowcytometric method using different effector target ratios. Serum was used to determine antibody responses in ELISA. Results & Conclusion: Immunisation with rHsp70-GFP fusion protein elicits cellular and humoral immune responses to GFP, GFP peptides and Hsp70-GFP but as assessed by chromium release assay and a flowcytometric assay, no cytotoxic T cell reactivity in cattle.

Detergent enrichment of mycobacterial envelope proteins

M D Lanigan, L Tsen, J A Vaughan, B J Shiell, Woytek P. Michalski

Abstract

Although *Mycobacterium avium* ssp. *paratuberculosis* (*M. ptb.*) has long been recognised as the causative agent of Johne's disease (JD), there is a growing body of evidence that implicates this organism in Crohn's disease (CD) in humans. Although no causal link between the two has yet been proved, *M. ptb.* represents a pathogen of enormous economic significance to agriculture world-wide as well as one with potential risks to human health. Our laboratory is applying proteomic methodologies to elucidate not only the molecular mechanisms underlying *M. ptb.* pathogenesis in animals but also proteins that might represent biomarkers of paratuberculosis infection. Whilst the interaction between *M. ptb.* and host cells is poorly understood at the cellular level, it is reasonable to suppose that surface-exposed mycobacterial proteins are one of many factors that mediate infection and transmission. The thick, waxy external coating of mycobacteria, while imparting to these organisms their characteristic robustness, necessitates analysis strategies that maximise extraction of cellular proteins without compromising the integrity of the proteomic milieu. We have used the non-ionic detergent Triton X-114, which partitions into aqueous- and detergent-rich phases, to enrich hydrophobic proteins from cell-surface extracts of *M. ptb.* and and the closely-related *M. avium*. Proteins were electrophoretically-separated, identified using mass spectrometry and subjected to bioinformatic analysis to determine their likely subcellular localisation. Thus far over three hundred proteins have been extracted from these two organisms and their usefulness as species-specific biomarkers or as a source of potential antigens is being evaluated through immunological screening.

Use of ante mortem tests to identify cattle with disseminated Mycobacterium avium ssp. paratuberculosis infection detected by post mortem culture of 15 tissues

Jason E. Lombard, M C Antognoli, H L Hirst, M M Dennis, S M Jensen, M D Salman, Franklyn Garry

Abstract

The objective of this study was to determine the association of ante mortem test results with presence of disseminated MAP infection at slaughter. Twenty adult lactating Holstein cows were obtained for slaughter from October 2003 to February 2004 from 4 Colorado dairies with a known history of clinical JD. Cows that were purchased had a history of at least one positive serum ELISA result. Prior to euthanasia, serum was collected for ELISA and feces for conventional and BACTEC culture and liver biopsy was performed for histologic exam. At necropsy 16 specimens, including feces, lymph nodes, and muscle were submitted for conventional culture. Cows with at least one positive tissue culture beyond the intestine and its associated LN were considered to have disseminated infection (DI). Sixteen of 20 cows had confirmed MAP infection via culture. Twelve of these had disseminated infection (DI). Of the 12 cows with DI, 5 had no evidence of clinical JD, 3 had weight loss without diarrhea, and 4 had obvious clinical JD. The most common tissue found culture positive for MAP in DI cows was the hepatic LN (11 of 12 DI cows). Fecal culture (FC) with BACTEC and conventional FC detected fecal shedding in 7 and 11 of 12 DI cows, respectively. Of 19 ante mortem liver biopsies collected, 4 were affected with multifocal granulomatous hepatitis, but no acid-fast bacteria were identified. Of the four cows that had negative ELISA results at euthanasia, 3 were infected and 2 had DI, despite previous positive serum ELISA results. If the perceived human health risk leads to mandatory exclusion of cows with disseminated MAP infection from the food supply, serum ELISA may be an option for detecting a large proportion of DI cattle ante mortem.

Bovine mucosal immune responses to systemic Mycobacterium paratuberculosis immunisation

S Marché, Marc Govaerts, V Rosseels, Jacques Godfroid, Kris Huygen, K Walravens

Abstract

Local and systemic immune parameters were investigated following subcutaneous immunisation of cattle with a gamma-irradiated *M. avium* subsp. *paratuberculosis* (Map) inactivated vaccine. The ATCC 19698 strain was exposed to 0.5 megarad and a single dosis of 4 mg dry weight was administered subcutaneously (SC) in a non-mineral Montanide ISA775^{O} water in oil suspension to five 2-week old Friesian-Holstein male calves. Animals were monitored fortnighly and sacrificed 7 months post-immunisation together with 3 non-immunised controls. Lymphocytes prepared from peripheral blood, spleen, the SC draining lymph node (LN), ileocecal lymph nodes, lamina propria (LP) and intraepithelial lymphocytes (IEL), were stimulated *in vitro* and antigen-specific IFN-γ responses measured in 20-h and 5-day cultures along with proliferative responses. Strong systemic IFN-γ responses were recorded at 20h in immunised animals versus no response in ileocecal, LP and IEL preparations, whereas IFN-γ was detected in 5-day cultures from LP, IEL and ileocecal LN together with enhanced systemic responses. Results are discussed in terms of induction of mucosal CD4 Th1 immunity, of effector and memory T cell functions, and of systemic vaccine potency.

Engulfment of MAP by bovine primary blood macrophages and the establishment of a reliable protein and RNA expression assay

P McWaters, J A Vaughan, G Beddome, M D Lanigan, Mark Tizard, Woytek P. Michalski

Abstract

The interactions of *M. avium* ssp. *paratuberculosis* (MAP) with host macrophages and the development of immune responses are not well understood. The pathogenicity of MAP is directly related to its ability to persist in host macrophages. Our overall aim is to study these mechanisms of survival at the cellular level. The aim of this work is to identify the specific MAP and macrophage proteins produced at immediate, early and sustained stages of MAP/ macrophage interaction and study the resultant changes in macrophage function. To perform these studies, a reliable, reproducible MAP/ macrophage engulfment assay was developed and optimised. In a series of pulse-chase experiments, the uptake of MAP, protein expression, engulfment dynamics, incubation times, multiplicity of infection (MOI), period of macrophage adherence and prestimulation of primary cells. The effects of bacterial growth phases, culture medium, and presentation at engulfment were also studied. Ziehl-Nielsen staining was used to evaluate uptake and survival of MAP within host macrophages. A MAP/ host cell MOI of 10 was the engulfment ratio of choice. Below a MOI of 10, engulfment was reduced. This preliminary work highlights the variability in engulfment of MAP by primary cells and the need to strictly standardise assay procedures to achieve uniform uptake of MAP. Studies of protein and gene expression at the cellular level require reproducible conditions and engulfment. Variability can be introduced through cell type, period of attachment of cells and variance in MAP culture parameters. Macrophages will be used for proteomic analysis and RNA extraction for micro array analysis through metabolic isotope-labelling (^{35}S and ^{14}C) of bacteria and macrophages.

PPE proteins of Mycobacterium avium subspecies paratuberculosis induce interferon-gamma production by peripheral blood mononuclear cells from infected cattle

Reiko Nagata, Y Muneta, Yusuyuki Mori

Abstract

Mycobacterium avium subspecies *paratuberculosis* (Map) genomic library expressed in Escherichia coli was screened for the identification of novel interferon-gamma (IFN-γ) inducing antigens using peripheral blood mononuclear cells (PBMC) from experimentally infected cattle. Two positive clones which induced IFN-γ in the PBMC were identified from 1,200 colonies examined. Three recombinant proteins, referred to as Map10, Map39 and Map41 according to their respective molecular sizes, were produced based on nucleotides sequences obtained from these clones. All recombinant proteins significantly elicited IFN-?γ production by PBMC from cattle infected with Map. Two of the three recombinant proteins, Map39 and Map41, appeared to be members of the PPE protein family of Map, due to the sequence homology of the deduced amino acids with those of the M. tuberculosis PPE family protein. *M. avium* subsp. *avium* also possessed similar PPE family protein genes, but no cross-reactions were observed among the other mycobacterial species tested. Monoclonal antibodies (Mab) against Map39 and Map41 strongly reacted with corresponding antigens in immunoblotting with the sonicated extract and PPD of Map, suggesting that these two PPE antigens are actually produced by this organism. Most of IFN-γ ?producing cells stimulated by these PPE antigens were CD4 positive cells, and they co-cultured with the dendritic cells which were stimulated by PPE antigens secreted IFN-γ far higher than that with macrophages. IL-10 was also detected in the culture supernatant of PBMC stimulated by PPE antigens besides IFN-γ, and addition of anti bovine IL-10 Mab increased IFN-γ ?production. These cytokine inductions by PPE antigens may be implicated in immunopathogenesis of Johne's disease

Clinical and subclinical diseases predisposing to Johne's disease

Eran Raizman, Scott Wells

Abstract

The objectives of this study were to assess the effect of clinical and subclinical diseases on incidence of clinical Johne's disease (JD), and to determine the effect of clinical and subclinical diseases on incidence of fecal shedding after 305 days in lactation. 1297 cows from two Minnesota dairies were enrolled in the study after fecal samples were obtained during the close-up period. A second fecal sample was obtained from cows after at least 305 after calving (DIM) or at time of leaving the herd (sold/dead). Between 3-21 DIM, blood samples were obtained for Beta Hydroxy Butyrate (BHB) and serum total protein testing. Body condition score (BCS) was evaluated during the close-up period, within 21 DIM and at end of lactation. Clinical disease (milk fever, retained placenta, metritis, ketosis, displacement abomasum, lameness, mastitis, abortion over 180 days of pregnancy, and JD clinical signs) and reproductive performance data (days to first breeding, times bred, and days open) were recorded. Average DIM when JD clinical signs cows were culled (n=66) was 209. Factors associated with culling due to JD clinical signs included pneumonia (OR=2.3, 95%CI=1.23-4.37), positive fecal culture during the closeup period (OR=35, CI=18-69), serum total protein levels 0.7 (0.46-1.1), BCS during the closeup period 0.45 (0.26-0.78) and in the beginning and end of lactation 0.54 (0.33-0.87), mastitis 0.50 (0.24-1.10), and retained placenta 0.36 (0.11-1.16). Onset of fecal shedding at the end of the lactation was associated with pneumonia (OR=2.4 CI=1.3-4.3), milk fever (OR=5.0, CI=1.7-17.8), mastitis (OR=0.46, CI=0.22-0.93), and BCS in the end of the lactation (OR=0.40, CI= 0.25-0.66). The results provide insights into the role of other diseases on JD clinical signs and fecal shedding, which may enable us to better manage the disease early in the lactation.

Anatomopathology of paratuberculosis in dairy cattle from Rio de Janeiro, Brazil

A B Rodrigues, P Ristow, C D Marassi, A S Santos, F Rocha, R Ferreira, W Lilenbaum, C B Carvalho, E C Carvalho

Abstract

The purpose of this study was to describe the anatomopathological findings of three cases of paratuberculosis in autochthonous dairy cattle from Rio de Janeiro, Brazil. Animals presented characteristic clinical symptoms of paratuberculosis as cachexia, diarrhea and leanness, and were first diagnosed by sero-reactivity to an in-house ELISA. Animals were sacrificed and samples of small and large intestines, mesenteric lymph nodes and ileocaecal valve were collected and processed for both histopathology and bacteriology. Tissues were fixed in 10% buffered formalin, processed for paraffin inclusion and stained by both HE (haemathoxilin-eosin) and ZN (Ziehl-Neelsen) methods. Macroscopic alterations such as small intestine wall segmental thickness, mucosal hyperaemia and prominent corrugation were observed; ileocaecal valve emaciation, evident mesenteric lymphadenomegally, and lymphangiectasis were also noted. The main histopathological findings were enteritis, lymphangitis and granulomatous lymphadenitis. Intestinal lesions were mainly restricted to mucosa and submucosa of jejune and ileum, and characterised by inflammatory infiltration of lymphocytes, eosinophils, epithelioid macrophages and scarce giant Langhans-type cells. Numerous acid-fast bacilli were observed into macrophages, either alone or forming aggregates, on the top of villi, lamina propria and lymph nodes parenchyma. Most of the tissues yielded Map, and culture was confirmed by mycobactin dependence and specific PCR IS*900*. In conclusion, anatomopathology yielded characteristic findings that were later confirmed by culture and PCR. Therefore, due to its rapid results, low cost and reliability, this method (group of gross and microscopic lesions) was considered as a valuable tool for the diagnosis of paratuberculosis.

Identification and immunological characterization of Mycobacterium avium ssp. paratuberculosis specific antigens with the goal to develop a vaccine

V Roupie, N Trinchero, I Danese, J - Letesson, I Noël-Georis, Ruddy Wattiez, V Rosseels, M Romano, Kris Huygen

Abstract

Paratuberculosis is responsible for major economic losses -particularly in the dairy sector- because of reduced milk production in affected animals. As the existing vaccine, composed of whole, killed mycobacteria, interferes with the PPD skin test used for the diagnosis of bovine tuberculosis, farmers are reluctant to use it. A sub-unit vaccine composed of immunodominant and specific antigens could offer a solution to this problem. Ninety-four *M. ptb.* specific antigens were identified using "in silico" comparison of *M. ptb.* genome with that of *M. avium*. Another seven *M. ptb.* specific antigens were identified by mass spectrometry of *M. ptb.* extract antigens, separated by 2D gel electrophoresis and immunoblotted with sera from diseased cattle. From these 101 candidates, 10 were selected on the basis of prediction programs for murine MHC Class I and MHC Class II epitopes. Sequences particularly rich in potential CD8+ and CD4+ T cell epitopes were selected. These 10 candidates were cloned in the eucaryotic expression vector V1J.ns-tPA and checked for expression in transiently transfected BHK cells. Immunogenicity was analyzed by plasmid DNA vaccination of BALB/c and C57BL/6 mice. *M. ptb.* specific Th1 type (IL-2 and IFN-γ) spleen cell cytokine secretion and antibody production was examined. Screening of the protective efficacy of these *M. ptb.* specific DNA vaccines is in progress. *Vi.R. is holder of a FRIA fellowship. R.W is Research Associate of the FNRS. This work is partially supported by the "region Wallonne" of Belgium*

Pathology and diagnosis of paratuberculosis in water buffaloes (Bubalus bubalis)

P Sivakumar, N Singh, Bhupendra Nath Tripathi

Abstract

The objectives of the present study were to characterize the rarely studied gross and histological lesions of paratuberculosis in water buffaloes and to study the efficacy of the different diagnostic methods. Intestinal tissues (n=405) including jejunum, ileum, ileocecal junction and associated mesenteric lymph nodes (MLN) collected from 1000 buffaloes screened at slaughterhouse and 5 buffaloes necropsied at the postmortem room of the institute were examined histologically for paratuberculosis lesions. Selected tissues (n=50) were subjected to bacterial culture and IS*900* PCR. On histological evaluation of sections, granulomatous inflammatory changes were observed in 20 animals that had shown gross lesions of intestinal thickening and mucosal corrugations, and enlarged and edematous mesenteric lymph nodes. Histological lesions were classified into 3 grades (Grade-1, 2 and 3) on the basis of type and severity of cellular infiltrates and presence of acid-fast bacilli (AFB) or acid-fast granular debris. Grade-1 lesions observed in 8 animals were characterized by scattered infiltration of epithelioid macrophages amongst large number of lymphocytes with macrophages containing acid-fast granular debris in the intestine and lymph nodes. Grade-2 lesions (n=8) consisted of microgranulomas in the intestine and large granulomas in the MLN with AFB or acid-fast granular debris. Grade-3 lesions (n=4) were characterized by the presence of multiple granulomas and multinucleated giant cells and definite AFB in the intestine and MLN. Positive bacterial culture and IS*900* PCR was observed in 6 and 14 animals, respectively. The partial sequence analysis of PCR products of bubaline isolate of *Mycobacterium avium* subsp. *paratuberculosis* (Accession No: AY 660657) showed more than 98% of homology with the published sequence of IS*900* gene (Accession No: X16293). From the results of the present study, it was concluded that the histological lesions observed in water buffaloes could be paucibacillary type and tissue PCR was found to have more sensitivity than bacterial culture and smear examination.

Evidence for a reduced transactivating capacity of CCAAT/enhancer binding protein (C/EBP) β in Mycobacterium avium ssp. paratuberculosis (MAP) infected macrophages

S Sommer, S Jeckstadt, Peter Valentin-Weigand, R Goethe

Abstract

Pathogenic mycobacteria circumvent host immune responses by disruption of macrophage effector functions. Here we examined cytokine production, gene expression and protein-DNA interactions of MAP-infected murine J774A.1 and RAW264.7 macrophage cell lines (MAPMac) and compared it to macrophages stimulated with LPS (LPSMac), a potent activator of macrophages. ELISA and Northern analyses revealed that the expression levels of TNFa, IL-1b and IL-6 were significantly lower in MAPMac than in LPSMac. IL-6 was almost not induced in MAPMac RAW264.7 cells, whereas its expression increased over the time in MAPMac J774A.1 cells. Since the transcription regulators NFkB and C/ebpb contribute to IL-6 gene expression electrophoretic-mobility-shift-assays (EMSA) were performed. EMSA revealed reduced NFkB and C/ebpb binding to the NFkB and C/ebp binding site of the IL-6 gene in MAPMac as compared to LPSMac. Interestingly, when compared to uninfected cells C/ebpb binding activity was almost not enhanced in MAPMac RAW264.7 cells but enhanced in MAPMac J774.A1 cells. In these cells, however, C/ebpb binding complexes appeared to be smaller than in LPSMac. C/ebpb protein complexes can be composed of two alternatively translated C/ebpb-isoforms; the larger 37 kDa LAP that is fully transactive and the smaller 20 kDa LIP that has reduced transactivating capacity. Thus, different LAP-LIP combinations will result in C/EBPb EMSA complexes of different sizes and biological function. Since C/EBPb mRNA expression was similar in LPSMac and MAPMac, we determined the Lap-LIP ratio in nuclear extracts from LPSMac and MAPMac by Southwestern analysis. We found LAP-LIP ratios similar to that of untreated cells in MAPMac, whereas the ratios were considerably higher in LPSMac. These data provide evidence for a reduced transactivating capacity of C/EBPb leading to reduced IL-6 expression in MAPMac.

Expression of CD5+ on lymphocytes in cattle with paratuberculosis

Judy R. Stabel, M S Khalifeh, K N Soenksen

Abstract

CD5+ is a cell surface molecule involved in antigen recognition and is present on all T cells and a subset of B cells. Recent work in our laboratory on cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) has shown the differential expression of the CD5 marker on peripheral blood B cells, with a shift in B cell expression from CD5^{dim} to CD5^{bright} as disease progresses from a subclinical to a clinical state. Previous research has also shown an increase in the number of peripheral blood B cells and a decrease in peripheral blood CD4$^+$ T cells as disease progression occurs. The purpose of this study was to examine lymphocyte subsets and CD5$^+$ expression on peripheral blood B and T cells from healthy cattle and cattle in the subclinical and clinical stages of paratuberculosis. Peripheral blood mononuclear cells (PBMC) were isolated, cultured in the presence or absence of live *M. paratuberculosis*, and then analyzed by flow cytometry within the three treatment groups. Three CD5$^+$ populations were identified: CD5^{dim}, CD5^{bright}, and CD5$^{extra\ bright}$. Analysis showed an increase in B cells in clinical animals compared to subclinically infected cows. In addition, a decrease in the CD5^{dim} B cell population along with a concomitant increase in the CD5^{bright} B cell population was observed in subclinically and clinically infected cows. No significant trends were observed in T cell populations or CD5$^+$ expression within T cell populations, and *in vitro* infection with live *M. avium* subsp. *paratuberculosis* did not result in significant changes in B cell or T cell populations. These results suggest that changes in CD5$^+$ expression on B cells in animals with paratuberculosis may play a role in progression of the disease.

Gene expression and survival of M. paratuberculosis infected macrophages

D Taylor, K de Silva, L Di Fiore, Richard Whittington

Abstract

We have used a murine macrophage model of infection in preliminary investigations to characterize alterations in host gene expression, protein secretion, induction of apoptosis, macrophage survival and bacterial persistence in response to long term infection with *M. ptb*. Murine macrophages (RAW264.7 cells) were incubated with *M. ptb* (MOI=10) for up to 7 days. Cells incubated with medium alone or heat-killed *M. ptb* were included as controls. Cells were collected prior to infection and at days 1, 2, 3, 4 and 7 post infection to determine the macrophage response at the cellular and molecular level. Cellular parameters assessed were cell number, apoptosis (caspase activity determined by flow cytometry), number of infected cells and number of *M. ptb* per cell. RNA was isolated from the macrophages at each time point and differential display PCR (DD-PCR) is being used to identify novel changes in gene expression in response to infection over time. Differences in expression of known immuno-responsive genes have also been determined using quantitative PCR. Our results demonstrate that the presence of *M. ptb* reduces proliferation of RAW264.7 cells, and significantly affects the expression of a number of gene products.

Bovine CD1 family contains functional group 1 CD1 but no functional CD1d

I Van Rhijn, Victor PMG Rutten, Adriana P. Koets

Abstract

CD1 presents a variety of antigenic structures to T cells, including lipids, glycolipids, small aromatic compounds, and lipopeptide antigens. CD1 proteins are structurally related to MHC class I proteins in terms of the overall structure of the three α domains and association with β2-microglobulin. Group 1 CD1 molecules (CD1a, CD1b, and CD1c) have been shown to exclusively present mycobacterial lipids. Group 2 CD1 molecules (CD1d) are known to present antigen to NKT cells, a T cell lineage that is characterized by a limited T cell repertoire and an antigen experienced phenotype, capable of rapidly secreting large amounts of γ-Interferon and IL-4. Understanding the development of CD1 restricted, T cell mediated immunity upon infection with mycobacteria is of key importance for the development of new or improved vaccines based on lipid antigens. Knowledge about the protective effects of CD1 restricted responses against infection, the interplay with other parts of the immune system, and whether CD1 restricted T cells can give rise to immunological memory is insufficient. B. taurus is the natural host of several pathogenic mycobacteria, among which *M. avium* ssp. *paratuberculosis* (MAP), causing Johnes disease, and *M. bovis*, causing bovine tuberculosis. In the present study we describe the CD1 family genes in B. taurus and provide evidence that B. taurus expresses CD1b and CD1e molecules, and has genomic sequences of CD1A and CD1D. The two CD1D genes that were found are pseudogenes. BoCD1b was cloned and transfected into 293T cells and CD1b expression was shown by antibodies SBU-T6, CC14, CC20, CC122, BCD1b.3, but not CC43. T cells isolated from cattle infected with MAP, but not from uninfected cattle, can be stimulated with mycobacterial lipids, and this stimulation is CD1b dependent. These findings suggest that mycolic acid based antigens can be presented by cattle, but that CD1d-restricted NKT cells are absent.

Variations in the pathological response of lambs experimentally infected with different Map strains

Andrea Verna, C García-Pariente, O Moreno, L E Reyes, J Benavides, Fernando A. Paolicchi, M I Romano, J Francisco Garcia Marin, V Pérez

Abstract

Infection by *Mycobacterium avium* subsp. *paratuberculosis* (Map) causes a granulomatous inflammatory response in the intestine and associated lymph nodes. Differences in the pathological features (organs affected or inflammatory infiltrate) have been observed between species and individuals. The host immune responses or the role of different Map strains have been suggested as possible explanations. A total of 30 lambs, 1 month-old, divided in 6 groups, were challenged with several strains of Map: groups 1 and 2, with two bovine strains showing different genetic pattern (A and E); groups 3 and 4, with a bovine strain, directly purified from the intestinal mucosa of a clinical case, or the same strain grown in culture media, respectively; group 5, an ovine strain, directly purified from the intestinal mucosa of a clinical case; group 6 was kept as control. Peripheral immune responses were assessed from 0 to 150 days-post-infection (dpi), when lambs were humanely killed. Pathological and bacteriological studies were performed in tissues from the intestine and lymph nodes. Lesional type and the inflammatory infiltrate were examined. Number of granulomas per section and area occupied by lesion in the lymph nodes were counted, as indicators of pathogenicity. All the lambs infected by bovine Map strains, regardless the type, showed a common lesional pattern, characterised by focal lesions, located mainly in the mesenteric lymph nodes, the presence of fibrous tissue, and occasionally necrosis, in the granulomas and the existence of numerous giant cells. Differences in lesion severity were seen among groups: lambs from groups 1 and 2 had the highest number of granulomas and lymph node area affected. In animals from group 5, lesions were more severe and appeared mainly in the intestinal lymphoid tissue. Necrosis, fibrosis or giant cells were never detected. Map was isolated from tissues from all the groups and showed the same pattern than the inoculum. The type of Map strain influences the pathological response of the infected lambs. *This work was founded by grant AGL-2004-07421-C0201 from MEC.*

MAP Super-Shedders: Another factor in the control of Johne's disease

Robert H. Whitlock, Raymond W. Sweeney, Terry L Fyock, Jerry W. Smith

Abstract

Objective: To determine the range of MAP cfu/gram of manure in cattle classified as heavy shedders. Cattle are typically classified as low, moderate or high shedders based on the visible colonies of MAP on the surface of solid media, typically, Herrold's egg yolk media (HEYM). Over the past five years most laboratories in the USA report the number of visible MAP colonies on each tube of HEYM, but rarely enumerate above 50 to 70 colonies per tube. Fifty cfu on each of four tubes represents an estimated 1,050 colony forming units of MAP per gram of manure. Materials & Methods: Fecal samples from cattle classified as high shedders were serially diluted: 1:5, 1:10, 1:50; 1:100, 1:500, 1:1:000, 1:5,000, 1:10,000 and 1:50,000. Then, a larger number of fecal samples from more than 200 cattle, classified as heavy shedders, were cultured and serially diluted at 1:100 and 1:1,000. Super-Shedders are defined as cattle with fecal samples having more than 10,000 cfu MAP per gram of manure. Results: The vast majority of heavy shedders would be classified as Super-shedders with more than 10,000 cfu MAP per gram of manure. Some cattle not showing clinical signs of JD shed more than 1,000,000 cfu of MAP per gram of manure. Conclusions: Super-shedders represent the greatest risk to spread Johne's disease among herd mates. Some Super-shedders would contaminate the environment with more MAP than 160 heavy shedders, or more than 2,000 moderate shedders and more than 20,000 low shedders. Based on this new dimension of Super-Shedders, a significant proportion of low shedders are likely to represent "pass-through" and not active true infections. The challenge to the Johne's academic community will be to develop diagnostic methods to detect these Super-shedders in a cost efficient manner and eliminate them from the herd prior to massive environmental contamination.

The prospects for herd level control of paratuberculosis in cattle: a consultant's view

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INTRODUCTION

Paratuberculosis, caused by infection with the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is an infectious disease of ruminants with socio-economic importance that has the potential to affect international trade (OIE 2004). Once clinical disease develops, animals usually have profuse diarrhoea and lose body weight, which necessitates culling on humane grounds and thus their early loss from the herd. Reduced productivity is also a feature of the later stages of the subclinical phase of the disease and paratuberculosis can have a significant financial impact in dairy herds (Ott et al. 1999). In some production systems, beef herds infected with MAP may also suffer significant losses as the peak age incidence of clinical paratuberculosis is three to five years and the productive life of beef cows is ten years. The possibility of a causal association between MAP and Crohn's disease of humans has long been considered (Chiodini and Rossiter 1996) and MAP has been isolated from the ileal mucosa (Bull et al. 2003) and the blood (Naser et al. 2004) of some humans suffering from Crohn's disease. As yet there is no consensus on whether MAP has a causal role in Crohn's disease and neither has any causal link between paratuberculosis in ruminants and Crohn's disease in man been demonstrated. However milk from infected herds can contain MAP. Although pasteurisation is highly effective in killing MAP, the organism has heat resistant characteristics that allow a small proportion of MAP organisms to survive pasteurisation (Grant et al. 2001). A precautionary approach is a responsible position to adopt, thus working to control paratuberculosis in the dairy herd and so limit one route of human dietary exposure to MAP (for example see UK Food Standards Agency web site, http://www.food.gov.uk). A positive argument for the control of paratuberculosis can therefore be made from production efficiency, animal welfare and food hygiene perspectives.

The principals of a national approach to the control of paratuberculosis have been described (Kennedy and Benedictus 2001) and rest on:
1. Herd assurance programmes to identify test negative herds that that can be the source of breeding replacements which constitute a low risk of introducing infection.
2. Providing effective advice to herd owners on biosecurity to reduce the risk of introducing the infection.
3. Providing effective advice on improving hygiene and husbandry to reduce within-herd transmission.
4. Test and cull programmes to reduce the impact of infection within the herd.

Several of the countries with significant dairy industries had previously adopted national control strategies, which embrace all four points, but where the initial focus was on points 1 and 4. As experience was gained the need for greater emphasis on points 2 and 3 has become clear.

Effective delivery of the advice on prevention and control is a critical component of programmes at the farm and regional level. This paper offers a consultant's view of the issues that affect translation of national control policy to the herd level and outlines the future prospects for herd level control, based particularly on experience within the British dairy and beef herds.

Background
As with most livestock production, there has been a need to increase efficiency in the dairy and beef industries by expanding the unit size to reduce the fixed costs of production (Wolf 2003). However, unlike the pig and poultry industries, this has not been accompanied by the recognition of the importance of animal health and biosecurity. Frequently herds have rapidly expanded with little consideration for biosecurity. It has been shown that herds finding the majority of their replacements from outside the herd are more likely to be infected with MAP (Chi et al. 2002). At this meeting, reports from France (Beadeau et

al. 2005) and the Netherlands (van Weering et al. 2005) reaffirm that increasing herd size and purchase of replacements are key risk factors for paratuberculosis in the dairy herd. An increase in the number of cows managed per worker has increased the potential for poor management in the critical areas of cow hygiene, dry cow management and calf rearing. Deficiencies in these areas favour the spread of paratuberculosis. This intensification, particularly of the dairy industry, has also meant that infertility, lameness and mastitis have continued to be sources of significant losses. Where there are restricted resources for improving animal health, the control of paratuberculosis is unlikely to feature high on the list of priorities until such times as the disease is established in the herd and causes significant losses. Until this point is reached the financial argument for controlling the disease is related to the price the farmer receives for milk. Modelling has demonstrated that for British conditions when the milk price is £0.16 per litre or lower it is difficult to find financial justification for controlling paratuberculosis in herds where the annual incidence of clinical disease is at or below 2% (Stott et al. 2001). Beef production is similarly under pressure and in the European Union (EU) this is likely to increase as the removal of headage subsidy for beef production in 2005 begins to reduce the income generated by beef herds. The pressures on the cattle industry favour the spread of MAP infection between and within herds and do not favour the implementation of control and prevention programmes.

How effective are the programmes?
Both the assurance programme and the test and cull programme (points 1 and 4 above) are based on tests that have significant limitations. In the pre-clinical stages the sensitivity of the antibody ELISA may be as low as 15% while that of faecal culture may be 30% (Whitlock et al. 2000). This hampers the ability to detect infected animals before they begin to excrete significant numbers of organisms into the environment and generate new infections. For the assurance programme the lack of a sensitive test for the pre-clinical phase of the disease means that herds with a low prevalence may go undetected. This problem is increased where herd size is low, a particular problem for pedigree beef herds in Europe. The consequence is that infected animals may be sold from test-negative herds that achieve the requisite standards for a particular assurance programme. Screening herd replacements before introduction, whether from an assured source or otherwise, can fail to detect the infection because of the inherent limitations of the tests and because many purchased breeding animals are at an age when they are unlikely to show any evidence of infection (under two years of age). This is further compounded by the probable long time lag between the introduction of an infected animal and either the appearance of clinical disease in the herd or a test failure in the assurance programme. In these respects assurance programmes for the control of paratuberculosis compare poorly with those for the control of bovine virus diarrhoea virus (BVDV) and bovine herpes virus 1 (BHV1) where there are more sensitive diagnostic tests and a serological response occurs earlier.

These limitations need to be managed. In most cases this is done through grading the degree of assurance on the basis of the number of years the herd has been test-negative and has complied with the biosecurity requirements of the programme. However an argument can be made for supporting this further by weighting each herd on the basis of herd size and the number of purchased replacements that have entered the herd over a set time period. That is to say the value of the assurance programmes can be increased by increasing its scrutiny of the herd, relating back to the concept of risk assessment in the biosecurity plan for herds purchasing replacements. The downside is that, in general, greater programme complexity deters farmers from participating and realizing its benefits. This applies both to participants in the assurance programme, but also to those purchasing the stock.

There is little information published to indicate whether the assurance programmes are useful for the purchasers of breeding stock although the numbers of herds participating in the assurance schemes of several countries are regularly made available and considerable progress has been made in several countries. Experience in Britain indicates that the concept has proven to be of little interest to dairy farmers. There is greater awareness on the part of beef farmers who seek to buy bulls from low-risk sources, perhaps because the purchase of a high value bull that subsequently develops paratuberculosis is a very visible loss. Relatively few beef herds producing commercial type breeding females for sale have enrolled in the assurance programme and the availability of female replacements of assured status is limited. This raises the possibility that the assurance programmes as they exist are too costly and that a lower level or alternative strategy for assurance may be required.

Indeed reducing the cost of programmes appears to be an important consideration. Setting aside the debate on the merits of faecal culture verses serology as the basis of assurance programmes, the screening of environmental samples for MAP has been shown to be of potential in reducing costs while achieving an accurate assessment of whether a dairy herd is infected with MAP (Raizman et al. 2004). Similarly in this meeting, Gardner and colleagues have indicated that targeting within the herd can reduce the number of animals tested and costs by up to 50% while achieving the same detection probability (Gardner et al. 2005). Stratman and colleagues also in this meeting have used bulk tank antibody detection in conjunction with follow up detection systems for MAP as a way of determining herd infection (Stratmann et al. 2005). An alternative targeted approach for large herds might be to screen only animals leaving the herd, i.e. adults being culled. This specifically targets the population that is most likely to be infected in the herd and reduces the number of animals tested and costs to around 25% to 35% (dairy herd) and 10% to 20% (beef herd). Such systems while superficially attractive can be difficult to manage to ensure that animals are sampled before they leave the farm. This approach would seem to be inappropriate for small purebred beef herds, which make up the bulk of sire producers in Western Europe. A lower level of assurance has also been considered to be appropriate for the beef herds in Australia, based on targeted sampling (Kennedy et al. 2002)

While the cost of assurance programmes may be a barrier to widespread uptake it should not be assumed that this is always the case. An indication of UK milk producers' attitude to quality systems can be taken from two areas. Despite there being a clear understanding on how milk hygiene could be improved, it was only once milk purchasers put in place penalty deductions and bonus payments in relation to somatic cell counts that the rate of improvement in milk hygiene increased significantly (Booth 2000). Progress with the implementation of whole herd quality systems was similarly slow until the National Dairy Farm Assured Scheme (NDFAS) was formed and the primary milk purchasers made participation a condition for their suppliers. Now 95% of dairy farms meet the assurance standards (pers com. Derek Kennedy). This suggests that the implementation of quality systems is related to immediate financial reward or penalty. A reluctance to spend money on a disease that is not perceived to be a problem by the breeder is to be expected. This reluctance is unlikely to be overcome merely be rationalising testing programmes and reducing costs of testing. To put this in context, in Britain the cost of the assurance programme excluding farm labour costs is estimated to be £7.00 per adult in the herd. After calf losses and internal replacements are considered there will be no more than 15 to 20 heifers per 100 dairy cows available for sale each year with the figure at 25 to 30 heifers in beef herds. The cost of the assurance programme per heifer sold is therefore in the region of £35 to £47 per head for dairy heifers and £24 to £28 per head for beef heifers. The current cost of dairy heifers in Britain ranges from £800 for commercial type (SAC 2004) to £1100 for high genetic merit animals (Anon 2005) and for commercial beef heifers £700 (SAC 2004) to £1000 for purebred maternal breeds. This would suggest that at least in Britain the cost of the assurance programme represents a small part of the sale price and so should not be a major barrier to participation. Evidence to support this comes from the experience of one specialised maternal beef breed, the Welsh Black. The breed society's support for membership in the assurance programme has led to this breed making up 65% of the beef herds that have achieved the monitored free status.

The British experience with assurance and test and cull programmes has shown that most interest has come from both dairy and beef cow herds with significant losses due to clinical paratuberculosis. Dairy farmers facing a deteriorating clinical status have also been influenced by the FSA position on MAP in relation to Crohn's disease. Implementing a test and cull programme supported by a hygiene programme has allowed them to address the problem. However the long time scale of the infection means that participants will see no progress for many years. Again this contrasts with control programmes for mastitis or BVDV, where improvements can be immediate or at least seen within the course of a year. Furthermore there is uncertainty concerning the effectiveness of test and cull programmes.

Modelling has been used to explore the use of the control programmes and the output has indicated that using a test and cull programme only will lead to a reduction in prevalence from 10% to 5% in 15 to 20 years (Collins and Morgan 1991). In a more recent modelling exercise the value of a test and cull programme appeared to be unattractive from economic and biological perspectives, but the hygiene programme would be more cost effective (Groenendaal et al. 2003). Output from this latter model also indicated that it would not be possible using any programme to reduce the within-herd prevalence in beef

herds to close to zero within 20 years. However these models have also indicated that the within-herd prevalence will rise if no control programme is put in place.

There are very few studies that offer progress reports from the field. Two such describe the experience in Victoria, Australia in dairy (Jubb and Galvin 2004 b) and beef herds (Jubb and Galvin 2004 a). These looked at a group of herds over a ten-year period. Clinical disease had been increasing before the programme started and diminished rapidly after testing and removal of test positives occurred. The decline in the number of reactors was slower and in some herds the control programme failed to control the disease. However in 47% of the 522 participating dairy herds there were no home born reactors after the start of the programme and by the end of the ten year assessment 6% of the dairy herds had completed three clear tests. There were only 14 beef herds that enrolled and continued to follow the testing programme and of these six herds achieved three clear annual herd tests.

The mean age of test positive animals was close to the mean age of clinical cases suggesting that testing identified only infected animals late in the pre-clinical phase of the disease. The authors were cautious in their conclusions, citing the short period of evaluation and also expressed concern that lack of progress in some herds may have been because of poor compliance with the programme or because the transmission of the disease was as yet not fully understood.

Despite the declared caution from the report on the field experience the results contrast sharply with the projections from the more recent modelling exercises. In the United Kingdom the control programme is based closely on that used in Australia and is run by several licensed operators. The experience of one of these operators (Premium Cattle Health Scheme, SAC) is that after seven years there are 608 member herds, with beef herds making up the majority, and 130 had achieved two or more clear herd tests. A third of these herds had experienced clinical cases and test positives before achieving three clear annual herd tests. Such apparent progress could not be made using a test with a sensitivity of 15%, but the sensitivity of the antibody ELISA in the later stages of the disease is considered to be close to 90% and therefore the sensitivity achieved by repeat testing at the herd level should also be close to 90%.

In the assessments that have been made it was not possible to separate the effects of the hygiene programme from the test and cull programme. It is clear that control is not easily achieved in some herds, but that in the majority a significant degree of control can be achieved over a ten year period and in some herds effective control is achieved much sooner. No attempt has yet been made to carry out a financial assessment of the value of the control programmes in infected herds. Such assessments are necessary to allow the veterinary consultant and herd manager to take decisions on implementing control, but as outlined above straight profit and loss are not the only concerns where the disease in question affects animal welfare as well as productivity and there is a potential human health concern.

Management of the test and cull programme at the herd level is also hampered by a widespread suspicion of the "accuracy" of test results. If sampled and tested again, ELISA-positive animals frequently present with a vastly different optical density (OD) reading and there can be poor agreement between antibody test results and detection of MAP in faeces by faecal culture or by direct polymerase chain reaction (PCR) testing. However experience with infected herds indicates that any animal that tests positive by the antibody ELISA is likely to do so again even if it may test negative on an intervening occasion. This is supported by the experience in Victoria where within the test and cull programme the specificity of the test was found to be 99.62% (Holmes et al. 2004). To overcome this uncertainty the herd owner participating in a test and cull programme must be made aware that it can be difficult to prove that an individual animal is infected, but for maximum progress all test positive animals should be viewed as high risk of being infected and managed accordingly. This extends to the offspring of test positive females where the two most recent calves born are considered to be at highest risk of being infected.

In infected herds, scheduling test positives for removal presents a difficulty, particularly where test positives represent 10% or more of the herd's production unit. In these situations the test positives are frequently retained, but scheduled for later culling. A system using likelihood ratios to further prioritise culling has been suggested to manage this problem (Collins 2002). The test positive animals are kept separate from the test negatives during the dry period and at parturition in the dairy herd. In the beef herd they are further separated until their calves can be weaned. Such pragmatic responses are required to allow the

programmes to operate, but may compromise the effectiveness of the programmes to some degree. Dorshurst and Collins have presented at this meeting a modelling approach to determining the best approach to control paratuberculosis at the individual herd level (Dorshorst and Collins 2005). This exercise has indicated that a test and cull approach is not desirable for all infected herds and improving hygiene alone may be the most suitable control option for some herds.

Providing advice on improving hygiene and husbandry to reduce within-herd transmission.
The basic advice on control has centred on the understanding that calves are most at risk of becoming infected and that this risk diminishes with age, adult cattle being relatively resistant to infection. The sources of infection are from the faeces of adult cattle, from colostrum and milk that may be directly or fecally contaminated, and from *in utero* transmission. There are other potential sources of infection, such as between young stock and adult-to-adult transmission or breeding technology techniques, however these are secondary sources and are probably insignificant in the spread of paratuberculosis within the infected dairy herd. Therefore reducing faecal contamination of the environment and the cows in the dry and periparturient periods along with colostrum management and hygienic rearing of young calves are the cornerstones of hygiene management for the control of paratuberculosis in the dairy herd. Such a message is simple and easily understood by the herd managers and their veterinarians, but there are several reasons why translation into effective control is not easily achieved.

Herds that have undergone rapid expansion frequently have inadequate dry cow areas and calving boxes. It can be difficult for improvements to be achieved without significant investment in new buildings. The pooling of colostrum was a measure designed to reduce the amount of neonatal calf disease, particularly rotavirus and coronavirus enteritis. It has the added benefit of saving labour and providing a low cost feed. Moving away from this may compromise calf welfare on some units and will increase the cost of calf rearing. Continuing to feed pooled colostrum or waste milk while excluding milk from test positive animals is occasionally advocated and may reduce risk as the heaviest shedders are eliminated, however the low test sensitivity of the screening tests should preclude this approach. Small pasteurisation units for the treatment of colostrum are also recommended, but while there is some reduction in the numbers of viable MAP (Meylan et al. 1996) the quality of the immunoglobulins is reduced (Godden et al. 2003) and calves may receive inadequate amounts of passively derived antibody.

The hygiene management approach to herd level control of paratuberculosis is advocated widely and is supported by various models of herd level control, frequently indicating that this alone is the most cost effective approach to the control of paratuberculosis, but there is little published to support this position. Currently the effectiveness of this is being examined in the Netherlands, but at this time the study has not been running long enough to allow an assessment (van Weering et al. 2005). Previously it has been shown that hygienic measures to prevent the spread of paratuberculosis were not widely practised in the Netherlands (Muskens et al. 2003) or Australia (Wraight et al. 2000) and at this meeting Cashman and others have shown that a similar situation currently exists in Ireland (Cashman et al. 2005). There is therefore room for doubt as to whether satisfactory improvements in hygiene can be achieved in the majority of infected herds. Personal experience indicates that where there is high staff motivation then the hygiene programme can be implemented satisfactorily, but the required degree of staff motivation is not the norm.

In the beef herd there is less opportunity to improve the hygiene of the herd with respect to paratuberculosis control. Calves stay with their dams until they are at least six months old. In many herds in Britain calving takes place inside and calving season may last longer than the nine weeks recommended for optimum efficiency. There is therefore considerable opportunity for calves to be exposed to infected faecal material. The main focus is to reduce the opportunity for faecal contamination of udders and coats by keeping cows clean. It is also important to minimise the length of time that young calves are housed and to provide calf creep areas away from the feeding areas of the cows. Secondary actions are to exclude cows and calves from pasture where there are natural water sources that may be subject to faecal contamination and to ensure that slurry is neither put onto pasture nor where possible onto grass scheduled for conservation. Manure should be composted for a minimum period of six months.

There is no report on the effectiveness of hygiene programmes for the control of paratuberculosis in beef herds and personal experience indicates that the opportunity to change or improve the situation on many,

but not all, infected farms is limited. There is therefore good reason to doubt whether hygiene management can be an effective tool in implementing paratuberculosis control in the majority of beef herds. Nevertheless in many herds small improvements in the hygiene will be possible and in occasional herds major deficiencies in the hygiene management can be identified and corrected.

The role of vaccination
Herd vaccination has previously been shown to be an effective tool in the control of paratuberculosis (Larsen et al. 1978; Wentink et al. 1994; van Schaik et al. 1996.) although infection rates within the herd may remain high (Wentink et al. 1994). In the UK the live vaccine continues to be used by a few herds and it would appear that its use is not an important source of confusion in interpreting herd test results. At this meeting there are encouraging reports on the efficacy of vaccination (Garcia-Pariente et al. 2005; Garrido et al. 2005). Despite these it will be some time before it can be shown that vaccines offer an effective solution to the large herds of high genetic merit animals that will make up an increasing proportion of our dairy herds.

Providing effective advice to herd owners on biosecurity to reduce the risk of introducing infection
Consideration of this aspect of control and prevention is clearly closely related to the previous discussion on the assurance programmes. Minimal participation in the assurance programme means that there is an inadequate pool of low risk animals available for purchase. Advice can be given to the herd owner to buy stock from assured sources, but this advice is of little practical value where the stock does not exist. In Britain the concept of herd health plans is widely accepted throughout the dairy and the beef herds and it is at this level that the veterinarian can begin to raise awareness. A biosecurity plan is an integral part of a health plan and here the issue of managing the risk of introducing MAP infected animals can be discussed. Motivated farmers will produce creative solutions where they receive the necessary level of technical support from their veterinarian.

The education of veterinarians and livestock farmers on the control and prevention of paratuberculosis
Control of paratuberculosis at the herd level is unlikely to progress without effective education of the veterinarians and livestock farmers. Herd health planning can be an effective medium in this respect as it requires veterinarians to be trained in a planned approach and the procedure ensures that important disease issues for the herd are discussed with the farmer and documented in the written plan. Despite the complexities of paratuberculosis and the questions that remain unanswered on the pathology and epidemiology of the disease there is sufficient agreement on the important factors to put together a clear and simple message on control and prevention and to translate that into effective training for veterinarians and farmers. It is not difficult to find effective examples of this training; three approaches are described at this meeting (McDonald et al. 2005), (Nielsen and Nielsen 2005), (Mainali 2005). It is difficult to fault the stated objectives of providing a uniform base of knowledge and a consistent message about diagnosis and control (McDonald et al. 2005), however some caution is required. The Alberta Johne's disease control programme has now accredited 119 veterinarians (Mainali 2005), but there are only eight dairy herds and nine beef herds enrolled in the programme. Providing effective education material is not in itself valuable if the industry is less than lukewarm to the initiative. A clear example of this was seen in Britain after the foot and mouth disease epidemic of 2001. A document on the biosecurity issues of restocking herds was sent out to all dairy herds and beef herds that were slaughtered in the epidemic and to all farm animal veterinary practices. This document gave clear advice on paratuberculosis; despite this paratuberculosis is a significant problem on restocked herds (Holliman 2003) and experience has shown that it is difficult to find an affected farmer or a veterinarian that can recall having seen the document in question.

CONCLUSION

Experience to date indicates that the tools exist to achieve herd level control of paratuberculosis when all involved are highly motivated. There are adequate systems in place to ensure that both farmers and veterinarians can obtain high quality training materials to support herd level control. The awareness of the importance of paratuberculosis and the application of risk assessment in managing biosecurity issues is increasing. Health planning must also embrace the need for flexibility in standards for both maintaining freedom from infection and controlling paratuberculosis when it is present. Infection monitoring even where

assurance of freedom from infection with MAP is not an objective can be helpful. The outcome from this monitoring can be used to evaluate the control and biosecurity elements of the health plan. This monitoring can be achieved through targeted testing of clinical cases and cull animals as well as environmental and bulk tank monitoring.

In the UK the beef industry has proved more receptive to the concept of paratuberculosis control than the dairy industry. This is assumed to be due to the demand for high value sire bulls that are at low risk of developing clinical paratuberculosis plus the initiatives of certain pedigree breed societies to promote the assurance and control programmes. In the dairy industry most interest has come from those herds experiencing a significant clinical problem where adoption of control programmes is considered essential on financial grounds, but here it can be difficult to effect control because of dairy production financial pressures. The dairy industry does not appear to consider biosecurity to be important and this may be because the sale of breeding stock is a low priority for the individual business, but also because the other significant areas of loss from disease such as lameness and mastitis have a negligible biosecurity component. This coupled to slow adoption of quality systems has meant that herds with no evidence of infection have shown no interest in joining assurance programmes. This is unlikely to change until the industry as a whole can recognise the significance of biosecurity in relation to the spread of paratuberculosis and communicate a clear message to the producers. In turn this must be supported by a financial incentive for the producers who become involved in paratuberculosis assurance schemes. Together these elements constitute a challenge for the dairy industry that is only likely to become more pressing as long as uncertainty remains over the zoonotic potential of MAP infection in cattle.

ACKNOWLEDGEMENTS

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The prospects for herd level control of paratuberculosis - A scientist's view

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INTRODUCTION

Control of Johne's disease (JD) in cattle herds historically has been based on empirical knowledge. Testing cattle and removal of test positives was the primary strategy first used in affected herds, with management changes to reduce transmission to susceptible cattle employed as ancillary control measures. However, this often was not an effective strategy since many herds initially did not recognize the disease, did not test for the disease, and did not change management to deal with the disease if they did recognize the disease in their herd (NAHMS, 1997; Wells, 2000).

In the US, a major step forward in control of Johne's disease was the development and implementation of the JD Herd Risk Assessment and Herd Management Plan. Along with a major educational effort, many herds have joined the US JD Herd Control Program, though many others remain outside of control programs. The focus of this report is to address the issue of control of Johne's disease with evidence from scientific literature and experience primarily from US dairy cattle herds and to identify knowledge gaps in our current understanding. Issues to be discussed are:

1. Does the cost of Johne's disease justify focused control programs?
2. Which methods of transmission are the most important in dairy herds?
3. What is the best control strategy for use in dairy cattle herds?
4. What is the best strategy for eradication of JD in dairy cattle herds?
5. What is the best strategy for reduction of human exposure to MAP in dairy cattle herds?

DISCUSSION

Does the cost of Johne's disease justify focused control programs?
Johne's disease is an economically important infectious disease in dairy cattle, causing chronic incurable diarrhea, reduced milk production, progressive weight loss, and premature culling (Nordlund et al, 1996; Johnson-Ifearulundu et al, 1999). The 1996 National Animal Health Monitoring System (NAHMS) Dairy Study (Ott et al, 1999) showed an average loss of $97 per cow in inventory per year for herds positive for Johne's disease (22% of US dairy herds from this study). Herds with an apparent high prevalence of Johne's disease (greater than 10% cull cows with clinical signs) lost an average of $245 per cow in inventory annually compared to test negative herds without clinical signs of JD. These studies show that Johne's disease can be a significant economic burden to dairy herds.

The effects of Johne's disease in animals, however, are usually hidden from view. This is in part due to the long incubation period from initial infection until the onset of clinical signs of disease years later. In addition, clinical Johne's disease appears as sporadic individual cases of disease within the herd rather than as an obvious herd outbreak. Additionally, results from the 1996 NAHMS study indicated that many infected herds do not experience measurable economic losses due to JD. These factors suggest that economic losses may not be sufficient motivation for widespread cattle producer participation in voluntary control programs.

In addition to its reducing animal health hend economic performance, Johne's disease also represents a potential public health concern due to its potential link to human Crohn's disease (NRC, 2003). As a result, the US National Research Council (2003) has indicated that "Johne's disease is a significant animal health problem that warrants high priority … for implementation of control programs."

Which methods of transmission are the most important in dairy herds?
Risk factors associated with Johne's disease in dairy herds include herd size, geographic location, percentage of cows born at other dairies, group housing for periparturient cows, and group housing for pre-weaned calves (Wells and Wagner, 2000). It is recognized that MAP is often brought into dairy herds through the purchase of infected but clinically normal cattle (Sweeney, 1996): introduction of purchased cattle plays a critical role in transmission from farm to farm. A key preventive measure for Johne's disease is the careful evaluation of purchased cattle (through screening of the herd of origin) to avoid introducing MAP to the operation.

Early reports indicated that young calves are more susceptible than older cattle for infection with MAP (Payne and Rankin 1961, Larsen et al, 1975). A Minnesota study (Kovich et al, in press) indicates that heifers born and raised in test-negative herds and later introduced into infected herds prior to first calving are less likely than herdmates to test positive later in life, although older cattle can become infected under some circumstances.

Most transmission of Johne's disease is thought to occur through fecal-oral transmission of the pathogen (NRC, 2003). Fecal shedding by infected cattle can occur at very high concentrations, though the distribution of typical shedding concentrations is not well defined. The role of heavy fecal shedding cattle to the overall MAP bioburden and transmission of Johne's disease is likely to be considerable, as suggested in a report by Fyock et al (2005). Pass-through shedding (shedding of the organism by an uninfected animal likely in a heavily contaminated environment) has been demonstrated in experimental studies in calves (Sweeney et al, 1992) and could serve as a method of transmission in young stock. Theoretically, pass-through shedding could also occur in older cattle; additional information is needed to clarify its significance.

In addition, MAP survives very well in the environment of many dairy farms, especially during cool and damp weather (Schroen et al, 2002) and potentially for many months in shaded conditions (Whittington et al, 2004). Survival in bovine slurry at 5 C has been demonstrated for over 8 months (Jorgensen, 1977). This survival can lead to high levels of exposure of susceptible cattle in intensively managed cattle operations. The distribution of MAP in the environment of dairy cattle farms has been demonstrated in a recent study of Minnesota dairy herds (Raizman et al, 2004). The farm environment was culture-positive on 95% of herds with at least one positive pool of cow fecal samples. In this study, the farm environment was culture-positive in samples collected from cow alleyways (77% of the culture-positive herds), manure storage (68%), calving areas (21%), and sick cow pens (18%).

While the most important route of Johne's disease transmission is generally considered to be through the ingestion of contaminated feces in the calf's environment, other potential sources of transmission include shedding (or post-harvest contamination) of MAP in colostrum or raw waste milk (e.g. transition milk or non-saleable milk from antibiotic-treated cows). One study reported that 27% of subclinically infected cows had culture-positive supramammary lymph nodes and 12% had culture-positive milk (Sweeney et al., 1992). Another study reported that up to 22% of infected cows shed the organism in milk and colostrum (Streeter et al., 1995).

A consideration is the concentration of MAP in milk and colostrum. Limited research to date suggests that the level of this pathogen in milk from hematogenous sources is much lower than the typical levels used in experimental studies, and suggests that pasteurization would be likely to eliminate this pathogen at this level. However the level of MAP in milk due to fecal contamination is unknown.

Finally, the prevalence and concentration of MAP in surface water sources has not been estimated. This may prove to be an important source of infection in certain situations in which standing water serves as primary drinking water source for cattle.

What is the best control strategy for use in dairy cattle herds?
The first step in control of Johne's disease is to determine the herd infection status. Herd testing strategies to identify herds likely to be free from infection have been developed in and adopted by many countries. In the US, the Voluntary Johne's Disease Herd Status Program (VJDHSP) for Cattle involves an annual test of

all or a subset of adult cattle within the herd. A test-negative status adds value to cattle marketed to other herds as herd replacements.

In this program, blood samples are collected from a random sample of 30 cows in second lactation or higher in the herd to identify the herd infection status. If at least one cow in the herd is test-positive using serum ELISA testing, fecal samples are collected from test-positive cows for confirmation of herd infection via bacterial culture. If test-positive, a State or Federal district veterinarian will visit the farm and perform a risk assessment to identify key risks for transmission and assist the dairy producer and herd veterinarian in developing a herd control plan. If test negative, the herd is eligible to participate in the Voluntary Johne's Disease Herd Status Program and gain the advantage of marketing low risk cattle replacements. A study evaluating the herd sensitivity of this 30 sample test strategy (Wells et al, 2002) showed that the probability of detecting infected dairy herds was affected by within-herd fecal culture prevalence. Only 33% of low prevalence herds were detected ($\leq$ 5% of cows had positive bacterial culture results), compared with detection of 84% of higher prevalence herds in which $\geq$ 10% of cows had positive bacterial culture results.

Other herd testing strategies involve testing of pooled fecal samples through which a larger number of animals are represented within a tested population for a fixed laboratory cost. Several studies (Kalis, 2000; Wells et al, 2002; Wells et al, 2003; McKenna, 2005) have shown that bacterial culture of fecal pools is effective in detecting nearly all fecal pools with at least one cow shedding at moderate to high levels. These data support the use of fecal pool culture in characterizing infection status in dairy cattle herds and estimating within-herd prevalence. A study of bacteriologic culture of fecal samples from the dairy cow environment (Raizman et al, 2004) indicated that testing of fecal samples from pooled samples from cow alleyways and manure storage provides an efficient alternative strategy for determining herd infection status. An additional recent study by Tavornpanich (2005) has demonstrated that the most efficient initial herd testing strategy includes bacterial culture of pooled fecal samples collected from the dairy farm environment. In the US, bacterial culture of pooled fecal samples from dairy cow environment is beginning to be utilized for initial herd screening.

Strategies to control Johne's disease within an infected herd are 1) to reduce transmission of the organism to susceptible animals and 2) to identify and remove animals known to test positive for the disease. Despite a general understanding of Johne's disease transmission, however, little information is available regarding the efficacy of specific herd control programs. A recent study evaluating costs of various control programs on mid-sized US dairy farms (Groenendaal and Galligan, 2003) using simulation models showed that test and cull strategies were not cost-effective and did not reduce disease prevalence. Vaccination of replacement heifers in the first month of life did show economic benefit due to reduced involuntary culling of productive cows from the herd but disease prevalence was not reduced. In comparison, improved calf management through a combination of hygienic maternity management, low-risk colostrum and milk feeding programs, and segregated heifer rearing systems reduced disease prevalence through time and were cost-effective. Assumptions used for these simulation models included reduction of transmission at calving and no horizontal transmission among heifers or cows.

Vaccines for control of Johne's disease are available in some countries. In the US, a commercial vaccine is available and adopted on occasion but only with State Veterinarian approval on a herd by herd basis. Evidence from a controlled clinical trial (Larson et al, 1978) showed that vaccination with whole cell bacterin reduces incidence of clinical JD. Another study showed that vaccination is cost-effective due through reducing the culling rate of clinically affected cattle (van Schaik et al, 1996). Longitudinal uncontrolled studies from several countries show a reduction in clinical disease with whole herd vaccination but less consistent results are available regarding the effect of vaccination on fecal shedding. Results from an Australian study using a vaccine in young sheep showed a reduction in mortality and fecal shedding (Eppleston et al, 2005).

1. Animal treatment.
Treatment of clinically affected cattle with antimicrobials has not been shown to be economically beneficial. A recent study, however, indicates the potential for use of monensin to reduce MAP fecal shedding in young cattle (Whitlock et al, 2005). More research is needed to further explore effects of monensin in Johne's disease control.

2. Animal removal.
Because current test sensitivities for various tests are less than 50%, reliance on test-and-cull strategies to control Johne's disease will not be completely successful, as shown by Groenendaal and Galligan (2003). Test-negative, clinically healthy yet infected cows can transmit infection to other cattle. Similarly, a study by Dorshorst et al (2005) indicates that testing is less critical than herd management in herd control programs. In addition, this study suggests that inexpensive screening tests are preferred over costly albeit more sensitive tests in control programs in known infected herds.

An issue for further consideration, however, is the removal of late stage cattle with the highest risk of transmission to susceptible young cattle (i.e., heavy fecal shedders). These cattle are at highest risk for contaminating the environment as well as shedding in the colostrum, milk, and through the placenta. More research is needed to identify the importance of these heavy fecal shedders in herd control.

3. Herd management.
Replacement heifers may have very high exposure levels from fecal contamination produced by adult cattle in later stages of infection. Recommended herd control measures focus on this dominant risk factor to minimize transmission. Johne's disease control programs frequently stress management practices designed to prevent transmission of MAP to newborn calves and youngstock through fecal-oral routes as well as infective colostrum and milk. It must be acknowledged, however, that the patterns of MAP transmission within cattle herds are inadequately understood.

One of the earliest potential postnatal exposures of dairy replacement heifers to contaminated fecal material from infected cows occurs in the first few hours of life within the maternity area. Because of the large potential risk, Johne's disease herd management plans emphasize maternity pen management. Dairy herd control plans often include the use of individual calving pens cleaned between successive uses (vs. calving cows in a group pen on a bedded pack; Rossiter and Hansen, 2000). Information from controlled clinical trials is not available however to substantiate this recommendation.

Another management recommendation designed to prevent transmission of MAP on infected farms is to raise youngstock segregated from cow feces in the environment. The practice of off-site heifer rearing, either by the owner or by a professional dairy heifer grower, is often recommended to help achieve this goal. Results from a simulation study by Groenendaal and Galligan (2003) suggest that calf separation from adult cattle at one day of age (until 12 mos. of age) is more effective in reducing transmission of MAP than removing the calf at older ages. Again, this has not been demonstrated in controlled clinical trials. Proper handling of infective fecal material is an important part of the herd management program. Promising results from a recent study (Gobec et al, 2005) show that composting of manure does eliminate viable MAP.

The risk of transmission of Johne's disease from cow to calf through colostrum feeding is not fully understood, and some producers use results from individual cow testing for making colostrum feeding decisions. As a result, Johne's control programs often include management recommendations such as avoiding feeding pooled colostrum and feeding colostrum from "test-negative" cows to calves. Limited research investigating the value of pasteurizing colostrum for pathogen control demonstrated that pasteurization was successful in eliminating all viable *Salmonella* spp., *Escherichia coli* 0157-H7, *Listeria monocytogenes*, and *S. aureus*, though MAP was not evaluated (Green et al., 2002). However pasteurization of colostrum also resulted in a 25-30% reduction in IgG (Godden et al., 2003), which makes widespread adoption impractical. Another option for avoiding MAP transmission in colostrum is to instead feed a commercial colostrum substitute. A new commercial colostrum substitute provides a higher concentration of bovine IgG than previously available products (Quigley, 2002). Articifical colostrum may thus be useful in some settings to prevent the transmission of pathogens, including MAP, to newborn calves although it is expensive (approximately $US 20 per dose). These control approaches have not been fully evaluated in a controlled clinical trial.

One of the benefits of feeding a commercial milk replacer is to prevent transmission of MAP, as well as other important pathogens in potentially infective waste milk. Similarly, the recent introduction of on-farm commercial pasteurization equipment has offered dairy producers an economically attractive method to feed pasteurized waste milk while controlling pathogen transmission. Some concerns remain, however, that pasteurization may not effectively destroy all viable MAP present in waste milk, especially if present at high

concentrations. Furthermore, even if some bacteria did survive the pasteurization process, it is not known if very low levels remaining in the milk are infective to cattle. A field conditions study is needed to evaluate the effect of commercial waste milk pasteurization on transmission of MAP.

Despite lack of research documenting the effects of specific herd management practices on JD control, information has been gained from uncontrolled longitudinal herd studies. An Australian report (Jubb and Galvin, 2004) provides evidence that herd control programs in dairy herds do reduce clinical disease and test prevalence. Preliminary data analyses (Wells, unpublished) show that after 4 to 5 years of a control program focusing on reduction of transmission to young cattle, clinical JD in Minnesota dairy and beef demonstration herds is reduced. These results indicate that motivated cattle producers can make progress in herd control, though it is impossible to separate the effects of herd management vs. removal of some test-positive cattle in these uncontrolled studies. Several clinical trials to evaluate the impact of specific herd management are currently underway in the US (feeding of colostrum vs. colostrum substitute to calves, individual cow maternity pens vs. group housing, segregated heifer rearing vs. calf rearing with adult cow contact), but results will not be available for several years.

What is the best strategy for eradication of JD in dairy cattle herds?
Limited information is available to address eradication of JD in cattle herds. Some dairy and beef cattle herds, however, are interested in eradication of Johne's disease from their operations. They have the financial incentive to do so, plus a desire to reduce their liability from potentially selling infected cattle to other producers.

For infected herds interested in disease eradication in addition to implementation of the management control program, additional testing is warranted to identify individual infected cattle for removal from the herd. Current tests do not identify all infected cattle. Best estimates are that the serum ELISA tests currently available detect fewer than 25% of subclinically infected adult cattle and falsely identify as positive up to 4% of uninfected cattle (Collins et al, 2005). These assay errors indicate that uninfected cattle in low prevalence herds may be culled in error which must be considered a cost of the eradication program. The cost-effectiveness of a test-and-cull program depends upon the specific herd situation, but test and removal has been shown not to be cost-effective for most dairy herds using one of the currently available serum ELISA tests (Groenendaal and Galligan, 2003). Herds willing to make this investment should avoid re-introduction of Johne's disease through purchased cattle either through maintaining a closed herd or by purchasing cattle only from low risk herds.

What is the best strategy for reduction of human exposure to MAP *in dairy cattle herds?*
Debate regarding the public health significance of Johne's disease continues, and if MAP is shown to be a human pathogen, costs of infection control will increase dramatically due to loss of market access and control program costs. Contingency planning is needed, utilizing risk assessment-based decision-making. The goal of a program considering Johne's disease as a public health issue would be to reduce human exposure to MAP to reduce transmission. Potential routes of human exposure to MAP include ingestion of milk, water, and infected or contaminated foods (meats, vegetables, fruits) and direct contact to infected fecal material, especially for those with occupational exposures (producers and veterinarians).

Potential control strategies for this situation have not been fully developed, but should focus on highest potential risks first. The highest risk of exposure is experienced by individuals with direct contact with clinically affected and other heavy fecal shedding cattle. Occupational exposures to cattle producers, veterinarians, and slaughter plant workers could be managed through identification of infected herds, pointing out the need to identify the herd status of all herds potentially infected. Within infected herds, precautions would be needed to reduce exposures from direct contact through use of latex gloves, facial masks, and effective handwashing.

For human exposures through consumption, education would be needed to encourage consumers to avoid unpasteurized products. Another consideration may be a change to pasteurization time-temperature protocols given studies indicating potential survival of MAP after pasteurization. To avoid consumption of meats and other foods contaminated with MAP, food safety education would also be needed to promote complete cooking of meats and wash fruits and vegetables with uncontaminated water prior to

consumption. To avoid ingestion of contaminated water, research is needed to ensure uncontaminated water delivery.

Because of the multiple routes of exposure to humans through various pathways, if a causal link between MAP and human disease is shown, an intense focus would be placed on control of the pathogen at the farm. This might include mandatory herd testing on a periodic basis (at least annually). Test-negative herds would be allowed to ship milk to processors and send cull cows to slaughter. Markets for negative herds would be available to sell excess replacement heifers. For test-positive herds, however, milk may be required to be sold by processors only under enhanced pasteurization conditions. Cull cows could be required to be sold for slaughter under different processing conditions only (e.g., cooked product), and sales of replacement cattle might be restricted. Herds in the control program (infected herds) would likely have further restrictions, including annual testing of all adult cattle with removal of test-positives from farm to rendering, movement controls for all cattle movements, a manure management plan required to avoid contamination of water and environmental sources with required monitoring, and enhanced hygiene requirements to minimize direct contact to people on-farm.

CONCLUSION

JD is worthy of our best control efforts, as well as continued research to better understand the efficacy of control options. Though complete information is not available, we have the tools available today to reduce the within-herd prevalence of infection on dairy and beef cattle operations to minimize economic loss to cattle producers. Further information from clinical trials and other research will assist in fine-tuning effective management programs. It is unclear whether we currently have the knowledge needed to effectively eradicate Johne's disease from cattle herds. Eradication will be necessary if MAP is conclusively shown to be a public health risk, since control of JD will not be adequate to protect human health and satisfy public demands.

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Farm factors associated with *Mycobacterium avium* subspecies *paratuberculosis* infections in Northern Italian dairy herds.

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ABSTRACT

Paratuberculosis has been described as one of the most important diseases affecting cattle worldwide. Studies conducted in various areas of Italy reported a high prevalence of paratuberculosis in dairy cattle. The study was carried out on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infected dairy herds located in Northern Italy. The aim was to identify management and environmental risk factors associated with three outcome variables: MAP seroprevalence (ELISA Herd Check, IDEXX), incidence of clinical disease, and animal age of disease onset. The three variables were monitored in each herd for three years (2002-2004). The risk factors were: number of cows/herd, purchase of animals, automatic barn scraper, manure spread on fields, feed grown where manure was spread, calving assistance, udder washing, calves fed with farm/herd milk, each calf housed separately, soil pH, and iron content of drinking water.

The survey involved 56 herds with an average number cows/herd 199 ± 204, median 116. The values (mean $\pm$ s.d.) of the three outcome variables were: seroprevalence 5.7% $\pm$ 3.4 (min 1.2, max 21.0), incidence of clinical disease 4.0% $\pm$ 1.9 (min 1.2, max 8.4), and age of disease onset in months 37.1 ± 2.7 (min 31.0, max 42.5). The seroprevalence was associated (t-test) with number of cows/herd (p=0.027, correlation 0.2954), purchase of animals (p=0.004, correlation -0.3786), and iron content of drinking water (p<0.001, correlation 0.4827). The incidence of disease was associated with the iron content of drinking water (p<0.001, correlation 0.7512). The age of disease onset was negatively associated with the number of cows/herd (p=0.019, correlation -0.3120), and with the iron content of drinking water (p<0.001, correlation -0.5803).

Key words: Paratuberculosis, dairy cattle, risk factors, iron, water.

INTRODUCTION

Paratuberculosis has been described as one of the most important diseases affecting cattle worldwide (McNab et al., 1991). In cattle, infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been associated with substantial economic losses as a result of decreased milk production, decreased value of culled cows, increased time to pregnancy, and increased replacement costs (Benedictus et al., 1987; Johnson-Ifearulundu et al., 2000). Estimates of the prevalence of MAP infection vary depending on the methods used to select cattle for testing and to verify infection.

Studies conducted in various areas of Italy indicated MAP infection prevalence at herd levels ranging from 1.7 to 10.4%, determined by fecal culture (Cagnasso et al., 1995; Colavita and Giaccone, 1995; Vicenzoni et al., 1999), and from 7.5 to 60.3% by serology (Colavita and Giaccone, 1995; Sacco and Gennero, 1997; Robbi et al., 2002).
Despite the broad awareness of the impact of MAP infection on the dairy cattle industry, at present in Italy only voluntary control programs at single herd levels are active. It is not possible to officially certify that a herd is paratuberculosis-free.

To determine the scope of the paratuberculosis problem and to improve the effectiveness of control measures, research has been conducted to identify management and environmental risk factors in an effort. Although several reports have identified management practices that are associated with an increased

risk of paratuberculosis, less information is available regarding environmental risk factors. Among them, potentially important environmental risk factors are soil pH and soil iron content (Johnson-Ifearulundu et al., 1999; Ward et al., 2004). The proposed mechanisms by which soil pH is believed to influence the prevalence of paratuberculosis is via modulation of soil iron availability (Johnson-Ifearulundu and Kaneene, 1997).

On this basis it is rational to hypothesize that even the iron content of drinking water could be regarded as a risk factor. Since the feed regimens of dairy cattle reared in Italy are based on dry roughage, the water intake is higher compared to grazing cattle. This may imply that the iron content of the water is of concern.

This study is aimed at detecting management and environmental risk factors that influence characteristics of paratuberculosis in MAP-infected dairy cattle in an area of Northern Italy.

MATERIAL AND METHODS

A voluntary control program was established in three provinces of Northern Italy, namely Parma, Reggio Emilia, and Modena. A herd was considered MAP-infected when at least one animal aged >24 months showed chronic diarrhea, decreased milk production, weight loss, normal appetite, and normal rectal temperature. The clinical diagnosis was confirmed by detection of enteric lesions at slaughter, plus culture and serologic tests. Among 145 dairy herds examined in the three provinces, 56 herds (overall 11,144 cows) were found to be MAP-infected. These herds were included in the study.

The number of cows/herd was 199 ± 204 (mean $\pm$ standard deviation), median 116. Eleven risk factors were considered: number of cows/herd, purchase of animals (yes/no), automatic barn scrapers (yes/no), manure spread on fields (yes/no), feed grown where manure was spread (yes/no), calving assistance (yes/no), udder washing (yes/no), calves fed with farm/herd milk (yes/no), each calf housed separately (yes/no). Data were collected by an extensive questionnaire on demographic, management, hygiene, and health factors. In addition, soil pH (acid/basic) and iron content in drinking water (mcg/l) were determined for each farm. Soil pH was determined by mixing soil samples 1:1 with distilled water and then testing by a pH-meter. For iron determination in drinking water, the spectrophotometric 1,10 phenanthrolin method (UNI 10510:1996) was utilized. In accordance to the standard parameters adopted in Italy, iron content was considered high for concentrations $\geq$200 mcg/l of water.

The 11 risk factors were evaluated in relation to three outcome variables, namely MAP seroprevalence (ELISA Herd Check, IDEXX), incidence of clinical disease (typical signs of paratuberculosis confirmed by culture and/or serologic testing), and age of disease onset. Data from each farm were collected once during the summer and fall of 2002-2004. Statistical analysis was performed by using NCSS statistical package (http://www.ncss.com/).

For each outcome variable, stepwise regression (probability to enter 0.05, probability to remove 0.20, method: forward), and multiple regression were performed. Continuous variables were checked for normality. To assure normality, the following variables were natural log-transformed: number of cows/herd, iron content in drinking water, seroprevalence, incidence of clinical disease, and age of disease onset. Qualitative dichotomous variables were processed assigning the value of 1 for "yes" and 0 for "no".

RESULTS

Overall, the values (mean $\pm$ s.d.) of the outcome variables were:
   i.   seroprevalence 5.7 ± 3.4 (min 1.2, max 21.0);
   ii.   incidence of clinical disease 4.0 ± 1.9 (min 1.2, max 8.4);
   iii. age of disease onset in months 37.1 ± 2.7 (min 31.0, max 42.5).

Statistical analysis pointed out that:

i.   seroprevalence was associated (t-test, $p<0.05$) with the following factors: number of cows/herd ($p=0.027$, correlation 0.2954), purchase of animals ($p=0.004$, correlation −0.3786), iron content of drinking water ($p<0.001$, correlation 0.4827). The final multivariable model was: 0.0746 + 0.1409 * number of cows/herd – 0.3007 * animal purchase + 0.2124 * iron content.

ii.   incidence of disease was associated with the iron content of drinking water ($p<0.001$, correlation 0.7512). The final model was: -0.4904 + 0.3941 * iron content.

iii.   age of disease onset was negatively associated with the number of cows/herd ($p=0.019$, correlation −0.3120), and iron content of drinking water ($p<0.001$, correlation -0.5803). The final multivariable model was: 48.0451 – 0.8480 * number of cows/herd – 1.5190 * iron content.

No association was demonstrated between the soil pH and the three outcome variables.

DISCUSSION

The study was carried out in the context of a paratuberculosis voluntary program granted by Emilia Romagna Region to improve the consciousness of dairy farmers about the adoption of effective control measures. Given the voluntary character of the program, the herds included in the study were not randomly selected. Thus the prevalence (56/145=0.386) has to be considered a biased estimate of the true prevalence in the area. The aim of the study was to assess the factors influencing clinical disease in MAP-infected animals so epidemiological data concerning seroprevalence, incidence of clinical disease, and age of disease onset were collected only in herds recognized as being MAP-infected.

The seroprevalence in MAP-infected herds was highly variable, ranging from 1.2 to 21.0% (mean 5.7 ± 3.4). Morbidity ranged from 1.2 to 8.4% (mean 4.0 ± 1.9) and clinical paratuberculosis usually occurred in animals aged >3 years. However, even in this case, variability was observed among the herds.

In order to set up effective control measures, an assessment of the main risk factors involving MAP-infected herds was performed. It was found that the number of cows/herd was associated with both seroprevalence and age of disease onset. This can be explained by the reduced management attention that usually occurs in large herds, in particular to calving practices. Poor calving sanitation facilitates calf exposure to and infection by MAP.

The purchase of animals was negatively associated with seroprevalence. This is contrary to the common assumption that purchasing of animals is a risk factor for introducing infection into the herd. However, given the herds being studied were known to be infected, home-born calves may have been more likely to be infected than purchased heifers coming from a dairy cattle population with lower paratuberculosis prevalence. With time, the introduction of healthy heifers could lead to a decrease of the overall herd prevalence, since to animals aged >12 months are usually less susceptible to MAP infection.

The data indicated that a high concentration of iron in water iron (≥ 200 mcg/l) was associated with increased seroprevalence and morbidity, and negatively associated with the age of disease onset. During the survey, we have found iron concentrations ranging from 50 to 600 mcg/l. There was a positive linear association between water iron concentration and the level of paratuberculosis in a herd.

Taken together, the results indicate that iron content in drinking water should be considered a main risk factor associated with paratuberculosis in MAP-infected dairy herds in the area of concern.

Fulfilment of the criterion of plausibility requires that the proposed causal relationship is biologically sound. In accordance with this criterion, there is evidence that iron availability is consistent with MAP growth. Iron is an essential trace element for most bacteria. MAP may be particularly sensitive to environmental iron content because of its poor capacity for iron uptake. As an inefficient iron chelator, MAP competes less effectively with other bacterial species for sequestration of available iron when iron exists in limited quantities. MAP is unable to produce mycobactin, which makes it unable to sequester iron outside of the host (Snow, 1970). This is demonstrated *in vitro* by the dependence of MAP on culture media with either a high concentration of iron or mycobactin supplementation. Thus, higher iron content, in our case in drinking water, may favour MAP survival, increasing the risk of transmission to a susceptible host and its ability to

replicate in infected animals. The relationship between soil iron content and paratuberculosis has been previously demonstrated (Johnson-Ifearulundu and Kaneene, 1997; Johnson-Ifearulundu and Kaneene, 1999). In this case, iron transport can pose a problem because at neutral pH, Fe^{+++} forms insoluble colloidal hydroxides (Davis, 1980).

The solubility of iron increases as pH decreases (Barclay, 1985). Therefore, iron is more readily available to all microrganisms, including MAP, in an acid environment. Competition with other microrganisms that are more efficient in their uptake of iron may inhibit growth of MAP at a high environmental pH. Thus, iron availability for MAP is influenced by both iron content and soil pH. Acid soil with high iron content has been demonstrated to be a risk factor for paratuberculosis in dairy cattle herds (Johnson-Ifearulundu and Kaneene, 1997; Johnson-Ifearulundu and Kaneene, 1999). In our case, iron content in drinking water has been demonstrated as a risk factor associated with seroprevalence, morbidity and age of disease onset, occurring independently from the soil pH. This evidence can be related to the availability of a high amount of soluble Fe^{+++} in water. In addition, the feeding regimens of dairy cattle, as is usual in the cattle industry in Italy, provide only dry roughage. This leads to a higher intake of drinking water (about 100 l/day) than in grazing cattle. Therefore the iron in drinking water has to be considered as a main source of iron for the animals.

CONCLUSION

Although the literature does not provide evidence for a causal relationship between iron content of drinking water and paratuberculosis, the strength of the evidence supporting the biological plausibility of this association demonstrates that this relationship warrants further studies. When the strong evidence of biological plausibility is considered in light of coherent evidence, it becomes clear that field-base epidemiologic and controlled experimental studies should be conducted to determine whether a causal relationship does exist between iron content in water and paratuberculosis in cattle.

It is intriguing to think that iron content in drinking water may be the key for the control of paratuberculosis and that the incorporation of a specific measure, such a device for reducing water iron content, into existing control measures may succeed whether other expensive and time-consuming methods have failed.

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Efficacy of a killed *Mycobacterium paratuberculosis* vaccine for the control of OJD in Australian sheep flocks

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ABSTRACT

A field trial was undertaken from 1999 until 2004 to determine the efficacy of a killed *Mycobacterium paratuberculosis* (MAP) vaccine, Gudair™, for the control of ovine Johne's disease (OJD) in Australian merinos run under Australian pastoral conditions. On each of three farms experiencing significant OJD mortalities (5 to 15% deaths per annum), 200 Merino lambs (age 1-4 months) were vaccinated with Gudair™, and 200 lambs were left unvaccinated. Animal assessments and sample collections were conducted twice yearly until 4 or 5 years of age. The impact of vaccination on mortality rate, faecal shedding of MAP (by pooled and individual faecal culture), lamb growth, condition score and wool productivity, vaccine injection site lesions and cellular (BOVIGAM™) and humoral (PARACHEK™) immunity were examined. Gudair™ reduced mortalities due to OJD by about 90% and delayed faecal shedding of MAP for the first year post-vaccination (pv). Thereafter, the prevalence of shedders among vaccinates was reduced by about 90%. The total numbers of MAP excreted by the vaccinated groups were also reduced by at least 90% at most sampling times. However, high levels of excretion by vaccinates were found on some occasions, and although overall only 7 vaccinates died of OJD, all 7 had multibacillary disease. Thus there remains a risk that some vaccinated sheep could transfer the disease. Small (less than 200g per animal) reductions in liveweight gain were found in vaccinated lambs in the first year pv, but not for condition score or wool productivity. Vaccine injection site lesions were detected in almost 50% of sheep 2 months pv, and these persisted for at least 4 years in 20-25% of vaccinates. The vaccine stimulated both cell-mediated and humoral immune responses in a high proportion of vaccinated lambs which declined over time, accompanied by a significant increase in the proportion of unvaccinated animals with positive immune reactions, presumably reflecting an increasing prevalence of OJD in this group.

Key words: Vaccination efficacy, sheep, OJD-control, cellular immunity.

INTRODUCTION

Ovine paratuberculosis or ovine Johne's disease was first diagnosed in Australia in 1980 on a property in the central tablelands region of New South Wales (NSW), gradually spreading to other areas of NSW and other states via movement of infected stock or local spread between neighbouring flocks (Seaman et al. 1981, Sergeant 2001). However it was not until the early 1990's, when Merino producers in the high prevalence region of NSW began reporting significant mortalities that the disease then became an industry issue. In 1999 following a call from some industry sectors to attempt to eradicate the disease by destocking all infected flocks, a 6-year $Aus40 million National OJD Program (NOJDP) was commenced. Its aim was to determine the best methods for managing the disease at both the flock and industry level.

A significant research and development effort was a major component of the NOJDP. Areas studied include detection, pathogenesis, epidemiology, cross-species transfer, disease eradication and control within flocks as well as the economic impact of flock infection. Several of these projects are continuing. Some of the major findings of this research to-date include: the development of pooled faecal culture methods (PFC – Whittington et al. 1999) and abattoir surveillance (Bradley and Cannon, 2003) as tools for better detecting infected flocks; proof that OJD infection can result in substantial mortality rates and economic losses (Bush

et al. 2005); the fact that most bacteria in the environment will die within 6 weeks but low numbers may survive for over 12 months in shaded areas (Whittington and Sergeant, 2001); evidence that exposure to a high level maternal or environmental contamination as lambs and after weaning are major risk factors for later development of disease (McGregor et al. 2005); destocking infected properties for 15 months over 2 summers before restocking with test-negative sheep does not reliably eradicate the disease (Taylor et al. 2005); vaccination was found to be an effective disease control tool.

The NOJDP finished in June 2004, concluding that movement controls and decontamination by destocking were not able to control the spread of the disease. A new national control program began in July 2004 with vaccination as the major focus for disease control. This paper presents the results of the major vaccination trial conducted that evaluated the efficacy of Gudair[tm] (CZ Veterinaria, Porrino, Spain) a killed MAP vaccine, in heavily infected merino flocks under Australian pastoral conditions. This trial has resulted in the registration of Gudair[TM] in Australia.

MATERIALS AND METHODS

The design of the trial and methods employed for farm selection, vaccination, sampling and testing were described previously (Eppleston et al. 2003). On three farms with a high infection prevalence, 200 merino lambs (aged 1-4 months) were vaccinated with Gudair[TM] while 200 lambs were left unvaccinated as controls. Both experimental groups were run together and when available, clinically affected adults were introduced to maximise exposure. Each trial farm was visited at vaccination, approximately 2 and 6 months post vaccination (pv), then every six months when blood and faecal samples were collected. The sheep were sampled up to an average age of 4.5 years.

Blood samples were assayed for gamma interferon (IFN-γ) to determine cell mediated immune responses and for plasma antibody levels by ELISA as described previously (Eppleston et al. 2003). Faeces from 10 to 40 sheep were pooled and cultured for the presence of MAP (Whittington et al. 2000). In addition, at samplings conducted from 8 or 15 months post vaccination (pv), individual faecal samples were stored at – 80° C and cultured if their respective pool tested positive for MAP. The individual faecal results were used to better determine the prevalence of shedding and to determine if the IFN-γ response measured soon after vaccination had any predictive value. An estimate of the total MAP excretion per group at each sampling point was made using the number of MAP per gram of faeces in each positive pool as determined from the timing of BACTEC growth index readings. Then the total excretion from each positive pool per day was calculated as follows: excretion of pool = excretion per gram x total grams of faeces (allowing for 1kg of faeces per sheep per day). Finally, the contamination contributions from each positive pool were summed.

Sheep that were either culled for normal flock management reasons, died or showed clinical signs of OJD were sampled for assessment of paratuberculosis by histology and at some samplings by culture of tissues.

A sheep was classified as a fecal shedder if at any sampling it had a positive individual fecal culture. A sheep was classified as infected if shed the organism, or if at necropsy it was positive by histology or by culture of tissues. Chi square analysis was used to analyse the effect of vaccination on mortality, lesion type, and subclinical disease. It also was used to analyse the association of immune responses amongst vaccinates with subsequent shedding, infection status and OJD-mortality.

Live-weight and body condition score were recorded at each sampling; at shearing greasy fleece weight and fibre diameter were measured. At each sampling all sheep were palpated at the site of vaccination and the incidence and diameter of any lesion were recorded.

RESULTS

Mortality due to clinical OJD
Vaccination was associated with both a delay and a reduction in OJD-related mortality (Fig. 1). OJD mortalities in control sheep began at 23, 14 and 19 months of age on farms 1, 2 and 3 compared to 41, 23 and 43 months respectively in vaccinates. This represents a mean delay in onset of clinical disease of about 17 months. Vaccination significantly reduced the total mortality due to OJD on all three farms by about 90% (a total of 80 controls and 7 vaccinates). Every vaccinate and 70 controls (88%) that died due to OJD had multibacillary disease (NS). Sheep with multibacillary lesions can excrete sufficient organisms to infect many thousands of susceptible animals (Whittington et al. 2000). Thus, the "breakdown" of a single vaccinated animal can have a disproportionate effect on potential transmission of the infection and the persistence of disease in the flock.
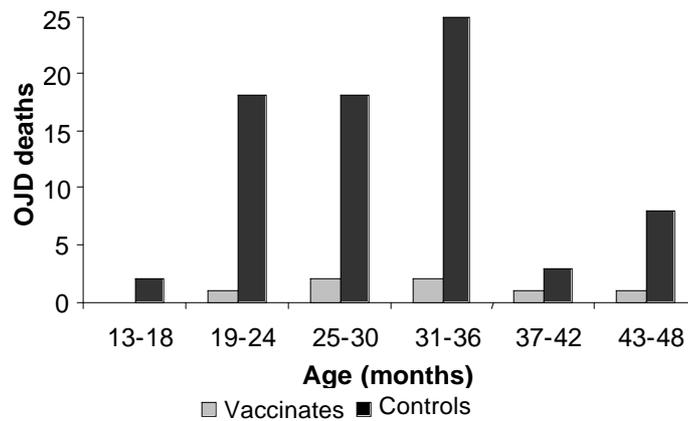
Fig. 1. Mortalities due to OJD across all 3 farms

Subclinical OJD
The prevalence of subclinical infection was assessed by histopathology in sheep sampled at abattoir slaughter at 2 years of age, and at 4-5 years of age. At both ages paratuberculosis lesions were found less often in vaccinated than control animals had. For 2 years the proportion of sheep with subclinical OJD was about 65% lower in vaccinates compared to controls (6 of 35 – 17% and 21 of 43 – 49% with lesions, for vaccinates and controls, respectively; P<0.01). At 4-5 years the proportion of sheep with subclinical OJD was about 50% lower in vaccinates compared to controls (24 of 163 – 15% and 41 of 144 – 28% for vaccinates and controls, respectively; P<0.01). Among sheep with lesions at either age, there was no significant difference between the percentage of vaccinates or controls with multibacillary lesions (2 of 6 – 33% and 4 of 21 – 19% for vaccinates and controls at 2 years respectively; 3 of 24 – 13% and 2 of 41 – 5%, for vaccinates and controls at 4-5 years respectively).

Shedding of MAP
On Farms 1 and 3 there was no detectable shedding of MAP by the vaccinated groups until 18 months pv (about 21 months of age), compared to 6 or 8 months pv (9 -11 months of age) in controls (data for Farm 3 is shown in Fig. 2). This reduction in the number of shedders averaged about 90%, and was maintained throughout the trial to 54 months pv (almost 5 years of age) on Farm 1, and to 42 months pv (almost 4 years of age) on Farm 3.
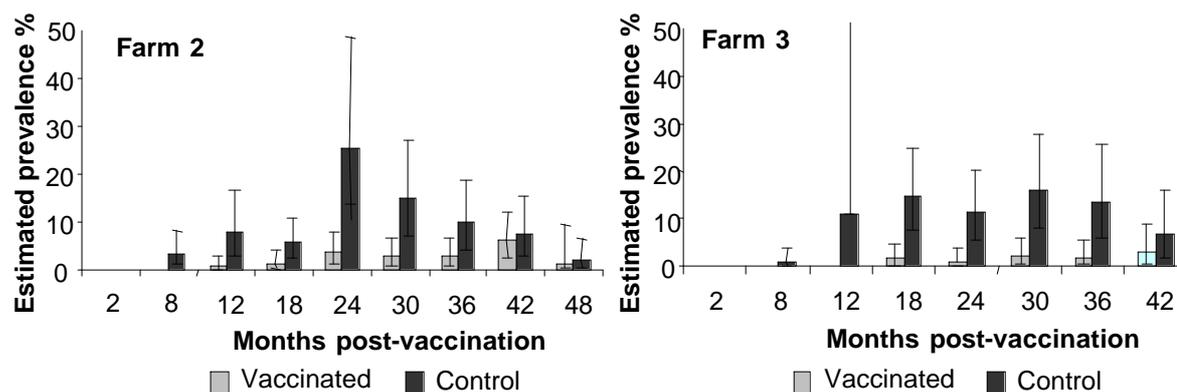
Fig. 2. Estimated prevalence of sheep shedding MAP in faeces on Farms 2 and 3. (error bars show the 95% binomial confidence interval)

Farm 2 (Fig. 2), had a higher prevalence of infection and less of a delay in the onset of excretion of MAP (15 months vs. 11 months for vaccinates and controls, respectively). The mean reduction in prevalence of shedders in the vaccinates was similar (86%) to Farm 1 and Farm 3 up to 36 mths pv. However, at the final 2 samplings at 42 and 48 mths pv, there was little difference in prevalence between the vaccinated and control groups, bringing the mean reduction in prevalence over the trial for Farm 2 down to 70%. However, the control group by this time was much smaller than the vaccinated group due to the large number of OJD mortalities that had occurred among the controls.

The pattern of total excretion of MAP is illustrated for Farm 3 in Fig. 3. At each sampling time throughout the trial on Farm 1, and up to 30 months pv on the other farms, the numbers of MAP excreted by the vaccinated group were at least one log lower than the controls. Note, however, that the actual level of excretion can be much higher than indicated by prevalence data alone. For example, at 18 mths pv on Farm 3 and Farm 1, the numbers of MAP excreted by the vaccinates equal or exceed the numbers excreted by the control groups at any previous sampling, but the number of individual shedders among the vaccinates was still very low. This reflects the large contribution that a single (or a few) multibacillary infected sheep may make to premise contamination. One sheep may excrete in excess of 10^{10} organisms per gram of faeces (Whittington et al. 2000), possibly equivalent to the excretion levels of many hundreds of sheep in the early stages of the disease or with paucibacillary infections.
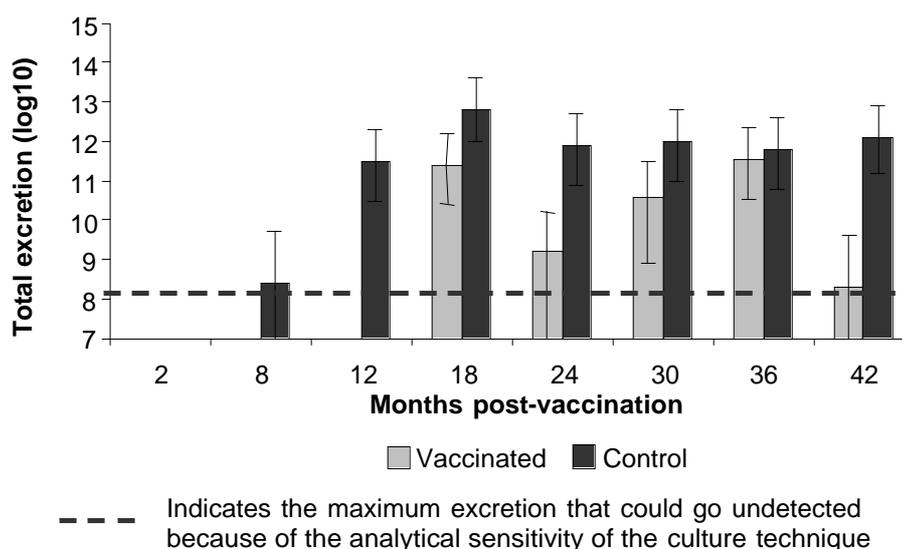
Fig. 3. Estimated total excretion of MAP by vaccinated and control groups on Farm 3.

Immunological responses
The stimulation of both the cell mediated (IFN-γ) and humoral (antibody) immune systems by vaccination is evident from the elevated IFN-γ reactions and ELISA antibody levels in vaccinates compared to controls.

Note also the rapid increase in these levels among vaccinates that occurred between vaccination and 2 months pv (Fig. 4).

Farm 2
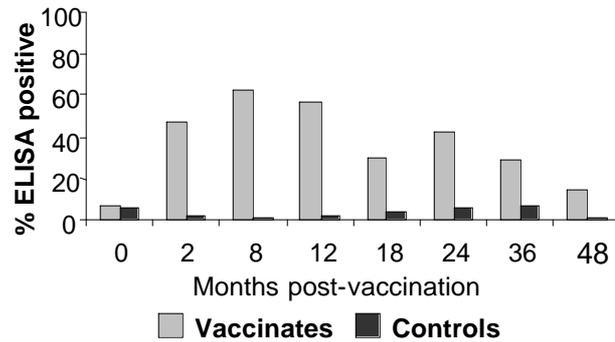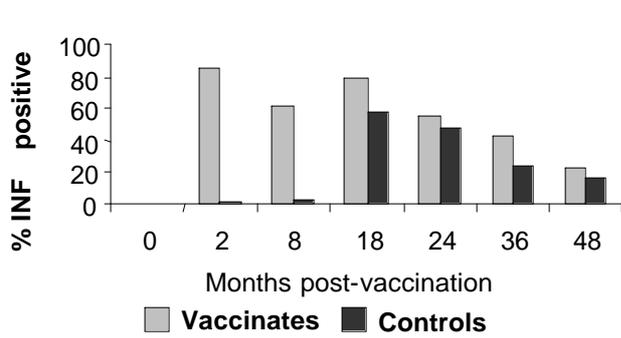
Farm 3
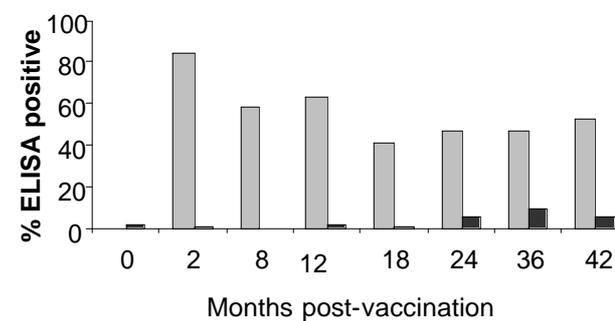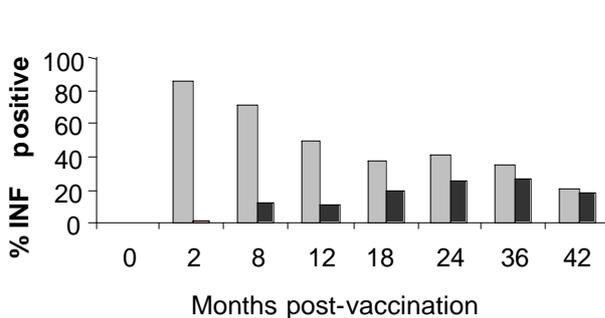
Fig. 4. Proportion of animals showing positive gamma interferon and ELISA responses for Farms 2 and 3.

In controls, positive IFN-γ responses were detected by 8-12 months pv and are likely to be due to environmental exposure to MAP. The proportion of vaccinates with positive IFN-γ responses was greatest at the first pv test on each farm, and then declined. These proportions remained higher than the controls at every subsequent sampling time, although at 24 and 48 mths on Farm 2, and 36 and 42 months on Farm 3, the differences between the two groups were not statistically significant. Farm 2 appeared to have had a very high level of environmental MAP challenge as evidenced by a high mortality rate and 58% IFN-γ reactors in controls at 18 months pv. This significant environmental challenge may also explain the rise in the number of IFN-γ test positives amongst vaccinates on Farm 2 at 18 months pv. Environmental MAP challenge of vaccinated sheep could boost a waning IFN-γ response. Positive pv IFN-γ responses were negatively associated with shedding ($P<0.05$) on all farms, and also negatively associated with infection ($P<0.01$) on Farms 1 and 2.

The pattern of ELISA results was similar on Farms 1 & 3, with high levels of seroconversion in vaccinates that declined slowly for about 18 months, and then levelled out. On Farm 2, the most severely affected farm, antibodies to MAP were present in 6-7% of lambs at the time of vaccination. This suggests the presence of passive maternal antibody which may have affected the ELISA responses to vaccination. On this farm fewer sheep responded initially, and by 8 months pv the percentage of reactors peaked at only 63%, in comparison with levels above 80% on the other two farms. At the 24 months pv sampling there was a large increase in the percentage of vaccinates with positive ELISA results, which may indicate an immune memory effect as vaccinates are exposed to increasing levels of exposure. Among control sheep, seroconversion occurred at about 12 months and the proportion of ELISA positives was greatest at 24 months pv. These seroconversions likely reflect the development of advancing disease. When stratified across all farms, a positive ELISA response in vaccinates was negatively associated with shedding ($P<0.0001$), with infection ($P<0.01$), and with OJD-mortality ($P<0.05$).

Vaccination site lesions
Injection site lesions were not identified in any control sheep, but 42% of vaccinated lambs developed lesions as assessed 2 months post vaccination (Fig. 5). The incidence of lesions decreased to a minimum

at 12 months pv and then remained steady at approximately 20% of vaccinates having palpable lesions until 3-4 years of age.

Production measurements
Liveweight at 2, 6 and 12 months and body condition score at 6 and 12 months pv were significantly lower in vaccinated compared to control sheep. However the magnitude of these differences were small (0.22, 0.34 and 0.75 kg liveweight at each time period respectively and 0.1 for condition score). There were no differences between controls and vaccinates in average greasy fleece weight at the first shearing across the three farms. However at the 2003 shearing on Farms 1 and 2, when sheep on were 3-4 years old, greasy fleece weight for vaccinates was higher (0.123 kg) than controls ($P<0.05$). There were no differences in fibre diameter at any sampling time.

DISCUSSION

This trial has been the first detailed, objective and controlled examination of the efficacy of vaccination of sheep against paratuberculosis. In previous work, properly controlled studies have not been done, and it has been difficult to isolate the effects of vaccination from other aspects of control programs. In Iceland, vaccination of sheep was introduced only after more traditional control measures, such as isolation and test and cull, had failed (Fridriksdottir et al. 2000). Vaccination proved to be an effective control measure and reduced mortality by 94%, but despite long term mandatory vaccination, OJD infection remains widespread in Iceland. The parallels with the Australian situation are clear.

Despite the rigour of the current trial, there were unavoidable limitations in the design that must be considered when translating the findings from this trial into management and control recommendations for the wider industry. Firstly, this trial investigated the effect of vaccination only against a very heavy challenge. The background level of infection on all three farms was high, all were experiencing mortality due to OJD, and clinically affected sheep from other mobs, rather than being culled immediately as normally recommended, were added to the trial mobs, further increasing the infectious challenge especially in the early stages. The trial was conducted under heavy challenge because of the difficulty in reliably detecting MAP infection in lower prevalence flocks, where few or no OJD mortalities are recognised.

Secondly, all sheep in the cohort were not vaccinated and these were excreting significant amounts of MAP, thus providing a high and on-going level of challenge. In the real world situation, the entire flock would be vaccinated and thus vaccination may be even more effective. In the current trial, the inclusion of the unvaccinated control sheep was essential to assess the effect of vaccination against controls that had had exactly the same MAP exposure under exactly the same management conditions. The alternative (having some wholly vaccinated and some wholly unvaccinated control flocks) is subject to bias as it is not possible to replicate conditions in different flocks.

Finally, in the current trial we examined only a single cohort of sheep over 4 to 5 years. In the real world situation, a new crop of lambs would be raised and vaccinated each year. The amount of MAP exposure would be expected to be successively reduced, with a reduction in the opportunity for transmission of the disease to future generations. Preliminary findings in a study of seven flocks after three years of vaccination indicate that this may be the case (Eppleston et al. 2005), and on-going more comprehensive studies are under way to confirm this hypothesis (Eppleston et al. 2004).

The above design limitations combine to give a disease challenge "worst-case scenario", but do assure a high level of infection against which any reduction due to vaccination can readily be measured. Intuitively, one may expect that vaccination will be more effective when other management procedures are used concurrently to reduce MAP challenge, both in the first and subsequent generations, but such a hypothesis was not tested in this trial.

All of the vaccinates that died had severe multibacillary infection, and would have been shedding enormous numbers of MAP leading up to their death. While there were very few of these sheep (only seven across the three farms), the contribution of just a single sheep with a multibacillary infection to environmental

contamination is significant and may outweigh many hundreds of sheep with less fulminant disease. If such a breakdown in OJD occurred at a critical time (e.g. in a ewe in the lambing paddock when pasture was short and many young lambs were beginning to graze) a whole cohort of young susceptible animals may be exposed to millions of MAP.

Another important observation was that the effect of vaccination on subclinical disease, although significant, was less than its effect on clinical disease. Clinical disease (i.e. mortality) in vaccinates was reduced by about 90%. In the yearling culls, 17% of vaccinates had lesions compared to 49% of controls, a 65% reduction in subclinical disease. Among the sheep at final slaughter, the reduction in subclinical disease in the vaccinates was less than 50% (19% of vaccinates with detectable infection or lesions compared to 31% of controls). However, the fact that many of the controls had already died (probably the most susceptible sheep) does confound the interpretation. In addition, vaccination did not decrease the proportion of subclinically affected sheep that had multibacillary lesions. This finding contrasts with previous overseas reports (Garcia Marin et al. 1997). These findings are probably not a concern on a heavily infected farm viewed in isolation, but they do have relevance where vaccinated sheep are moved to areas of low prevalence for OJD. Because vaccination is less effective in reducing subclinical disease, and because subclinical disease is more difficult to detect, it follows that an accurate quantification of the risk of vaccinated sheep to transmit OJD is not possible.

In this trial, vaccination was seen to provide life-long protection. However, because of the on-going high challenge with MAP, a natural boosting effect may have been operating. Whether vaccinated sheep in a low or zero challenge environment would still be protected if suddenly exposed to high levels of MAP after several years is unknown.

MAP shedding was detected in control sheep by 9 to 11 months of age, a little earlier than generally assumed. This is important information when young sheep are used to prepare low risk pasture for lambs and weaners. Vaccination extended the interval without excretion to 15 months on the worst affected farm, and 21 months on the other two farms, thus providing a much larger window of safety for management of pasture contamination.

CONCLUSION

Gudair™ reduced mortalities due to OJD by about 90% and delayed faecal shedding of MAP for the first year post-vaccination (pv). Thereafter, the prevalence of shedders among vaccinates was reduced by about 90% when compared with controls. The total numbers of MAP excreted by the vaccinated groups were also reduced by at least 90% at most sampling times. However, high levels of excretion by vaccinates were found on some occasions thus there remains a risk that some vaccinated sheep could transfer the disease. Vaccine injection site lesions were detected in almost 50% of sheep 2 months pv, and these persisted for at least 4 years in 20-25% of vaccinates.

ACKNOWLEDGEMENTS

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The potential impact of vaccination site lesions on the value of sheep previously vaccinated against ovine paratuberculosis in Australia

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ABSTRACT

Since 2003 the Australian sheep industry has increasingly relied on the use of vaccination for the control of ovine paratuberculosis. Given the reported incidence of lesions at the site of vaccination, and reports of discounts being applied in New Zealand, the Australian meat industry was concerned about a potential reduction in carcass value as vaccine use expanded. This paper presents the outcomes of a pilot survey on the prevalence of lesions and actual discounts applied to 20 lines of vaccinates slaughtered in Australia. The prevalence of lesions observed in the slaughter survey was 18% for mutton and 65% for lamb carcasses. The value of the trim removed was insignificant, the labour cost of its removal was nil and no carcass was downgraded to a lower value grade. This study was conducted at a time of low sheep supply, in carcasses vaccinated at the recommended site high on the neck and in these circumstances OJD vaccination site lesions are not a significant cost to producers or to the processing industry and will represent only a very small proportion of the total cost of OJD control by vaccination.

Key words: Vaccination, injection site lesions, OJD, sheep.

INTRODUCTION

A six-year OJD Control and Evaluation Program conducted in Australia concluded that vaccination was the preferred method of reducing the on-farm impact of OJD and the risk associated with the purchase of re-stocker sheep. Gudair[TM] (CZ Veterinaria, Porrino, Spain), a killed vaccine imported from Spain, was registered for use in Australia in 2002 and its use has expanded rapidly with 3.45 million doses being sold in 2004.

While the efficacy of Gudair for controlling OJD in Australian sheep is high (Eppleston et al. 2005), almost 50% of vaccinates developed injection site lesions within 2 months of vaccination. Moreover, anecdotal and survey reports from New Zealand had suggested that discounts are applied to vaccinated sheep at slaughter because of the need to trim affected carcasses. In one survey (Buchanan et al. 1998) discounts of $NZ8-9 were recorded in carcasses downgraded due to lesion trimming.

The Australian sheep industry was concerned that similar discounts may be applied in Australia particularly as the use of vaccine was expanding. As a result the industry commissioned a pilot survey of the prevalence and likely economic impact of vaccination site lesions at slaughter. This paper presents the outcomes of that survey.

MATERIALS AND METHODS

Consignments of Merino sheep previously vaccinated with Gudair[TM] that were destined for slaughter were identified and trucked separately to a commercial abattoir. Details of each consignment including age at slaughter, age at vaccination and time since vaccination were obtained from the owner of the sheep.

At slaughter, research personnel located near the beginning of the slaughter chain recorded the occurrence of injection site lesions and classified them by diameter. Further down the chain abattoir staff trimmers were

asked to carry out routine trimming of the neck region and to retain any trim taken due to the presence of lesions. These were later counted and weighed at the abattoir. The weights recorded comprised both granulomatous lesion and surrounding tissue, but no attempt was made to separate and weigh each component of lesion trim. In addition abattoir workers involved in trimming prescapular lymph nodes were asked to retain any enlarged nodes for later inspection, weighing and sampling them if required. A total of 18 consignments of adult mutton sheep and 2 consignments of lambs killed at 6 different abattoirs in eastern New South Wales were examined. Amongst mutton consignments the effect of age at vaccination on lesion prevalence was assessed by analysis of variance and the relationship between time since vaccination and lesion prevalence was tested by regression analysis.

Freshly excised vaccination site lesions from 16 mutton sheep collected from 3 consignments were examined and subjected to histopathological and bacteriological examination to determine the nature of the lesion and whether secondary infection due to poor vaccination technique could have been involved. The lesions examined were collected from sheep killed 18 months (2 lots) or 3 years (1 lot) after vaccination. Bacteriology consisted of a Gram stain and routine aerobic and anaerobic culture from 2 consignments and aerobic culture from a single consignment. The lesions were preserved in 10% formalin and prepared for histology by routine methods, sectioned and stained with haematoxylin and eosin and Ziehl-Neelsen methods and microscopically examined.

The magnitude of discounts due to the presence of lesions at slaughter can be attributed to 3 potential factors. These include the value of meat removed as trim, the labour cost of removing this trim, or the downgrading of the carcass to a cheaper market grade as a result of trimming. The proportion of trim that was non-lesion carcass tissue was not available. However an examination of the collected trimmings suggested that an estimate of 20% was appropriate to value the loss of carcass weight due to trimming. Hence the formula used to estimate the value of trim removed per slaughter lot was:

> Total weight of trim x 20% x carcass value (average price paid per carcass divided by average carcass weight).

Abattoir management was consulted to determine the additional labour cost, if any, associated with the trimming conducted on each consignment and the prevalence and cost of carcass downgrading due to trimming.

In addition to the physical survey described above, 8 sheep abattoirs located in south-eastern NSW servicing the region where most vaccine had been used were contacted. They were asked a series of questions designed to assess their experience and attitude concerning the risk of vaccination lesions entering their processing chain.

RESULTS

A higher proportion of lamb carcasses than mutton carcasses had injection site lesions present at slaughter (79 of 122 - 65% vs. 585 of 3199 - 18%, P<0.01). However there was considerable lesion prevalence variation among consignments of mutton sheep (Fig. 1; range 1 – 41%). Although only 2 consignments of lambs were assessed, the higher incidence of lesions probably reflects the short interval of around 6 months between vaccination and slaughter. The prevalence of injection site lesions was not affected by age at vaccination (P=0.72) for mutton consignments however, nor was there a significant association between the prevalence of lesions and the interval between vaccination and slaughter (P=0.73). A high proportion (average 34%) of the lesions identified were large, exceeding 25 mm in diameter (Fig. 2). Grossly these were filled with a thick caseous material that if broken or cut would adhere closely to the surrounding carcass.
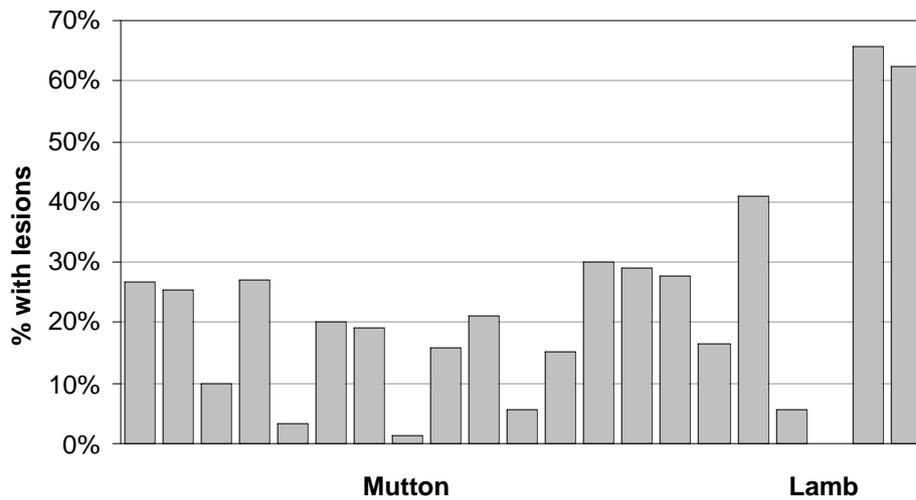
Fig. 1. Prevalence of injection site lesions by consignment.

Fig. 2. Two injection site lesions excised from Merino carcasses 3 years following vaccination, demonstrating the caseous content of these injection granulomas.

The value of non-lesion trim per carcass trimmed, calculated using the average carcass value, ranged from 0.4-6.9 (average 2.3) cents for mutton, and from 3.9-4.6 (average 4.3) cents for lambs. When expressed as a cost per carcass slaughtered (which accounts for the prevalence of lesions), this discount range fell to 0.02 to 2.2 (average 0.5) cents for mutton and from 2.4-3.1 (average 2.7) cents for lamb. On a per carcass basis this represents an average discount of only 0.01% for mutton and 0.04% for lamb. The value of excised enlarged prescapular lymph nodes was not calculated because of the small amount of trim recorded and because export abattoirs routinely removed these lymph nodes during processing.

Bacteriological examination of the lesions identified scant numbers of acid-fast bacilli, with no gram-positive organisms or bacterial growth in aerobic or anaerobic culture. Histology revealed that all lesions examined consisted of a central area of caseous necrosis containing scant numbers of acid-fast bacilli surrounded by a thick fibrous capsule. The inner surface of the capsules contained macrophages and low numbers of giant cells surrounded by moderate numbers of lymphocytes. The central necrotic area contained holes and spaces consistent with the presence of lipid or oil and these contained a higher concentration of acid-fast bacilli.

The abattoir phone survey indicated that abattoirs that had knowingly killed vaccinates did not consider the lesions a problem. However one processor did report 2 occurrences of poorly vaccinated sheep that required extensive trimming and he stressed the need to educate farmers to vaccinate using the location and method recommended. All respondents agreed that their existing quality assurance system for removing carcass blemishes would accommodate the OJD vaccination site lesions and that no additional

labour would be needed. However all abattoirs indicated that they would monitor the impact closely as the proportion of vaccinates killed expands.

DISCUSSION

The prevalence of vaccination site lesions observed in this survey is similar to that reported previously in Australia (Eppleston et al 2005) where lesions were detected in almost 50% of sheep 2 months after vaccination persisting for at least 4 years in 20-25% of vaccinates.

The bacteriological and histological examinations of the injection lesions confirm that they were not the result of secondary infection of the injection site. The presence of necrosis and granulomatous inflammation accompanying oil droplets and acid-fast bacteria suggest the lesions resulted from the irritant oil material or possibly the cell mediated immune response to the presence of killed mycobacteria combined with oil. These observations are similar to previous descriptions (Collett and West 2001; Eppleston et al. 2003) and are consistent with the expected tissue response following injection of a vaccine with a Freund's complete adjuvant, a mixture that also contains oil and mycobacteria (Windsor et al. 2005).

The prevalence of lesions requiring trimming in this survey, especially in lambs, was of a level that would require close inspection on the abattoir chain. Despite this, the amount of trim removed and the labour cost of its removal was very small and had miminal to no impact on abattoir labor cost, procedures or carcass market rating. This may reflect the fact that very little of Australian mutton is exported in the form of a whole carcass. In New Zealand most of the recorded discounts are associated with the trimming required for the small proportion of high value whole lamb carcasses destined for export (Brett 1998).

In the Australian context this study has identified that the cost of OJD vaccination lesions at slaughter represents a minor proportion of the total individual or industry cost of implementing OJD vaccination control programs. However it should be noted that these conclusions relate specifically to the market conditions existing at the time this survey was conducted, in sheep vaccinated high on the neck. Caution should be exercised in extrapolating these observations to times of an oversupply of sheep, and in sheep vaccinated at alternative sites.

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Influence of different sheep manure treatments on survival of *Mycobacterium avium* subsp. p*aratuberculosis*

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ABSTRACT

It is well known that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is highly resistant to physical and chemical factors. The survival of MAP ATCC 43015 in three manure handling systems was evaluated over 21 days. Bacterial survival was determined in sheep manure that was (1) composted with pine bark and water and placed in a bioreactor vessel with active ventilation, (2) composted with pine bark and water and piled on a raised pallet (passive aeration) and (3) piled directly on the ground without addition of bark. During the experiment, 5cm long open-ended holders inoculated with MAP and compost material at 2.0×10^6/g bacteria were placed in the three handling systems in direct contact with the composted manure/manure. In the bioreactor, MAP was isolated from three of 18 samples through 16 hours of the composting process after which no other isolations were made. In the pallet compost pile, no MAP culture isolates were made at any time. In the manure pile on the ground, MAP was isolated through 24 hours. Evidence of MAP DNA as shown by PCR positives was found in bioreactor samples until day 7 and in the pallet and ground piles through day 21. Bioreactor composting methods successfully eliminated *Mycobacterium avium* subsp. *paratuberculosis.* Composting can eliminate or at least drastically reduce the number of MAP and could therefore minimize environmental contamination and transmission risks caused by animal manure.

Key words: Composting, *Mycobacterium avium* subsp*. paratuberculosis* (MAP)*,* survival, PCR, sheep manure

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is highly resistant to physical and chemical factors due to a special cell wall containing mycoside C, mycolic acid, peptidoglycans and lipopolysaccharides (Levy-Frebault and Portaels, 1992, Hostetter et al., 2003). MAP causes paratuberculosis, an intestinal infection of domestic and wild ruminants. The main route of MAP dissemination is fecal contamination of the environment, followed by milk and in utero transmission (Sweeney, 1996, Benedictus et al., 2000). Subclinial cases pose transmission risk because the bacteria can be disseminated for a long period of time without noticeable signs in the infected animal (Whitlock and Buergelt, 1996, Kennedy et al., 2001).

Composting is a widely applied waste treatment method that could also be used to decontaminate biological material (Watanabe et al., 1997, Böhm, 1998, Watanabe et al., 2002 , Vinneras et al., 2003). A resistance to physical and chemical factors and the type of growth medium play an important role in MAP survival and its isolation from samples (Reviriego et al., 2000). Most of the research on MAP survival in a complex environment was performed before 1985 (Turner et al., 2000, Collins et al., 2001). In our study, we tested the usefulness of bacteriological and molecular assays in the assessment of MAP survival during composting.

MATERIALS AND METHODS

Three manure-handling approaches were used in this study. For two of them, a mixture of sheep manure, pine bark and water was created, resulting in a moisture content reaching approximately 65%. This mixture was placed into a bioreactor container and also piled on an open-weave pallet (or trellis) 15 cm above the ground ("pallet pile"). Uncomposted sheep manure was piled directly on the ground ("ground pile"). (Fig. 1) The large insulated bioreactor container (1m^3 volume - 1x1x1 m) included active ventilation while passive aeration occurred for the composted sheep manure raised above the ground by the pallet. The pallet and ground piles were 2m×2m×1.5 m containing approximately 3m^3 of material. The temperature in the bioreactor was controlled with PT 100 probes using the computer program "Visi DaQ"® (Advantech, USA) and was measured at three levels (16 cm, 50 cm and 66 cm above the bottom). The temperature was not allowed to exceed 68°C and was maintained in the containers by fans that also aerated the material. The temperature was measured in three levels in the pallet and ground piles at 25 cm, 75 cm and 125 cm above ground level by data loggers (Testo 175 T3, Germany):

The moisture of composting material was determined by sample weighing (Exacta, Tehtnica Železniki, Slovenia) before and 24 h after drying at 105°C - 110°C. The pH was determined using a calibrated pH meter (Iskra, Slovenia) in a compost extract: compost sample dried at 105°C - 110°C (5g) with distilled water (25g) and let stand for 10 minutes. The ammonia content was determined by a titration method: in a round flask, 10g of sample, 250 g H_2O, 3g MgO, 50 ml of 0.1 M H_2SO_4 and metal red dye were mixed. After distillation of 150 ml of fluid it was titrated with 0.1 M NaOH. Ash was determined with a beam method at 550°C 30 min weighed with the accuracy of 0.0001g.

In this study the type strain *Mycobacterium avium* subsp. *paratuberculosis* ATCC 43015 was used. A suspension of bacteria was prepared to dilute the mycobacterium colonies. Middlebrook 7H10 medium was used to determine the number of MAP cells in serial dilutions from 10^{-1} to 10^{-7} and inoculated media were incubated at 37°C for 6 weeks before the colonies were counted.

Bovine bones were used as holders for the test bacteria mixed with compost. The marrow cavity of long bones sawn to 5 cm in length were filled with approximately 5g of composting material and 200 µl of MAP suspension (2,0 x 10^6/g). Two holders were each placed in open-weave metal cases and packed with additional composting material. The cases were then put into bioreactor container at least 20 cm away from the walls and in the middle of the pallet and ground piles; thus the holders were in direct contact with composting material/manure in each system.
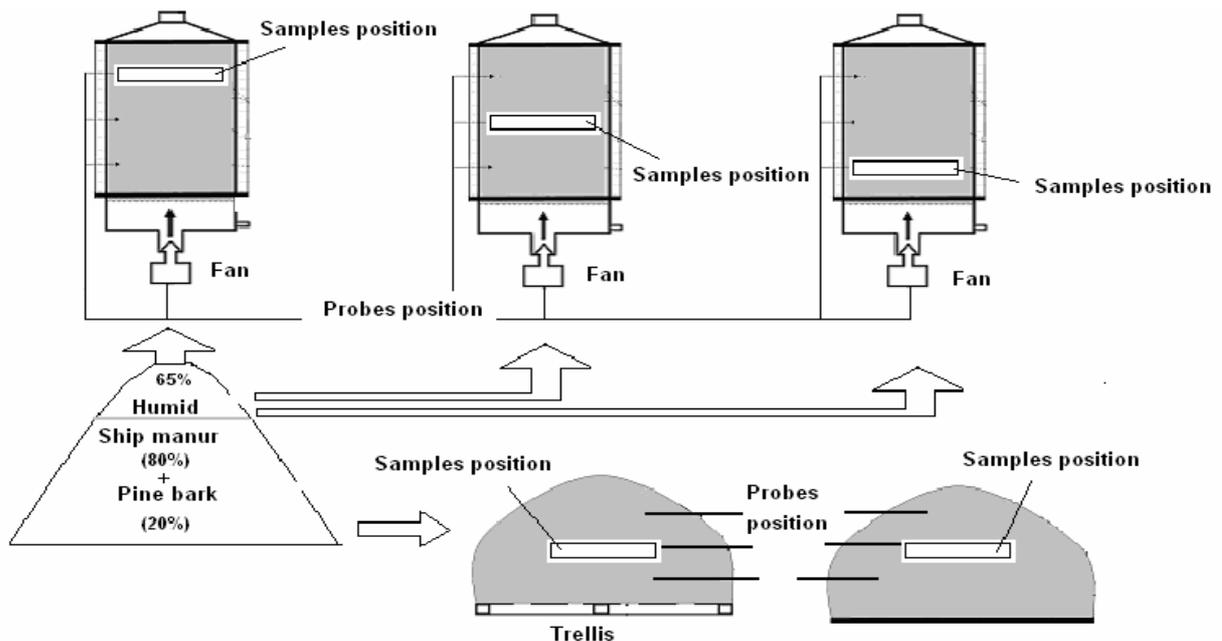
Fig. 1. Samples and probe positions

Nine cases (with two test bacteria holders each) were placed into the bioreactor at three different levels for a total of 27 bioreactor testing cases (Fig. 1). In each of the two manure piles nine cases were placed at one layer. Sampling was performed at nine different time periods after beginning composting (at 4, 8, 16, 24, 48, 72, 96 hours; then at 7 days and 21 days.) One case from each of the three layers in the bioreactor and one case each from the pallet pile and ground pile were removed at each of the 9 sampling periods. They were sealed in plastic bags and kept below 6°C until arrival at the laboratory. Two samples for each detection method (culture, direct PCR) were taken from each of the two bone holders in each case, resulting in (2 samples x 2 holders x 3 bioreactor levels) + (2 manure piles x 2 holders x 2 samples) or 20 samples per sampling period for culture and 20 for PCR. This resulted in a total of 180 cultures and 180 direct PCRs during the study.

Sample treatment for bacteriological investigation
Approximately 2 g of composting material inoculated with MAP was removed from the holders and 50 ml of H_2O were loaded in plastic bags and put into the stomacher (IUL) for 30 seconds. The homogenate was then transferred into 50 ml plastic tubes (Sarstedt, Germany), shaken for 30 minutes and left at room temperature for 30 minutes to permit sedimentation. 5 ml of supernatant were mixed with 25 ml of 0.9% hexadecylpyrimidum chloride (Sigma, USA). One ml of the supernatant was then transferred into a 15 ml tube, shaken again for 30 minutes and left at room temperature for 18 hours. Samples were centrifuged 20 min at 1400g (Heraeus 1.0 R, Germany). The supernatant was discarded and the sediment diluted with 1500 µl of H_2O.

Culture
200 µl of diluted sediment were cultivated on Herrold's Egg Yolk medium with supplements: (i) mycobactin (2 ml), (ii) mycobactin (2 ml, egg yolk 6 ml, malachite green 2.1 ml, penicillin 0.5 g, amphotericin 5.05 g) and on Becton-Dickinson medium.

Inoculated media prepared in 25 ml tubes were incubated in a horizontal position at 37°C for two weeks, then vertically for another 3 months. Colonies were tested by PCR for MAP identity confirmation. Each sample was analyzed in duplicate.

DNA extraction
DNA extraction was performed with QIAamp DNA Stool Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions.

PCR was performed with IS*900*-specific primers P90 (5' GTT CGG GGC CGT CGC TTA GG 3') and P91 (5' GAG GTC GAT CGC CCA CGT GA 3'). After the amplification, 400-bp PCR-products were separated with electrophoresis on a 2% agarose gel stained with ethidium bromide and analyzed by scanning and a visualization system (Gel Doc 1000, BioRad, USA).

RESULTS

Isolation of MAP through culture was made three times from the bioreactor container samples during the first 16 hours of composting (Table 1): once in the upper position after 16 hours and twice in the lower position at eight and 16 hours. No MAP was ever isolated from the samples in the middle position of the bioreactor container and no MAP was isolated from any bioreactor sample after 16 hours.

In the pallet compost pile with passive aeration no MAP was isolated through culture at any time. In samples from the manure-only ground pile, MAP was isolated in five samples within the first 24 hours (Table 1). After this period, no further isolations MAP were made from the ground pile. In three cases, mycobacteria other than MAP were isolated.

Direct IS*900* PCR detected MAP genetic material in 78 out of 80 samples from the three composting systems through day 7 (Table 1). On day 21 of composting process, the presence of IS*900* was shown in one of the two samples from the pallet pile and in one of the two samples from the ground pile while none of the samples from the bioreactor vessel contained detectable IS*900*.

Table 1. Number of MAP positive samples (of 6 bioreactor, 2 pallet,
2 ground pile samples collected at each sampling period)

Sampling period	Diagnostic method	Bioreactor vessel	Pallet pile	Ground pile
4 hrs	PCR	6	2	2
	Culture	0	0	1
8 hrs	PCR	6	2	2
	Culture	1	0	1
16 hrs	PCR	6	2	2
	Culture	2	0	1
24 hrs	PCR	6	2	2
	Culture	0	0	2
48 hrs	PCR	6	2	2
	Culture	0	0	0
72 hrs	PCR	6	2	2
	Culture	0	0	0
96 hrs	PCR	5	1	2
	Culture	0	0	0
7 days	PCR	5	2	2
	Culture	0	0	0
21 days	PCR	0	1	1
	Culture	0	0	0
Total	PCR	46 / 54	16 / 18	17 / 18
	Culture	3 / 54	0 / 18	5 / 18

After the beginning of the composting process the temperature in composting mixture increased quickly. The average hourly temperature of the bioreactor 16 hours after the beginning of composting exceeded 50°C in the upper and lower position and 45°C in middle position. Within the next 8 hours the temperature in the upper position of bioreactor vessel reached the target upper temperature limit of 68°C (Fig. 2). Temperature in the middle position increased at a slower rate. The temperature in the ground pile decreased 2.7^{o}C from the initial temperature, while the pallet pile's temperature after 24 hours exceeded 61°C (Fig. 2). The temperature in ground pile (sheep manure) increased to 27.3^{o}C after 24 hours of the experiment (Fig. 2).
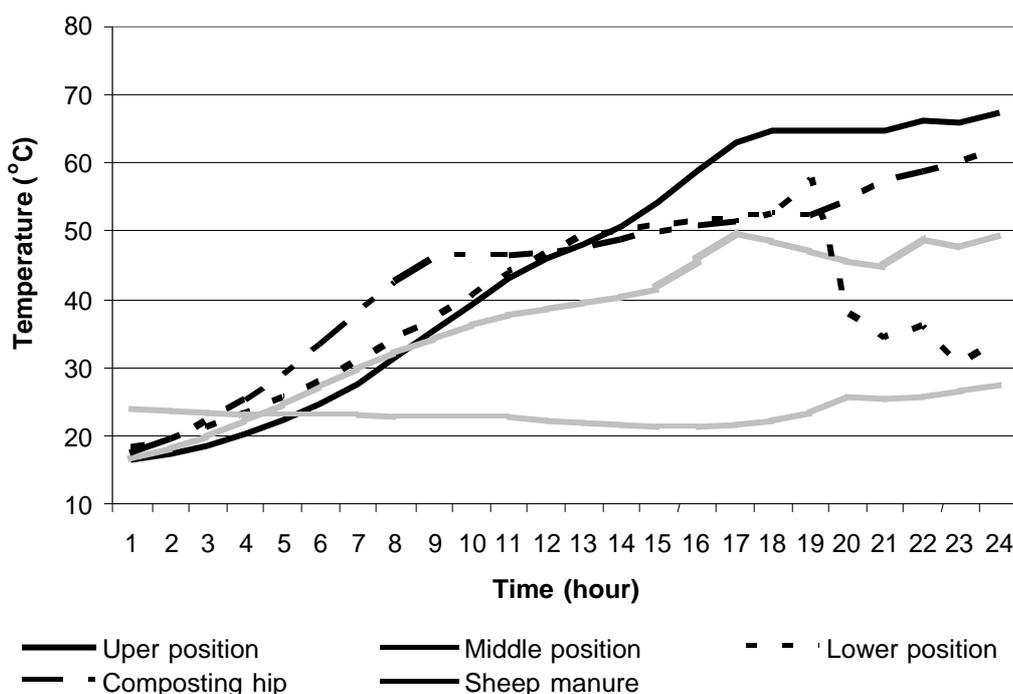
Fig. 2. Average hourly temperatures in the bioreactor, pallet pile ("composting hip") and ground pile ("sheep manure") at different sample positions in the first 24 hours.

The average daily temperature in the bioreactor upper position decreased below 50°C on day 9 while in the middle and in the lower position the same drop occurred after day 7 and day 6, respectively (Table 3). In the pallet pile the average daily temperature decreased below 50°C on day 8 (Table 3).

The temperature in the ground pile increased after the 1st day, the average daily temperature on day 5 exceeded 50°C, and reached 70°C on day 7. In the next four days average daily temperature dropped to 39.1°C (Table 3).

The moisture content of the bioreactor composting mixture initially was 65%. A decrease was seen with the greatest reduction noted for the lower position of samples from bioreactor vessel at the last sampling period (Table 4). During the experiment the moisture content in the pallet pile also decreased; this did not occur in the ground pile (Table 4).

Table 3. Average daily temperatures (^{o} C)

Day	Bior. upper	Bior. middle	Bior. lower	Pallet pile	Ground pile
1	43.8	36.5	37.9	43.3	23.0
2	67.1	51.2	43.7	66.3	36.4
3	67.1	55.0	51.8	71.2	59.4
4	67.4	54.3	52.2	66.0	66.8
5	65.8	53.9	50.7	63.4	70.3
6	65.1	51.0	47.1	68.7	69.3
7	62.1	47.9	41.7	60.3	67.1
8	53.3	43.1	37.6	48.0	51.9
9	48.0	38.8	32.7	36.9	31.2
10	43.8	36.5	29.2	38.0	34.1
11	43.8	35.7	28.2	44.2	41.8
12	39.5	32.9	25.5	43.4	42.8
13	38.8	32.7	25.5	44.5	38.0
14	36.6	32.4	25.6	49.0	44.3
15	33.3	29.8	22.8	35.7	47.3
16	30.5	30.2	23.6	28.7	46.6
17	27.9	30.1	23.8	30.3	48.9
18	25.5	31.1	25.0	34.2	49.0
19	23.9	30.7	24.9	38.8	48.8
20	22.5	30.8	25.0	40.9	46.2
21	21.4	30.5	24.4	43.7	44.7

Table 4. Moisture content (g/kg)

Sampling period	Bior. upper	Bior. middle	Bior. lower	Pallet pile	Ground pile
1	639.5	646.9	643.0	637.3	630.9
2	649.0	657.9	647.6	622.5	671.7
3	644.7	627.3	649.9	697.3	631.2
4	646.2	659.6	632.9	635.7	660.2
5	623.9	644.5	585.4	619.8	612.2
6	574.2	646.3	592.7	606.5	605.2
7	491.1	610.9	522.0	622.7	573.4
8	568.0	596.1	522.3	543.2	612.0
9	319.4	308.3	210.1	378.8	651.5

During the experiment, pH values varied from slightly acidic to slightly basic (Table 5). Sample pH values increased during the first 24 hours of the experiment and then persisted in nearly neutral range (Table 5). A deviation from that trend was observed in pH values of the ground pile on days 7 and 21 (Table 5).

Ammonia content was high in at the start of the study in the sheep manure and in compost samples (Table 6). By day 21, values in the compost material whether in the bioreactor or on the pallet pile were reduced

on average by more than 80%. The ammonia content in the ground pile (sheep manure only) on day 21 dropped by only 20% over the same period (Table 6).

Table 5. pH values

Sampling period	Bior. upper	Bior. middle	Bior. lower	Pallet pile	Ground pile
1	6.6	6.6	6.8	6.7	6.6
2	7.0	6.9	7.0	6.9	6.7
3	7.0	6.9	7.0	7.0	7.0
4	7.4	7.0	7.3	6.9	6.3
5	6.9	6.8	7.0	6.7	6.9
6	6.9	6.6	6.9	6.4	6.9
7	6.9	6.7	6.9	6.6	6.9
8	6.8	6.6	7.1	6.7	7.7
9	6.9	6.7	6.6	6.7	7.6

Table 6. Ammonia content (mg/kg)

Day	Bior. upper	Bior. middle	Bior. lower	Pallet pile	Ground pile
0*	3529.6	3529.6	3529.6	3529.6	4691.9
1	4942.6	3908.2	2593.6	3548.1	2961.3
7	3245.8	4379.3	3157.8	4073.3	3605.9
21	381.9	868.7	558.3	814.4	3752.4

* Common sample of composting mixture before repartition in bioreactor vessels

DISCUSSION

In each composting systems, MAP ATCC 43015 was exposed to rapidly rising temperatures, high concentrations of ammonia and an increase in bacterial activity. Appropriate homogenization, optimization of the composition and aeration of the composting material are needed for effective composting (Mathur et al., 1990). An increase of the temperature of the composting material is a result of microbial oxidative degradation of organic mater and indicates the intensity of the process (Liang et al., 2003). In our case, the mixture presented good conditions for microbial biodegradation. A rapid increase in temperature in the composting material (above 50°C in 14 hours) is a result of microbial oxidative degradation of organic matter due to the sheep manure where biodegradation had started already in the stable. Sample preparation and persistence of fast-growing microorganisms on the medium are identified as important factors affecting isolation MAP (Klawonn et al., 1996, Pislak et al., 1998, Pislak, 2000, Nielsen et al., 2001). It is likely that the main reason for absence of positive results was overgrowth of MAP by contaminants. The other important factor seems to be the influence of sample processing methods which can reduce the number of MAP in samples by 2.7 log 10 or even 3.1 log 10 (Reddacliff et al., 2003). The slow temperature changes in the ground pile indicated pure biological activity in first 24 hours. It is possible that contaminants were not present in a high enough number to prevent isolation of MAP from these samples.

High temperature is an important sterilization factor in the composting process (Watanabe et al., 1997, Tiquia et al., 1998). As the temperature rises the number of temperature-sensitive (mesophile) microorganisms is reduced which, to our belief, improves the possibility of isolating MAP until the critical point where the temperatures reached values lethal for MAP. This could be the explanation for the two isolates obtained 16 hours after the composting began. After 24 hours MAP was isolated only from the manure pile on the ground. Although the sheep manure was just transported from the stable by front loader and put into the pile, this simple manipulation encouraged biodegradation of the material as indicated by a temperature increase on the third day of the experiment. In their study, Fiesinger and Harrison (2003) used manure naturally contaminated with MAP and obtained results similar to those in this study. After the first day of the composting they did not find MAP in the substrate. Similar results were described by Olsen et al.

(1985) which could explain the surviving time in moderate temperature conditions for 21 days in comparison to only some hours in high temperature conditions.

Previous research in our laboratory showed that direct PCR had a higher sensitivity than classic cultivation on media of 1 log 10 (Pislak, 2000). Blocking of persistent inhibitors and optimization of DNA extraction considerably improve assay sensitivity (Garrido et al., 2000, Chui et al., 2004). The molecular method used in our study did not enable us to demonstrate the viability of MAP so we are not able to compare the PCR results with culture results. However, negative culture results in the samples on day 21 could indicate the destruction or strong reduction of MAP in the samples, raising the question of DNA persistence in the compost after MAP death. Limited data on DNA destruction are available in the literature. In a study on DNA stability in compost, Koschinsky et al. (1999) mention six days duration after cell death. The ammonia concentration in samples at the beginning of the experiment was high (0.9-1.7 % in a dry matter). The role of ammonia in compost on MAP survival is not clear and only limited information on the subject is available from the literature. Some authors state that 3% ammonia reduces the amount of MAP in hay with a stronger effect seen with drier material (Katayama et al., 2002).

CONCLUSION

We believe that composting animal manure can eliminate or at least drastically reduce the number of pathogenic microorganisms and could therefore be used to minimize the microbiological risks of animal manure. The role of a number of factors in the bactericidal processes that occur during composting is not clear and need further evaluation.

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Efficacy of a killed vaccine (SILIRUM®) in calves challenged with MAP

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ABSTRACT

A killed vaccine against bovine paratuberculosis (SILIRUM®, CZ Veterinaria) was evaluated in calves experimentally challenged with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Ten calves out of 18 were injected subcutaneously when two months old with a single dose of the vaccine. The remaining 8 calves were vaccine controls, injected with PBS. Two months after vaccination 8 and 6 calves (from the vaccinated and control groups respectively) were challenged with 6 doses of 6.9×10^{10} cfu of MAP. Peripheral cellular and humoral immune responses were assessed as well as MAP fecal shedding between 0 and 330 dpv. Three vaccinated and two control calves were slaughtered at 180 dpv and the remaining 13 calves at 330 dpv. Pathologic and bacteriologic evaluation of intestine and lymph nodes samples were completed. The number of granulomas was counted in sections from both locations. No adverse reactions to the vaccine were observed in any of the calves. Humoral responses appeared in vaccinated groups at 90 dpv, whereas cellular responses were detected at 30 dpv, reaching the highest values at 120 dpv. A significant reduction in the number of granulomas present in the tissues was observed in vaccinated calves. These calves showed either no or only focal lesions confined mainly to the lymphoid tissue, except in one case of the diffuse form of infection. In non-vaccinated control calves, diffuse lesions extended to the intestinal mucosa. In this study the administration of a single dose of SILIRUM® in calves was safe and able to control the progression of disease. Vaccinated calves had fewer lesions, less severe lesions and a lower tissue burden of MAP than unvaccinated calves. Fecal shedding of MAP was not detected in any animal.

Key words: Paratuberculosis, calves, vaccination, SILURUM®, experimental study.

INTRODUCTION

Vaccination has been considered as a successful measure for controlling paratuberculosis. After its first description at the beginning of the last century (Vallée and Rinjard, 1926), different studies assessed the efficacy of vaccination in cattle (Wilesmith, 1982; Benedictus et al., 1988) or small ruminants (Nisbet et al., 1962; Pérez et al., 1995; Corpa et al*.,* 2000b), by the evaluating the number of clinical cases or the level of fecal excretion.

The most commonly used vaccines are suspended in mineral oil to provoke a higher and more persistent immune response. The major disadvantage of this type of vaccine is the formation of a subcutaneous nodule at the site of inoculation that can be large or even ulcerated (Doyle, 1964; Chiodini et al., 1984).

The subcutaneous injection of either killed or live vaccines induces both cellular and humoral peripheral immune responses (Juste et al., 1994; Corpa et al., 2000a) and reduces the number of animals that develop clinical disease and the level of MAP excretion (Wilesmith, 1982; Merkal, 1984; Benedictus et al., 1988; Körmendy, 1994). Other studies (Kalis et al., 2001) indicate that hygienic measures and the culling of shedding animals can add to vaccination efficiency.

Different experimental studies have shown that paratuberculosis vaccination does not prevent the actual infection of animals (Nisbet et al., 1962; Larsen et al., 1974; Juste et al., 1994) but instead modifies the response to infection by limiting the progression of granulomatous lesions. Pathological methods have been previously used to assess the efficacy of vaccines mainly in small ruminants (Nisbet et al., 1962; Juste et

al., 1994), focusing on the presence, extension and type of lesion. Recently, a classification of natural paratuberculosis lesions in sheep (Pérez et al., 1996) and cattle (González et al., 2005) associates the type of lesion with the phases of MAP infection and the resultant immune response. Infected animals may show a range of pathology from focal forms (with lesions confined exclusively to the intestinal or lymph node lymphoid tissue linked to initial or latent forms of the infection and high cellular immune responses) to diffuse forms that display a severe granulomatous enteritis affecting different areas of the intestine. The latter are associated with clinical disease and high humoral (antibody) immune responses in most animals.

The main aim of this study has been the assessment of the safety and efficiency of a killed vaccine made with highly refined mineral oils as adjuvant in experimentally challenged calves.

MATERIALS AND METHODS

Animals
Eighteen two-month-old Friesian calves were used. They were selected from a tuberculosis- and paratuberculosis-free herd in which no clinical cases of paratuberculosis had been reported for 10 years. The calves were the offspring of Johne's disease test-negative dams.

Vaccination
When two months old, ten of the calves were subcutaneously injected with one dose of 1 ml of SILIRUM® (CZV, Porriño, Spain in the brisket) (V); the non-vaccinated (NV) control group (8 calves) received 1 ml of sterile saline solution. SILIRUM® is a heat-inactivated vaccine containing 2.5 mg of the culture of strain 316F of MAP combined with an immunological adjuvant consisting of highly refined mineral oil.

MAP Inoculation
Two months post-vaccination, 8 vaccinated animals and 6 not vaccinated animals were inoculated with 6 oral doses of 6.9×10^{10} cfu of a virulent wild-type strain of MAP at 2 day intervals (VI vs. NVI). This challenge strain was directly isolated from the intestinal mucosa of a bovine clinical case. Two animals from the vaccinated group were kept as vaccination controls (VNI), and the remaining two calves from the control group (NVNI) served as negative controls.

Humoral immune responses
Post-vaccination and post-infection antibody response was measured by indirect ELISA in sera samples taken before vaccination (day 0) and at 15, 30, 45, 60, 90, 105, 120, 135, 150, 180, 210, 240, 270, and 330 days post-vaccination (dpv). This ELISA was performed following the methodology described by Pérez et al. (1997) and González et al. (2005), using the MAP protoplasmatic antigen PPA-3 (Allied Lab, Fayette, Missouri, USA) and protein G as a secondary antibody. The optical density (OD) result was transformed to an index value by division of the mean OD for each serum by the mean OD for the positive control for each plate. The result was considered positive when the index value was equal to or greater than 800.

An agar-gel immunodiffusion test (AGID) using the PPA-3 antigen was performed as described by Pérez et al. (1997). Samples were considered positive when a clearly definable precipitation line of identity appeared with the reference serum.

Cellular immune response
The comparative intradermal tuberculin test (ITT) was carried out at 0, 45, 150, and 330 dpv by the injection of 0.1 ml of bovine PPD tuberculin (CZV, Spain) and 0,1 ml of *M. avium* PPD tuberculin (CZV, Spain) on the left and right sides of the neck, respectively. After 72 h, increases in skin thickness equal or greater than 2 mm were considered as a positive reaction. For the evaluation of the interference of vaccination with tuberculosis, the Annex B of Council Directive 64/432/ECC was applied.

The interferon-γ production test (IFN-γ) was performed with samples of whole blood obtained at the same dates as that for the humoral response, as described by Pérez et al. (1999), using both *M. avium* and *M. bovis* tuberculins and the BOVIGAM™ commercial kit (CSL Veterinary, Australia). The OD results were transformed to an index value by dividing the mean OD of the plasma from the *M. avium* and *M. bovis* PPD-

stimulated blood by the mean OD of the same plasma incubated with PBSS. A result was considered positive when the index value was equal or greater than 2.

Pathology studies
At 180 dpv (120 dpi) three calves from the vaccinated-infected group (VI) and 2 calves from the non vaccinated-infected group (NVI) were killed by intravenous injection of barbiturate. The remaining calves were sacrificed at 330 dpv (270 dpv). Complete necropsies were performed and gross lesions were recorded with special attention to the gut and related lymph nodes. Samples for histopathologic examination consisted of duodenum, jejunum (a 5-cm sample from each of the cranial, intermediate and distal zones, each sample including areas with and without Peyer´s patches), ileum (three 5-cm samples, taken at 20, 40 and 60 cm from the ileocaecal valve), ileocaecal valve, caecum, colon, rectum, jejunal lymph nodes (two sections from the most caudal part), isolated ileal lymph nodes (two samples), and ileocaecal lymph nodes (two samples). Tissues were fixed in 10% neutral buffered formalin and dehydrated through a graded alcohol series before being embedded in paraffin wax. Sections 4-µm thick were cut from each sample and stained with hematoxylin-eosin (H-E) and the Ziehl-Neelsen (ZN) method for acid-fast bacteria (AFB).

Lesion intensity was assessed by counting the number of granulomas present in three different sections of each sample from the intestine. The number of granulomas in the lymphoid tissue and in the lamina propria of the associated mucosa was counted separately.

Bacteriology studies
Faecal samples, collected at the same dates that blood samples, and tissue samples (ileocaecal valve, distal ileum, jejunal Peyer´s patches and ileal and jejunal lymph nodes), were cultured in Herrold medium, as indicated by González et al., (2005). Isolates were identified as MAP on the basis of mycobactin dependency, acid-fast staining and the appearance of typical growth appearing at least after 6 weeks of incubation.

Polymerase chain reaction (PCR) in tissues
A PCR technique was performed for the same sites as used for culture using both frozen and paraffin-embedded samples by the method used by González et al., (2005).

Statistical analysis
The results of the OD index obtained in the ELISA and IFN-γ tests were logarithmically transformed to make them suitable for analysis of variance. The means from the OD index of each diagnostic test and the results of granuloma count were compared among the groups, through a Student-*t* test at each time of sampling.

RESULTS

Clinical follow-up
No adverse clinical reactions to vaccination were observed in any case. One calf from VI group died suddenly due to an abomasal ulcer at 250 dpv.

Vaccination nodules
A persistent nodule at the injection site that did not affect the animals' overall health appeared at 15 dpv, reaching the largest size around 210 dpv (Fig. 1). All nodules were hard, cool, loosely attached and with a smooth surface. These nodules were round, oval or semi-spherical and showed no ulceration or other adverse reactions at the inoculation site at any time during the study.
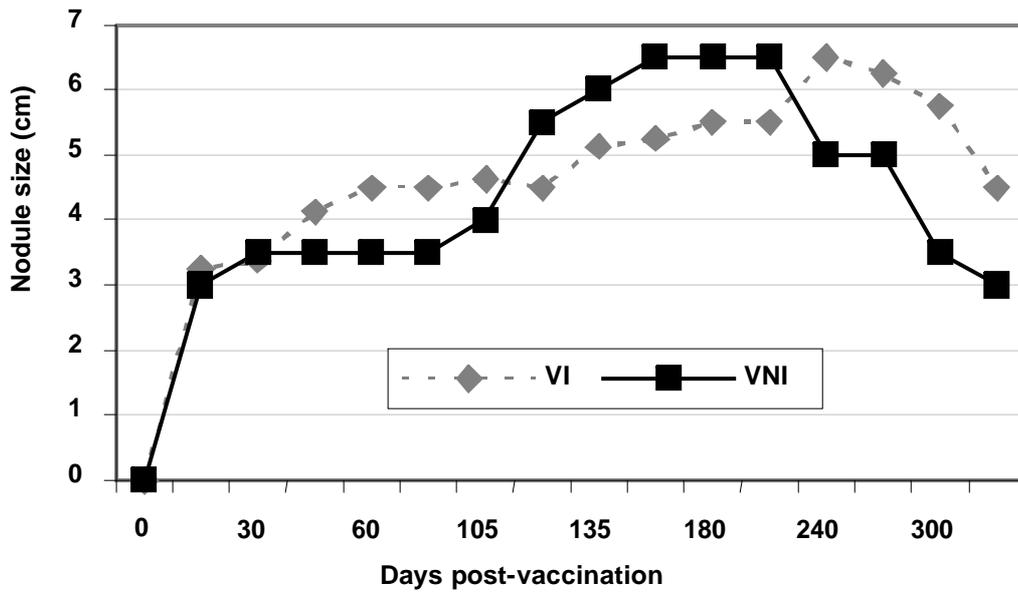
Fig. 1. Nodule evolution in cm for calves vaccinated with SILIRUM®
The size of the nodule is expressed as the maximum value in cm reached in any of its three dimensions.
Data are the mean values for the VI (vaccinated, inoculated) and VNI (vaccinated, not inoculated) treatment
groups.

The microscopic examination of the vaccination nodules at necropsy showed that they were mainly
composed of various foci of caseous necrosis, with abundant neutrophils in the center. Often calcified and
surrounded by a severe granulomatous reaction, they were formed by macrophages and giant cells plus
abundant lymphocytes and a fibrous reaction. Only one cow from group VI had a smaller nodule and a less
robust lymphocytic reaction in comparison with other calves from the group; this animal was found to have
diffuse gastrointestinal lesions at necropsy.
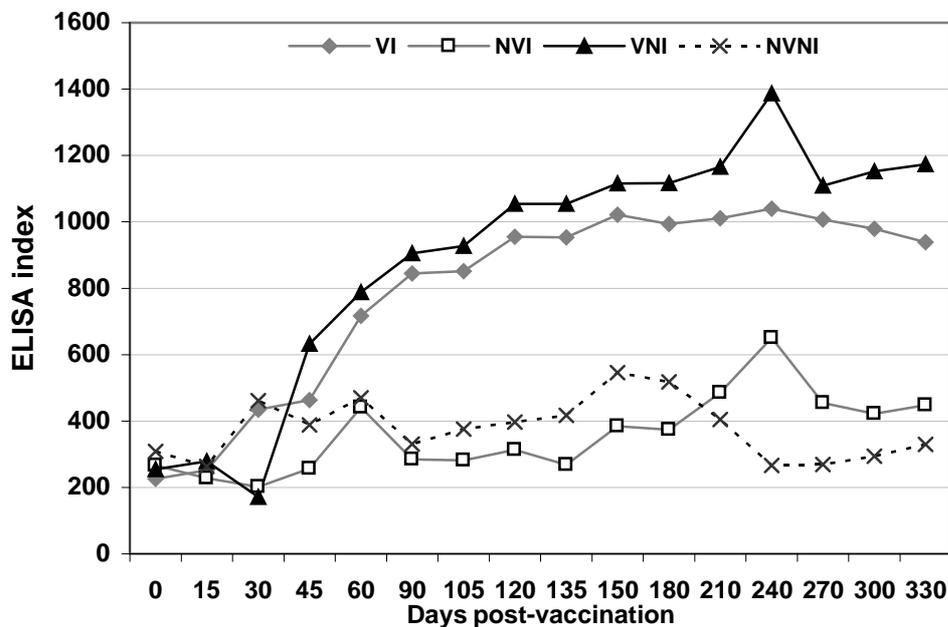
Fig. 2. Development of antibody production in calves vaccinated with SILIRUM® and challenged with MAP
at 60 dpv. by ELISA

Humoral immune response
Vaccinated calves' antibody production by 45 dpv differed significantly from that of unvaccinated calves
(Fig. 2). The OD level increased progressively up to 150 dpv. From then on, values remained high until the
end of the experiment. In the group NVI, index values reached significant differences at 210 dpv.

Vaccinated calves were ELISA-positive (index $\geq$ 800) at 90 dpv whereas the non-vaccinated, inoculated (NVI) animals never reached the threshold interpreted as antibody ELISA positive.

Only vaccinated calves were AGID positive (data not shown). This occurred at 60 dpv and was at a lower percentage than by ELISA.

Cellular immune responses
At 30 dpv, vaccinated calves IFN-γ production was noted after stimulation with *M. avium* PPD (Fig. 3). Production remained significantly higher than in the controls up to about 240 dpv. An increase in this cytokine after the stimulus with *M. bovis* PPD was also observed, although the values were not as high. The unvaccinated animals produced little to no detectable IFN-γ except for inoculated calves at one sampling period (120 dpv; 60 dpi).

For the comparative intradermal tuberculin test, all vaccinated animals were test-positive every time they were tested (Fig 4). The level of response decreased progressively however. A positive reaction against *M. bovis* PPD tuberculin was also observed, but it was always weaker than the response to *M. avium* PPD. In the unvaccinated calves (groups NVI, NVNI), a positive response was observed at 150 dpv (90 dpi) to both tuberculins, and again response to *M. avium* PPD was significantly stronger.
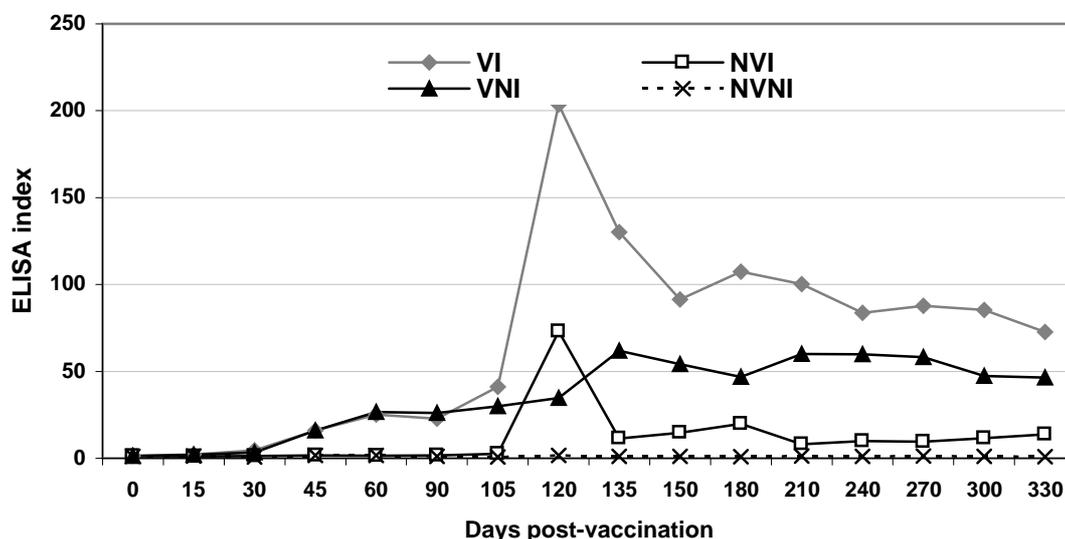
Fig. 3. IFN-γ production using *M. avium* PPD in calves vaccinated with SILIRUM® and challenged with MAP at 60 dpv. Results are expressed as an OD index. Positive: index $\geq$ 2.

Taking into account the interpretation criteria used in the tuberculosis eradication programs, none of the vaccinated calves would have had a positive result for tuberculosis, since in each case the reactions to *M. avium* PPD were stronger than those to bovine PPD.
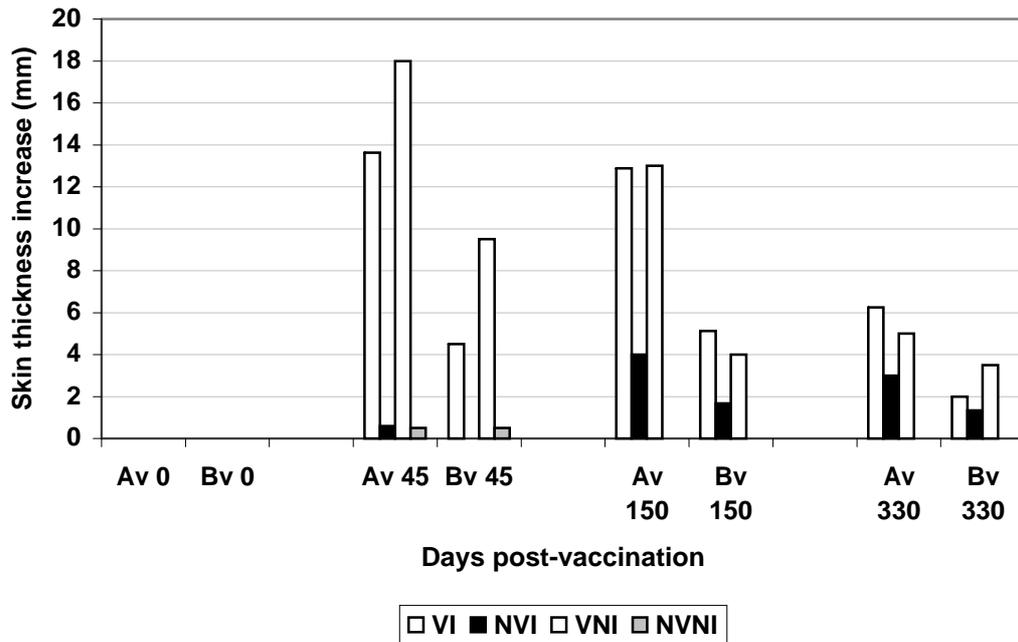
Fig. 4. ITT results in calves vaccinated with SILIRUM® and challenged with MAP at 60 dpv. Data are expressed as skin increase in mm recorded at 72 h after inoculation with the tuberculins, at 0, 45, 150 and 330 dpv. Av: reaction to *M. avium* PPD; Bv: reaction to *M. bovis* PPD.

Pathology results
Lesions showed by infected calves were classified into three categories according to González et al. (2005). The categories were *focal:* characterized by the presence of small and well-defined granulomas formed by macrophages and some giant cells with scant or no AFB, mainly in the cortical areas of the mesenteric lymph nodes or in the interfollicular areas of the Peyer´s patches; *multifocal:* with granulomas with scant AFB, spreading to the intestinal lamina propria, without modifying significantly its microscopic architecture, or *diffuse:* characterized by a severe granulomatous enteritis with diffuse thickening of several parts of the ileal and jejunal mucosa, usually having large numbers of AFB. Tables 1 and 2 show the distribution of calves according to the final lesion category. Except from one calf (Nº 5931) killed at 330 dpv, all the vaccinated animals had focal or no lesions. However, the majority of unvaccinated animals had lesions that were multifocal or diffuse.

Table 1: Granuloma counts and lesion classification in calves killed at 180 dpv (1st sacrifice), vaccinated with SILIRUM® and infected experimentally at 60 dpv with MAP.

1st SACRIFICE (180 dpv/120 dpi)			
Animal ID	**Mucosal granulomas**[1]	**Total granulomas** [2]	**Overall classification**
Vaccinated group (inoculated at 60 dpv)			
3303	0	2	Focal
5953	0	0	No lesions
9108	0	0	No lesions
Mean ± SD	0	0.67 ± 1.15	----
Control group (Not vaccinated, inoculated at 60 dpv)			
5933	22.73	58.46	Multifocal
7460	0	34.66	Focal
Mean ± SD	11.37 ± 16.07	46.56 ± 16.83	----

The cumulative granuloma counts corresponding to the lymph nodes and intestinal compartments examined in calves killed at 180 dpv and 330 dpv are shown in Tables 1 and 2 respectively. Significant differences (p<0.001) were noted in the number of granulomas between vaccinated and control groups, as well as the number of lesions present in the intestinal mucosa.

Bacteriology and PCR results

In tissues from two animals (No 5931 and 9109) belonging to VI group, MAP could be isolated, as well as in four calves from NVI group (No 3300, 7460, 5956 and 9107). Culture isolates were made in more organ types and the number of colonies was higher in calves from the NVI group than those from VI group. No positive isolation was made from fecal samples in any of the animals.

By PCR, only one unvaccinated animal (No 5956) had test-negative results for all tissue samples, while in the vaccinated group, three animals had all negative results (No 3303, 5953 and 7461).

Table 2: Granuloma counts and lesion classification in calves killed at 330 dpv (2nd sacrifice), vaccinated with SILIRUM® and inoculated experimentally at 60 dpv with MAP. Four cows were lesion-free (VNI n=2; NVNI n=2) and are not shown.

2nd SACRIFICE (330 dpv/270 dpi)			
Animal ID	**Mucosal granulomas[1]**	**Total granulomas [2]**	**Overall classification**
Vaccinated group (infected at 60 dpv)			
7461[3]	0	0	No lesions
3301	0	3.29	Focal
7930	0	83.26	Focal
9109	0	9.6	Focal
5931	416	1287	Diffuse
Mean ± SD	83.2 ± 186	276.63 ± 565.85	----
Control group (Not vaccinated, inoculated at 60 dpv)			
3300	0.66	8.59	Focal
3302	193.37	326.9	Multifocal
5956	7.99	83.26	Multifocal
9107	495	1741	Diffuse
Mean ± SD	174.26 ± 231.67	548.94 ± 812.16	----

DISCUSSION

The main aim of this study was to assess the efficacy of a killed vaccine (SILIRUM®) against bovine paratuberculosis in experimentally infected calves. From the results obtained, a protective effect of the compound was demonstrated even when taking into account a challenge dose that was considerably higher than usually experienced by an animal under field conditions.

No noticeable discomfort due to the presence of the vaccination nodules was observed in any of the vaccinated calves and no ulceration occurred. Other experiments have reported large and fistulated nodules (Doyle, 1964; Pérez et al., 1995). The quality and amount of the mineral oil used plus the site of inoculation for this vaccine differs from vaccines in previous studies. These factors apparently contributed to the reduction of vaccination nodule formation (Hanly, 1995).

Another well-known adverse effect of vaccination is interference in the official diagnostic test for tuberculosis. (Vallé and Rinjard, 1926; Stuart, 1962; Gilmour and Brothersson, 1966; Merkal, 1984) In this trial, as in the majority of the cases in other studies, the *M. bovis* tuberculin reaction was lower than the *M. avium* tuberculin for both the INF- production and ITT test. (Stuart, 1962; Huitema, 1967) The conclusion may be made that vaccination against paratuberculosis may not interfere with the official diagnostic test for tuberculosis when comparative tests are used. A stronger reaction to *M. bovis* PPD may be due to actual *M. bovis* infection rather than paratuberculosis vaccination. However, further studies would be needed to evaluate the immune response to the vaccine and its effect on diagnostic testing in tuberculosis-infected herds.

The main goal of vaccination is to elicit protective immune responses in animals. As has been previously observed in other experiments (Spangler et al., 1991; Juste et al., 1994; Corpa et al., 2000a), the vaccine used in this study stimulated both a cellular and humoral peripheral immune response in all the vaccinated calves. Immunity to all mycobacterial infections is dependent on cell-mediated immune responses and must be elicited by a vaccine for it to be effective (Gilmour, 1976). Humoral immune factors have little or no protective value (Chiodini, 1996). The strong and long-standing cellular immune responses induced by this

study's vaccination protocol in all the calves as measured by INF- or ITT are evidence of the protective effect of the vaccine. Moreover, high levels of INF- have been associated with an increase in the macrophage ability to limit the intracellular growth of MAP (Zhao et al., 1997) and with the presence of focal lesions, interpreted as latent or "resistant" forms (Perez et al., 1999). Although the antibody response may not have any protective effect, it would be an indicator of the degree of activation of the immune system against mycobacteria, since both cellular and humoral responses are mounted after the processing of mycobacteria by macrophages and antigen presentation to CD4+T cells (Munk and Emoto, 1995). In natural paratuberculosis, cellular immune responses appear in the first stages of the infection (Stabel, 2000), as was observed in the NVI group of this study, whereas in vaccinated animals both humoral and cellular appeared simultaneously. This pattern could be related to the immune system's exposure to large amounts of antigen (Abbas et al., 1996).

Histopathologic methods have been commonly used in the evaluation of the vaccination efficacy (Nisbet et al., 1962; Juste et al., 1994; Wentink et al., 1994; García Marín et al., 1995; Pérez et al., 1995; Corpa et al., 2000b). In this work, a quantification method for granulomatous lesions showed significant differences between study groups, with the vaccinated animals showing less gastrointestinal tract and lymphatic tissue pathology.

In experimental studies, the first lesions usually associated with MAP infection appear in lymphoid tissue (intestinal Peyer´s patches or lymph nodes) (Payne and Rankin, 1961; Nisbet et al., 1962; Larsen et al., 1975; Juste et al., 1994; Clarke, 1997; Kurade et al., 2004). Animals with these lesions are usually subclinical and the lesions are of the tuberculoid type. They may remain even until the death of the animal from other causes (Pérez et al., 1996). This lesion type is associated with strong cellular immune responses (Pérez et al., 1999; 2002) capable of controlling multiplication of the bacillus and progression of the lesions toward more severe forms. When this effective immune response is overcome, the lesions progress first toward the lamina propia of the mucosa associated with the lymphoid tissue, and then to different section of the intestinal mucosa causing severe diffuse granulomatous enteritis.

In our study, except for one case, vaccinated animals were either free of lesions or developed only the focal form. In contrast, multifocal or diffuse lesions were described in NVI calves. This finding supports the hypothesis that the vaccine limits granulomatous lesions to a tuberculoid form in which regressive granulomas with no or scarce mycobacteria are confined to the organized lymphoid tissue, as previously suggested in sheep (Juste et al., 1994; García Marín et al., 1995).

The above findings suggest a considerable protective effect of vaccination for bovine paratuberculosis. The protection linked with limiting the progression of pathology may be even greater than the observed in this study because the challenge dose was so large. Nevertheless, the protection provided by the vaccine product was not absolute since one vaccinated calf (No 5931) had diffuse lesions. This absence of absolute protection seems to be a common finding in paratuberculosis vaccines (Doyle, 1964; Larsen et al., 1978; Körmendy, 1994; Pérez et al., 1995; Corpa et al., 2000b) and is believed to be a function of factors that predispose an individual animal to a weaker or incomplete immune response (Doyle, 1964). One vaccinated calf in this study may be such a case, given its small nodule and meagre inflammatory response to vaccination. Coupling this calf's muted immune response with the high challenge dose resulted in a clinically severe infection.

Fewer MAP isolates were obtained from VI calves' tissues than what was seen with NVI calves. This finding was expected since diffuse forms of pathology, mostly seen in calves from the NVI group, usually bear high numbers of the bacilli. These results are in agreement with previous studies (Brotherston et al., 1961; Stuart, 1962; Juste et al., 1994; Pérez et al., 1995) and suggest that MAP multiplication is being suppressed. However, there are field studies (Kalis et al., 2001) that have not found a clear reduction in MAP shedding after a number of years in vaccinated flocks. In this study, no fecal shedding was detected in any animal.

CONCLUSION

SILIRUM®, a killed vaccine against paratuberculosis, was shown to be safe in calves experimentally challenged with MAP. It induced strong cellular and humoral immune responses. Although cellular immune response cross reactions to *M. bovis* PPD appeared, reactions were always higher to *M. avium* PPD suggesting that if comparative TB assays are used the paratuberculosis vaccine need not interfere in surveillance for tuberculosis.

The vaccine had a marked, although not absolute, protective effect against paratuberculosis. Vaccinated and challenged calves had fewer lesions, less severe lesions and a lower tissue burden of MAP than unvaccinated calves. The histopathological method employed has been shown to be useful in the assessment of the efficacy of a vaccine against paratuberculosis.

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The use of risk assessment, test and management strategies to control paratuberculosis and *Salmonella* Dublin in dairy herds

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ABSTRACT

Infections with *Salmonella* Dublin and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are among those, which receive most attention in the Danish cattle industry today. They share many common features with regard to control of infection in cattle herds. The infections cannot be controlled efficiently by treatment with antibiotics or other types of medicine, nor by test-and-cull procedures alone. They are mainly controlled by changing management to reduce transmission of bacteria between cattle via contaminated faecal matter and milk, and from the environment to susceptible cattle. Both infections are spread with asymptomatic carriers as well as with clinically diseased animals. Thus, control and prevention of both infections can be obtained using similar strategies. However, it is important that the person in charge of implementing the control strategies understand the basics of the transmission dynamics to achieve correct and consistent compliance necessary for intervention to be successful.

A tool for advisors to aid in conveying effective herd-specific control and prevention strategies to the herd manager has been developed. It is a manual which is based on risk assessment of important routes of transmission in Danish dairy herds. Steps are included in the manual to assure that learning is part of the decision making in planning intervention strategies. The principles of the manual are illustrated in this paper together with suggestions on how to use laboratory test results (antibody measurements in milk and blood samples by ELISA) to motivate and support control and possibly eradication of *Salmonella* Dublin and MAP in dairy herds and to evaluate progress in the intervention.

Key words: Paratuberculosis, *Salmonella* Dublin, control, management, risk assessment

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is widespread in the Danish cattle population, and paratuberculosis has received increasing attention in the cattle industry over the last decade due to losses in productivity and reduced health in infected herds. The zoonotic potential of *Salmonella enterica* subspecies *enterica* serovar Dublin (*S.* Dublin) in cattle is well-established as it causes severe invasive infections in humans (Fierer, 1983; Helms et al., 2003). Although it is not as widespread as MAP in Denmark, it causes both outbreaks of disease and persistent infections in cattle herds. Disease losses caused by *S.* Dublin are mainly due to calf mortality following diarrhoea, pneumonia, septicaemia and dehydration, veterinary expenses, abortions in adult cattle and increased workload during and following the initial outbreak (Peters, 1985; Visser et al., 1997).

In October 2002, a surveillance program was initiated for *S.* Dublin in all Danish cattle. This program classifies cattle herds into three different infection levels. If an animal is bought from a herd with a worse infection level than the receiving herd, temporary trade restrictions are placed on the buyer. Thus, it has become much more difficult to sell animals from herds classified in level 2a (probably infected), 2b (un-clarified) or 3 (bacteria detected). If a herd is not in salmonella level 1 (most likely un-infected), it is barred from participating in cattle shows. Therefore, the motivation to control and eradicate the infection has greatly increased in Denmark since the introduction of the surveillance and classification program. By July 2005, approximately 83% of all Danish dairy herds were classified level 1 in the national salmonella surveillance program. Since the introduction of the program, many level 2 and 3 herds have wanted to

become level 1. This can usually only be achieved through a thoroughly planned effort. We used the initiative for plans to manage *S.* Dublin to simultaneously focus on MAP in the herd, even in herds where MAP was not considered a problem at the time.

MAP and *S.* Dublin share several similarities, making it advantageous to coordinate intervention strategies against both simultaneously. Their similarities include:

a) Both infectious agents give rise to long-term intestinal infections with survival of the bacteria inside host macrophages, and with the main route of transmission between hosts through faecal contamination of the environment. Acute infection outbreaks may be seen with *S.* Dublin which is not a characteristic of MAP infection.

b) For both infections, age-related resistance is important, with calves below the age of six months being the most susceptible to infection. However, *S.* Dublin more frequently leads to new infections in all ages of cattle than does MAP, and this difference needs to be taken into account when planning control strategies for the two infections.

c) Asymptomatic carrier animals that cannot easily be identified with high accuracy by current diagnostic techniques are most likely critical to the persistence of these infections in cattle herds. Therefore, detection and management of high risk animals are essential parts of successful intervention.

d) Intervention strategies for both infections must be focused mainly on management to reduce the infection load in the environment of susceptible animals, as neither infection can be controlled efficiently by treatment with antibiotics or other types of medicine, nor by test-and-cull procedures alone. To succeed in controlling these infections, people involved in management of the herd therefore need to understand the most important issues in the transmission dynamics of the infections.

A tool for advisors to aid in conveying effective herd-specific control and prevention strategies to the herd manager has been developed (Nielsen and Nielsen, 2005). It is a manual based on risk assessment for important routes of transmission in Danish dairy herds. It has been adapted from a similar manual used in the United States (Rossiter et al., 1999). Steps are included in the manual to assure that learning about the infections is part of decision making and planning.

The Didactic Triangle
Risk factors for within-herd and between-herd transmission of infection are known from research studies and experience in the field (Counter and Gibson, 1980; Wray and Snoyenbos, 1985; Wray et al., 1989; Wray et al., 1990; Wray et al., 1991; Vaessen et al., 1998; Wedderkopp et al., 2001; Warnick et al., 2001; van Schaik et al., 2002; Warnick et al., 2005). Control of paratuberculosis and *S.* Dublin can be accomplished if the most important risk factors are recognised and subsequently managed. There are various challenges associated with managing the risk factors, but these challenges are no greater than most herd managers can learn to handle. Advisors may have a tendency to simply inform the herd manager what they think needs to be done in the herd, but the subsequent compliance is often disappointing. The herd manager's continued motivation, knowledge about transmission dynamics and participation in the actual decision making are key factors for compliance and thus for control of the infections. Therefore, the advisor's role can be seen as that of a teacher. This implies that the advisor uses techniques and tools to aid actual learning by the herd manager instead of providing just a one-way communication of facts. Broad understanding of complex issues may be hard to accomplish. It is a central feature of educational training. In Fig. 1, this is illustrated by the didactic triangle which suggests that the advisor takes the responsibility of bringing the knowledge into play along with the herd manager. It is adjusted to fit that of learning about infections such as paratuberculosis and *S.* Dublin. The arrows below the triangle illustrate that obtaining the knowledge necessary to prevent transmission in the herd requires not a one-way communication from the advisor to the herd manager, but a continuous exchange of knowledge and facts between the teacher and the student.
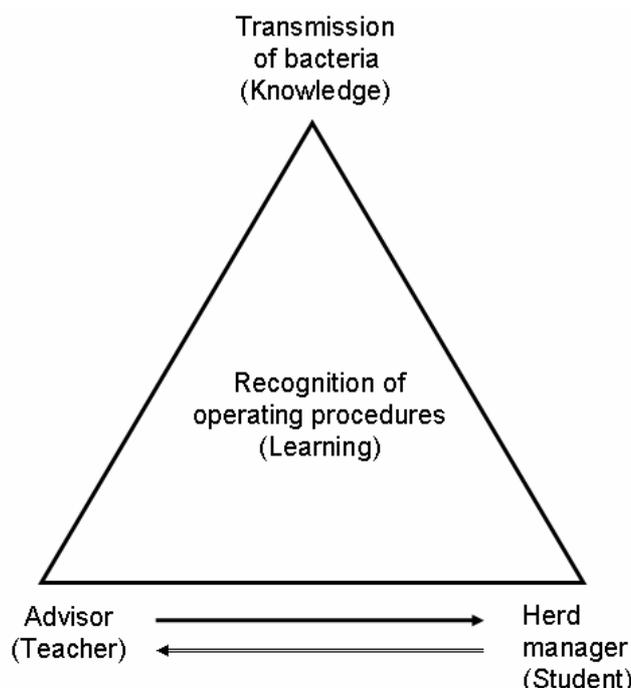
Fig. 1 Didactic triangle: learning about paratuberculosis and *Salmonella* Dublin intervention

The advisor also needs to understand a manager's short and long-term goals, possible economic, ethical or other limitations or possibilities, future plans, etc.   The advisor also needs to discover in which areas the manager most needs training and information. Learning becomes a learning-by-doing procedure that runs over several months if not years, when the learning objective is how to intervene and successfully control or eradicate MAP or *S.* Dublin from a cattle herd. Therefore, the strategies need to be evaluated and revised repeatedly over reasonable intervals, for instance every half a year or yearly.

Manual for Advisors
All important transmission routes in the herd must be recognised and closed for intervention to be successful. It is therefore important to go through the herd systematically to determine all possible transmission routes and evaluate the importance of each of them. This is not an easy task. Therefore, a tool has been developed to assist the herd manager and his advisors. It is a manual for advisors based on ideas from an United States manual concerning paratuberculosis control (Rossiter et al., 1999), but changed to match Danish farming practices and regulations and extended to include *S.* Dublin as well. The manual guides the learning process for both advisors and herd managers. The editors of the manual are responsible for providing the knowledge and framework. The herd manager and advisor(s) are responsible for filling in the framework with farm-specific information and bringing the knowledge into play, a process that enhances learning for everybody involved.

This advisory tool is both a risk assessment and an educational tool for developing intervention schemes on infected premises. An example of steps in the risk assessment is shown in Table 1 for paratuberculosis and Table 2 for *S.* Dublin. The maximum scores and transmission routes to be assessed for the two infections differ to some extent. This is because age-related resistance is not the same for the two infections, and because *S.* Dublin may multiply in the environment while MAP does not.

Table 1 Examples of risk assessment scores for MAP transmission in the barn area with calves before weaning. The risk assessment is an important part of a Danish manual for advisors for intervention against paratuberculosis and *Salmonella* Dublin in dairy herds.

Area	Risk factor	Score and criteria for paratuberculosis	
Calves before weaning	2.1 Feeding with pooled colostrum	0 3 5 7 10	Never happens 1-2 times per year 1-2 times per month Most of the time All of the time

	2.4	0	No contact at all (no possibility for carrying over of faecal matter between barn sections etc.)
		3	Only close to adult cattle when necessary. Separate barn sections.
	Physical separation between calves and cows	5	Calves in contact with cow manure during cleaning etc.
		7	Calves are frequently in contact with manure storage or cow/heifer barn sections
		10	Always close contact between calves and adult cows/heifers or their manure

Table 2 Examples of risk assessment scores for *Salmonella* Dublin transmission in the barn area with calves before weaning.

Area	Risk factor	Score and criteria for *Salmonella* Dublin	
Calves before weaning	2.1 Feeding with pooled colostrum	0 3 5 7 10	Never happens 1-2 times per year 1-2 times per month Most of the time All of the time
	2.4 Physical separation between calves and cows	0 5 10 15 20	No contact at all (no possibility for carrying over of faecal matter between barn sections etc.) Only close to adult cattle when necessary. Separate barn sections. Calves can get in contact with cow manure during cleaning etc. Calves are frequently in contact with manure storage or cow/heifer barn sections Always close contact between calves and adult cows/heifers or their manure

When the risk assessment for all barn areas is completed and summarized, it is usually quite clear to the herd manager and advisor which areas need particular attention. The manual encourages making a plan with clear and concrete management changes anad that all the changes recommended in the plan are prioritized. The sections on risk assessment and decision making in the manual include clear background theory and knowledge about the infections, diagnostic tests and epidemiology for the two infections.

Test strategies
The manual includes a section in which the advisor and the herd manager agree on test strategies for the herd during the intervention period. Such test strategies should be evaluated and revised at least yearly to assure that only necessary resources are spent on diagnostic testing (e.g. to help make decisions or evaluate the intervention process). It is important for the herd manager to understand that it is not sufficient just to test animals. The herd manager think ahead about what actions can be taken depending on the laboratory results received. In an intervention study currently running in 18 herds in Denmark, diagnostic test results are used to: 1) classify the animals into risk groups, and 2) evaluate the intervention progression.

High transmission risk animals should be treated with higher levels of caution than low risk animals when they come into contact with susceptible animals, particularly newborn calves and calves before weaning. An example of a laboratory report with animals grouped into paratuberculosis risk groups is shown in Table 3.

On the first page(s) of the report, animals that are assumed to be of any risk of shedding bacteria in faeces or milk are listed. Results are colour-coded to draw the manager's attention to the animals most likely to be shedding (colouring not shown here). These are the most important candidates for culling, and milk from these animals should never be used for calves. Their own calves should be removed immediately after birth and the calving area cleaned properly before any other animals enter the area. If possible these calves should be fed colostrum and milk from other cows than their own mother. Separate calving areas for high risk animals are preferred.

Table 3. Partial list of paratuberculosis high risk animals from one intervention herd. The list is based on antibody measurements and culture of bacteria from faecal samples. The date and result of faecal culture are given. Production losses are predictions based on the ELISA results.

| Cow ID	Parity	ELISA	Level	Previous ELISA	Faecal culture	Production losses	Infection group[§]
1241	4	Neg	0.0	0.1	0 (25/01/05)	Possible	5
1371	2	Doubt	0.1	0.1	1+ (JAN04)	Unknown	3
1498	5	Neg	0.0	0.1	0 (25/01/05)	Possible	5
1570	5	Pos	1.3	0.9	0 (25/01/05)	Very likely	9
1580	5	Neg	0.0	0.0	0 (25/01/05)	Unknown	3
1606	4	Neg	0.1	0.1	0 (25/01/05)	Possible	5
1619	5	Doubt	0.2	0.3	1+ (JAN05)	Very likely	2
1880	2	Neg	0.0	0.0	1+ (JAN04)	Unknown	3

Etc.

[§] "Infection group" is a classification based on consecutive ELISA measurements (4 times per year). Cows testing repeatedly positive on ELISA (group 9) or cows with a sudden rise in ELISA result (group 2) are cows which are "highest risk". Cows which cycle in antibody levels (group 5) or had a positive faecal culture more than 1 year ago (group 3) but are still sero-negative are considered medium-risk cows. Cows that have had a positive ELISA more than one year ago are considered potential false-positive (group 3). Cows of infection group 9 are recommended culled prior to next calving. Cows of infection group 2 may also be culled, but more diagnostic information could be obtained prior to making a final decision.

A similar list is made for *S.* Dublin. However, the risk groups are more difficult to define than for paratuberculosis. It is known that some animals are asymptomatic carriers of *S.* Dublin and that these usually have a long term high antibody response in blood or milk samples (Spier et al., 1990; Spier et al., 1991; Smith et al., 1992). Unfortunately, it is likely that the long term high antibody responses found in numerous cattle indicate memory in the immune system or repeated re-infection rather than actual persistent infection (Hoorfar et al., 1996). On the other hand, animals without a high antibody response may also spread the infection. Therefore, the use of high risk lists for culling of *S.* Dublin suspected carriers should not be used unless transmission is fully under control. Little is known about transmission of *S.* Dublin bacteria through milk from dam to calf, so the main control strategies for *S.* Dublin usually focus on preventing contact with faecal matter from other animals, e.g. between neighbouring calves and calving cows.

Motivation: Quantifying production losses due to paratuberculosis
Herd manager motivation is necessary for an intervention scheme to be accomplished satisfactorily. Motivation may come through quantification of "losses". Clinical disease is a parameter that can be monitored to assess the efficiency of intervention. However, in terms of control and eradication of infection, this parameter is not useful as the relationship between control of infection and eventual clinical disease is difficult to measure. Transmission of both *S.* Dublin and MAP can take place without clinical disease present in the herd. Quantification of production losses due to paratuberculosis is fairly easy to do however. It can be based on data from literature in the initial intervention process, and on the specific farm data later in the process as repeated diagnostic test results become available on the individual cow level. An example of reduced milk production attributable to MAP infection calculated from data collected in 19 intervention herd is shown in Fig. 2. In addition to milk losses were decreased value at slaughter, loss of genetic potential, premature culling, continued transmission of MAP and increased workload due to management of diseased animals. Such losses are harder to assess in a production setting, and simulations may be the only method for estimating total losses. However, while the losses shown in Fig. 2 may not appear large to some herd managers, summarising the losses of all affected cows can have a great effect. As an example: one herd manager in the intervention project did not really consider paratuberculosis to be a problem, and thought he might have had a loss of 1000 kg energy corrected milk (ECM) during the past 6 months. He

was quite surprised to be informed that the estimated losses were actually approximately 45,000 kg ECM, an estimate obtained simply by comparing cows in the various groups.
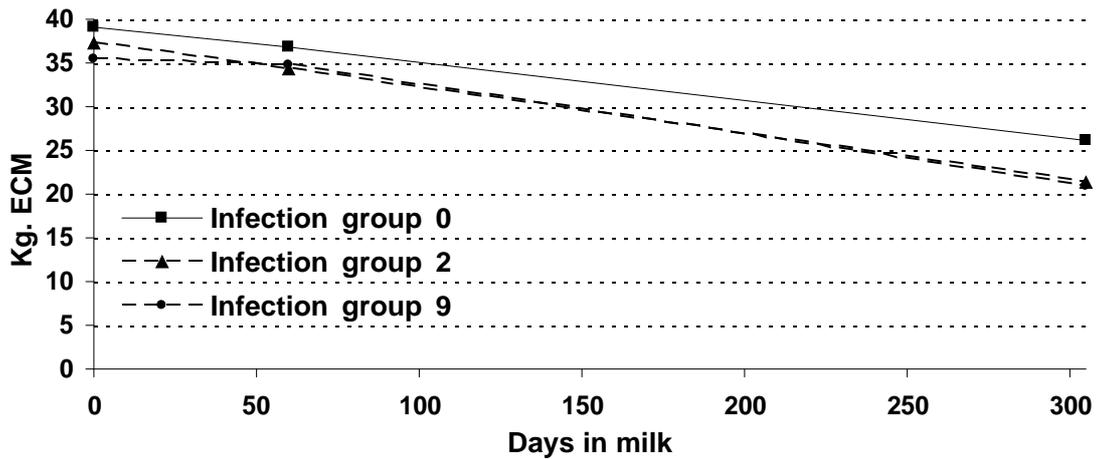
Fig. 2 Lactation curves of 610 Danish Holstein cows in their 2nd parity. The number of cows contributing to the lactation curves were as follows: Infection group 0: 486 cows; Infection group 2: 78 cow; Infection group 9: 63 cows. The losses of both infection group 2 and 9 correspond to a loss of 10%. Cows of groups 3 and 5 are not shown.

Evaluation of intervention strategies Salmonella Dublin and paratuberculosis
Eradication of *S.* Dublin has been successful in dairy herds with benefits for the general health of the animals in those herds (Jensen et al., 2004). Successful eradication of *S.* Dublin in a herd with concomitant *S.* Dublin and MAP infection may indicate successfully reduced transmission of both bacteria types in the calving area. There are strong indications that test-and-cull strategies only work in combination with improvement of the management and housing of young calves, the calving procedures and the hygiene of the housing environment. Test-and-cull procedures are most important towards the end of the eradication period.
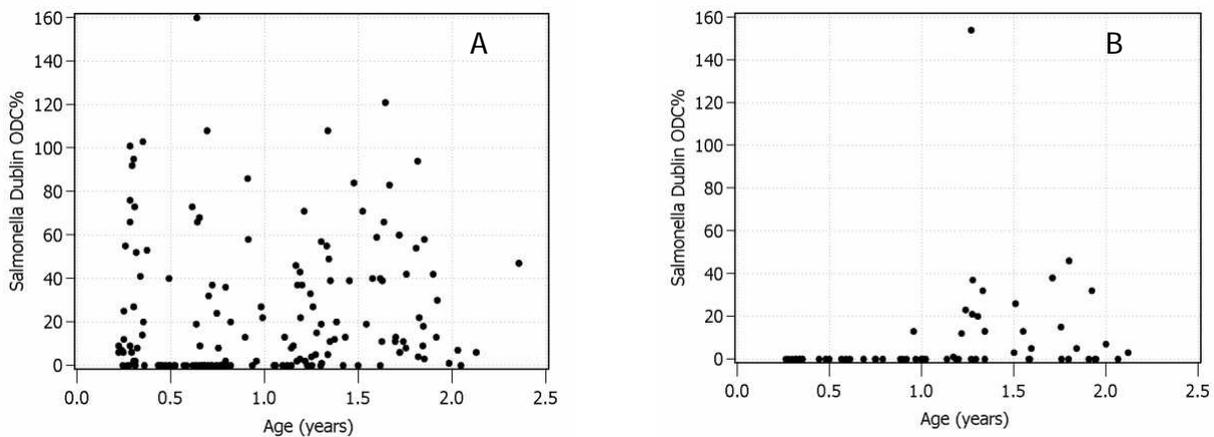
Fig. 3 *Salmonella* Dublin antibody levels measured by ELISA in blood samples from animals from 3 months of age to first calving in A: a herd with transmission of infection among young calves, B: a herd that performs active intervention against spread of infection among young calves by keeping calves in clean single huts outdoors until 8 weeks of age. This intervention strategy was initiated approximately 15 months before the samples were collected and appears to be very successful according to the screening results.

However, the herd manager is usually interested in knowing whether transmission has ceased after changing management strategies and whether to continue to follow the progression of the intervention. Achieving eradication takes daily and persistent effort from the herd manager and motivation can be enhanced by illustrations of cross sectional screening of the whole herd or certain age groups with diagnostic tests determining the antibody levels for *S.* Dublin in blood and/or individual milk samples

(ELISA). Diagnostic test results of such screenings of two herds are shown in Fig. 3. In one herd (A), the transmission routes to the newborn calves and/or horizontal spread between very young calves are problematic. It takes approximately 14 to 30 days for the calves to mount a high antibody response, so high levels of antibodies in blood samples from calves aged 3-6 months indicate transmission of infection in the pre-weaning calf barn section of the herd. Antibody measurements from calves below the age of 3 months are difficult to interpret because of possible transfer of maternal antibodies, and because calves below 11-12 weeks of age do not produce antibodies very well (Da Roden et al., 1992; Nielsen and Ersbøll, 2004). In the other herd (B), management changes in the calf barn were initiated 15 months prior to the screening date shown and were successful. There is no sign of transmission of *S.* Dublin in the calves within the last 15 months prior to screening. Antibodies in animals above the age of 15 months may be from earlier infection and do not necessarily indicate a risk of transmission. Only repeated samples on the same animals or another screening for instance 6 months later can tell if transmission is still present in the herd. If *S.* Dublin transmission has stopped there may be reason to believe that MAP transmission during the calving period has also been brought to an end. It does not prove that other important routes of infection such as that from milk feeding have ceased. Data of this sort cannot provide evidence but do provide indicators that a herd manager may use to monitor the usefulness of the ongoing procedures.

CONCLUSION

Control and eradication of *Salmonella* Dublin and paratuberculosis is possible, but are often difficult tasks that require knowledge about transmission dynamics and long term, persistent effort. A tool is available for Danish herd managers and their advisors to help plan and organise intervention strategies in individual herds, so that the best results can be obtained within the limitations of that particular herd. The manual is constructed to plan intervention strategies based on a systematic evaluation of the most important routes of transmission and the infection load in different barn sections of the herd. Didactic principles are used to increase knowledge about pathogenesis and infection dynamics. Knowledge about the infections has been incorporated in the scoring system to enhance learning, encourage compliance and increase motivation.

ACKNOWLEDGEMENTS

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Simulating control strategies for Johne's disease in New Zealand dairy herds

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ABSTRACT

A simulation was completed to model the effect of various Johne's disease control strategies using New Zealand dairy herd characteristics. The JohneSSim computer model is a stochastic, dynamic model developed in The Netherlands. It was adapted to represent the New Zealand dairy production system. Recent case/control and longitudinal studies in New Zealand, industry data, expert opinion and international data provided model input. Control strategies included Improved Farm Management (IFM), Vaccination, Genetic Resistance and Annual Test-and-Cull based on faecal culture or ELISA. Simulated within-herd true prevalence rose from 15% to 49% and annual losses from NZ$2,300 to NZ$23,400 in the absence of control over 20 years. All strategies slowed the rate at which prevalence increased, but only IFM reversed this rate resulting in prevalence of <1% after 18 years. IFM and Vaccination were the most economically attractive strategies. A decision to attempt either control or eradication must be made and the likely effects of each control strategy must be clearly understood before selecting a control program. IFM reduced the infection to very low levels and would probably be a low cost strategy, thus appearing the most attractive means of control. Valuable insight into the strengths and weaknesses of a variety of control strategies was obtained.

Key words: Johne's disease, dairy, simulation, model, JohneSSim, New Zealand

INTRODUCTION

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic wasting syndrome in ruminants that is endemic in many countries. It is thought to occur in about 70% of New Zealand dairy herds causing economic losses due to premature culling and reduced milk production. Widespread speculation about the possible role of MAP in Crohn's disease in humans has led to concern within the dairy industry, particularly since some studies have shown that the organism can survive pasteurization. A national control strategy would promote and protect New Zealand dairy export sales and match efforts by other major dairy producers such as the US, Australia and some EU countries. The insidious nature of JD and poor performance of diagnostic tests suit computer simulation methods for investigation of potential control strategies. JD was first modelled by Walker in 1988 and progressively more complex models have since been developed (Collins and Morgan 1991). JohneSSim is a stochastic, dynamic simulation model developed to evaluate control strategies for JD in The Netherlands (Groenendaal 2002; 2003; Weber, 2004) and later the United States (Groenendaal 2003). The aim of this study was to adapt the JohneSSim to model JD in the New Zealand dairy system and simulate possible control strategies.

MATERIALS AND METHODS

The JohneSSim model
JohneSSim has been thoroughly described by (Groenendaal 2002). In brief, a population representing New Zealand dairy herds was simulated over 20 years at 6 monthly intervals. This population had a specified range of infection levels and a specified proportion of herds used two high risk management practices (risk profiles) with respect to Johne's disease. These risk practices were a long (>12hr) period of contact

between the calf and dam and grazing all stock aged 3-9 months on-farm. Results were a distribution of values for the whole population. For some of the economic results, a range was included corresponding to the central 80% of the population.

Model input was derived from recent case/control and longitudinal studies, the national dairy industry database, expert opinion and international data. True prevalence (henceforth prevalence), costs and benefits were simulated for the situation without controls and for the different control strategies.

The average New Zealand dairy herd was described as seasonal calving with 280 milking cows (central 80% range from 100 to 450 milking cows), an annual increase in herd size of 3% and replacement rate of 23%. Milk production was 311kgMS per cow per year. The cost of voluntary culling was based on a retention pay-off value (RPO), calculated by an existing economic model (Groenendaal 2004), representing future expected profits of an individual cow. Heifers calved at two years of age. The mortality rate of calves born to heifers was 8% and 5% for older cows.

Johne's disease was assumed to be present on 70% of New Zealand dairy herds at a within herd sero-prevalence of 3-6%, resulting in an estimated true prevalence of approximately 15%. Cows occupied one of six possible infection states (Table 1)) and transmission could occur by one of six possible routes (Table 2). Test sensitivity varied depending on the infection state of the individual.

Table 1. Infection status modelled by JohneSSim

Infection state	Description
1	susceptible calves
2	resistant animals (not infected and >1 year old)
3	latent infection but not shedding
4	low infectiousness, shedding for 2 months post calving
5	highly infectious, infected and shedding
6	clinical, infectious and constantly shedding

Table 2. Transmission routes modelled by JohneSSim

Transmission route	Description
1	fetal infection
2	infection during birth
3	consumption of contaminated colostrum
4	consumption of contaminated milk
5	infection from environmental contamination
6	infection due to introduction (purchase) of an infected animal

Milk production was assumed to be reduced by 5, 10-15 and 20% for cows in infection states 4, 5 and 6 (Table 1). There was assumed to be a 20% probability that pooled milk was contaminated after milk/colostrum from a constantly shedding cow was added. Ninety five percent of calves fed from a contaminated pool became infected.

The purchase of bulls was not considered a major risk factor for the introduction of Johne's disease. Bulls were assumed to be kept for a short mating period (October-February), separate from young stock, and then sold. Bulls are mostly between 2-3 years of age and selected on the basis of good body condition.

All financial values were reported in New Zealand dollars (NZ$). Input parameters describing economic values were derived from industry statistics and, where no data source was available, from expert opinion.

The economic benefit of a control strategy was defined as the reduction in the losses caused by Johne's disease at the herd level relative to the no-control situation. The Net Present Value (NPV) is a standard economic measure for valuing investments with an extended time component. It represents the economic attractiveness of each strategy, defined as the benefits minus the costs, with future financial values discounted to their present-day value. Thus it is the value of the strategy to the herd owner at the time it commences.

Net Present Value = total discounted reduction in losses – total discounted costs

Control strategies
A total of ten control strategies were derived from suggestions by the expert group and from strategies used by other countries. The six strategies included in this report (Table 3) represent the range of effects observed.

Table 3. Control strategies for New Zealand dairy herds simulated by JohneSSim

	Strategy	Test-and-cull	Calf management	Other
1	Annual test-and-cull, ELISA (AnELISA)	Annual ELISA (cows>3yrs) confirmed by faecal culture	None	None
2	Annual test-and-cull, faecal culture (AnFC)	Annual faecal culture (cows>2yrs)	None	None
3	Improved Farm Management (Mgt)	None	Improved management	None
4	Vaccination (Vac)	None	None	Vaccination
5	Genetic Resistance (GenRes)			
6	Bi-annual test-and-cull (pooled faecal culture), partial hygiene improvement and off farm grazing (BiAnFCMO)	Bi-annual pooled faecal culture	Improved mgt and hygiene	Off farm grazing

Vaccination was assumed to increase the average period between infection and onset of heavy shedding from 5 to 7 years.

Improved Farm Management was improved calf hygiene during birth and in the first few days of life, provision of non-contaminated pooled milk/colostrum and reducing the likelihood of faecal/oral contact by off-farm grazing. Off-farm grazing is the practice of shipping weaned stock off-farm to contract raisers who provide pasture not grazed by adult cattle until calves join the adult herd.

Genetic Resistance was represented by a gradual reduction in the probability of infection between generations of calves, such that the probability of an individual becoming infected in year 20 was 50% less than in year 1. In addition, resistant but infected cows were assumed most likely to begin shedding at 7.5 years old, while non-resistant cows were most likely to begin shedding at 5.5 years old.

For strategy 6 we duplicated strategy 3, with the exception that feeding potentially contaminated pooled milk/colostrum was still practiced and biannual test-and-cull using faecal culture occurred.

Sensitivity analysis
There was substantial uncertainty surrounding the values of many input parameters. Sensitivity analyses were conducted on ten infection transmission parameters including culling rate, importing of stock, feeding of pooled milk or colostrum, the milk production of infected cows, foetal infection rate and faecal oral contact rate to illustrate their effect on model predictions.

RESULTS

Epidemiology
Without control, prevalence in the average infected herd increased from 15% to 48% over 20 years (Fig. 1). Improved Farm Management was the only strategy to successfully reverse the trend of increasing prevalence, reaching 1% after 18 years. All other strategies slowed the rate at which prevalence increased. The rate of increase of the prevalence slowed to zero under the Genetic Resistance strategy and given a longer simulation period, may have become negative.
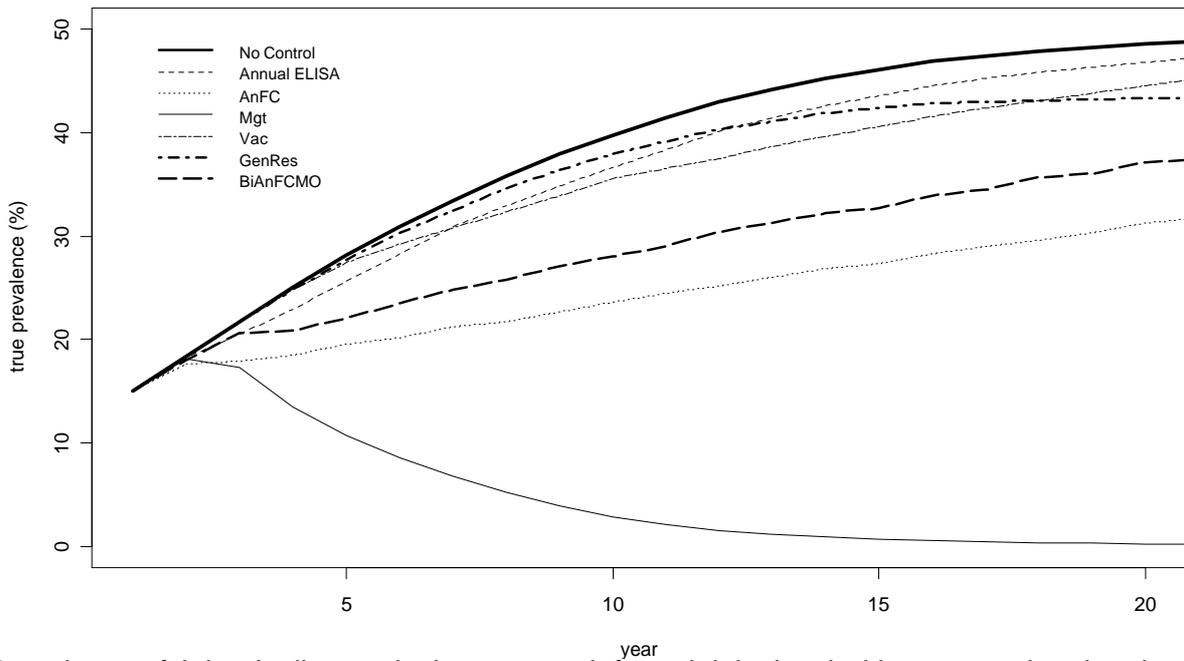
Fig. 1. Prevalence of Johne's disease in the average infected dairy herd without control and under six control strategies

Economic impact of control strategies

In the absence of control, annual losses due to Johne's disease for the average infected herd increased gradually from NZ$2,300 to NZ$23,400/yr after 20 years. Benefits (reduction in losses relative to no control) were generally proportional to the reduction in prevalence provided by a control strategy, thus were greatest for Improved Farm Management. However vaccination also provided considerable benefits, despite having little effect on prevalence (Fig. 2) because it reduced losses associated with sub-optimal culling and reduced milk production. Biannual testing in strategy 6 resulted in oscillating losses.
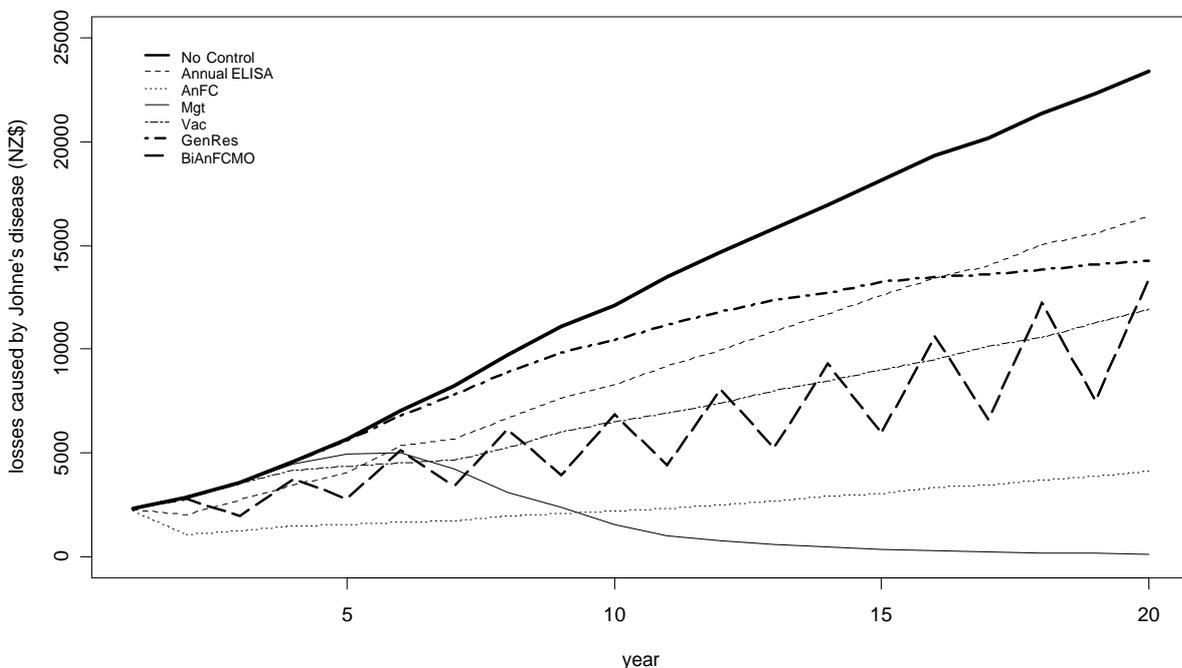
Fig. 2. Annual losses for the average infected dairy herd under Johne's disease control programs

Annual costs of control strategies steadily increased for those that failed to stop the increase in prevalence. Highest costs were associated with the test-and-cull strategy using faecal culture (NZ$29,500 at year 20) but were also high using an ELISA test (NZ$12,900 at year 20). The one exception was vaccination, which remained a low cost option despite increasing prevalence, being only NZ$2,200 after 20 years.

A positive Net Present Value (NPV) (Table 4) represents an economically attractive option to the average infected dairy herd. Costs of the Improved Farm Management and Genetic Resistance strategies were not included in the analysis, and therefore for these strategies the NPV is equivalent to the benefits of that strategy and represents the maximum cost at which a strategy remains economically attractive.

Table 4. Net present value (NZ$) of simulated control strategies for Johne's disease to the average infected New Zealand dairy herd and range containing the central 80% of dairy herds.

Control strategy	Average NPV (NZ$)	Range (NZ$)
No control	0	0
Annual test-and-cull (ELISA)	-61,798	-78,136 to -41,086
Annual test-and-cull (faecal culture)	-141,000	-182,132 to -50,447
Improved Farm Management	105,275	0 to 221,662
Vaccination	38,563	-19,964 — 103,494
Genetic Resistance	27,641	0 to 58,361
Bi-annual test-and-cull (pooled faecal culture), off farm grazing and partial hygiene improvement	-7,482	-37,779 to 56,709
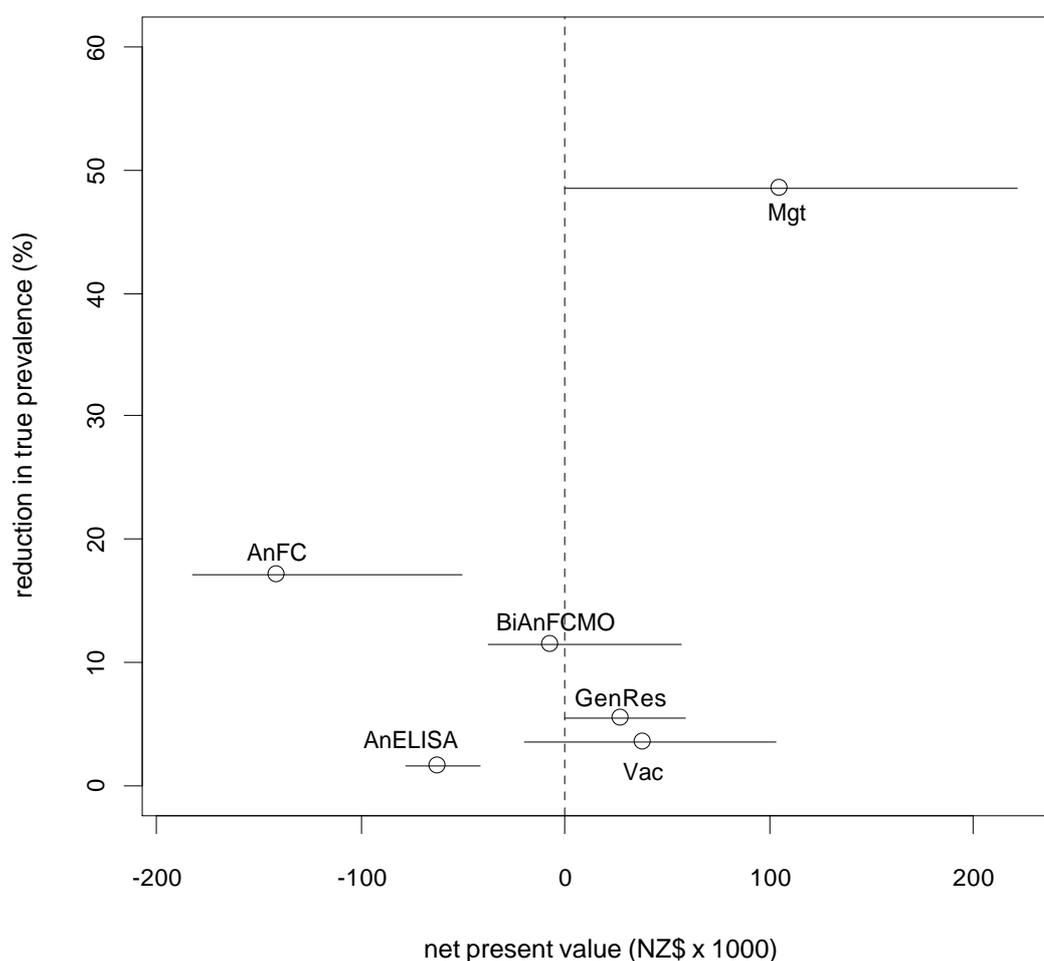
Fig. 1. Comparison of prevalence reduction of Johne's disease and net present value (to average infected herd and interval containing central 80% of herds) after 20 years for simulated control strategies.

The effects of using each strategy for 20 years were ranked by the reduction in prevalence and NPV (Fig. 3). Strategies toward the top right of the figure were both economically attractive and effective at reducing the prevalence of disease while strategies toward the bottom left were both expensive and ineffective at reducing prevalence.

Improved Farm Management was clearly the superior method for infection control. The interval containing the NPV for the central 80% of herds was wide for strategies that caused a large reduction in losses. Under

such strategies, the difference between herds maximising the benefit and those getting no benefit was large. In contrast, there was little NPV difference for strategies that had minimal effect on reducing prevalence.

Sensitivity Analysis
A limited amount of New Zealand data was available and therefore data from other countries with dairying systems that differ from those used in New Zealand were used. Expert opinion was incorporated as well although opinions were not always in agreement. Therefore, a sensitivity analysis was performed on ten of the more uncertain variables, of which two were found to influence model predictions.

The first influential assumption was that pooled milk/colostrum was never contaminated in contrast to the default situation of its being rarely contaminated. This caused a slower increase in prevalence, to 27% after 20 years compared with the default situation of 50%.

The second assumption affecting the outcome was that 70% of herds infrequently buy small numbers of replacement animals, whereas the default was that herds bred all their own replacements. This caused the prevalence to increase more quickly in the second half of the simulation period to 65% after 20 years compared to 50% in the default situation.

Model output varied little in response to changes in culling rate for all cows, milk production of infected cows, foetal infection rate, start of shedding by infected cows, faecal oral contact rate for calves and peri-natal infection rate.

DISCUSSION

The JohneSSim model illustrated a fundamental question in regard to the control of Johne's disease: whether the aim of control is reduction in the economic losses of disease, or eradication of the infection. Based on the results, eradication seems a lofty goal requiring a lengthy process, and in reality be equivalent to reducing the infefction to very low levels. Reduction in the economic effects of disease, however, is a comparatively achievable goal and was demonstrated to varying levels by five of the six control strategies.

Improved Farm Management was the most effective strategy by a large margin and the only one to reverse the trend of increasing prevalence. Subsequently, the costs associated with the disease also decline, eventually to negligible levels. The costs of implementing this strategy were not estimated but it would be economically attractive to the average infected herd provided it cost less than NZ$105,000 over 20 years. Combining Improved Farm Management with an annual test-and-cull strategy using faecal culture (data not shown) caused losses to decline more quickly, but hugely inflated the cost such that the combination was not economically attractive. In the New Zealand dairy production system the exceptionally high workload during the calving period represents a challenge to implementing the additional measures required to meet the Improved Farm Management criteria in the absence of clear benefits to the herd owner. A single whole herd test-and-cull operation for herds beginning a control program may be of benefit.

Of the non-management strategies that were simulated, an annual test-and-cull protocol using faecal culture provided the best infection control. However, this strategy was prohibitively expensive, with a net value to the average infected herd of NZ$-141,000 over 20 years. In addition, the logistic challenges associated with laboratory capabilities suitable for handling a national scale testing program must not be underestimated. Substituting an ELISA test provided a slightly lower cost, but also slowed the decrease in the prevalence and economic losses. Diagnostic tests are important tools in infection control, for example screening herds for infection (Muskens, Barkema et al. 2000; Quist, Nettles et al. 2002; Pence, Baldwin et al. 2003), but for a sustained control strategy more cost effective options should be sought.

We assumed that vaccination would extend the period prior to shedding thus increasing the lifespan and milk production of infected cows and reducing premature culling. This strategy was economically attractive due to a relatively low implementation cost and provided substantial reduction of Johne's disease associated losses. However, it provided only a minor reduction in prevalence.

Optimum infection control could be obtained if this vaccination strategy was combined with a low cost strategy that effectively reduced infection prevalence (Improved Farm Management perhaps). Vaccination has been effective against ovine Johne's disease in Australia and Iceland (MacDiarmid 1989; Bull, Brooks et al. 2004) and against caprine Johne's disease in Norway (MacDiarmid 1989). Unfortunately current vaccines can cause substantial injection site lesions, must be administered with extreme care and must be proven compatible with current test methods for tuberculosis in cattle.

The Genetic Resistance strategy provided a unique approach to the control of Johne's disease. While successful in stopping the increase in prevalence, it took the full 20-year period to achieve it. Given the current debate over the link between Johne's disease and Crohn's disease in humans, a control strategy that produces results more quickly is preferable. The costs of implementing this strategy were not estimated but are likely to be vastly greater than the benefits to the average infected herd of NZ$27,641 over 20 years. We assumed that the first cohort of resistant calves were born in year one of the simulation period and did not include a period or costs for identifying and developing resistant genes to the point where they were included in a calf's genetic makeup.

A thorough sensitivity analysis showed the model behaved rationally within the normal range of operation. Unfortunately no data were available with which to validate the model output under New Zealand circumstances. Of the ten parameters analyzed for sensitivity, infectivity of pooled milk/colostrum caused the greatest change in model predictions while the frequency of reintroduction (through purchase) of infection to a herd had the second largest effect. More detailed data describing purchase patterns would make the model more representative of actual conditions.

Assuming that contaminated pooled milk/colostrum was not infectious to calves due to dilution by pooling with uncontaminated milk substantially reduced the rate at which prevalence increased such that it more closely resembled anecdotal evidence. Further modelling will explore the effects of this assumption on the simulated control strategies.

More detailed information on the infectivity of pooled milk/colostrum would be valuable but such a study would face considerable challenges. Most importantly, the minimum infective dose for a calf and the route of infection is not known with certainty, while the concentration of MAP in infected milk prior to pooling (2-8 CFU/50mL, (Sweeney, Whitlock et al. 1992)) is generally at the lower limit of existing detection methods (Sweeney, Whitlock et al. 1992; Streeter 1995). Further, contamination rates in faeces may be in the order of 10^6 colony forming units per gram (Sweeney 1992) indicating that this material is a much greater force of infection than contaminated milk. Currently no broadly accepted techniques are available to accurately enumerate MAP levels in milk (Grant and Rowe 2001).

Future use of JohneSSim will be improved by adjusting input parameters with additional data. While these updates may result in slightly lower infection levels, the trends reported here are expected to remain largely unchanged.

In conclusion, the JohneSSim model has provided insight into Johne's disease control in New Zealand dairy herds. Improving farm management was clearly the most effective method for reducing the prevalence of infection and is potentially of low to moderate cost. The benefits of vaccination were also evident. Test-and-cull approaches were neither economically or epidemiologically attractive. Uncertainty in many of the input parameters requires that results be interpreted as trends rather than specific values. Sensitivity analyses showed that the infectiousness of pooled milk/colostrum and the frequency of reintroduction of infection had strong influences on prevalence of infection. Additional information to raise the descriptive accuracy of these parameters would be valuable.

ACKNOWLEDGEMENTS

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The distribution of *Mycobacterium avium* subsp*. paratuberculosis* in the environment surrounding Minnesota dairy farms

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ABSTRACT

The objective of this study was to characterize the distribution of *Mycobacterium avium subsp paratuberculosis* (MAP) in the environment of infected and noninfected Minnesota dairy farms. One hundred and eight Minnesota dairy herds were sampled during the summer of 2002, including 80 herds likely to be infected based on previous testing in the Johne's Disease Control Program (JDCP) of the Minnesota Board of Animal Health (MBAH) and 28 herds likely to be noninfected based on previous testing in the Voluntary Johne's Disease Herd Status Program (VJDHSP) of the MBAH.   Fecal samples were obtained from up to 100 cows in each herd and were cultured in pools of five cows per pool based on age order. Environmental samples were obtained from each farm, with up to 2 samples collected from each various. Fecal pools and environmental samples were tested using bacterial culture for MAP at the Minnesota Veterinary Diagnostic Laboratory. Sixty-four of the 80 JDCP herds (80%) had at least one positive pool;
16 of these herds did not have any positive pools. The farm environment was detected as contaminated on 61 of the 64 (95%) herds with positive pools and in one of the 16 JDCP herds with negative pools. Twenty-six of the VJDHSP herds (93%) had no positive pools; 2 herds had one positive pool each. The environment of one of these herds was also detected as contaminated. The farm environment was detected as contaminated in cow alleyways (77% of the herds), manure storage (68%), calving area (21%), sick cow pen (18%), water stream edge and water runoff (6%), and postweaned calves area (3%), but not in preweaned calves or fields near cow area.   The results indicate that targeted sampling of cow alleyways and manure storage may be a useful alternative strategy for herd screening and Johne's infection status assessment and for estimating herd fecal prevalence.

Key words: Johne's disease, *Mycobacterium paratuberculosis*, environment, dairy cow, pooling.

INTRODUCTION

Although *Mycobacterium avium subsp. paratuberculosis* (MAP) does not propagate in the environment, it survives for long periods in different environmental conditions. Several reports describe long-term in-vitro bacterial survival in water, urine, manure and extreme temperatures (Vishnevskii et al., 1940; Lovel et al., 1944; Larsen et al., 1956; Jørgensen, 1977). These findings suggest that MAP survives well in conditions that would be expected on dairy farms. Whitlock et al. (1992) sampled 11 infected herds in Pennsylvania. Obvious manure samples, which contribute to environmental contamination, were not sampled. The authors found that 45% of the farms had positive environmental samples but only one farm had a high prevalence of environmental contamination (22%). Similarly, Pavlik et al (2002) sampled 2906 environmental samples from 20 infected herds and found that only 2% tested positive for MAP.

Johne's disease control programs have been developed in different countries (Kennedy and Allworth, 2000; Kalis et al, 2000) and in several states in the USA such as Wisconsin and Minnesota (Bulaga, 1998). These programs aim to test and classify herds of cattle as infected or presumptively noninfected with maximum accuracy and least cost. Generally, these programs utilize recognized laboratory tests such as enzyme-linked immunosorbent assays (ELISA) or direct microbiological individual fecal culture. However, these tests have several disadvantages, especially when applied in herds with subclinical disease or low prevalence. The pooled fecal culture method, which aggregates several cows' fecal samples to one culture

unit, has been recently suggested as a good alternative strategy for lowering the procedure cost in herd screening programs for dairy cattle (Kalis et al. 2000a, Wells et al, 2003). The approach is reported however to have with variable sensitivity depending on phase of infection, herd prevalence and fecal shedding rates.

The objectives of study were: 1) To describe MAP distribution and prevalence in the environment on Minnesota dairy farms, 2) To assess the relationship between culture status of MAP in the farm environment and fecal-pooled culture status.

MATERIALS AND METHODS

Herd Selection and sampling
Herds were selected from the database available for two Johne's disease (JD) programs in Minnesota. These herds were known to be either infected according to previous testing in the Johne's Disease Control Program (JDCP) of the Minnesota Board of Animal Health (MBAH), or noninfected based on previous testing in the Voluntary Johne's Disease Herd Status Program (VJDHSP) of the MBAH. All herd owners were contacted by letter to request voluntary participation in the study. One hundred eight Minnesota dairy herds were sampled between May to September 2002, including 80 from the JDCP and 28 from VJDHSP. Up to 100 cows were selected to represent the distribution of cows within each herd using systematic sampling whenever possible. In the free-stall herds where cows were grouped based on their lactation stage, a proportion of cows from each pen was sampled. Approximately 30 grams of fecal sample were obtained via rectal retrieval with a plastic disposable rectal examination glove lubricated with sterile water. Samples were placed in a 95 ml plastic covered specimen container and stored in a cooler with ice during transport to the laboratory.

From each study farm environmental samples were obtained from each of the following locations if available in the farm: calving area, dry cow area, cow alleyways, manure storage, soil from crop fields near cows open areas (dry lot or pasture) where possible drainage from cow area to the fields is possible, edge of streams where cows have access, water runoff from the parlor or cow barn, preweaned calves, postweaned calves housing, and sick cow pen. Two samples were collected in each location with two exceptions: in preweaned calf areas where a small number of calves were available, only one sample was obtained and in small sick cow pens also only one sample was collected. Each sample contained approximately 20 g of fecal material with bedding or soil from 3-4 different sites within each sampling location. The material was collected with a disposable latex glove for each location and placed in a 95 ml plastic covered specimen container and stored in a cooler with ice in a separate plastic bag from the rest of the cows' fecal samples during transport to the laboratory.

In freestall barns (61 farms), each cow alleyway sample was obtained from 3-4 sites across 1-2 alleyways. In tie-stall barns (46 farms), samples from cow alleyways were obtained from the gutter on each side of the alleyway. Each sample from the manure storage lagoon was obtained by sampling 3-4 locations at the edge of the lagoon around its perimeter and by submerging the sampling container up to 10 cm under the water surface. Each sample from manure piles was obtained from 3-4 different sites up to 10 cm in depth. Each sample from the manure pit contained 2-3 sterile 4x4 gauze pads tied to fishing line with weight and soaked at least 10-15 cm below the manure surface. In two herds, closed slurries were not sampled due to safety reasons. Samples from preweaned calves were collected from the floor, or if not available from the environment directly from the rectum, and each sample contained fecal material from 1-3 calves. Samples from postweaned calves were obtained from the floor of calf housing areas.
The samples from stream banks included fecal material in contact with the water and moist soil. Samples from water runoff from the parlor or barn contained moist soil or sediment collected from the bottom of the stream as well as the water. Samples from fields near cow open areas contained soil from locations where drainage from the cow area was possible.

Laboratory testing
Fecal samples from cows were pooled in groups of five cows per pool based on age order for a total of 10 grams per pool. The procedure involved mixing 2 grams of manure from each of the five cows with wooden

stick, thereafter weighing 2 grams for the pooled fecal culture. Fecal pools and environmental samples were tested using bacterial culture for MAP at the Minnesota Veterinary Diagnostic Laboratory using the method previously described with Herrold's egg yolk medium. Colony counts were recorded on a weekly basis for 16 weeks and final results were scored as negative, light (mean of 0.25 to 9 colonies/tube), moderate (mean of 10 to 49 colonies/tube), and heavy (mean of ≥ 50 colonies/tube) fecal shedders. Herd or farm environment was defined as infected if at least one pool or environmental sample respectively was positive for MAP, assuming that the fecal culture method has 100% specificity.

Statistical Analysis

Descriptive statistics were used to describe the distribution of the environmental samples using Excel (Microsoft Office 2000) and SAS (Statistical Analysis Systems Institute Inc., 2001).

A chi square Fisher exact test with statistical level $p<0.05$ was used to determine the association between environment sampling and fecal pool status on the herd level. To assess the correlation between the number of positive fecal pools and different positive environment samples, a Spearman rank order correlation was applied. To determine the best environmental sampling strategy to assess herd infection status, a multivariable logistic regression model was developed using SAS PROC LOGISTIC, where the dependent variable was herd status based on fecal pool culture (positive or negative) and the independent variables were environment culture status by location. To assess the prevalence of infection in the herd based on environmental sampling areas used in the selected logistic regression model, PROC MIXED with the EMPIRICAL option was used to fit the modeling for herd fecal pool prevalence. The dependent variable was percent of positive fecal pools and the independent variables were: maximum colonies per tube (CPT) by environment location sampled (negative, light, moderate and heavy), number of positive samples (0,1,2) from the selected farms areas, herd size (<100, 100-200, >200), and housing type (freestall or tiestall).

RESULTS

Sixty-four (80%) of the 80 JDCP herds had at least one-pool positive; 16 did not have any positive pools. Twenty-six herds of the VJDHSP herds (n=28) were found to be culture negative, with no positive pools; 2 herds had one positive pool each with maximum level of shedding of 10-50 colonies per tube in one herd and >100 CPT in the other.

The environment around the JDCP farms was found to be contaminated for 61 of the 64 herds with positive pools and in one of the 16 herds with negative pools. In the latter herd with 210 milking cows, only partial sampling occurred (100 cows). The environment among the VJDHSP herds was found to be contaminated in one herd (cow alleyways and manure spreader); this herd had one positive fecal pool as well. A highly significant relationship was found between the environment and fecal pool culture status (OR= 636, 95% C.I.= 69-5890, $p<0.0001$).

The most common areas found to be contaminated on the farms were cow alleyways (77% of herds with positive pools or environment) and manure storage areas (68%). The distribution of positive environment samples among the 67 farms with positive environment or pools is presented in Table 1.

Most of the positive environmental samples had moderate and low numbers of CPT (including cow alleyways and manure storage), whereas most of the pooled fecal cultures had high numbers of CPT.

At least one sample from cow alleyway or/and manure storage was positive for MAP in 90% of the herds with positive environment or pools. Heavily contaminated samples (>50 CPT) from cow alleyways and manure storage areas were found only in highly infected herds (>30% positive pools). In highly infected herds (>30% positive pools), all cow alleyways samples were MAP-positive.

To assess herd infection status based on environmental sampling, the best-fitting logistic regression model included the sampling of cow alleyways hend manure storage (-2 LL= 42.72). No other additional variable could be fit between cow alleyways and manure storage because of collinearity (i.e. since most of the environmental samples included these two areas, no other environmental sample could contribute additional information to the model).

Table 1. Distribution of environmental samples among dairy herds with culture positive pool or environment

Area in the farm Cattle housing	# Samples Taken	% Positive	# Farms Tested	% Farms Positive	Distribution of Positive Farms
Cows Alleyways	122	70%	66	77%	86% - Free Stall
					14% - Tie stall
Calving Area	108	17%	59	24%	98% - CA only
					2% - CA+ Sick Pen
Dry cow Area	122	19%	61	25%	53% - Dry lot
					40% - Pasture
					7% - Pen
Sick Pen	38	45%	21	57%	
Preweaned	125	0%	63	0%	
Post Weaned	98	3%	52	4%	
Environment					
Manure Storage	120	55%	60	68%	68% - Lagoon
					15% - Manure Pit
					10% - Manure pile
					7% - Manure spreader
Water Access	18	17%	13	7%	
Water Runoff	8	38%	4	75%	
Soil of Crop Fields	30	0%	15	0	

The best-fit model to predict herd fecal pool prevalence included the maximum CPT from cow alleyways and manure storage area. Because of the presence of collinearity, interaction terms were not estimable. Given the model, herds with negative environment have 2% (CI: 0.3-4%) positive fecal pool prevalence; heavy contamination in both areas result in the highest positive fecal pool prevalence (63%, CI: 53-73%; Table 2).

Table 2. Estimation of fecal pool prevalence (95% CI) based on maximum shedding level of two samples from each two environmental location (n=60).

		Cow alleyways			
	Max. level of shedding	Negative	Light	Moderate	Heavy
Manure storage	Negative	2% (0.3-4)	18% (12-24)	20% (12-28)	36% (27-45)
	Light	12% (7-17)	29% (22-36)	30% (22-39)	46% (38-55)
	Moderate	13% (3-23)	29% (16-42)	31% (21-39)	47% (36-58)
	Heavy	29% (23-35)	45% (36-57)	46% (36-56)	63% (53-73)

DISCUSSION

This is the first large scale study evaluating the distribution of MAP in cattle and the environment surrounding dairy farms using different variables such as types of cattle housing, herd sizes, a variety of locations in the farm environment, and diverse geographical distribution.

The limitations of this study are related to fecal culture sensitivity constraints. In the current study, the limited pool test sensitivity for light shedders (1-10 CPT) may have failed to detect some of the infected JDCP herds. Wells et al. (2002) found that the sensitivity of detection for MAP was greater with a smaller pool size (i.e. 5 vs. 10 samples per pool) and pool sensitivity was 44% in low bacterial shedding samples and 94% in high bacterial shedding samples. In the current study, presumably, if undetected these truly infected herds would have a very low prevalence. Additionally, since in the current study a maximum of 100 cows per herd were sampled, it is possible that infected cows (especially low shedders) were present and were not detected in herds where not all of the cows were sampled. Interestingly however, all herds with negative fecal pools, except one, had test-negative environments as well. Furthermore, the environmental sampling procedure used in the current study, which involved the collection of 3-4 scoops of feces from each location, is a form of a pooling procedure. However, it is possible that environmental samples

containing small numbers of bacteria (<10 CPT) fell below the sensitivity of the culture procedure and thus were considered test-negative (as happened in four farms that were pool-culture positive but all environmental samples were negative). Nevertheless, herds with high pool prevalence were detected with environmental sampling.

The environmental sampling results in this study suggest that the initial assessment of farm infection status does not necessarily require individual cattle serology testing, which despite its limitations (low sensitivity, especially in low shedding/subclinical cows, requires individual handling cattle) has been used in both the JDCP and VJDHSP. The results of the current study suggest that an assessment of herd infection status can be performed by targeted sampling of the cow alleyway and manure storage areas. This approach suggests a convenient and promising alternative strategy for herd screening and Johne's infection status assessment, and could potentially replace the first screening ELISA tests for level one of the VJDHSP or for JDCP herds that were never tested before. Furthermore, the statistical models developed in this study offer a practical way to estimate the herd fecal pool apparent prevalence by using the maximum CPT.

Based on this approach, it is estimated that 30 minutes of sampling and US$100 laboratory fees is able to estimate a herd's infection status with reasonable (approximately 90%) accuracy. Achieving the same goal using ELISA test requires at least $180 (U.S.) laboratory fees in addition to veterinary labor fees. Increasing the number of samples obtained from cow alleyways or manure storage may increase the probability of detecting low prevalence herds still with relatively low costs and time.

Finally, since the study was performed during the summer time and only in Minnesota, and herd sampling and infection status assessments occur all year around across the country, it is important to obtain further information about MAP distribution throughout the year, especially during the winter months and in different parts of United States and worldwide.

CONCLUSION

The association between infected herds and MAP contamination of their environment emphasizes the critical importance of farm management strategies to reduce environmental MAP contamination that occurs through cattle manure.

Targeted common and contaminated areas in the farm environment, specifically cow alleyways and manure storage (especially the lagoon), suggest a promising alternative strategy for herd screening and Johne's infection status assessment and for estimating herd fecal prevalence. This strategy has the potential of saving significant economical resources in terms of cost and time.

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Bovine paratuberculosis in dairy herds: A two-step testing approach combining bulk milk ELISA and bulk milk capture PCR

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ABSTRACT

The estimated herd prevalence of bovine paratuberculosis in Germany is 10 to 15%, resulting in high economic losses for dairy farmers. Further, the possible link between *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and Crohn's disease has not been elucidated to date, and this supports the need for a control and surveillance program. Laboratory diagnosis of paratuberculosis using fecal culture as confirmatory method is labor intensive and time consuming. Extensive area-wide testing with this method would not be possible. An alternative and affordable option for the determination of herd prevalence would be a two-step testing of bulk milk samples. In this approach, the first step would be the determination of antibody titers in the bulk milk with a highly sensitive test (Svanovir®), the second confirmatory step on bulk milk from "non unsuspected", i.e. ELISA-positive herds would be a peptide-based capture PCR. The applicability of this approach is supported by our results indicating a correlation of bulk milk ELISA and MAP detection via peptide-based capture PCR: we predominantly detected MAP-DNA in milk that was categorized by ELISA as "recommended for surveillance" (PP [percent positivity] value 5-15) or "highly prevalent" (PP value >15). Based on these findings, the suggested combination of milk ELISA and molecular biology-based methods seems to be suitable for herd classification. The suggested combination of test systems would facilitate continuous surveillance as well as herd classification. The program provides the opportunity to protect the consumer by excluding severely MAP-contaminated milk from the dairy manufacturing process.

Key words: Bulk milk ELISA, peptide-mediated capture PCR, herd prevalence.

INTRODUCTION

Paratuberculosis, also called Johne's disease, is a severe and incurable chronic enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP; Kreeger 1991). Paratuberculosis is prevalent worldwide (Manning and Collins 2001) and has a considerable economic impact on the livestock industry (Harris and Barletta 2001). The zoonotic potential of MAP is not yet fully elucidated (Romero et al. 2005, Hermon-Taylor and Bull 2002). Control and eradication of paratuberculosis is aggravated because of the chronicity of the disease, the long incubation period with intermittent shedding of the organism, and the late seroconversion of infected animals (Nielsen et al. 2002). Attempts to eradicate the disease in infected herds have not been economically feasible (Jubb and Galvin 2004, Beyerbach et al. 2001). This is most likely due to the sudden onset of high shedding of MAP upon stress by infected animals, its hardiness (Whittington et al. 2004, Hammer et al. 2003), and its ubiquitous presence in the environment (Raizman et al. 2004).

As eradication of the infection seems not economically feasible, an alternative would be a control and classification program aiming at the identification of, on the one hand, non-infected herds and, on the other hand, non-negative herds with a highly sensitive and inexpensive bulk-milk ELISA followed by a highly specific confirmatory test for the detection of the pathogen itself (i.e. identification of herds containing not only infected animals but animals shedding MAP in high numbers). In the next phase, fecal culture or a

highly specific ELISA would identify individual heavy shedders in bulk milk test-positive herds thereby reducing environmental contamination and the infectious pressure on the herd.

In the study presented here we investigated the correlation of a bulk milk ELISA licensed in Germany and a peptide-mediated capture PCR. The goal was to evaluate an economically feasible approach for the surveillance and control of bovine paratuberculosis.

MATERIAL AND METHODS

Samples.
Bulk milk samples were obtained from 533 herds mainly located in eastern and northern Germany. Upon receipt, the samples were transferred to sterile 50 ml conical tubes (Sarstedt, Nümbrecht, Germany) and stored at -70°C. Prior to testing samples were thawed and the cream was removed using a water jet pump.

Testing of bulk milk samples.
Enzyme-linked immunosorbent assay (ELISA) testing of the bulk milk samples was performed using the Svanovir$^®$ Para-TB-Ab test (Svanova Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. This indirect ELISA relies on MAP-derived lipoarabinomannan as the immobilized antigen and was originally validated for serum samples using not fecal culture but lymph node culture as the reference method (Jark et al. 1997). Subsequently the test was validated on individual milk samples (Winterhoff et al. 2002) and licensed for bulk milk sample testing. Interpretation of the ELISA results is based on the percent positivity (PP value) of the positive control serum used.

$$PP\ value = \frac{mean\ OD_{450}\ value\ of\ the\ sample\ x\ 100}{mean\ OD_{450}\ value\ of\ the\ positive\ control\ serum}$$

Herd prevalence for paratuberculosis was classified based on the PP value as follows: PP< 5, "unsuspected" herd; PP 5-15 herd with low prevalence and recommended for surveillance; PP>15 herd with high prevalence. Since the ELISA was to be used as a screening test, the cut-off values were set in order to obtain maximum diagnostic sensitivity.

Culture isolation of MAP
Processed samples were inoculated to Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich., USA) supplemented with 10% of oleic acid-albumin-dextrose-catalase enrichment (OADC; 100 ml contain sodium chloride [145 mM], bovine serum albumin [fraction V; 0.5 g], dextrose [1.1 M], catalase [3 mg], and oleic acid [60 µl]), glycerol (0.2%), and mycobactin J
(2 mg l^{-1}; Synbiotics, Lyon, France). For further use as a positive control in the peptide-mediated capture, MAP colonies were harvested from the Middlebrook 7H10 agar by careful removal from the agar and suspended in phosphate-buffered saline (PBS [pH 7.2]; NaCl [150 mM], KH$_2$PO$_4$ [1.5 mM], Na$_2$HPO$_4$ [9 mM], KCl [2.5 mM]). The suspension was homogenized by vortexing for five minutes with glass beads (30 beads of 3 mm diameter per 5 ml of bacterial suspension in a polypropylene tube), and 1 ml samples of pasteurized milk were contaminated with 10^2 MAP as described previously (Stratmann et al. 2002).

Peptide aMptD-mediated capture PCR
Peptide aMptD (GKNHHHQHHRPQ) was synthesized (Fa. Affina Immuntech, Berlin, Germany) and coupled directly to paramagnetic beads using the carbodiimide method (Fa. Chemicell, Berlin, Germany). Carbodiimides react with the terminal carboxylate groups from the magnetic beads to highly reactive O-acylisurea derivatives which then form a covalent bond with the free amino group of the lysine of peptide aMptD. Peptide-mediated capture PCR and spiking of pasteurized milk was performed as described previously (Stratmann et al. 2004, Stratmann et al. 2002). In contrast to the previously described method the peptide-mediated capture PCR was modified. Bulk milk samples (1 ml) were distributed on a deep well plate (DWP) and 10 µl peptide aMptD-coupled paramagnetic beads were added to each well; the DWP was then incubated overnight at 4°C with light agitation. The paramagnetic beads were sedimented using the MagnaBot$^®$ 96 magnetic separation device (Promega, Madison, WI, USA). After removing the supernatant (milk) the beads were resuspended in 200 µl of 0.1 x TrisHCl-bufferd saline with Tween (TBST; 1 x TBST is 50 mM Tris-HCl [pH 7.5] and 150 mM NaCl containing 0.05% Tween$^®$20) and transferred to a U-bottom

mikrotiter plate. The beads were sedimented again and were washed 10 times with 0.1 x TBST using the Nunc-Immuno® washer (Nunc, Roskilde, Denmark). After removing the washing buffer carefully, the beads were resuspended in 50 µl 0.1 x TE buffer (1x TE buffer is 10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) and boiled in a microwave oven for 15 min at 180 W. After sedimenting the beads again, the supernatant was transferred into a new mikrotiter plate and 5 µl of the supernatant was used as template in a PCR using IS*Mav2*-derived primers with internal amplification control. Agarose gel electrophoresis and PCR were done by standard procedures. Taq polymerase and primers were purchased from Invitrogen (Groningen, The Netherlands).

Table 1: Results obtained testing 533 bulk milk samples with the Svanovir® ELISA and the capture mediated PCR.

ELISA (OD%)	Bulk milk samples	PCR positive	PCR negative	% PCR positive per category	% PCR positive
"unsuspected" (< 5%)	89	1	88	1.1%	0.2%
Recommended for surveillance (5 – 15%)	324	15	309	4.6%	2.8%
Highly prevalent (> 15%)	120	15	105	18%	2.8%
Total	533	31	502		5.8%

RESULTS

Bulk milk samples were collected from 533 herds and simultaneously tested with the Svanovir® ELISA, licensed in Germany for the testing of bulk milk samples, and the peptide-mediated capture PCR. The results are summarized in Table 1. The analytical sensitivity of the capture PCR was 10^2 CFU ml^{-1} and the capturing agent, peptide aMptD, has been shown to be highly specific for the capture of MAP (Stratmann et al. 2005). All samples were classified in ELISA categories by PP value. Eighty-nine samples were ELISA "unsuspected" (PP value <5 OD%); in this category a single sample was PCR positive for MAP. Fifteen of the 324 samples ELISA-classified as "recommended for surveillance" (PP value 5-15 OD%; n=324) were PCR positive. Of the 120 samples with "highly prevalent" results (PP value >15 OD%), 15 samples were PCR-positive. These data were analyzed to calculate the probability of a sample testing positive in the capture-mediated PCR. The probability of a positive PCR result is 4 times higher in herds "recommended for surveillance" by ELISA and 16 times higher in "highly prevalent" herds when compared to ELISA-negative herds.
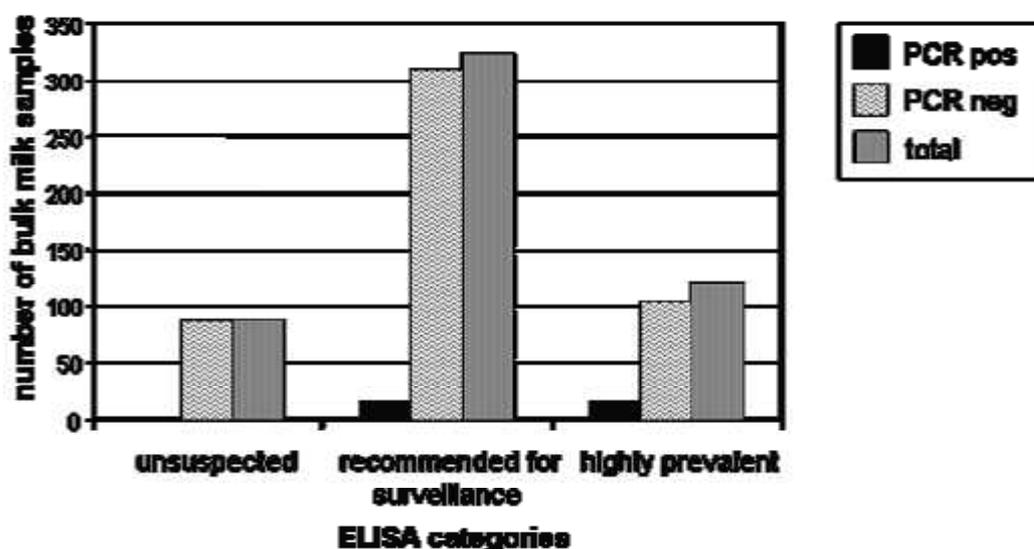
Fig. 1: Bulk milk ELISA interpretation vs. peptide-mediated capture PCR results (n = 533)

DISCUSSION

This study investigated the practicability of a two-step testing approach for a paratuberculosis control and surveillance program using a bulk milk ELISA licensed in Germany and a bulk milk peptide-mediated capture PCR.

The study was performed with randomly taken samples from different regions in Germany representing a cross section of different herd sizes (ranging from 20 to 2000 cows) and herd management practices. Therefore representative data was obtained even though only a limited number of samples (533) could be tested. The bulk milk ELISA used was evaluated not against fecal shedding, but lymph node culture as a "gold standard" (Jark et al. 1997). Subsequently it was validated for individual milk samples (Winterhoff et al. 2002) and was later licensed for use on bulk milk samples. Since the test was to be used as a screening ELISA the cut-off values were set in order to obtain maximum diagnostic sensitivity values. Further, as on the single animal level this ELISA does not correlate with shedding but with infection, herds with negative results should have a high probability of freedom from infection and therefore should not require the more expensive PCR testing. These herds could serve as a relatively reliable source for uninfected herd replacements.

Bulk milk from a large proportion of herds (80%) were ELISA-positive and therefore they were likely to have been exposed to MAP. Since testing of all these herds on a single animal level was not feasible, a confirmatory test identifying herds with high shedders was established (Stratmann et al. 2005). Testing of the 533 bulk milk samples using the peptide-mediated capture PCR resulted in 15 PCR-positive samples in both of the ELISA categories "recommended for surveillance" and "highly prevalent" and only one PCR positive sample in the ELISA category "unsuspected". These data show that the probability of a PCR positive sample is 4 times higher in the ELISA category "recommended for surveillance" and 16 times higher in the category "highly prevalent" compared to the ELISA category "unsuspected". Thereby a clear correlation between the ELISA OD% and the outcome of the peptide-mediated PCR was shown, supporting the feasibility of this two-step testing approach.

Based on these results a more defined scheme for a possible control and suveillance program was devised (Fig. 2). The first step is to find suspect herds using the highly sensitive and inexpensive bulk milk ELISA. This is followed by the highly specific peptide-mediated capture PCR in these ELISA-positive (i.e. likely infected) herds thereby identifying herds with heavy shedding into milk. Therefore, the suggested combination of ELISA and PCR would allow the identification of herds not exposed to the organism (i.e. unsuspected); these herds would be a relatively reliable source for restocking. Among the herds at risk, those with high shedding can be detected with the second step of the testing regime using the peptide-mediated capture PCR. Only if MAP is detected in the bulk milk using the PCR does individual animal testing occur to identify and eliminate shedders in the herd to reduce environmental contamination and the risk of new infections in the herd. This individual animal, vs. bulk mik, testing could be done by using a highly specific ELISA or fecal culture. This would, on the long run, lead to a reduction of paratuberculosis prevalence.

Unsuspected	Recommended for surveillance	Highly prevalent
One year negative and ELISA unsuspected or 4 x ELISA unsuspected	One year negative and ELISA recommended for surveillance or 2 x ELISA recommended for surveillance and PCR neg.	ELISA highly prevalent

Suggested procedure depending on the herd status

1 x per year ELISA	2 x per year PCR	4 x per year PCR
	Testing on single animal level if PCR pos	

Fig. 2: Diagram presenting the suggested application of the two-step testing approach combining bulk milk ELISA and peptide-mediated capture PCR.

CONCLUSION

We investigated a practical, two-step testing approach for a paratuberculosis control and surveillance program using a bulk milk ELISA licensed in Germany and a bulk-milk peptide-mediated capture PCR. A combination of these two independent test systems may present a powerful tool aimed at identifying non-infected herds and infected herds containing heavy shedders.

ACKNOWLEDGEMENTS

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The effect of biosecurity measures for paratuberculosis on the seroprevalence in Dutch dairy herds

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ABSTRACT

In the Netherlands, a feasible control programme is desired for paratuberculosis. The basis for control is biosecurity. The purpose of this study was to determine the effect of biosecurity on the serostatus of dairy herds.

Blood samples were taken over a 3-year interval from cows originating from 1,023 dairy herds and tested for antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The herds participated in a voluntary control programme in which their management was assessed on a yearly basis during the study period. Eight factors related to calf-management and purchase of cattle were tested in two logistic-regression models, one for the serostatus at the start of the study and one for the serostatus after 3 years as the dependent variables. Backward elimination was used to exclude non-significant management factors from saturated models.

Larger herds were more likely to be infected than smaller herds. No other management factors were found to be related to the serostatus of the herds at the start and at the end of the study. The serostatus at the end of the study was strongly related to the serostatus at the start. Only purchase of cattle during the study from herds with an unknown status of paratuberculosis was associated with a positive serostatus at the end of the study.

A three year study period may have been too short to evaluate the results of calf rearing management during the study on the serostatus of a herd. Purchase of cattle is a risk factor for introducing MAP in a herd. The on-going increase in herd size in the Netherlands can lead to an increase in herd level seroprevalence.

Key words: *Mycobacterium avium* subsp. *Paratuberculosis*, Paratuberculosis, Cattle, Dairy, ELISA, Longitudinal study, Biosecurity, Transition model.

INTRODUCTION

In the Netherlands, a feasible control programme is desired for paratuberculosis. The main reasons are the economic damage in infected herds and the ongoing discussion about the zoonotic implications of paratuberculosis.

In the previous century, several control approaches were attempted in the Netherlands (Benedictus and Kalis, 2003). These approaches indicated that preventive management that limits the transmission of the causative bacteria*, Mycobacterium avium* subsp. *paratuberculosisis* (MAP), is essential for the control of paratuberculosis. Control, based on test-and-cull programmes only, was not successful because the sensitivity of the available diagnostic assays is too low to detect all infected animals before they become infectious (Whitlock et al. 2000, Groenendaal et al. 2003)

High shedders of MAP and cattle with clinical signs of paratuberculosis are responsible for the greater part of the contamination of their environment, the economic damage in infected herds and the presence of bacteria in milk (Grant 2003, Raizman et al. 2004). To monitor the progression of a control programme the herds need to be tested. The most practical method for this is serology. The enzyme-linked immunosorbent

assay (ELISA) is a suitable diagnostic tool to detect serum antibodies against MAP on a large scale, because it is possible to test large numbers of samples with this assay with a high reproducibility (Collins 1996, van Maanen et al. 2002). In general, commercially available ELISA-kits for paratuberculosis have a low sensitivity (Whitlock et al. 2000, Stabel et al. 2002, Collins et al. 2005) But for the detection of high shedders and animals in a pre-clinical stage the assays have a reasonable good sensitivity. (Sweeney et al.1995, Whitlock et al. 2000, Schaik et al. 2005, Collins et al. 2005). An ELISA can identify heavy shedders for culling to reduce the pressure of infection in infected herds and thus enhance the efficacy of the preventive measures (Kennedy and Benedictus 2001, Benedictus and Kalis, 2003).

To our knowledge no longitudinal studies have been performed that used ELISA testing on a regular basis to monitor the efficacy of preventive management for paratuberculosis. The objective of the study was to evaluate the results of testing of a large number of dairy herds in a 3 year period in relation to management procedures applied in those herds. These screening tests were carried out in a large field trial as part of the Dutch paratuberculosis programme to investigate if and to what extent dairy farmers were prepared to take preventive and biosecurity measures to reduce the introduction and spread of MAP within their herds.

MATERIALS AND METHODS

Herds
The large field trial ran for three years and consisted of 1,083 Dutch dairy herds. It is briefly described as the "extensive programme" by Benedictus and Kalis (2003). The herds in the study were selected by veterinarians from their clientele based on the following criteria:
- Interest of the farmer for the prevention of the spread of paratuberculosis by biosecurity measures;
- A herd with   20 animals of   2 years old;
- No indication that the farmer would go out of business within the near future;
- Willingness to carry out two whole herd blood tests (at the start and the end of the project);
- Willingness to fill in four questionnaires with their veterinarian about their management with one year interval.
- Willingness to participate in study groups with 8 to 10 other dairy farmers to review results of blood tests and questionnaires under guidance of a practitioner and an employee of the Animal Health Service.

Questionnaire
The questionnaire consisted of 41 questions dealing with management practices in the previous 12-months. Only "yes or no" answers were possible to indicate if the particular procedure was present or absent. In the analysis nine management factors were taken into account because they were expected to be most influential on the transmission within a herd. The following factors were considered:
1. The presence of a separate calving area;
2. Cleaning of the calving area after each birth;
3. Removal of the calves from the dam immediately after birth and housing the calves separately from each other and from their dam;
4. Feeding the calves colostrum only from their own dam;
5. Feeding only milk replacer after the colostrum period;
6. A separate water supply for the calves to avoid contamination with faeces of the older animals;
7. Housing of calves   3 months old separately from the animals   2 years old;
8. Pasturing of calves   3 months old;
9. Purchase of animals from other herds in the previous 12 months.

Only management information for the 12 months prior to the start of the project were included in the analysis with one exception: the purchase of animals during the study period was also considered.

Testing protocol
The veterinarians of the participating farmers collected the blood samples from all cattle   3 years of age. The samples were sent to the Animal Health Service for analysis on antibodies against MAP. This was

done twice, the first time (t1) at the start and the second time three years later at the end of the study period (t2).

All blood samples were analysed by a commercial indirect ELISA using the instructions of the manufacturer (ELISA Bovine Paratuberculosis Serum Verification, Pourquier Institute, France). The specificity of this test was increased by pre-absorption with *Mycobacterium phlei* antigen (Couquet et al., 1999). Wash steps were completed with an automated washer. Plates were read with an automated plate reader linked to a computer. On each 96-well plate, 88 serum samples were tested in single wells. In addition to the negative and positive control samples provided by the manufacturer two internal control serum samples were run on each plate. The cut-off as defined by the manufacturer was Sample to Positive ratio greater than or equal to 110% counting half of the samples with an inconclusive result S/P ratio between 90 and 110% as positive). With this cut-off previous data provided an overall specificity of 99.8% (CI95%: 99.6 – 100.0) and an overall sensitivity of 40.8% (CI95%: 35.3 - 46.7) (van Maanen et al., 2002).

In each sampling, a herd was test-positive when there was at least one positive or inconclusive ELISA-result; all results had to be below the cut-off for a herd to be considered test-negative.

Statistical analysis

All management factors were tested in two logistic-regression models (one each for the serostatus at the start of the study and the serostatus after three years in the study as the dependent variables). Backward elimination was used to exclude non-significant (P>0.05) management factors from the saturated models. The following scheme was used:

1. The serostatus t1 (first herd test at the start of the study) in relation to management procedures in the 12-month period before t1.
2. The serostatus at t2 (second herd test three years later at the end of the study) in relation to management procedures in the 12-month period before t1 without taking the serostatus at t1 in account
3. The serostatus at t2 given the serostatus at t1 and the management of the herd in the 12-month period before t1.

The transition model includes both incidence and prevalence at the same time and is described in more detail by Schukken et al. (2004).

In this study, the logistic transition model was used to determine factors associated with both new and remaining infections with MAP. In the transition model the infection status at the start of the intervention period was included. The intercept is the baseline risk of a new infection and the sum of the intercept and the estimate for the previous serostatus is the baseline risk of remaining infected. Eight internal management factors and purchase of cattle were tested for significance (p 0.05) with the backward elimination procedure from the saturated model.

All analyses were carried out in SAS for Windows version 9.1 (SAS Inc, 2002). The independent management variables were categorised into binary outcomes and "present" was compared with "absent". Measures of central tendency and dispersion were used to investigate continuous variables and frequency distributions to investigate categorical variables. The associations between herd size and the binary variables were determined with a T-test.

The fit of the models was based on the scaled deviation (that should be close to 1) and the predictive value of the model. Results were checked by plotting the standardised predicted values against the standardised residuals. Outliers with large residuals (<-2 and >2) were excluded from the model to check the robustness of the models. Collinearity between the covariates was checked by T-tests or 2-by-2 tables. A significant difference was assumed when the two-side P<0.05 unless mentioned otherwise.

RESULTS

The serostatus of 1,023 herds in this study was determined at the start (t1) and 3 years later at the end (t2) of the study. The frequency distributions of these herds in relation to the number of seropositive samples are presented in Table 1.

There were no significant differences in the frequency distributions after three years. About 40% of the herds was seropositive on both occasions. Half of the seropositive herds had only one seropositive sample, a quarter two seropositive samples and the remaining quarter three or more seropositive samples.

Table 1. The sero-positive frequency distributions within herds at t1 and t2.

Number of sero-positives per herd	Percentage of herds (t1)	Percentage of herds (t2)
0	60.2	61.7
1	19.6	15.7
2	7.0	8.8
3	3.2	4.4
4	2.4	2.8
5	1.2	2.0
6	1.6	1.3
7	4.7	3.3
Total test-positive	39.8	38.3
Total	100.0	100.0

Of the 616 seronegative herds at the start of the study 135 (22%) had one or more seropositive cattle three years later at the end of the study. Of the 407 herds with one or more seropositive samples at the start of the study 150 (37%) were seronegative 3 years later.

The mean number of cattle 3 years increased during the study from 59 (SD = 26) cows at the start to 63 (SD = 30) at the end of the study. The mean herd size of the participating herds in this study was larger than the mean herd size of the average Dutch dairy herd with 48 cattle 3 years in the same period. In this study larger herds were more likely to be seropositive than smaller herds as demonstrated in Fig. 1.
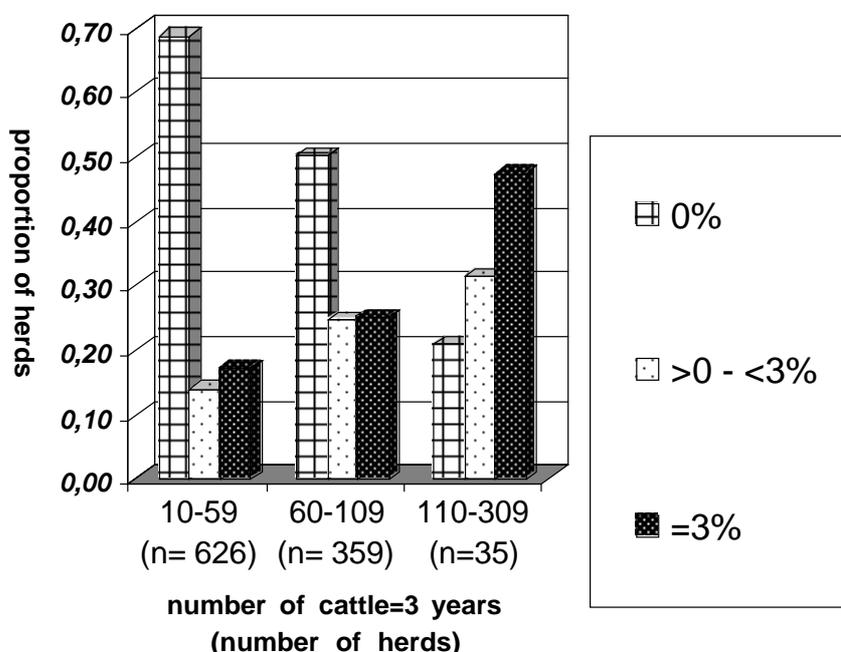
Fig. 1. Herd seropositive rate vs. herd size.

The proportion of seropositive herds increased from 0.31 for herds with 10-59 animals 3 years to 0.79 for herds with 110-309 animals 3 years.

Data from the first questionnaire administered at the start of this study were compared with the initial serostatus. The distribution of eight management variables is presented in Table 2 for all participating farms, both seropostive and seronegative.

Table 2. Presence of management factors for all herds in the first year of the study.

Variables	All herds (n=1,113) %	% Sero-positive (n=448)	% Sero-negative (n=665)
A separate calving area was available	74	78	72
The calving area was cleaned after each birth	16	15	17
The calves were separated from the dam immediately after birth and housed separately from each other or from their dam	44	42	44
The calves were fed only colostrum from their own dam	84	84	84
The calves were fed only milk replacer after the colostrum period	50	58*	46*
The water supply for the calves was completely separated to avoid contamination with faeces of the older animals	97	96	98
The calves 3 months old were housed separately from the animals 2 years old	84	83	84
The calves 3 months stayed indoors	95	95	95

*significant difference at P 0.05

With the exception of the variable "The calves were fed only milk replacer after the colostrum period", there were no significant differences in the presence of the management factors amongst the seropositive and seronegative herds.

Purchase of cattle from herds with an unknown paratuberculosis status occurred in about 24% of the herds in the 12 months prior to the start of the study and decreased to 12 -14% in the first year of the three-years intervention period. The proportion of herds that purchased cattle in the intervention period is presented in Table 3.

Table 3. Percentages of herds purchasing cattle of unknown infection status in the study period.

Purchase of cattle of unknown status:	N	% All Herds	% Sero-positive	% Sero-negative
The year prior to the study period compared with serostatus at t1	1,113	24	26	22
The year prior to the study period compared with serostatus at t2	1,038	23	27	21
1st year of the study compared with sero-prevalence at t2	1,023	12	18*	9*
2nd year of the study compared with sero-prevalence at t2	907	13	16	11
3rd year of the study compared with sero-prevalence at t2	1,008	14	16	12

*Significant difference at P 0.05

The percentage of seropositive herds that purchased cattle with an unknown status for paratuberculosis was higher than in seronegative herds for the whole study period.
The association between all management factors and the serostatus of the herds at the start of the study period with a logistic regression model are presented in Table 4.

Table 4. Logistic regression model for the herd serostatus at the start of the study.

Parameter	Estimate	SE	P	OR
Intercept	-2.22	0.22	<.01	
Herd size at t1	0.03	0.00	<.01	1.03
Calves are fed with milk replacer only	0.43	0.14	<.01	1.54

The odds of being infected increased by 1.03 for every extra cow in the herd. Herds that fed milk replacer to their calves were 1.5 times more likely to be infected. No other management measures or interactions were found to be significant.

Herd size was significantly associated with the herd serostatus as well as several other management practises. Larger herds more often had a separate calving area, more often fed milk replacer to the calves, more often separated the calves 3 months and less often gave colostrum of the dam to her own calf. However, in a model without herd sizes these variables were not significantly associated with the serostatus.

Table 5: Results of the logistic transition model for the herd serostatus at the end of the study

Parameter	Estimate	SE	P	OR
Intercept	-0.72	0.25	<0.01	
Negative sero-status at t1	-1.61	0.16	<0.01	0.20
Purchase of cattle n 1st year of the study	0.68	0.23	<0.01	1.98
Herd size at t2	0.02	0.00	<0.01	1.02

The logistic regression model for the serostatus at the end of the study without inclusion of the serostatus at the start resulted in the same risk factors (herd size, feeding milk replacer and purchase) and estimates as in the other models (results not shown).

Finally, the association between all management factors and the serostatus of the herds at the end of the study period given the serostatus at the start with a logistic transition model are presented in Table 5.

A herd's serostatus at the start of the study was strongly related with the serostatus after three years: seropositive herds at the start were five times more likely to be seropositive after three years than herds that were seronegative when entering the programme. Purchase of cattle from herds with an unknown status for paratuberculosis in the first year of the study was associated with a positive serostatus at the end of the study. However, purchase in the 2nd or 3rd year did not increase the odds any further. Herds that purchased in the first year of the study were twice as likely to be seropositive. Larger herds were also more likely to be infected. All two-way interactions were added to the model but none were significant, and no risk factors for remaining infected could be determined.

DISCUSSION

Results of this study demonstrated the dynamics of ELISA results at the herd level for dairy herds participating in a three year intervention programme for paratuberculosis. The herd level prevalence did not alter significantly over the period of the programme. The percentage of herds moving from seronegative to seropositive was 22% (135/616); the percentage moving from seropositive to seronegative was 37% (150/407).

Intervention measures are supposed to have their main effect on the chance that calves < 6 months become infected (Sweeney 1996). In this study, samples of cattle 3 years were used to determine the herd-status because in general infected cattle below the age of 3 years do not have a detectable level of antibodies in their blood (van Maanen not published data, Huda et al. 2004). This means that the effect of intervention measures cannot be measured with an ELISA until after more than 3 years. At that time, more of the herd is comprised of animals that grew up under conditions of preventive management. Therefore, only the relationship between the herd serostatus and biosecurity practices prior to the study was analysed in this study, not the relationship between the herd serostatus at the end of the study and preventive measures implemented during the intervention period. However, buying in cattle from other herds during the study period was included in the analysis, because purchase of infected animals that are seropositive at the moment of herd testing can have a direct effect on the herd serostatus. In this study, only purchase in the first year of the intervention period was significantly associated with a seropositive herd status. The reason for this may be that purchased cattle usually are pregnant heifers. The heifers purchased in the first year may have become seropositive at the last herd sample while heifers purchased later were not yet seropositive after two years.

In this study, herd size was strongly associated with a seropositive herd status. The odds of being seropositive increased by 1.02 -1.03 for each cow in the herd. This is in accordance with other studies that have investigated the relationship between herd status for paratuberculosis and management practices (Wells et al. 2000, Muskens et al. 2003). In this study the association between herd size and serostatus may be confounded by sample size, as more samples were collected from large herds than small herds. However, the specificity of the ELISA used in this study was estimated to be at least 99.8% (van Maanen et al. 2002) and even 100% in a evaluation study of five ELISA kits of Collins and colleagues (2005). Therefore, the contribution of sample size in regard to relatively small herd sizes of Dutch dairy herds is limited. In addition, certain management practices were correlated with herd size. Thus a protective measure such as "feeding only milk replacer to calves after the colostrum period", a more common practise in large herds, was significantly associated with a seropositive status. For this variable herd size may act as a strong confounder of the data. Another possibility is that farmers of infected herds familiar with the disease have changed management in an effort to control the infection. In the years prior to the field trial dairy farmers had been repeatedly told about the risks for new MAP infections caused by feeding pooled colostrum and raw milk.

Other management practices in this study, with the exception of purchase of cattle, were not significantly associated with the herd serostatus even when herd size was excluded from the model. Other studies also found no or a limited association between single management practices and the serostatus for paratuberculosis. (Wells et al. 2000, Muskens et al. 2003, Collins et al. 2005). Apparently, the interaction among the presence of the infection, environmental circumstances and management practices that determine animal contact is complicated. The impact of each management practicef alone is not. It appears that a combination of management practises plus the herd structure in larger herds affect the probability to be infected with MAP.

CONCLUSIONS

Purchase of cattle from herds with an unknown status for paratuberculosis is a risk factor for introducing the infection and acquiring a seropositive status. The serostatus of the majority of the dairy herds remained the same after three years of a Johne's disease control programme. It is likely that a longer interval is needed to investigate the influence of preventive measures on the herd serostatus for paratuberculosis and a whole-herd ELISA test needs to be repeated in 1-2 years time.

ACKNOWLEDGEMENTS

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Control strategies in a caprine herd with mixed tuberculosis-paratuberculosis infection

J Álvarez, L de Juan, J Bezos, A Aranaz, B Romero, P Díez de Tejada, J M Fernández, A Mateos, L Domínguez

Abstract

Paratuberculosis in goats causes decrease in milk production, loss of weight and increased culling of infected animals. Caprine paratuberculosis and tuberculosis are endemic important infectious diseases in Spain. Mixed infection with these two mycobacterial diseases is present in cattle and goat. In this work we describe the control strategies implemented for the last three years in a goat farm with a dual infection to obtain a free flock. In 2001 both tuberculosis and paratuberculosis were detected in a goat farm in Madrid. In 2002 segregation of neonates immediately after birth (to avoid direct contact with the does and vice versa) was implemented, feeding the kids with artificial colostrum and milk. All these kids were placed in a different area separated from adults, and strict control measures were introduced to avoid contamination between the two groups. Periodically a blood extraction was performed in both groups for detection of bovine γ-IFN (tuberculosis diagnosis) and antibodies against Map (paratuberculosis diagnosis). Since the study started around 300 hundred animals have been culled to confirm the bacteriological status based on serological results. Samples have been taken from these animals and cultured for the detection of *Mycobacterium bovis* and Map. When this work began, 78.1% of the animals of the group not subjected to control measures were positive to either tuberculosis, paratuberculosis or both. After three years, the controlled group is believed to be free of tuberculosis, and only 9 out of 203 (5.4%) of the animals were seropositive to paratuberculosis. The study is still ongoing to clarify possible ways of transmission for this small percentage, such as vertical transmission. However, control measures implemented have demonstrated their usefulness so far. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903.*

The impact of Johne's disease on performance in an Irish dairy herd

Damien Barrett, Margaret Good, M Hayes, S J More

Abstract

A case study of the economic impact of Johne's disease in an Irish dairy herd is described. An epidemiological investigation concluded that the purchase of 20 heifers from the Netherlands in 1993 introduced Johne's disease to the herd. The practice of feeding pooled colostrum/milk was considered to have disseminated *Mycobacterium avium* subspecies *paratuberculosis* (MAP) widely within the herd. Farm performance between 1993 and 2003 declined substantially, as a result of reduced milk yields, increased culling and reduced cull cow values. This in turn reduced the profit margin per litre milk sold and per cow. The performance relative to peers also deteriorated over the study period, where the farm performance was superior to that of peers until the late 1990's, but was markedly worse than that of peers by 2002.

Variation in milk yield associated with the cow-status to Mycobacterium avium subspecies paratuberculosis (Map) infection in French dairy herds

F Beaudeau, M Belliard, A Joly, H Seegers

Abstract

This study aimed at quantifying the variation in test-day milk yield (TDMY) of dairy cows according to Map-infection status of cows. The cow-status was determined combining (i) her testing(s)-result(s) (ELISA, faecal culture (FC), PCR, Ziehl staining), (ii) the Map-status of her herd, and (iii) her possible vaccination against Map. When combining this information, a total of 14 cow-statuses were defined. A total of 23,219 cows in 569 herds located in Bretagne was considered. The effect on TDMY of the cow-status to Map, adjusted for herd-year (random), lactation number, days in milk and breed was assessed using mixed linear models. The average TDMY was significantly lower in cows from herds with at least one Map-infected cow (defined as positive herds). Individual TDMY showed a reduction of 1.6, 2.5, 2.1 and 6.2 kg/day (P<0.001) for cows tested negative in a positive herd, not vaccinated and ELISA-positive, PCR- or FC-positive, and Ziehl-positive, respectively, in comparison with cows in Map-negative herds. ELISA-tested positive but vaccinated cows had a smaller loss in TDMY than those not vaccinated, suggesting the coexistence of infected and not-infected animals among ELISA-positive cows. These estimates will be used to further assess the economic impact associated with Map-infection in dairy herds or to enlighten the culling decision making process regarding infected cows.

Risk factors for Mycobacterium avium subspecies paratuberculosis infection in dairy herds located in Brittany (Western France)

F Beaudeau, D Ledoux, N Poumerol, A Joly, H Seegers

Abstract

The aim of this study was to identify herd-level factors associated with the presence of cows infected by *Mycobacterium avium* subspecies *paratuberculosis* (Map) in dairy herds. Farm-management data were collected in 192 dairy herds located in western France according to a specific questionnaire administered during a farm visit from April to July 2003. Herd-status to Map-infection was defined depending on the presence (Map-positive) or the absence (Map-negative) in the herd of at least one positive-tested (ELISA, faecal culture (FC), PCR or Ziehl staining) lactating cow, aged more than 24 months. Logistic regression was used to quantify the relationships between herd-status to Map and farm-management factors. Factors associated with an increased risk for a herd of being Map-positive were cattle purchase (RR=1.60), late separation of the calf from its dam with possible suckling (RR=1.30), systematic distribution to calves of their dams' colostrum (RR=1.44), non hygienic milk supply (one bucket for several calves and/or cleaned less than once per day) (RR=1.24), long lasting bulk milk supply to the calves (RR=1.40), possible long-lasting contact of calves (RR=1.33) and heifers (RR=1.20) with adult cows, and absence of cleaning and disinfection procedure in the cows shed (RR=1.24). These results, which are in agreement with previous findings, suggest that main measures to prevent Map-infection must rely on calf care and on purchase of presumably non-infected cattle.

Modelling the on-farm financial impact of ovine Johne's disease in Australia

R D Bush, Peter Windsor, J - Toribio, S R Webster

Abstract

A gross margin (GM) model is being developed to predict the on-farm financial impact of ovine Johne's disease (OJD) for a range of wool and sheep-meat enterprises and disease scenarios within Australia. Individual GM budgets will be developed for Merino fine (19mm), medium (21mm) and strong (23mm) wool enterprises as well as for 1st and 2nd cross lamb production. Disease scenarios include non-infected, infected (status quo) and infected (vaccination). The infected scenario reflects low (<3%), medium (3 to < 7%) and high (7% and greater) OJD mortalities. The status quo scenario assumes no control and the vaccination scenario simulates a reduction in OJD mortalities, based on Australian mortality and vaccine research. Sheep numbers are modified for each enterprise to reflect mortality throughout the year. Income accounts for sales of wool, sheep and fodder while variable costs include sheep health, wool and stock selling, supplementary feed, pasture, weeds and pests and the cost of replacement sheep. Commodity prices are based on five-year median prices obtained from the NSW Dept. of Primary Industries. A discount value is applied to account for debt levels and capital effects. The model will estimate changes in profits due to increased mortalities, as a result of OJD, and the cost of control through the use of Gudair™. Gross margins are reported as total, per ewe or wether, per dry sheep equivalent (DSE) and per hectare (ha). The cost of OJD is reported as reduced wool income and sheep sales whereas the benefits of control are reported as increased wool income and sheep sales. The output will be presented as net present value (NPV) at 5, 10, 15 and 20-year intervals. This paper will present the model and its outputs, an important tool for determining the financial impact of OJD on individual farms and developing cost effective strategies for disease control and management.

Surveillance of epidemiological factors associated with the potential spread of Mycobacterium avium subsp. paratuberculosis (Map) on selected dairy holdings in Ireland

W J Cashman, J F Buckley, S Fanning, Michael T. Rowe, K J O' Farrell KJ

Abstract

A subset of 57 liquid milk production holdings representing 3800 cows (9700 total cattle) were surveyed as part of an emerging pathogen prevalence study based on milk filter residue (MFR) analysis. 10% of MFR samples from clinically normal herds demonstrated Map DNA positive signals on IMS-PCR analysis. Epidemiological factors relevant to the development of a Map control programme were assessed. 49% of farmers clipped and cleaned cows' udders pre-calving. 61% of calvings were supervised. Post calving, 14% removed calves immediately, 16% allowed suckling >24 hours and 9% allowed communal suckling. 81% of farms fed an average of 4.6L of dam's colostrum in the first 24 hrs, and 88% fed 8.3L between 24 and 72 hrs. 25% of farms (14/57) used pooled colostrum in the first 24 hours and 88% fed pooled milk, while 51% of farms feed pooled milk mixed with colostrum. 25% of farms used milk replacer. 35% wash udders pre-milking all year, 35% pre-milking wash only during winter. 84% clip cows udders routinely. 57% spread slurry on calf pasture, and 53% allow calves graze adult grazed pasture. 9% of farmers purchase cattle from multiple farms, 14% use agents or marts and 8% had purchased imported animals. In 2003, 19% of farms had progeny of imported animals on their farm, and 28% had boundary contact with neighbouring cattle. 32% of farms had common water sources. Rabbit faeces and water gave PCR positive Map signals on 41% and 33% of farms respectively. These findings disclose a serious challenge in terms of controlling the threat posed by Map imported into Irish dairy farms since 1994.

Stop perinatal transmission of M. a. paratuberculosis to control paratuberculosis

L de Juan, J Álvarez, A Aranaz, B Romero, J Bezos, N Montero, A Santos, A Mateos, L Domínguez

Abstract

The objective of this study was to determine the effect of perinatal transmission in the control and eradication programs of paratuberculosis. This study was performed in a Spanish flock of Murciano-Granadina breed in Toledo, where paratuberculosis was first microbiologically diagnosed in 1999. A control program was established in the farm, with the main objective of interrupting the biological cycle of *M. a. paratuberculosis*, especially during the perinatal period. The program is based on two main aspects: 1) implementation of control and management guidelines, segregation of neonates immediately after birth and artificial feeding system; and 2) seroprevalence study of paratuberculosis. Based on the results a physical segregation of the positive and negative animals is carried out. During a three year period, paratuberculosis seroprevalence in the farm decreased from 28.7% to 13%. A genealogic study was carried out to determine the influence of mother's status in the transmission of the disease. Serological results of 72 mothers not included in the control program were compared with the results of their offspring (129 and 27 included or not in the control program). The results obtained revealed the importance of immediate segregation of neonates after birth, because the percentage of positive animals from positive mothers was higher (72.7% vs. 26.4%) in kids not included in the control program than the ones segregated from their mothers. This higher percentage reveals the importance of perinatal transmission since kids can be infected from several routes (vertical, faecal-oral, environment, etc.). Due to the interesting results obtained in this study, we recommend the application of this type of control program in caprine flocks to decrease the rate of contamination in the flock. ***Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903.***

Decision analysis model for paratuberculosis control in commercial dairy herds

N C Dorshorst, Michael T. Collins

Abstract

Despite improved diagnostic tests, extensive education programs, demonstration of the infection's significant costs, and continued investigation of *M. paratuberculosis*' potential as a food-borne zoonotic agent, paratuberculosis control programs are not being widely adopted by dairy producers. Possible reasons include a failure of experts to justify the economics of control programs, to appreciate how costly control programs are to producers, and an inflexibility in program design that does not incorporate differing dairy size, infection prevalence, productivity, or milk prices. A previous economic test-and-cull decision analysis model was strengthened and updated with current epidemiologic information. Created using Excel® and PrecisionTree® software, the model incorporates costs and benefits of herd management changes, diagnostic testing, and different management actions based on test results to control paratuberculosis in commercial dairy herds. This novel "JD-tree" model includes a herd management decision node (4 options), a test/no test decision node (2 options), a diagnostic test choice decision node (5 options), test result chance nodes (4 levels of possible results), and test action decision nodes (3 options; cull, manage, no action). The model culminates in a chance node for true infection status. Outcomes are measured as a net cost-benefit value to the producer. The model herds demonstrate that herd management to control infection spread (hygiene) is often more economical than testing; not all herds should test as part of a paratuberculosis control program. For many herds, low-cost tests are more useful than higher cost, more sensitive tests. The model also indicates that test-positive cows in early stages of infection may be retained in the herd to generate farm income, provided they are managed properly to limit infection transmission. JD-tree is a useful instructional tool for veterinarians wanting to understand the complex interactions affecting the economics of paratuberculosis control and for defining the specifications of better diagnostic tests.

Whole herd fecal pools to assess herd MAP bio-burden compared to composite Environmental Samples

B A Fyock, Jerry W. Smith, E Hoving, Ynte H Schukken, Robert H. Whitlock

Abstract

Assessment of the herd MAP bio-burden is a relatively new method of monitoring effectiveness of herd management practices, especially culling of the most infectious MAP positive cattle on the farm. Two primary methods are deemed appropriate; a) culture of environmental manure samples from high cow traffic areas such as walk ways, feeding areas and milking parlor entrance; b) culture of 1 to 5 pools of manure samples from all adult cows. Objective: To compare the results of composite environmental manure samples from high cow traffic areas to composite manure sample of all adult cows in the herd.Materials & methods: Individual fecal samples from several known MAP infected herds have been cultured and pools of 1:5, 1:10, 1:50, 1:100 and then the entire herd have been cultured for MAP. These larger pool sizes have been compared to composite environmental manure samples. Typically 4-6 manure samples from high cow traffic areas are mixed together for one composite sample. Then, the 6 to 8 composite samples environmental sample culture results are compared to the entire herd composite in addition to individual cows MAP cfu. Results: To date composite herd fecal pool MAP cfu correlates well with MAP cfu from environmental fecal samples. Removal of super-shedders reduces both the herd composite MAP cfu and environmental MAP cfu. One herd had three composite environmental samples with 100 cfu/tube with a herd culture prevalence of 3.2% (4 pos/125). One super-shedder cow contributed to this massive environmental contamination. Conclusion: Pools with all adult cattle in a herd offers quantitative assessment of MAP cfu from the entire adult herd that is not available when culturing individual cows. Composite environmental fecal samples offer a second method to verify the extent and to quantify the environmental MAP cfu bio-burden. *This project was supported in-part by the USDA-ARS-RDQMA project.*

Inactivation of Mycobacterium avium subsp. paratuberculosis (MAP) in bovine slurry

Orla Flynn, John Egan, Donald O'Grady, Margaret Good

Abstract

In the Republic of Ireland slurry from herds infected with notifiable diseases, including tuberculosis and brucellosis, must be treated with Calcium Hydroxide suspension -20kg $Ca(OH)_2$/m3 before spreading on pasture to minimise the risk of spreading disease. As the incidence of Johne's Disease is increasing it was desirable to see if a similar treatment was effective for inactivating *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in slurry. Method: 300 ml quantities of sterile slurry (autoclaved at 130°C for 60 mins) were spiked with a suspension of MAP type strain NCTC 8578 to give a concentration 1×10^{-9}cfu /ml. and incubated at 19°C with shaking. Liquid Calcium Hydroxide was then added to the containers to achieve a pH of 12.7, and samples were subsequently taken from the treated containers and an untreated control at 6, 10, 24, 48 and 72 hrs, and 7 and 14 days post treatment and cultured. To prepare inoculum for culture 5ml of sample was suspended in 20ml of sterile phosphate buffered saline (PBS) and centrifuged briefly at 1000rpm for 60secs to precipitate large particles. The resulting supernatant was then centrifuged at 3000pm for 30mins to pellet the organisms, the pellet was washed 3 times with PBS, and finally resuspended in 2ml of PBS. 100µl of suspension was inoculated into Bactec B12B medium and HEY slants, incubated at 37°C, for 12weeks and examined at weekly intervals for 12 weeks for growth. Results: Colony counts on the solid medium showed a 10^7 fold reduction in the number of viable organisms after 72hrs treatment compared to the untreated control, and in the liquid B12B medium time growth increased substantially up to 72hours. No organisms were recovered from either medium after 7days. Conclusion: Survival of MAP after 24hrs and up to 72 hrs at pH 12.5 was unexpected and would render this method of treatment impractical. It is proposed to repeat the experiment using raw slurry and sample intervals of 12hrs for seven days, enumerating MAP colonies on solid media without decontamination with CPC. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

The efficacy of a killed vaccine against paratuberculosis (SILIRUM®) in cattle. A field study

C García-Pariente, V Pérez, Marivi Geijo, O Moreno, M Muñoz, M Fuertes, E Puentes, J Doce, M C Ferreras, J Francisco Garcia Marin

Abstract

The efficacy of a killed vaccine against paratuberculosis in cattle has been assessed in a field study, using a histopathological method for the evaluation of paratuberculosis-associated lesions in culled animals. Based on previous natural and experimental studies, lesions present in the intestine and associated lymph nodes, were classified as focal-multifocal -related to initial or latent forms, low bacterial load and subclinical-, and diffuse or severe, usually with high levels of mycobacteria in the tissues. The study has been carried out in a Friesian dairy herd, showing approximately a 12% of annual clinical cases of the disease, and formed by 468 adult cows. A 75% of the total, regardless the age or clinical status, was vaccinated with a single dose of SILIRUM® (CZ Veterinaria) and the remaining 25% kept as unvaccinated controls. For a period of 30 months post-vaccination (mpv), all the culled animals, for any reason, have been recorded and pathological studies carried out in 79 of them. Cellular and humoral peripheral immune responses have been also assessed by IFN-γ production and ELISA tests respectively. Vaccination induced strong cellular and humoral responses that persisted in more than 90% of them until the end of the study. No new clinical cases appeared later than 6 mpv. The total number of culled animals (n=192), in the evaluated period, was higher in the control (48.1%) than in the vaccinated group (39%). A significant reduction in the percentage of culled animals that showed lesions associated with paratuberculosis infection has been also observed in vaccinated (56.6%) with respect to control (92.3%) cows. A clear decrease has been also observed in the number of cows with diffuse or severe lesions (15.1% of vaccinated vs 34.6% of controls) although some of them have appeared, at 30 mpv, in vaccinated cows. Vaccination has shown to be a valuable method for achieving a significant reduction of paratuberculosis in a herd with high percentage of losses. *This work was founded by grant LE24/04 from Junta de Castilla y León.*

Targeted sampling of subpopulations can improve detection of Mycobacterium avium subsp. paratuberculosis (Map) in dairy herds

Ian Gardner, Saraya Tavornpanich, T E Carpenter, W O Johnson, R J Anderson

Abstract

The objective of the study was to investigate financial aspects of targeted sampling of subpopulations of cows on classification of herd paratuberculosis status, as part of level 1 of the Voluntary Johne's Disease Herd Status Program. All cows from 3 known infected herds with low to moderate prevalence, and from a non-infected herd in California were tested. The infection status of each cow was determined using ELISA results alone, or ELISA followed by fecal culture. Simulation methods were designed to randomly sample cows based on their lactation number, stage of lactation, and milk production. Sample sizes were ranged from 30 to 300 in herds that had 329 to 2474 cows. Simulation results showed that testing 30 cows in lactation ≥ 2 with ELISA followed by fecal culture detected 3% and 34% of paratuberculosis infected herds with estimated within-herd prevalences of 4% and 15%, respectively. ELISA testing alone provided a higher detection probability; however, it would misclassify 5%-89% of non-infected herds, depending on the number of samples collected. Sample size and costs could be decreased by up to 50% with targeted sampling of cows while achieving the same detection probability. In most situations, samples from cows in lactation ≥ 2 and DIM ≥ 200 days provided the highest detection probability. Findings from the study indicate that detection of Map infected herds could be improved, and cost of testing could be substantially reduced by sampling targeted groups of cows.

Control of paratuberculosis in dairy farms in the Basque Country

Joseba Garrido, J M Plazaola, I Sevilla, Marivi Geijo, N Elguezabal, G Aduriz, J Doce, E Puentes, Ramon A. Juste

Abstract

Bovine paratuberculosis is increasing in importance in the Basque Country and in other regions of Spain. The Gipuzkoa provincial government has considered necessary to undertake a voluntary program. Since experiences with culling strategies have been rather disappointing in other regions, a mixed approach using vaccination and culling has been submitted and approved by the Spanish Animal Health and Drug Authorities. Due to fears of interference with tuberculosis diagnosis, this program is considered experimental and restricted to a maximum of 50 herds. In order to qualify to this program, the farms need to meet a series of epidemiological and trade conditions. The main points are having been free of tuberculosis for at least ten years, having a paratuberculosis prevalence of 5% or higher and selling no breeding animals. A commercial vaccine (SILIRUM™, CZ Veterinaria, Porriño, Pontevedra, Spain) will be given to all cattle at the beginning, and then to all replacer calves within their first 2 months of life. Fecal culture, ELISA, and comparative intradermal test are carried out prior to the first vaccination, and then yearly for, at least 5 years. A pilot study in a herd of 113 cattle has been run for one year and a half. This herd had a clinical incidence of paratuberculosis of about 8%, and a prevalence of 5.5% by fecal culture and 8.0% by ELISA at the beginning of the study. After the first year of control, no new clinical cases have appeared and the prevalence is 3.6% by fecal culture and 23.0% by ELISA. No interferences with the diagnosis of bovine tuberculosis by the IDR test have been found after vaccination. These results are very encouraging and farmers and veterinarians are highly motivated to join the program, which would be the largest vaccination trial in cattle in Spain up to date.

Prevalence of infection caused by Mycobacterium avium subsp. paratuberculosis (Map) in dairy herds in Rio Grande do Sul -South Brazil

Marcos JP Gomes, W Asanome, V R Ribeiro

Abstract

The prevalence of Map infection was estimated by whole-herd (all cows > 3 years of age) testing in 36 bovine dairy herds in Rio Grande do Sul State by absorbed ELISA using PPA-3 commercial antigen. The herds were selected at random. The ELISA test identified 558 positive animals (44.6%) among 1316 samples tested. The infection was detected in 35 (97.2%) from 36 herds sampled and was present in all 25 municipal districts performed. The Map infection was disseminated in bovine dairy herds in Rio Grande do Sul, showing the necessity for adoption of control measures for the protection of State dairy herds.

Efficacy of spheroplastic and cell wall competent vaccines for Johne's disease in experimentally challenged baby goats

Murray E. Hines II, S Stiver, D Giri, L Whittington, C Watson, J Johnson, M Pence, C Baldwin, L Sangster, Sharif S Aly

Abstract

Current vaccines for Johne's disease (JD) are not as effective as we might wish. A *Mycobacterium avium* subsp. *paratuberculosis* (MAP) vaccine that reduced the rate of fecal shedding and/or eliminated disease would be useful in control of JD. The efficacy of four vaccine combinations, including cell-wall competent (CWC) alum adjuvant, CWC-QS21 adjuvant, cell-wall deficient (CWD) alum adjuvant and CWD-QS21 adjuvant vaccines were evaluated at both six and nine month post challenge. Goats were vaccinated at one and four weeks of age with each vaccine or a sham control vaccine consisting of alum adjuvant. Kids were challenged orally with 6.0×10^9 organisms in four divided doses (1.5×10^6 organisms per dose) using a confirmed goat isolate of MAP. Eighty kids in total were evaluated, with each experimental group consisting of 10 kids; each control group contained 6 kids. Half of the kids within each group were necropsied at six months post challenge and the remaining kids were necropsied at nine months post challenge. Gross and microscopic lesions were evaluated and scored at necropsy as were the relative number of acid-fast bacilli. Results indicated that all challenged kids had some lesions compatible with JD suggesting none of the vaccines prevented infection. Results also suggested that three vaccines (CWC-alum, CWC-QS21 and CWD-QS21) affected lesion development: lesion scores were reduced by 45.6 - 50.6% at the nine-month period. The CWD-alum vaccine resulted in a more severe (+33.5%) lesion score than what was seen for sham-vaccinated challenged controls. Lesion scores were greater for the six to nine-month necropsy period in the sham-vaccinated challenged group and CWD-alum vaccinated group. Lesion scores were generally stable with remaining vaccines. Mean fecal CFUs/g were significantly different across time from challenge to 9 month necropsy (p=0.043) and the CWD-QS21 vaccine group had a marked reduction in fecal CFU/g at all time points post challenge. A reduction in MAP CFU/g was also detected in necropsy tissues from kids given the CWC-alum, CWC-QS21 and CWD-QS21 vaccines. Greater CFUs/g were detected in tissues from kids given the CWD-alum vaccine.

Evaluation of test strategy for detection of cows shedding Mycobacterium avium subsp. paratuberculosis (Map) in milk fed for calves

L Juhl, Soeren Saxmose Nielsen

Abstract

Background: Map can be shed in milk and thus infect calves fed milk containing Map. Objective: To evaluate the ability to detect cows shedding Map in milk based on a test scheme with ELISA and faecal culture. Materials & Methods: Milk and faecal samples from 668 cows from six dairy herds were assessed in a test scheme with four annual herd screenings of milk and one annual herd screening of faecal samples. Milk samples were analysed for antibodies with ELISA and faecal samples were cultured for presence of Map. The cows were classified into three risk-groups defined as follows: 0) Low risk (never test-positive (ELISA or faecal culture)); 1) Medium risk: test-negative, but only tested once; 2) High risk: Test positive on at least one sampling. Presence of Map in milk samples was estimated by use of a peptide-mediated capture PCR (IS*Mav2*-based). Results: The cows were distributed in the risk groups as follows: 0) 386 cows; 1) 138 cows; 2) 144 cows. The PCR test was positive on milk samples from nine cows. None of these cows had diarrhoea or low body condition scores. Four were from risk group 0, one was from risk group 1, and four were from risk group 2. Discussion: Four cows classified as low risk apparently had Map in their milk, and farmers would use their milk for feeding. Given the test-positive responses in the PCR were caused by Map-infection of the animals, these animals may pose a serious risk of further transmission of Map and the approach selected for classification of cows is insufficient. A major problem of this study is the unknown accuracy of the PCR. Contamination may have occurred during sampling, and the sensitivity is unknown.

The effect of sub-clinical paratuberculosis on the fertility of dairy ewes and goats varies with parity

Poychronis Kostoulas, Leonidas Leontides, C Billinis, G Amoiridis

Abstract

Objective: Toevaluate the impact of sub-clinical *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection on fertility of dairy sheep and goats, defined as lambing or kidding in the year before testing. Materials and methods: One hundred sera and faecal samples were collected from each of four dairy sheep and goat flocks with a history of clinical paratuberculosis. At sampling, individual animal data were recorded. Faecal samples were cultured in Herrold's Egg Yolk medium supplemented with mycobactin J and antibiotics. Sera were tested by a commercially available serum ELISA. An animal was considered sub-clinically infected when found either seropositive or culture-positive. Rogan–Gladen estimates of the true prevalence (TP) of sub-clinically infected animals were calculated separately for sheep and goats. To assess the association between fertility and MAP infection, a multi-level logistic regression model was fitted to the data using the GLIMMIX macro in SAS[O]. Results: TP was 10% (95%C.I: 1–25%) and 36% (9–47%) for sheep and goats, respectively. The association between MAP infection and fertility did not differ between species and was modified by parity. Low parity (<4) animals were more likely to be fertile when infected than non-infected. In animals of medium parity (4≤parity<7) this association was not significant. High parity (>7) animals were more likely to be infertile in the year before sampling when infected than non-infected. Conclusions: High milk production stress and immune regulation during pregnancy may favour proliferation of sub-clinical infection in low parity animals that were probably infected with high numbers of bacilli early in their life. The farmers usually culled these animals when paratuberculosis became clinically evident in subsequent lactations. The detection of a negative association between sub-clinical infection and fertility in high parity stock was probably due to the long incubation period and subsequent induction of negative energy balance secondary to slow proliferation of enteric lesions.

Production effects of Mycobacterium avium subspecies paratuberculosis based on diagnostic test results

Jason E. Lombard, B A Wagner, B J McCluskey, Franklyn Garry

Abstract

The National Animal Health Monitoring System's Dairy 2002 surveyed dairy operations in 21 states representing 82.8% of U.S. dairy operations and 85.5% of U.S. dairy cows. A subset of operations allowed collection of biological samples for fecal culture and serum- and milk-ELISA testing for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection, and access to Dairy Herd Improvement Association production records. Mature equivalent (ME) milk production in the lactation in which testing occurred was evaluated using Proc Mixed in SAS. There were 2,832 cows from 23 herds evaluated using fecal culture results, 7,614 cows from 38 herds evaluated with serum ELISA and 11,874 cows from 33 herds using milk ELISA. Cows classified as heavy shedders produced significantly less ME milk compared to all other cows. Heavy MAP shedders produced almost 5,000 lbs less ME milk compared to moderate shedders and 8-9,000 lbs less than low, very low and culture negative cows. Cows that tested strong positive on serum ELISA produced 2,000 lbs less than test-positive cows and more than 2,500 lbs less than test-negative cows. For both ELISA testing methods, ME milk production was almost identical for each testing category. Cows that tested strong positive via serum ELISA produced significantly less ME milk in the current lactation compared to cows that tested positive, inconclusive or negative. The same was true for cows tested via milk ELISA. There were no significant differences in ME milk production between test-negative and test-positive cows for either ELISA. Results of this study suggest that the decline in milk production for cows with MAP infection occur in cattle that are shedding the largest number of bacteria or have developed the greatest immune response.

Protective effect of DNA vaccines encoding different mycobacterial antigens against Mycobacterium avium subspecies paratuberculosis in Sardinian lambs

L Mara, S Ortu, P Cappai, A Leoni, R Frothingam, S Zanetti, L A Sechi

Abstract

Paratuberculosis, or Johne's disease, is a disease of domestic and wild ruminants that culminate with a chronic enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis*. The aim of this work was to evaluate the type of immune response, Th1 or Th2, induced by DNA vaccinations in lambs of Sarda breed. Twenty-five lambs, seronegative for paratuberculosis, were selected at birth from equally seronegative mothers. The lambs were inoculated with three different mycobacterial antigens cloned into a mammalian expression vector as fusion protein with the enhanced green fluorescent protein (pEGFP-N1). The animals at 5 months were divided in 5 groups containing each 5 lambs. Each group was vaccinated as following (A: physiological solution, B: Gudair™, C: p-85A-Mav, D: p-85A-BCG, E: p-Hsp65). Immune response was evaluated by measuring the expression of INF-γ (Th1 type response) and IL-10 (Th2 type response) by Real Time PCR. Gene expression was estimated by comparing the results with that of b-actin. Each animal was vaccinated by intramuscular inoculation with three doses. RNA extracted from peripheral lymphocytes stimulated with Johnin PPD (purified protein derivate) and from lymphocytes not stimulated, was extracted and used as templates in reverse transcription reaction. The cDNA obtained was investigated in order to evaluate gene expression by Real Time PCR. INF-γ expression level was increased in lambs vaccinated with plasmids codifying mycobacterial antigens (in particular with Hsp65) in comparison with the controls suggesting a Th1 immune response as that supported by natural infection by *M. paratuberculosis*. Moreover, vaccinated animals were challenged after 3 months with *M. paratuberculosis*. Animals were infected orally with a single dose of 20 ml of 10^8 bact/ml. Sheep were sacrificed after one year from the challenge, histological analysis was performed and correlated with expression of INF-γ and IL-10 in order to verify *in vivo* the protection level of the different vaccines. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

Comparison of fecal pooling strategies for detection of Mycobacterium avium subsp. paratuberculosis

Shawn McKenna, H W Barkema, Greg Keefe, Donald C. Sockett

Abstract

Feces collected from cattle at slaughter were used to estimate the sensitivity of fecal pooling for detection of *Mycobacterium avium* subsp. *paratuberculosis*. Results from tissue culture using the TREK ESP® Culture System II on the same cattle were used to determine which cows were to be included in each pool. Pool sizes of 3, 5, 8, 10, and 15 were used with varying number of tissue positive animals in each pool ranging from 0 to five. An individual fecal culture was also performed on each animal. All fecal cultures were performed using the TREK ESP® Culture System II. Overall, there were 49, 52, 48, 49 and 75 pools of 3, 5, 8, 10 and 15 animals, which included at least one fecal culture positive animal, respectively. Some pools had more than one fecal-culture positive animal. Using pools with minimally one fecal shedder to determine the sensitivity of pooling, the results for pools of 3, 5, 8, 10, and 15 were 46 (95% CI: 33.0-61.0), 67 (95% CI: 54.5-80.1), 43 (95% CI: 29.7-57.8), 59 (95% CI: 45.4-72.9), and 39% (95% CI: 28.1 - 50.3), respectively. For screening purposes, pools of 10 animals appeared to perform adequately, however, a pool of five was optimal. There were two pools that only had fecal culture-negative animals that were positive, however both had at least one tissue culture-positive animal included.

Comparison of the methods used for the diagnosis of paratuberculosis infection in herds of small ruminants in Portugal

S Mendes, T Albuquerque, Augusto Afonso, F Boinas, Alice Amado

Abstract

In the absence of exceptional diagnostic tests for the evaluation of paratuberculosis infection in small ruminants the methods available have to be evaluated for specific epidemiological situations to design an adequate testing protocol. A serological survey of 66 sheep and goat herds in the region of Lisbon identified 27% of the flocks with seropositive animals. The methods used for the diagnosis were the Agar gel Immunodifusion test used in parallel with the ELISA test (Paracheck). 164 animals from positive herds were followed-up of by IFN-γ (Bovigam) and isolations of *Mycobacterium avium* spp. *paratuberculosis* were attempted using in parallel Herrolds media and Bactec with mycobactin. Further evaluation was made in a sample of 17 seropositive animals by necropsies and histopathology and by Polymerase chain reaction PCR (IS*900*) of the tissues and faeces. The present project allowed the validation of the PCR in faeces to use in paratuberculosis survey. Comparison was made of the several tests and suggestions are made about an optimal testing protocol that can be used in the identification of infected animals. Considering that in Portugal vaccination is presently not allowed, the laboratory testing together with the sanitary measures are the only tools that can be used for control and eradication of paratuberculosis in the small ruminant herds.

Using ELISA adjusted optical density (OD) measures to predict Mycobacterium avium paratuberculosis shedding status of individual dairy cattle

R Pillars, John B. Kaneene, D L Grooms

Abstract

Fecal culture remains the standard for diagnosing *Mycobaterium avium paratuberculosis* (MAP) in individual cattle, however it generally requires 8-16 weeks to obtain results. The enzyme-linked immunosorbent assay (ELISA) has rapid turn-around time, although its sensitivity is poor, especially in subclinically infected cattle. It is generally accepted that as infection progresses mean ELISA response and sensitivity increases. Cattle infected with MAP have a long prepatent period in which no shedding of the organism occurs followed by intermittent then continuous shedding increasing in volume as disease progresses. While any cow infected with MAP is undesirable, only cattle shedding the organism pose an immediate threat to other cattle. These cattle generally have higher ELISA adjusted optical density (OD) values compared to cattle not shedding MAP. The objective of this study was to compare mean ELISA adjusted OD values of cattle with negative fecal cultures to cattle classified as either low ($\leq$10 cfus) or high (>10 cfus) shedders and determine the positive likelihood ratios for each. Fecal culture results and ELISA adjusted OD values were evaluated from 2,578 adult cattle from six Michigan dairy herds over two years. Prevalence of MAP in these herds based on fecal culture ranged from zero to 42% with an average of 9.8%. The mean ELISA adjusted OD value for fecal culture negative cattle (n=2,362) was 0.023 (95% CI: 0.02-0.03). That for cattle classified as low shedders (n=158) and high shedders (n=58) was 0.201 (95% CI: 0.12-0.28) and 0.784 (95% CI: 0.52-1.05), respectively. Using the mean as the ELISA cutoff for each group, likelihood ratios for a positive test for fecal culture negative, low, and high shedders were 3.2, 9.7, and 1600, respectively. Given the ELISA adjusted OD, the likelihood ratios can be used to predict the probability of a cow shedding MAP, provided herd prevalence information is available.

Sheep paratuberculosis study in Portugal, Serra da Estrela Region (preliminary results)

C Seixas, F Esteves, T Albuquerque, Augusto Afonso, Ana Botelho, C 4 Santos, H Vala, Alice Amado

Abstract

The aim of the project, started in September 2004 and with the duration of three years, is the study of paratuberculosis incidence in sheep herds in one of the best Portuguese cheese production region. Every year producers suffer great economic losses due to the lack of control of the disease in their herds. Thirteen sheep herds were selected based in clinical signs of paratuberculosis disease. A serological survey by ELISA test, performed in 1220 animals, revealed that 5.6% were positive. Fourteen positive (9.7%) AGID tests, performed so far in 145 animals, were obtained. Post-mortem examination of these 14 animals revealed typical macroscopic lesions of paratuberculosis in all of them. Histophathological analysis showed suspected paratuberculosis microscopic lesions in seven animals. Bowel tissues with lesions, from the same 14 animals, were cultured in selective media for bacteriological analysis and also for direct detection with the ADIAVET kit. Seven tissue samples processed until now allowed the isolation of six strains, identified as *Mycobacterium avium* subsp. *paratuberculosis* by bacteriologic conventional methods. To start the molecular epidemiological study of paratuberculosis these six strains, and others isolated in the future, will be typed by molecular methods using IS*900* restriction fragment length polymorphism (RFLP) analysis. This molecular approach will allow the detection of sources and routes of infection, essential for disease control. After these preliminary results, we intend to develop methods to rapidly detect *Mycobacterium avium* subsp. *paratuberculosis* in milk samples from infected and non infected sheep. With all the field and laboratory information and results gathered, it is our perspective, in three years from now, to have controlled paratuberculosis in sheep herds from Serra da Estrela region, and also to have a safe guaranty for the consumer of Serra da Estrela cheese.

The risk of paratuberculosis in Finnish suckler herds and the impact of various interventions

J Seppänen, P Tuominen, E Seuna, S Pelkonen, R Maijala

Abstract

In Finland, paratuberculosis has been detected in five beef herds in 1992 – 2000. In order to plan how the spread of paratuberculosis both at the national and farm level could be prevented, a qualitative risk assessment was made. The main focus was laid on the domestic spread of the disease. The assessment was done in accordance with the risk assessment code of the World Organization for Animal Health (OIE). Suckler herds were the primary target and data from 2001 was used. The assessment focused on paratuberculosis as an animal disease, the possible role as a zoonosis was not taken into account. The risk of paratuberculosis spreading into Finland is dependent on the number of imported cattle, prevalence in the herd of origin and importing country as well as the monitoring methods of respective countries. It was estimated that the risk associated with importing has reduced since 1995 because the prevalence of paratuberculosis in the herds of origin has been reasonably well known. The current risk was considered to be moderate. The choice of the country and the herd of origin are crucial in reducing this risk. The interaction between Finnish suckler herds, e.g. animal sales, was considered to pose a moderate risk for the spread of paratuberculosis between the herds. The infection may also spread to dairy farms. The most effective ways to prevent the spread into individual herds are embryo transfers, abandoning the practice of sharing grazing, water sources, machinery and transport vehicles, and closed herds. When paratuberculosis has been detected on a farm the best methods to prevent the spread are the monitoring for symptoms, testing all animals and the removal of infected animals. One of the conclusions was also that the benefits of statutory or voluntary national control program should be evaluated. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

Survey of ruminant population of Northern India for the incidence of Mycobacterium avium subsp. paratuberculosis infection

Shri N. Singh, A V Singh, R Singh, K S Sandhu, V K Gupta, S Misra

Abstract

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection wasestimated in the ruminant populating of Uttar Pradesh (33 villages) and organized herds of Punjab (bovines) in India. In UP villages were selected randomly from the total number of villages in the country. Of the total 33 villages surveyed, 21 were in South UP and 12 in Western UP. In all 879 animals (buffaloes, cattle and goats) belonging to 700 farmers in these 33 villages were screened for paratuberculosis infection using serum samples. Prevalence of MAP infection in UP using plate ELISA (MAP Bison type antigen) was 31.9%, 37.6% and 33.3% in buffaloes (601), cattle (125) and goats (153), respectively. Prevalence in South UP was 40.3%, 42.6% and 15.0% in buffaloes (260), cattle (75) and goats (93), whereas in Western UP, it was 25.5%, 30.0% and 50.0%, in buffaloes (341), cattle (50) and goats (14), respectively. Bovine (buffaloes and cattle) and caprine samples from Ludhiana region of Punjab were from organized herds and consisted of fecal (32), milk (27), and serum (699). Prevalence of MAP infection using MAP Bison type antigen in plate ELISA was 25.0% and 15.25% in bovine (699), and caprine (177) serum samples, respectively. In milk ELISA, of 27 bovine samples 14.8% animals were positive. However, in culture 62.9% and 70.3% animals were found positive in culture of sediment and fat layers of bovines milk samples. On the basis of culture of bovine fecal samples (32), 62.5% were found positive in culture, whereas in IS*900* PCR 31.25% fecal samples were positive. Culture was most sensitive than milk ELISA and PCR for the diagnosis of MAP infection in bovines and caprine population.

Prevalence and molecular characterization of Juvenile form of paratuberculosis in Indian goatherds

Shri N. Singh, P Kumar, A V Singh, A K Srivastava, I Sevilla, Ramon A. Juste, V K Gupta

Abstract

Kids (0 to 6 months) from organized and farmer's herds were screened by culture and PCR (fecal and tissues) and plate ELISA (serum). Of 50 fecal samples, 56.0% kids developed colonies. Whereas, direct microscopy revealed only 38.0% kids infected with MAP. Screening of 60 kids tissues from slaughterhouse by culture showed 58.3% positive. (Organized herds- 18.1% and farmer's 67.3%). Of 60 tissues MLN (38.3%) and small intestine (43.6%) were positive. However, direct microscopy revealed only 31.6% kids positive. Typical MAP colonies usually appeared around 75 days of inoculation in most of the fecal samples, while in case of tissue samples the maximum number of colonies was observed between 60-90 days post inoculation. Serum samples (334) collected from kids belong to organized and farmers' herds (slaughter house). Screenings of 179 serum samples of kids from organized herds 58.6% were positive in plate ELISA using MAP (bison type) antigen. Whereas, screening of 155 kids from farmer's herds, 35.4% kids were positive. While using MAP bovine type antigen none of the kids could be detected as positive. MAP culture were identified on the basis of primary and molecular characterization using specific IS*900* and IS*1311* PCR and REA analysis and showed that MAP isolates were bison type. Early lesions were also seen on the histo-pathological examination of some of the tissues.

Diagnosis of paratuberculosis in goats by the interferon gamma test

A K Storset, S Kulberg, Ingrid Olsen, I Berg, Berit Djønne

Abstract

To obtain more information about the interferon gamma test used for diagnosis of paratuberculosis in goats, three categories of herds were tested: non-vaccinated herds without paratuberculosis; herds that had been vaccinated against paratuberculosis but had never shown clinical signs of paratuberculosis nor given positive *M. a. paratuberculosis* fecal samples and herds that had a history of paratuberculosis, had given positive *M. a. paratuberculosis* fecal samples and were vaccinated. Heparinized blood was stimulated with purified protein derivate (PPD) from *M. a. paratuberculosis* for 24 h and plasma was assayed for the presence of interferon gamma. Results were recorded as the difference between OD values of PPD stimulated and control samples. Due to geographical matters, blood samples were set up in the gamma interferon assay the day after they were sampled. In order to test the viability of the blood samples, positive stimulations with SED were set up. Only 1% of the 1200 animals originating from herds free of paratuberculosis gave weak positive responses in the interferon gamma test (delta OD exceeding 0.2). However, about 5% of the animals had positive responses in control samples and had to be re-sampled. Vaccinated animals from herds with paratuberculosis showed significant higher responses than animals from vaccinated herds without paratuberculosis. Some of the vaccinated animals had a response lasting for several years, which demonstrate a long lasting interference with diagnostic testing. In both these groups responses were correlated to age with higher responses in younger animals. Although more information about the early interferon gamma responses of naturally infected goats and the presence of false negative tests in infected animals is needed, we conclude that the low responses of non-infected animals indicate that this test may be useful in monitoring the paratuberculosis status in non-vaccinated goat herds. *Attendance to this conference was sponsored by the EU-funded co-ordination action SSPE-CT-2004-501903*

Production impact of sub-clinical manifestations of bovine paratuberculosis in dairy cattle

M A Villarino, E R Jordan

Abstract

Bovine paratuberculosis (Johne's Disease) is a chronic debilitating disease of cattle. The disease is widely distributed and dairy veterinary practitioners and dairy producers are becoming aware of the benefits of establishing Johne's Disease control measures. Although there is no doubt that the clinical disease can cause significant economic losses, the sub-clinical consequences of the disease are not well documented. The objective of this investigation was to evaluate the production impact of sub-clinical manifestations of Johne's Disease in dairy cattle based on sero-conversion using an ELISA test. To accomplish this objective, a retrospective cohort study in a commercial dairy located in northwest Texas was conducted. Production data were collected starting April, 2001 using a paired comparison scheme. The cohort consisted of cows of similar age (+/- 30 days), lactation, and origin. Production parameters (lifetime milk production, milk per lactation) and individual performance parameters (days in milk, days in dairy, cull date and reasons for culling) were collected from 120 ELISA positive and 120 ELISA negative for Johne's Disease cows, for as long as the animals were maintained on the premise. Statistical comparison (t-test) and linear regression analysis on the obtained data were conducted. Our results to date indicate a significant reduction in milk production (4,090 kg lifetime milk production per animal) from ELISA positive cows when compared to ELISA negative cows. Also, more ELISA positive cows became lame (5x), developed respiratory disease (1.25x), digestive disease (1.83x), and mastitis (2x) than ELISA negative cows. Currently, 28.33 % of the ELISA positive and 60.34 % of the ELISA negative animals remained in the herd. The reduction of milk production in the ELISA positive cow started in the second lactation and remained for the rest of her productive life, regardless of when the cow was detected as ELISA positive.

Johne's disease: The effect of feeding Monensin to reduce the bio-burden of Mycobacterium avium subspecies paratuberculosis in neo-natal calves

Robert H. Whitlock, Raymond W. Sweeney, Terry L Fyock, S McAdams, Ian Gardner, D G McClary

Abstract

Objective: The current experiment was designed to assess the efficacy of monensin to reduce pass-through fecal shedding and to reduce tissue bacterial load (Bio-burden) of MAP in calves. Materials and Methods: Twelve neonatal Holstein heifer calves (1-3 days of age) were randomly assigned to receive a carrier containing 35 mg. Monensin (n=6), or placebo (n=6), added to the milk replacer at each twice-a-day feeding. The trial was conducted as a randomized double blind trial. All calves were administered two oral doses of viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on two consecutive days between day 7 and 9 of the trial. Calves were euthanized between days 65 and 67 days. Results: Calves fed monensin had fewer culture positive (55%) fecal samples, fewer total HEYM positive tubes (63%) and less MAP cfu (72%) detected in the manure compared to controls. Monensin fed calves had fewer culture positive tissues (66%), fewer total culture positive HEYM tissue tubes (68%) and lower MAP cfu (87%) in the tissues compared to controls. Significance: Monensin effectively reduced tissue colonization with MAP following oral challenge, and also reduced fecal pass-through shedding of MAP. Monensin may act directly on *Mycobacterium avium* subsp. *paratuberculosis* by inhibiting growth of the mycobacterial cell as preliminary evidence in our laboratory has shown or it may enhance phagocytic killing of mycobacteria or both. The amount of monensin (70 mg) administered per day to calves in this study is higher than the amount that would normally be consumed by a neonatal calf in a calf starter. This study was a proof-of-concept study to determine the efficacy of monensin in controlling infection with MAP in the neonatal calf. *Monensin[TM], Elanco Animal Health, a Division of Eli Lilly & Company, Indianapolis, ID 46285*

Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in foods and the impact of milk processing on its survival

Grant IR[1]

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Key words: Surveillance; survival; milk; cheesemaking; dairy processing; pasteurisation.

INTRODUCTION

Whilst the role of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in human disease (whether Crohn's or other disease) has remained the subject of continuing debate over recent years, the possibility that MAP could be transferred to humans via foods of animal origin has been the focus of considerable research effort. Published studies relating to MAP in food over the past five years (2000-2005) fall into two broad categories, those which investigated the natural occurrence of MAP in milk, other dairy products and beef (surveillance studies) and those which described the impact of various dairy processes on MAP in milk (survival studies). This Review and Perspectives paper summarises the information arising from both types of study.

SURVEILLANCE STUDIES

MAP in raw and pasteurised cows' milk

Several studies reporting the presence of MAP, by PCR and/or culture, in raw cows' milk from individual animals sub-clinically or clinically affected by Johne's disease (Giese and Ahrens 2000; Pillai and Jayarao 2002; Maher et al. 2004; Ayele et al. 2005), from bulk tanks at farm level (Corti and Stephan 2002; Sevilla et al. 2002; Stabel et al. 2002; Jayarao et al. 2004), and from bulk silos at processing level (Grant et al. 2002a; O'Doherty et al. 2002; O'Reilly et al. 2004) have been published over the last five years. Due to limitations in culture methods, accurate enumeration of MAP in milk is still not possible and, therefore, MAP counts reported in two of these studies (4-20 CFU/50 ml (Ayele et al. 2005) and <100 CFU/ml (Giese and Ahrens 2000)) are underestimates and need to be cited by others with due acknowledgement of this fact. It should also be noted that non-recovery or low recovery of viable MAP may have occurred in some studies (despite very high levels of IS*900* PCR positivity) because the decontamination methods employed would have damaged or killed the MAP present (Pillai and Jayarao 2002; Stabel et al. 2002; Jayarao et al. 2004). Whilst it is likely that MAP numbers in raw milk will decrease as contaminated milk from individual animals is mixed in the bulk tank at farm level and then milk from herds of differing Johne's disease status is mixed prior to processing, current evidence indicates that detectable levels of MAP may still be present in raw cows' milk prior to processing in various parts of the world.

The first survey to confirm the presence of low levels of viable MAP in commercially pasteurised cows' milk was carried out in the United Kingdom; 1.8% pasteurised milk samples tested MAP culture positive (Grant et al. 2002a; United Kingdom Food Standards Agency 2003). Whilst it was acknowledged that these culture positives could have arisen as a consequence of post-process contamination of milk by MAP, all the indications were that the MAP isolates obtained from pasteurised milk were genuine survivors (Grant et al. 2002a). Viable MAP has since been cultured from commercially pasteurised milk during surveys in California, Minnesota and Wisconsin, USA (Ellingson et al. 2005), Czech Republic (Ayele et al. 2005) and Argentina (Paolicchi et al. 2005), but not during surveys in Republic of Ireland (O'Doherty et al. 2002; O'Reilly et al. 2004) and Ontario, Canada (Gao et al. 2002). Table 1 summarises the key features of the published milk surveys. The decontamination method adopted for many of the surveys - 0.75% hexadecylpyridinium chloride for 4-5 h - has been shown to be the optimal method for recovery of MAP from milk when compared to other decontamination protocols (Dundee et al. 2001; Gao et al. 2005).

Table 1. Overview of surveillance studies to determine the presence of MAP in commercially pasteurised cows' milk.

Study / Country	Volume of pasteurised milk tested	No. (%) IS*900* PCR positive	No. (%) culture positive	Chemical decontamination before culture
Grant et al. (2002a) / UK	50 ml	67 / 567 (11.8%)	10 / 567 (1.8%)	0.75% HPC[a]/5h
Gao et al. (2002) / CAN	1 or 5 ml	110 / 710 (15%)	0 / 244[b]	None
O'Doherty et al. (2002) / IRE	50 ml	-[c]	0 / 77	0.75% HPC/5h
O'Reilly *et al*. (2004) / IRE	50 ml	35 / 357 (9.8%)	0 / 357[b]	0.75% HPC/5h
Ellingson et al. (2005) / USA	40 ml	452 / 702 (64%)	20 / 702 (2.8%)	None
Ayele et al. (2005) / CZ	50 ml	-	4 / 244 (1.6%)	0.75% HPC/5h
Paolicchi et al. (2005) / ARG	50 ml	2 / 70 (2.9%)	2 / 70 (2.9%)	0.75% HPC

[a] HPC, Hexadecylpyridinium chloride; [b] Suspect MAP positive cultures containing acid-fast cells that tested IS*900* PCR positive were encountered but sub-culture was unsuccessful; [c] IS*900* PCR was only used to confirm identity of acid-fast colonies, not to test milk samples directly.

MAP in raw sheep and goats' milk

DNA evidence of MAP in raw goats' milk in the UK (1.1% positive, Grant et al. 2001), Norway (7.1% positive, Djonne et al. 2003) and Switzerland (23.0% positive, Muehlherr et al. 2003) has been reported, but either culture was not attempted or the presence of viable MAP was not confirmed in any of these studies. In the UK study (Grant et al. 2001) raw sheep's milk was also tested but there was no cultural or PCR evidence of MAP, whereas in the Swiss study a similar percentage of raw sheep's milk samples (23.8%) as goats' milk samples (23.0%) tested MAP positive by PCR (Muehlherr et al. 2003). Detection rates observed probably reflect Johne's disease prevalence in the goat and sheep populations in the three countries concerned.

MAP in dairy products

The presence of viable MAP in dairy products other than liquid milk has not been extensively studied to date. The first reports of viable MAP being isolated from retail cheese and powdered infant formula were published recently. Gazouli et al. (2003) tested 42 samples of retail Feta cheese (made from a mixture of sheep and goats' milk) from Greece and reported the detection of MAP by PCR in 50% of samples and isolation of viable MAP from one sample. Hruska et al. (2005) tested 51 samples of powdered infant formula marketed by 10 manufacturers from seven European countries and detected MAP DNA in 49% (n=25) samples and cultivated viable MAP from one sample. More extensive surveillance of these and other dairy products is warranted in order to establish potential risk posed to consumers.

MAP in ground beef

In the advanced stages of Johne's disease in cattle, MAP infection is likely to be widely disseminated throughout the animal including muscle, lymph nodes and blood. It has, therefore, been suggested that meat from old dairy cows, used to make ground beef for human consumption in some parts of the world, may represent a source of MAP infection for consumers (Rossiter and Henning 2001). It is hypothesised that when ground (minced) beef is prepared, localised infection (for example in a lymph node) could be spread throughout a whole batch of ground beef. To date, there is no scientific evidence to substantiate this theoretical risk of MAP in beef. The only reported survey of 113 minced beef samples collected from a single meat processing plant in the Republic of Ireland over a 4-month period found no evidence of viable MAP (Maher et al. 2004). It should be noted, however, that details of the culture methodology adopted in this survey are not published and it is possible that a sub-optimal recovery method may have been employed for testing the beef. More extensive surveillance is needed to determine if beef is a significant potential vehicle of transmission of MAP to humans.

SURVIVAL STUDIES

HTST pasteurisation

Over the past five years there have been at least 10 pasteurisation studies published (Table 2). It is interesting to note that most researchers have moved away from laboratory studies (which have in the past been viewed as not accurately simulating commercial pasteurisation conditions), choosing instead to investigate pasteurisation of spiked or naturally infected milk in pilot- or commercial-scale plant. In recent studies, HTST pasteurisation has generally achieved a substantial $\log_{10}$ kill (4-7 $\log_{10}$) of MAP in milk. However, survival of low numbers of MAP after pasteurisation has been observed in the majority of studies involving both artificially spiked (7 of 8 studies) and naturally infected (2 of 2 studies) milk (Table 2) under a variety of time/temperature conditions.

Estimates of the number of MAP surviving HTST pasteurisation were 10-20 CFU/150 ml (Grant et al. 2005a) and 0.002-0.004 CFU/ml (McDonald et al. 2005). Factors such as the volume of milk tested, whether or not chemical decontamination was applied, and time lapse between pasteurisation and testing (immediate testing or after refrigerated storage) are now known to be important for the successful recovery of low numbers of viable MAP from pasteurised milk (Grant 2004; Grant and Rowe 2004; Gao et al. 2005).

The mechanism that enables MAP to survive pasteurisation remains to be fully elucidated. Protection of cells within clumps from the lethal effects of heat is widely discounted since heat penetration into clumps should be instantaneous. However, given that greater inactivation of MAP cells was reported when clumps were disrupted by homogenisation prior to pasteurisation (Grant et al. 2005a), clumping appears to have some influence on survival. A further hypothesis put forward to explain the presence of low numbers of MAP in pasteurised milk is heat activation of MAP cells above certain temperatures and extended holding times (Hammer et al. 2002; Herman et al. 2005).

Table 2. Overview of studies assessing the efficacy of HTST pasteurisation with respect to inactivation of MAP, 2000-2005.

Study / Country	Volume of pasteurised milk cultured	Chemical decontamination before culture	Time between pasteurisation and testing	MAP survival observed
(A) Laboratory-simulated pasteurisation of MAP spiked milk				
Gao et al. (2002) / CAN	600 μl	None	-[a]	Yes
Stabel and Lambertz (2004) / USA	50 ml	None	-	Yes
(B) Pilot/commercial-scale pasteurisation of MAP spiked milk				
Pearce et al. (2001) / NZ	50 ml	1% HPC/50 min	≤ 2 h	No
Hammer et al. (2002) / GER	50 or 100 ml	None	≤ 1 h	Yes
McDonald et al. (2005) / AUS	1500 ml	0.75% HPC/4h	-	Yes[b]
Grant et al. (2005a) / UK	150 ml	None	48 h	Yes[c]
(C) Commercial-scale pasteurisation of naturally infected milk				
Grant et al. (2002b) / UK	50 ml	0.75% HPC/5h	24 h	Yes
Ayele et al. (2005) / CZ	50 ml	0.75% HPC/5h	-	Yes

[a] Time lapse not stated in published paper but milk samples were apparently processed for culture within same working day; [b] Number of MAP surviving pasteurisation reported to be 0.002-0.004 CFU/ml (note: decontamination applied before culture); [c] Number of MAP surviving pasteurisation estimated to be 10-20 CFU/150ml (note: no decontamination applied before culture).

Homogenisation

Homogenisation is a process applied to milk to break up the milk fat globules by forcing them through a fine aperture under pressure. This prevents formation of a cream layer during distribution and storage. An additional effect of homogenisation on MAP cells was demonstrated recently (Grant et al. 2005a). Using a Mastersizer X spectrometer (Malvern Instruments Limited, Malvern, England) the cell size distribution in homogenised (2500 lb/in^2) and non-homogenised (control) MAP suspension was assessed. Homogenisation caused large MAP clumps to be disrupted into single cells or mini-clumps. However, clumps were shown to reform within a few minutes, particularly if the suspension was mixed. McDonald et al. (2005) reported a one $\log_{10}$ increase in numbers of MAP present when spiked milk was homogenised at 27,000 kg/cm^2, which provides further evidence of clump disruption by homogenisation.

Homogenisation is commonly applied to liquid milk prior to pasteurisation in many countries. Grant et al. (2005a) investigated the combined effects of homogenisation and pasteurisation on MAP inactivation. They

reported that heat treatments incorporating homogenisation (2,500 lb/in^2), applied upstream (as a separate process) or in-hold (at the start of the hold section), in conjunction with a 25 s hold time resulted in significantly fewer culture positive milk samples than pasteurisation treatments without homogenisation ($p<0.001$ for in-hold treatments and $p<0.05$ for upstream treatments). Given that homogenisation breaks up clumps, MAP cells would have been present as predominantly declumped cells when HTST pasteurisation was subsequently applied and this may explain the greater MAP inactivation achieved by the combination of homogenisation and pasteurisation. However, it should be noted that 100% inactivation of MAP is not guaranteed by combining homogenisation and pasteurisation since in that and previous studies low numbers of viable MAP have been isolated from pasteurised homogenised milk (Grant et al. 2002b; Grant et al. 2005a; McDonald et al. 2005).

Centrifugation
Centrifugation (or bactofugation) is used by the dairy industry for the pre-treatment of cheesemilk to remove clostridial spores that can otherwise cause problems during ripening. Essentially, bactofugation involves the application of centrifugal force (typically 7000 x g for a dwell time of 7s in commercial practice) to milk, which forces heavier particles (such as spores) out of suspension. Centrifugation is generally applied to milk preheated to 50-65°C to aid the separation process. Clarification and separation are two other processes used by the dairy industry for liquid milk processing which also rely on centrifugal force. The application of centrifugation could have the potential to physically remove clumps of MAP from milk. The impact of laboratory-simulated commercial bactofugation conditions on MAP in spiked milk samples was assessed recently (Grant et al. 2005b). Centrifugation at 7,000g for 10s (after pre-heating the spiked milk to 60°C) resulted in 85-93% removal of MAP cells when de-clumped broth suspensions of two strains (NCTC 8578 and B4) were used to spike whole milk, and 74-79% removal when untreated (clumped) MAP suspensions were employed. The differences between numbers of clumped and de-clumped NCTC 8578 cells (0.26 $\log_{10}$), and clumped and de-clumped B4 cells (0.42 $\log_{10}$), removed approached statistical significance in both cases (paired t-test, $P=0.0532$ and $P=0.0829$, respectively), indicating that single MAP cells may be more readily sedimented during centrifugation than clumps of MAP cells, possibly as a consequence of the effects of entrapped air in intercellular spaces impacting on the buoyant density of clumps. However, as the data were acquired by laboratory methods simulating commercial centrifugation conditions, the efficacy of centrifugation for removal of MAP still needs to be verified by experiments employing appropriate pilot-scale plant and spiked or naturally infected milk.

Microfiltration
Membrane technologies were introduced into the dairy industry around 1970. Initially fouling problems hindered commercial application within the dairy industry but with the advent of ceramic membranes microfiltration of skim milk became a viable commercial process. Filtered milk is available commercially in a number of countries under the PurFiltre™ brand name. Typically for skim milk a pore size of 1.4 μm is used commercially. In theory, clumps of MAP should be efficiently removed from skim milk by microfiltration using a pore size of 1.4 μm. In a recent study simulated microfiltration using a 1.2 μm porosity filter achieved a reduction of between 1.3 and 3.4 $\log_{10}$ in numbers of MAP present in broth suspensions depending on strain, corresponding to the removal of 94.6-99.9% of cells present (Grant et al. 2005b). Although attempts were made to filter spiked skim milk, the 1.2 μm filter clogged up instantly preventing the filtration of no more than a few drops of spiked milk. In commercial microfiltration equipment this fat clogging problem would be overcome by cross-flow plant configuration and a pressure differential across the membrane, which it was not possible to simulate in the laboratory. The efficacy of microfiltration for removal of MAP from skim milk would need to be verified by experiments employing appropriate pilot-scale plant and spiked or naturally infected milk. However, it is important to note that microfiltration can only be applied to skim milk so MAP in the cream fraction would still need to be inactivated by heat treatment.

Cheesemaking
Cheese manufacture may be considered as a dehydration process in which the casein (protein) and fat of milk are concentrated, approximately tenfold. Conversion of milk into cheese involves several distinct but interrelated operations including coagulation, acidification, syneresis (separation of whey), dehydration, moulding, pressing and salting. A 10-fold concentration in MAP numbers occurs upon curd formation such that a level of 10 MAP/ml in milk ends up as 100 MAP/g of cheese (Donaghy et al. 2004). Donaghy et al. (2004) also reported that 1-4% of MAP cells present were lost in whey fraction. Survival of MAP in four varieties of laboratory-produced cheese has been studied: Queso Fresco (Sung and Collins 2000), Swiss

Tilsiter and Emmentaler (Spahr and Schafroth 2001), and Cheddar (Donaghy et al. 2004). All three studies reported that MAP counts decreased slowly but steadily over the ripening period in each of the cheese varieties, the rate of which was influenced by factors such as temperatures applied during cheesemaking, pH, water activity (a_w) and NaCl concentration. Survival D_{10} values (time required for MAP numbers to decrease by 1 log_{10}) reported for MAP during ripening were: 36.5-59.9 d for Queso Fresco (Sung and Collins 2000), 45.5 d for Swiss Tilsiter (Spahr and Schafroth 2001), 27.8 d for Swiss Emmentaler (Spahr and Schafroth 2001) and 90-107 d for Cheddar (Donaghy et al. 2004).

Other potential processes for liquid milk
The impact of three processes that may have future commercial application for liquid milk processing on MAP inactivation has been investigated: gamma irradiation (Stabel et al. 2001), pulsed electric fields (Rowan et al. 2001) and high hydrostatic pressure (Sevilla et al. 2005). Stabel et al. (2001) reported that 10^6 MAP in raw milk were destroyed by 5, 10 or 15 kGy doses of gamma radiation. Rowan et al. (2001) reported that a 5.9 log_{10} kill of MAP in milk was achieved by 2500 pulses at 30 kV/cm in a 25 min period. A pressure treatment of 500 MPa for 10 min at 5 or 20^oC was shown by Sevilla et al. (2005) to effect a 4.8 log_{10} kill of MAP in milk. Whilst in theory all three processes could be possible alternatives to pasteurisation for milk, the impact of the processing conditions stated above on the organoleptic properties, and hence consumer acceptability, of milk would need to be assessed before commercialisation. Processing costs, relative to HTST pasteurisation, would also be an important consideration for the dairy industry.

DISCUSSION

To my knowledge, the above represents all the information on the occurrence of MAP in foods and the impact of milk processing on its survival currently in the public domain. As liquid milk has been the main focus of surveillance and research over recent years we know most about this food commodity. The results of recent studies indicate that MAP is present in raw cows' milk at detectable levels in various parts of the world and that HTST pasteurisation may not achieve 100% inactivation of MAP cells present on every processing occasion. Unfortunately, as current cultural methods do not permit the accurate enumeration of MAP in milk, we are unable to state with any confidence that if milk contains less than 'x' CFU/ml then HTST pasteurisation, or HTST pasteurisation in combination with some other process (such as homogenisation, centrifugation, microfiltration, cheesemaking), will completely inactivate this potential human pathogen. Information about the occurrence of viable MAP in other dairy products is not very comprehensive at present. Whilst the first reports of viable MAP in retail Feta cheese and powdered infant formula have recently been published, many more dairy products exist which have not been looked at, for example spray-dried milk, yoghurt, other cheeses (particularly those manufactured from raw milk) and UHT milk. Future surveillance/research efforts should, in my opinion, focus on dairy products and also on beef, rather than on liquid milk.

Clearly, if a potential food safety issue exists with a particular foodstuff the food industry needs to know about it to be able to take action to resolve the situation. It is vitally important that methods used to recover MAP from foods other than liquid milk are thoroughly evaluated prior to use to ensure maximal recovery of MAP from a different food matrix and sufficient detection sensitivity. When methods used to culture MAP from animal faeces were originally used to recover MAP from milk many false negative results were obtained because decontamination was too harsh and recovery of MAP from milk was not optimal. As decontamination methods for milk were evaluated and optimised the real picture about MAP in milk has emerged. Methods adopted in future studies of dairy products and beef must be capable of detecting MAP if present, otherwise false negative results could lull the dairy or beef industries into a false sense of security about the safety of their products.

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PCR evidence of MAP in Blau syndrome patient tissues

Dow CT

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ABSTRACT

Blau syndrome is familial, juvenile, systemic granulomatosis with primary clinical findings of uveitis, arthritis, and dermatitis. Although rare, Blau syndrome shares aspects of the more common diseases sarcoidosis and Crohn's disease. The clinical findings of Blau syndrome are similar to those of juvenile sarcoidosis and mutations of Blau syndrome are on the same chromosome 16 gene that confers susceptibility to Crohn's disease, CARD15. The product of this gene is a component of the innate immune system involved in bacterial pathogen surveillance. *Mycobacterium avium ss. paratuberculosis* (MAP) has been implicated as a causative agent of sarcoidosis and Crohn's disease. The presence of MAP in Blau syndrome tissue was postulated and archival paraffin block tissues of five individuals with Blau syndrome were acquired to test for the DNA of MAP. Six tissues of five patients representing three family members were tested. The tissues were granulomatous lesions of skin, synovium, liver and kidney. The six tissues were IS*900* positive and three were also hspX positive.   The presence of MAP DNA in this multi-system disease extends the argument for the disease-causing role of MAP beyond the gut and positions MAP for consideration as a "superantigen".

Key Words: MAP, CARD, Crohn's, Blau

INTRODUCTION

Blau syndrome is familial juvenile systemic granulomatosis (Blau, 1985). Although rare, Blau syndrome has been of interest in current medical literature because of the discovery that its genetic defect is on the same gene that confers susceptibility to Crohn's disease (Hampe et al., 2002, Miceli-Richard et al., 2001). Linkage studies by Hugot et al. (2001) have placed the gene on chromosome 16; originally referred to as the NOD2 gene, it is now known as the CARD15 gene. The Blau susceptiblility component of the CARD15 gene resides in the nucleotide binding domain (Hampe et al. 2002; Wang et al. 2002) while the Crohn's susceptibility is in the N-terminal leucine-rich repeat (Hugot et al. 2001; Lesage et al., 2002). In addition to Crohn's disease and Blau syndrome, mutations of the CARD15 gene have been linked to psoriatic arthritis (Rahman et al., 2003). The CARD15 gene is part of the ancestral innate immune system that senses and eliminates bacteria (Inohara et al. 2003; Girardin et al. 2003) and is part of the newly recognized CATERPILLER gene family that acts as sensors to detect pathogens and regulate inflammatory and apoptotic responses (Ting and Davis, 2005). Blau syndrome is unique in that it is the only systemic granulomatous disease that has a recognized Mendelian pattern of inheritance: autosomal dominant (Tromp et al., 1996).

The landmark progress represented by associating this gene with systemic granulomatous disease prompted studies of other granulomatous diseases to look for a similar defect (Miceli-Richard et al., 2001: Ogura et al., 2001) While the CARD15 defect confers susceptibility to Crohn's disease, Blau syndrome and psoriatic arthritis no similar defect was found in patients with sarcoidosis (Martin et al., 2003), ankylosing spondylitis (van der Paardt et al., 2003) Wegeners' granulomatosis (Newman et al., 2003), systemic lupus erythematosis (Ferreiros-Vidal et al., 2003a) or rheumatoid arthritis (Ferreiros-Vidal et al., 2003b).

Mycobacterium avium ss. paratuberculosis (MAP) is an obligate intracellular organism that causes a transmural enteric granulomatous disease in ruminant animals known as Johne's disease (Harris and Barletta, 2001; Collins et al., 1994). MAP has been implicated in Crohn's disease, a transmural,

granulomatous disease of humans (Hermon-Taylor, 2000; Greenstein, 2003; Shafran et al., 2002; Ogura, 2003; Torok et al., 2003).

The traditional methods of detecting bacteria, i.e. culture and stain, are largely ineffective in detecting MAP in humans. The bacteria are very difficult to isolate through culture and MAP is able to exist in a spheroplast (cell-wall deficient) form in humans (Sechi et al., 2004; Wall et al., 1993; Hines and Styer, 2003). The advent of bacterial DNA detection with polymerase chain reaction (PCR) has greatly aided the detection of mycobacteria (Lachnik et al., 2002; O'Mahony and Hill, 2002). A report by Bull et al. (2003) detected the DNA of MAP in greater than 90% of biopsy specimens from individuals with Crohn's disease. Naser et al., (2004) reported that when employing newer culture methods he detected MAP circulating in the blood (bacteremia) of a substantial number of patients with Crohn's disease.

In addition to Crohn's disease MAP is also implicated in sarcoidosis as reported by el-Zaatari, et al. (1996). Because of the association of Blau syndrome with the gene conferring susceptibility to Crohn's disease and because clinical findings of Blau syndrome are evocative of juvenile sarcoidosis, it was postulated that MAP might play a role in Blau syndrome.

Materials studied
Several clinicians who had published articles featuring Blau syndrome granulomas were contacted about providing representative Blau tissue for MAP DNA probing. Three clinicians sent tissues: paraffin blocks of Blau skin, synovium and liver granulomas (Saini and Rose, 1996; de Chadarevian et al., 1993) and unstained slides of Blau skin and renal granulomas (Ting et al., 1998). Six different tissues from five patients representing three family members were received and subjected to an DNA probe for MAP (IS*900* and hspX) at the Marshfield Clinic, Marshfield, Wisconsin. Insertion sequences are small genetic elements that are used to identify organisms. IS*900*, the most commonly recognized DNA sequence associated with MAP, has multiple copies within the MAP genome (Harris and Barletta, 2001; Greenet al., 1989). An experimental probe, hspX, has been characterized by Ellingson (1998); this marker is more specific for MAP as there is only a single copy within the MAP genome.

MATERIALS AND METHODS

(As performed by the Food Safety Lab at the Marshfield Clinic, Marshfield, Wisconsin, USA)
Patient tissues mounted on slides were prepared as follows: each tissue section was scraped from the glass slide into a sterile microcentrifuge tube using a sterile scalpel. Sections of the same tissue from different slides were pooled. Patient tissues embedded in paraffin blocks were effaced until complete tissue sections were obtained, then five, 5 micron sections of each paraffin-embedded tissue was cut and placed into a sterile microcentrifuge tube. TE buffer (100 μl; pH 7.5) was then added to each tissue sample and vortexed for one minute. The tubes containing samples were then placed into a boiling water bath for 10 minutes and then immediately vortexed for two minutes. The 10-minute boiling step followed by two minutes of vortexing was repeated twice (a total of three times). Tubes were boiled for an additional 5 minutes, cooled to room temperature and then centrifuged at 10,000 rpms for10 minutes. DNA was extracted from 100 ul of the supernatant using the Magna Pure LC DNA Isolation Kit III (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. DNA was amplified according to the parameters described by Miller, et al. (1999). Amplified PCR reactions were analyzed by agarose gel electrophoresis (1.5 % agarose gel). All products were compared to a standard molecular weight ladder and the positive control to determine amplicon size. A sample was considered positive if amplified product was noted at 229 bp for the IS900 primer set and 211 bp with the hspX primer set.

RESULTS

Results of the PCRs for MAP using six paraffin block tissues from five patients with Blau syndrome are found in Table 1 and in Fig. 1. All samples were test-positive for MAP IS*900*. Three of six tissues tested positive for hspX; these samples came from patients that were related.

Table 1. Results of DNA probing for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Blau syndrome tissues. Gel lanes referenced in Fig. 1.

Source	Rose		DeChadarevian		Ziegler	
Patient	Mother	Child	Both from same patient		Mother	Child
Tissue	Liver	Synovium	Synovium	Skin	Kidney	Skin
IS*900*	+	+	+	+	+	+
hspX	-	+	+	-	+	-
Gel lanes	2, 3, 4	5	6	7	8, 9, 10	11
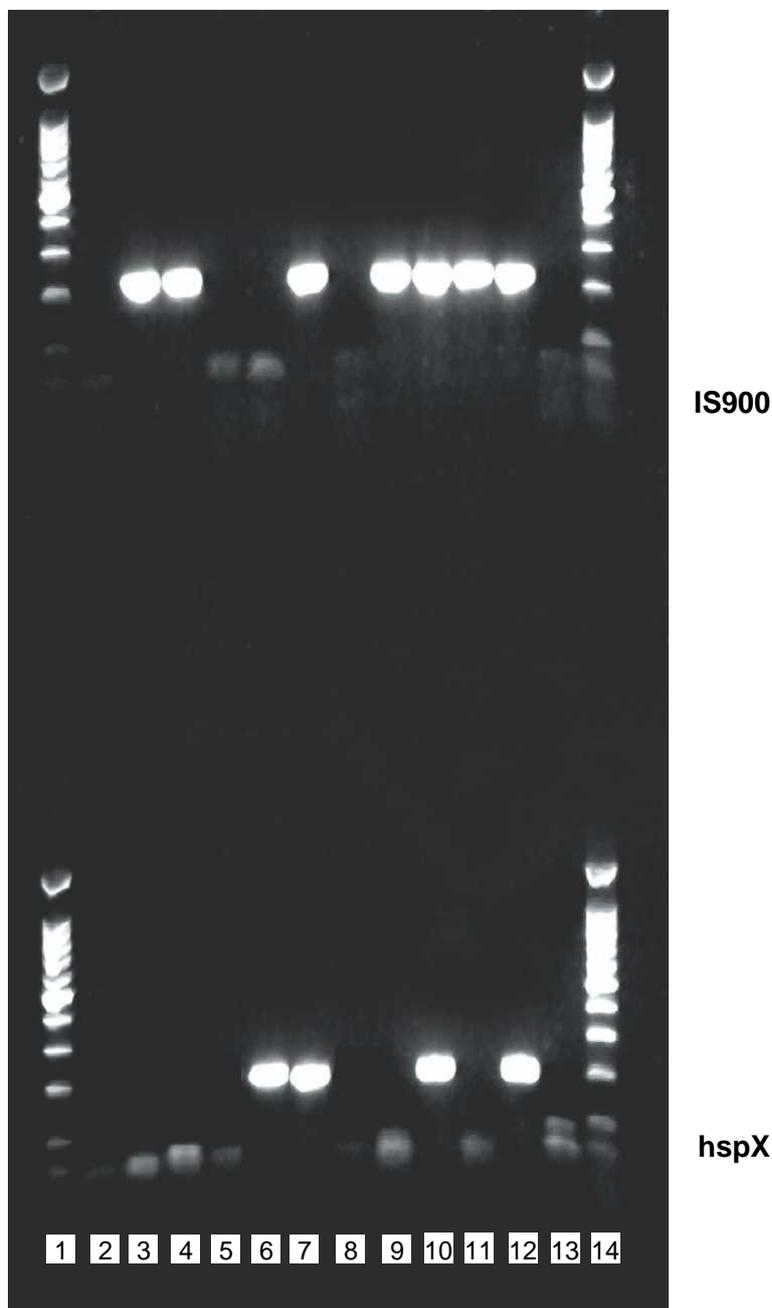
Fig. 1. Lane 1: 100 bp ladder; Lanes 2 to 11: See Table 1; Lane 12: Positive control MAP - ATCC 19698; Lan[...] Negative control; Lane 14: 100 bp ladder

DISCUSSION

The presence of MAP in Blau syndrome tissues was considered possible because of the genetic connection to Crohn's disease and because of its clinical similarities to sarcoidosis. DNA probes have provided a tool to investigate the link between MAP and Crohn's disease. The elucidation of CARD15

mutations (limiting an effective response to invading pathogens) strengthens this link. Recent studies featuring genetic testing of patients with "sporadic" Blau syndrome show CARD15 defects similar to families with autosomal dominant graulomatous disease (van Duist et al., 2005, Rose, 2005). Although the data in this study are small in number and patient controls were not included, they are important for two reasons: 1) the presence of MAP DNA in affected Blau syndrome tissues was predictable because of the Blau genetic defect and 2) the presence of MAP in this disease can be used to justify studying other inflammatory diseases, particularly those that share genetic defects with Crohn's disease to determine if MAP is present in the affected tissues. Further study of Blau patients' fresh tissues and blood for evidence of MAP as Naser et al. (2004) has done from Crohn's patients will provide additional data concerning the link between this bacterium and human inflammatory disease.

CONCLUSION

The presence of MAP DNA in Blau syndrome (an autosomal dominant, systemic, inflammatory disease) connects genetic and environmental aspects of "autoimmune" disease and positions MAP for consideration as a "superantigen".

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A proposed link between MAP and Type I Diabetes - Mapping the TRIGR

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is an autoimmune disease having complex genetic inheritance and proposed environmental factors contributing to its etiology. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the cause of bovine Johne's disease and also is thought to act as an immune antigen in Crohn's disease and other granulomatous diseases. This essay discusses three separate links that may tie mycobacteria to T1DM: shared genetic susceptibilities, epitope homology allowing molecular mimicry and epidemiologic studies. The paper postulates a causative role for MAP in T1DM in the genetically at risk.

Key words: T1DM, MAP, NRAMP, VDR, GAD, Hsp65, TRIGR.

INTRODUCTION

Type 1 diabetes (TIDM) is thought to be caused by genetic and environmental factors. It is an autoimmune disease in which T lymphocytes infiltrate the islets of pancreas and destroy the insulin producing beta cell population (Akerblom et al., 2002). This paper postulates a causative role for *Mycobacterium avium ss. paratuberculosis* (MAP) as an environmental agent that could trigger T1DM in a susceptible individual. Three links are offered to support this postulate: 1) recent immunogenetic discoveries that tie several autoimmune diseases, including T1DM, to susceptibility to mycobacterial disease, 2) epitope homologies between mycobacterial elements and pancreatic glutamic acid decarboxylase (GAD), and 3) an alternative interpretation of the epidemiologic findings that launched a large study, the Trial to Reduce Type 1 Diabetes in the Genetically at Risk (TRIGR).

MAP
Mycobacterium avium ss. paratuberculosis (MAP) is an obligate intracellular organism that causes an enteric granulomatous disease, Johne's disease, in ruminant animals (Harris and Barletta 2001; Collins, et al., 1994). MAP has been implicated in Crohn's disease, a transmural, granulomatous disease of humans (Hermon-Taylor, 2000; Greenstein, 2003; Shafran, et al. 2002; Ogura, 2003; Torok, et al., 2003).

Traditional methods of detecting bacteria, culture and stain, have largely been ineffective in detecting MAP in humans. The bacteria are very difficult to culture and MAP is able to exist in a spheroplast (cell wall-deficient) form in humans (Sechi, et al., 2004; Wall, et al., 1993; Hines and Styer, 2003). The advent of bacterial DNA detection with polymerase chain reaction (PCR) has greatly aided the detection of mycobacteria (Lachnik, et al., 2002; O'Mahony and Hill, 2002). Bull, et al. (2003) detected the DNA of MAP in greater than 90% of biopsy specimens from individuals with Crohn's disease. Naser, et al., (2004) reported that by employing newer culture methods he detected MAP circulating in the blood (bacterimia) in a number of patients with Crohn's disease; and, viable MAP was recently shown by Ellingson (2005) to survive pasteurization in retail milk.

Genetics and T1DM
Onkamo, et al., (1999) analyzed multiple populations and showed that TIDM is increasing at an incidence of 3% per year since 1960. Historically, genetic association with TIDM has been established for three chromosomal regions: HLA DQ/DR (IDDM1) and INS VNTR (IDDM2). (Eerligh, et al., 2004). and CTLA-4 (cytotoxic lymphocyte antigen-4), (Hornum, 2004).

More recently, susceptibility genes for T1DM have expanded to include the NRAMP-natural resistance-associated macrophage protein gene (also known as SLC11A1) (Bassuny, et al. 2002, Takahashi, et al., 2004) as well as the VDR gene (vitamin D receptor). (Motohashi, et al., 2003), (Hayes, et al., 2003)

NRAMP
Nramp (natural resistance-associated macrophage protein) is a gene that encodes a divalent cation transporter in phagosomes of macrophages (Blackwell, et al., 2000). NRAMP modulates the cellular environment in response to cell activation by intracellular pathogens by acidifying the phagosome (Lapham, et al., 2004). As such, it plays a role in host innate immunity (Wyllie, et al., 2002). Mutation of Nramp1 impairs phagosome acidification yielding a permissive environment for intracellular bacteria as shown by Hackam, et al., (1998).

VDR
In addition to a role in the regulation of bone and mineral metabolism, Vitamin D is a potent modulator of the immune system. (Zella and DeLuca, 2003). Vitamin D activity occurs via the vitamin D receptor (VDR). VDR is part of the steroid receptor super-family and is widely express in many cell types including lymphocytes, macrophages and the insulin producing pancreatic beta-cells as reviewed by Hayes, et al., (2003).

Vitamin D and its receptor, VDR, have been implicated in the pathogenesis of TIDM: VDR gene polymorphisms have been described in TIDM in Taiwanese (Chang, et al., 2000), Indian Asians (McDermott, et al., 1997), Germans (Pani, et al., 2000), Spaniards (Marti, et al., 2004), Japanese (Ban, et al., 2001) and Croatians (Skrabic, et al., 2003). Additionally, DeLuca and Cantorna (2001) report that calcitriol, the hormonal form of vitamin D, can either prevent or markedly suppress experimental TIDM.

In addition to T1DM, NRAMP and VDR polymorphisms are also associated with other autoimmune diseases (DeLuca and Cantorna, 2001), (Hayes, et al., 2003) and, most notably, with mycobacterial infection (Bellamy, 2003, Hill, 1998, Hayes, et al., 2003).

Molecular Mimicry
Davis (1997) proposed that epitope homology between infectious agents and host proteins allow molecular mimicry that can induce autoimmune disease and, though noting a lack of evidence, included T1DM in his discussion. Wucherpfennig and Strominger, (1995) postulated that cross-reactive microbial antigens in a genetically susceptible host is the critical event leading to the autoimmune destruction of insulin-producing beta cells of the pancreas.

Hsp65 is a highly conserved heat shock protein unique to mycobacteria (Zugel, 1999). Child, et al., (1995) noted the important role that heat shock proteins (Hsp) play in autoimmunity and infection. He also notes that glutamic acid decarboxylase (GAD) is the prime antigen of Type 1 diabetes, has similar amino acid sequences to Hsp65. He concluded that Hsp65 "should not be completely discarded as having a possible role in the development of Type 1 diabetes".

Scheinin, et al., (1996) studied children with newly diagnosed Type 1 diabetes and found responses to mycobacterial Hsp65 in all 47 individuals tested. He found significant correlation between anti-GAD antibodies and proliferation of peripheral blood mononuclear cells to Hsp65.

Epidemiologic Evidence
Several studies indicate an association between early exposure to dietary cow's milk proteins and an increased risk of TIDM; representative are: Akerblom and Knip, (1998), Gerstein, (1994) and Gimeno and deSouza, (1997). These studies centered on an observation: children at risk for TIDM who were breast fed exclusively for more than six months were less likely to have TIDM later in life than similar risk children who were weaned onto cow's milk based formula at an earlier age. This observation lead to the TRIGR study: Trial to Reduce IDDM in the Genetically at Risk. Driving the study is the postulate that there is something about cow's milk protein that is the immunologic trigger for TIDM.
The TRIGR study (www.trigr.org) is an ongoing 17-country study enlisting 6200 infants genetically at risk to develop TIDM. Children weaned early from breastfeeding are randomized into two groups, one receiving

traditional cow's milk-based formula and the other receiving formula in which the protein has been hydrolyzed.

DISCUSSION

This paper postulates that the presence of MAP may contribute to the pathogenesis of T1DM. Genetic evidence suggests that there are states of macrophage dysfunction that promote both T1DM and mycobacterial infection. These states can be viewed as templates of macrophage incompetence that individually or in combination allow obligate intracellular pathogens to persist and serve as immune antigens. Homology between the diabetic marker, pancreatic GAD, and mycobacterial Hsp65 permit the possibility of a mycobacterial tie to T1DM.   Viable MAP has been found in commercial milk; MAP has been suggested as a possible cause of Crohn's disease. The epidemiologic association of TIDM with early exposure to cow's milk has prompted the large TRIGR study. The hypothesis offered here is that MAP may be viable in infant milk formula and can act as an immune antigen, a trigger, of TIDM.

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High frequency of *Mycobacterium avium* subsp. *paratuberculosis* DNA in inflammatory bowel disease patient blood and controls detected by nested PCR

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ABSTRACT

Crohn's disease (CD) is one form of inflammatory bowel disease (IBD), a chronic intestinal disease which also include ulcerative colitis (UC) and indeterminate colitis (IC). IBD is becoming a major health problem in developed societies as incidence rates rise and the age of onset decreases. Although the etiology of CD has never been fully clarified, the most accepted theory recognises CD as an autoimmune disease. Its similarity in symptoms to ruminant paratuberculosis and the isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from gut, lymphoid tissue and blood of CD patients suggests that MAP could play an important role in the pathogenesis of this illness. This study was aimed at finding microbiological and immunological evidences of an association between MAP and IBD in the patients and controls from Gipuzkoa in the Basque Country, Spain. A blind assay was designed and carried out to include DNA extraction of buffy coat blood cells and nested PCR for the amplification of IS*900* from 61 patients and 80 healthy blood donors, as well as a -IFN stimulation ELISA test with PPA-3 MAP antigen. The study found that 41.0% of IBD patients (CD: 40.7%, UC: 41.2%) and 42.5% of controls had buffy coat cells that were MAP PCR positive to IS*900*. These findings are generally in agreement with previous works even though they show strikingly higher test-positive results among healthy controls. IBD patients showed increased -IFN production compared to controls when lymphocytes were incubated with MAP antigen, whereas no significant differences were found when incubated with concanavalin A (ConA). A ratio of the MAP to ConA response showed no significant differences between controls and IBD patients. However, a significant MAP/ConA ratio difference was noted between PCR-positive and PCR-negative IBD patients. These results indicate the existence of a clear type I specific immune response among IBD patients related to the presence of MAP DNA in their peripheral blood.

Keywords: inflammatory bowel disease, Crohn's disease, *Mycobacterium avium* subsp. *paratuberculosis,* PCR, gamma interferon

INTRODUCTION

The term "inflammatory bowel disease" (IBD) groups a set of illnesses that have in common a localized inflammation of the intestines. Depending on the areas affected and other clinical signs, three main types are recognized: Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC). Although inflammation in UC is restricted to the intestine, patients with CD can have any part of their digestive tract affected, from the mouth to the anus. In contrast to other types of enteritis of known infectious origin, the aetiology IBD is not established. The most widely accepted hypothesis is a multifactorial disease where an anomalous immune response induces a regional inflammation. Current therapy protocols consist of anti-inflammatory and immunosuppressive medication, failure of which frequently ends with the surgical removal of the affected digestive tract. The number of cases of IBD is increasing all over the world (Grant, 2005; Wells and Blennerhassett, 2005) and the age of onset is decreasing (Armitage et al., 2001). This might be due to a change in the epidemiology of these diseases, but also to the use of more sensitive diagnostic methods and an increased awareness of the disease.

Johne's disease (JD) is an intestinal disease that affects ruminants that is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Because of the similarities between JD and human inflammatory bowel disease there is a concern that MAP might be a causative agent of some cases of IBD, specifically in Crohn's disease (CD) (Bull et al., 2003; Chioodini, 1996; Collins et al., 2000; Grant, 2005; Greenstein, 2003, Lisby et al., 1994; Suenaga et al., 1995). Actually this putative connection was already made in the original description of the disease by Crohn and colleagues, back in 1932 (Crohn et al., 1932). Attempts made since then to demonstrate a link between both diseases have never been successful.

IBD and JD raise important economic problems. Cattle with Johne's disease are culled as soon as the disease is detected. Mortality from IBD is low, but the associated problems of chronic ill health, hospital admission, drug toxicity, and surgery present an important cause of morbidity. The recent isolation of MAP from blood of IBD patients (Naser et al., 2004) indicating bacteraemia could be interpreted as this infection's being a zoonosis, but this hypothesis is not yet widely accepted. Since we have previously studied the presence of MAP DNA in blood of cattle and sheep (Juste et al., 2005), and there has been a recent report of PCR detection of MAP in blood of IBD patients (Naser et al., 2004), we aimed to confirm these findings in a wider patient and control group setting. We also decided to analyse whether there was a Th1 MAP-specific immune response related to the detection of MAP DNA in blood (Collins et al., 2000; Suzuki et al., 2003; Wells and Blennerhassett, 2005).

MATERIALS AND METHODS

Subject recruitment
A total of 145 subjects were recruited in the Quirón Donostia Clinic and the Gipuzkoa Blood Bank as follows: 65 IBD patients belonging to the Gipuzkoa (Spain) local patient association (ACCU Gipuzkoa), and 80 healthy controls (blood donors). The IBD patients presented the following pathology: 27 with CD, 34 with ulcerative colitis, and 4 with indeterminate colitis. Informed consent was obtained in accordance with the relevant national and European regulations. The diagnosis of IBD was established based on standard clinical endoscopic, histological, and radiographic criteria by the treating physician and treatment. The current status of the disease was confirmed via a standard questionnaire completed by each patient in the presence of the clinician.

Blood sampling
Three 4 mL whole blood samples were obtained from every subject, of which two were drawn into sterile EDTA and one into heparin-litium Vacutainer tubes (BD). All blood samples were coded to conceal the patient's identity and diagnosis. All samples were processed within 4 hours after extraction in a class II biosafety cabinet.

Nested PCR
Genomic DNA was extracted from cells in the buffy coat. In order to obtain the buffy coat, blood was incubated with 0,83% ammonium chloride (1:2) for 20 minutes to lyse the red blood cells. Then the tube was centrifuged for 10 minutes at 200 x g and the cell pellet was washed twice with PBS by centrifugation under the same conditions. QIAamp DNA Blood Mini Kit (QIAGEN) was used. Purified DNA was stored at –20ºC until use.

A nested PCR protocol for amplification of insertion sequence IS*900* was used according to Naser et al. (2004). Briefly, primers P90 and P91 were used in the first round to amplify a 398 bp fragment of IS*900* and primers AV1 and AV2 in the second round to amplify a 298 bp internal sequence. For the first PCR reaction, 10 ng of genomic DNA were added to 40 µl of PCR buffer mixture. The PCR buffer mixture consisted of 5mM $MgCl_2$, 0.2 mM dNTP, 6% DMSO, 2 µM primers and 2.5 U of Taq Polymerase (Invitrogene Ltd., Paisley, UK). In the second round all conditions were the same except that 5 µl of the PCR product from the first round were used as DNA template. The PCR cycling conditions were 95ºC for 5 min, 34 cycles of 95ºC for 1 min, 58ºC for 1.5 min, 72 ºC for 1.5 min, and a final extension phase of 10 min at 72 ºC. The products were resolved by gel electrophoresis in a 2% agarose gel for 50 min at 150 V. A band of the expected 298 bp size matching the positive control was considered as a positive result. Primary and secondary rounds of PCR were done in separate rooms.

To double check the identity of the amplified products, the target 298 bp band from 2 positive healthy controls and 2 IBD patients was cut out, purified with a GFX PCR DNA and Gel Band purification kit (Amersham Biosciences, Buckinghamshire, UK) and nucleotide sequencing was used to verify the specificity of the amplified IS*900* fragment by BLAST and alignment sequence analyses.

Gamma-interferon ELISA
Three 1400 µl aliquots of blood were transferred to 1.5 ml Eppendorf tubes**.** To each tube, 100 µl of either 30 µg/ml MAP PPA-3 antigen (Allied Monitor, Fayette, MO, USA), 30 µg/ml concanavalin A (ConA) (SIGMA, USA), or PBS, respectively, were added. All tubes were incubated overnight at 37°C. After incubation, plasma was collected and stored at –20°C until use with a commercial -IFN ELISA assay. Upon plate validation, PPA-3 and ConA OD readings as well as the MAP PPA-3/ConA ratio (M/C) were used for statistical analysis.

Statistical analysis
Comparisons of frequencies for overall IBD, IBD specific form, activity, lesion location and PCR results were made using the Fisher exact test. Gamma-interferon tests were related to these variables and were analyzed by different General Linear Model Procedures. The means comparisons were carried out by a Duncan test at a $p < 0.05$ significance level or a Student t test for marginal means. All statistical analyses were made with the SAS statistical package (SAS Institute Inc., Cary, NC 27513, USA).

RESULTS

The code concealing subjects' identities and diagnoses was broken and data were compared after the conclusion of all experiments. The IBD subjects ranged from 16-65 years, the average age being 38.7 ±11.3. Time from onset of disease was 10.9±8.5 years at the time of study, ranging between 1 and 47 years. Females accounted for 64.6% of the IBD patients. 27/65 IBD patients were CD (41.5%) and 34/65 were UC (52.3%). Four patients with indeterminate colitis (6.2%) were excluded from the study. Forty two patients (31.1%) had inactive IBD disease. Twelve of the IBD patients were not on medication when the study started (7 CD, 5 UC and 1 IC). The rest of IBD patients were being treated with a variety of drugs including mesalazine (47.7%), azathioprine (10.2%), prednisolone (10.2%), budesonide (9.1%) and others (9.1%).
Blood samples that were positive for the MAP-specific IS*900* element clearly showed a single bright 298 bp band on 2% agarose gel (Fig. 1). The sequenced bands showed a 100% similarity to the published IS*900* sequence (data not shown). MAP DNA was detected in 59/141 (41.8%) of all blood samples. The fifty nine IS*900* (+) samples were distributed in the different groups as follows: 11/27 (40.7%) patients with CD, 14/34 (41.2%) patients with UC, and 34/80 (42.5%) of healthy controls.

Table 1. PCR results according to type of disease, activity and lesion main location.

	Overall IBD		
	IBD		**Control**
PCR +ve	25 (41.0%)		34 (42.5%)
PCR -ve	36 (59.0%)		46 (57.5%)
	Specific Form		
	CD	**UC**	**Control**
PCR +ve	11 (40.7%)	14 (41.2%)	34 (42.5%
PCR -ve	16 (59.3%)	20 (58.8%)	46 (57.5%)

	Activity			
	Moderate	**Mild**	**Inactive**	**Control**
PCR +ve	2 (28.6%)	5 (41.7%)	18 (42.9%)	34 (42.5%
PCR -ve	5 (71.4%)	7 (58.3%)	24 (58.1%)	46 (57.5%)

	Lesion Main Location		
	Colon	**Ileum**	**Rectum**
PCR +ve	11 (52.4%)	7 (35.0%)	7 (35.0%)
PCR -ve	10 (47.6%)	13 (65.0%)	13 (65.0%)

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: ulcerative colitis
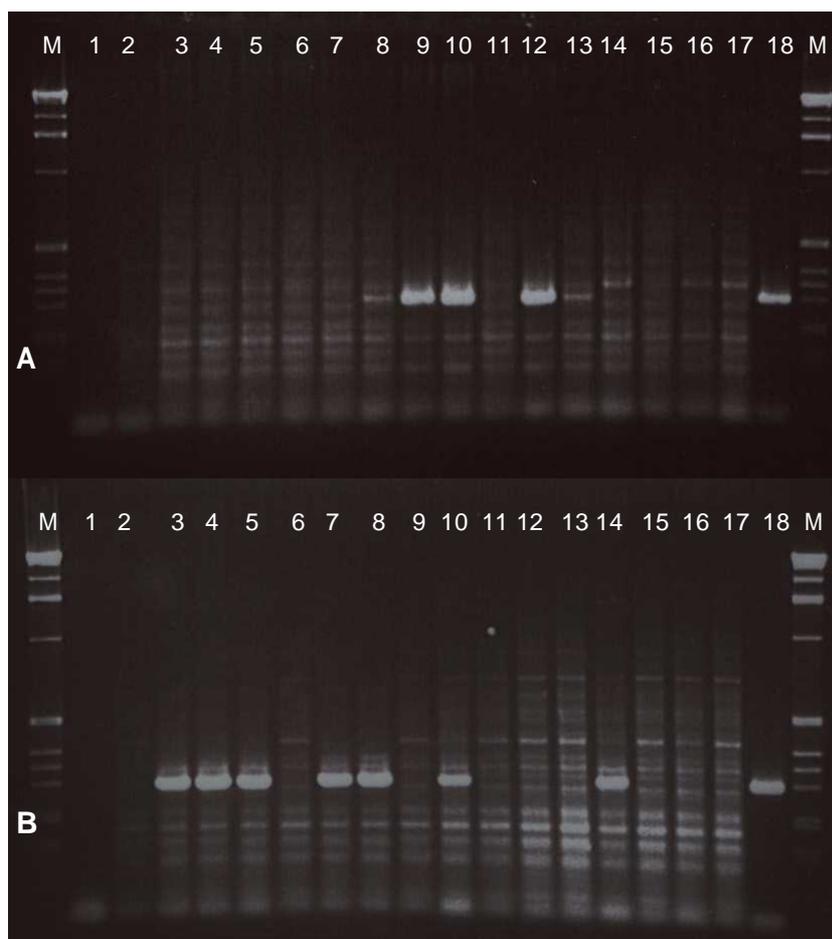
Fig. 1. Nested PCR results of MAP DNA from peripheral blood samples. (A) Controls (B) Patients. (A) M=molecular weight marker, Lane 1 negative control of first round PCR, Lane 2 negative control of second round PCR, Lanes 3-17 healthy controls, Lane 18 DNA from MAP strain ATCC 19698. (B) M=molecular weight marker, Lane 1 negative control of first round PCR, Lane 2 negative control of second round PCR, Lanes 3-17 IBD patients, Lane 18 DNA from MAP strain ATCC 19698.

In total, for IBD patients 25/61 (41.0%) showed IS*900* positive reactions (these results are summarised in Table 1). None of these frequencies were significantly different.

Table 2. Results of -IFN ELISA according to disease type and PCR results.

IBD type			IFN *Map*		IFN ConA		*Map*/ConA	
		Mean	0.083[a,b]		0.190[a]		0.951[a]	
	CD	N	27	0.088*	27	0.149	27	0.989
IBD		SD	0.070	61	0.548	61	0.466	61
		Mean	0.092[a]	0.078	0.120[a]	0.389	1.018[a]	0.460
	UC	N	34		34		34	
		SD	0.085		0.193		0.460	
Control		Mean	0.061[b,*]		0.076[a]		1.013[a]	
		N	80		80		80	
		SD	0.030		0.081		0.552	
PCR result			**IFN *Map***		**IFN ConA**		***Map*/ConA**	
+		Mean	0.071		0.075		1.104*	
		N	59		59		59	
		SD	0.047		0.070		0.631	
Neg		Mean	0.074		0.130		0.929	
		N	82		82		82	
		SD	0.064		0.341		0.394	

IBD: Inflammatory bowel disease; Control: Healthy blood donors; CD: Crohn's disease; UC: Ulcerative colitis. Means with the same letter are not significantly different. * P<0.05

The general linear models were unable to explain all of the variability of the data. (R^2 below 0.10). Since Crohn's disease and ulcerative colitis behaved similarly in this analysis, they were treated together as a single IBD group for all remaining analyses (Column 4, Table 2). No significant IFN production differences between IBD patients and controls were observed when ConA was used for lymphocyte stimulation. However, significant IFN production differences between IBD and control groups were observed when MAP antigen was used (p=0.006). These differences disappeared when the ratio MAP/ConA (M/C) was used.
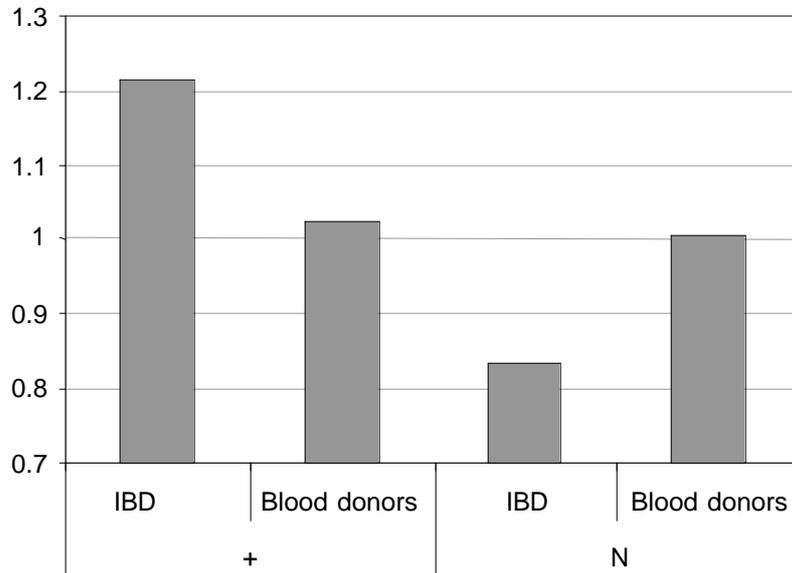
Fig 2. MAP/ConA ratio expression of -IFN results in IBD patients and controls by buffy coat PCR result.

When blood PCR results were introduced into the model, the R^2 values increased substantially, but no change in the pattern of differences was observed. That is, significant differences were observed for IBD types versus controls only when MAP antigen was used (p=0.051 and p=0.014, for CD and UC, respectively). For neither the ConA data nor the M/C ratio were significant differences observed. However, the PCR result had a clear effect on the M/C ratio (p=0.022): the PCR-positive group showed a higher M/C ratio than the PCR-negative group. There was also a strong association between PCR result and IBD status: (p=0.007) PCR-positive IBD patients' M/C ratio differed significantly from PCR-negative IBD ratios (p= 0.004). This was not true for controls whose M/C ratio was independent of their PCR test status (Fig. 2).

DISCUSSION

The data presented in this study indicate that genetic and environmental factors might be more relevant for IBD susceptibility and progression than simple exposure to MAP, since MAP DNA could be detected in the bloodstream of an important proportion of both IBD and healthy subjects through nested PCR. The absence of correlation of IS*900* positivity with onset of disease, however, could be due to methodological problems: in order to achieve statistically significant results, our study was designed to be unbiased. It included all available patients, with no pre-selection made according to medical records, disease status, medication prescribed, etc. All relevant clinical data were collected with the hope of detecting potentially meaningful associations. Because of the approach selected, we can not discard the existence of an association at specific disease stages or in the absence of medication.

Recently published work by Naser et al. (2004) demonstrated a significant association between MAP bacteremia and Crohn's disease. Our results are in agreement with those of Naser et al. (2004) regarding the detection of IS*900* sequence in a proportion of IBD patients (45.9% in that study *vs* 41.0% in this study). This agreement extends to the two forms of IBD considered, CD and UC, which in both studies had a similar proportion of PCR-positive results. However, a major discrepancy was found in the control subjects; this study found double the PCR-positive frequency in healthy controls than was found by Naser et al. (2004) (20% vs 42.5%). We think that this discrepancy can be explained by the number of samples and type of controls used in both studies. In the present study there were 1.6 times more IBD patients and 7.2

times more controls. Our controls were all healthy blood donors while in the other publication no details were given about all but four controls with colon cancer, diverticulitis and gastroesophageal reflux.

Our results show that MAP DNA in blood samples is uniformly distributed among IBD patients and controls. This finding is consistent with the known environmental distribution of mycobacteria (30-50% of samples) in water, soil and air (Hisamatsu et al., 2003), suggesting a wide exposure of humans to these infections that remain mostly free of disease. In another study *Mycobacterium avium* subsp *silvaticum* (a species closely related to MAP) was found to be equally distributed among tissues of patients with CD, colon cancer or completely healthy people (Moss et al., 1992). However, other laboratories have found that CD patients tested positive significantly more often than controls (Lisby et al., 1994; Suenaga et al., 1995).

The interpretation in our case is that no association between MAP DNA detection in peripheral blood and clinical disease can be demonstrated from the simple comparison of frequencies. However, our experience in ruminants indicates that such a simple relationship is not to be expected in paratuberculosis, where it is widely accepted that clinical cases are only a small fraction of the total number of infected individuals. In a recent work we found that more than 25% of cows in an affected herd and 12% of ewes from flocks without a history of paratuberculosis had a positive result in blood using a single round PCR (Juste et al., 2005). Other works also indicate that clinical and subclinical animals account for about 50% of the individuals (Peréz et al., 1996; van Schaik et al., 1996). In this sense, if the pathogenesis model of paratuberculosis was applied to IBD, clinical cases would represent only a small fraction of the total infected population. Therefore, if cases are defined only on the basis of clinical manifestations, a high prevalence of positive results is to be expected among non-cases.

Regarding the high proportion of patients with PCR-negative results, it can be pointed out that bacterial isolation, which seemed to be slightly more sensitive in CD (Naser et al., 2004) was not included in this study. On the other hand, MAP DNA PCR detection in adult cattle and sheep blood with an ELISA positive result was only 5% and 33%, respectively, in a recent study (Juste et al., 2005). It is possible that bacteraemia is intermittent, and therefore even if MAP were involved in IBD, a close matching between MAP detection in blood and disease is not likely to occur.

Interpretation of -IFN results is controversial. In a study on cattle expression of -IFN encoding genes, expression was higher in ileal tissues from MAP infected cattle than in uninfected cattle (Coussens et al., 2001) whereas in a study with human samples, the -IFN release by peripheral blood leukocytes after MAP PPD antigen stimulation was significantly lower in CD patients than in UC patients or controls (Suenaga et al., 1995). The -IFN test was included in this study to seek further specific associations. These results indicated that IBD patients had a significantly stronger reactivity against MAP antigens than the healthy controls, whereas no significant differences were found when a non-specific stimulus was used (ConA). The use of the M/C ratio and the introduction of blood PCR results in the analytical model showed that the pattern of relative -IFN reactivity was associated with the PCR result. PCR-positive individuals produced higher M/C ratios, an increase believed to be due to the interaction between the blood PCR result and IBD status. That is, patients believed to be bacteremic (PCR positive) had a significantly increased response than did non-bacteremic patients (PCR negative). For controls, the M/C results were not associated with PCR results.

In our opinion, all these results are compatible with the existence of a link between MAP and IBD, but this link is not sufficient to establish a causal relationship. Genetic factors like NOD2 mutations, already associated with CD (Behr et al., 2004; Hisamatsu et al., 2004; McGovern et al., 2001; Ogura et al., 2001; Wells and Blennerhassett, 2005), should be considered of prime importance in the development of an altered immune response. Other genes like TLR4 and CD14 also appear to show an association with CD (Gazouli et al., 2005; Suzuki et al., 2003). Regarding the environmental factors, food, water or other routes re-exposition to MAP should also be taken into account. Lack of association with disease or lesion location seems more likely to be related to these factors.

In summary, the results of the current study, although not conclusive, lend further support to the hypothesis of an aetiological association between IBD and MAP. Most importantly experimental approaches for demonstrating such an association should take into account that the presence of MAP might be necessary for IBD, but that it may not be sufficient.

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Heat resistance of *Mycobacterium avium* subsp. *paratuberculosis* in skim milk and cream tested in a pilot plant pasteurizer

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ABSTRACT

Heating experiments reported in the literature show differing results about the heat resistance of MAP in milk. Presently, it seems to be clear that small numbers may survive HTST treatment. Most of the experiments were performed with whole milk, whereas in practice skim milk and cream are very often treated separately. The main objective of this study was to investigate the heat resistance of MAP in the substrates by applying a heating technology most comparable to commercial systems. Skim milk and cream were inoculated with a cocktail of five MAP strains of bovine, caprine and human origin at colony counts between 10^3-10^5 cfu ml^{-1}. Both liquid substrates were subjected to heat treatment in a pilot plant pasteurizer. Skim milk was heated between 67-90 °C and cream between 85-100 °C at holding times between 15-60 s. Detection by culture of surviving MAP was performed with 6-months' resuscitation/enrichment in modified Dubos medium and subsequent culture onto Herrolds egg yolk medium for another 6 months. Survivors were identified by acid fast staining and IS900 based PCR. In skim milk 54 of 93 experiments showed small numbers of survivors at any time-temperature combination tested. The reduction achieved from initial MAP concentration was between 3-7 log cycles. In 9 of 57 experiments with cream, small numbers of MAP also survived despite a reduction of up to 7 log cycles.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, heat resistance, pilot plant pasteurizer, skim milk, cream

INTRODUCTION

Heating experiments reported in the literature show differing results about the heat resistance of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk. Presently it seems to be clear that small numbers may survive HTST treatment (reviewed by Gould et al. 2004). Most of these experiments were performed with whole milk. However, in practice skim milk and cream are very often treated separately. The main objective of this study was to investigate the heat resistance of MAP in different liquid milk substrates by applying heating technologies most comparable to commercial systems. A wide range of temperature-time combinations was tested in order to reflect the processing protocols as they occur in industrial practice. The protocols were evaluated to find temperature-time combinations that best lead to a destruction of MAP in the different substrates under practical conditions.

MATERIALS AND METHODS

A short list of materials and methods except those differing from the exact descriptions are given in Hammer et al. 2002.

Bacterial strains
For the heating experiments a cocktail of the following MAP-strains was used:

Strain-no.	type	origin
DSMZ 44133	reference strain	bovine
OL	field strain	bovine, faeces
Niebuell	field strain	bovine, milk
ATCC 43015	reference strain	human (Linda)
M100/C	field strain	caprine

Field strains of bovine origin were identified by Ziehl-Neelsen stain, mycobactin J dependency and IS*900* based PCR. The Niebuell strain was isolated from an aseptically collected milk sample of a clinically diseased cow, so its presence was due to secretion vs. faecal contamination. The caprine strain was kindly supplied by Dr. Karen Stevenson, Moredun Research Institute, Scotland UK; the OL strain by the "Veterinäruntersuchungsamt Oldenburg" (Lower Saxony, Germany.) Strains were kept on Herrold's egg yolk medium (HEYM) in permanent culture and subcultured every eight weeks. In addition, stock cultures of each strain were stored at -80 °C in Dubos medium + 10% glycerol.

Pilot plant pasteurizer
Design. Exact dimensions of the pilot plant are given in Hammer et al. 2002. Figure 1 displays the construction scheme. The process water is heated by steam. As an electronic air pressure driven valve regulates the steam supply, the precise selected temperature can stably be maintained. In order to keep any possible risk of recontamination as low as possible, a counterflow of heat-treated and non-heated milk is prevented.
Basic technical data:
- holder for HTST: spiral shaped, inside tube diameter 7 mm
    a) total volume 0.223 l, tube length 3.5 m,
        for holding up to 30 s
    b) total volume 0.770 l, tube length 1.60 m,
        for holding up to 60 s
- heating: 60-135 °C, variable at 0.1 °C steps
- flow rate: 16-80 l/h, variable at 1 l/h steps
- holding time: 12-60 s average holding time, variable change of flow rate

$$average\ holding\ time = \frac{holder\ volume \times 3600}{flow\ rate}$$

Table 1 displays data on the residence time distribution for the two holding sections. The maximum residence time scattering means the time interval between the most rapid and the slowest milk components as they pass through the holding section outlet. The table shows calculations for some exemplary temperature-time combinations. The corresponding Reynolds numbers show that turbulent flow was achieved under all experimental conditions used. (The Reynolds number is a parameter for the turbulence of flow. Numbers > 2000 indicate that turbulent flow is present in a system.)

Table 1. Examples for residence time distributions for the holding sections of the pilot plant

Holding section	Average residence time	Minimum residence time	Standard deviation (%)	Reynolds-no.
223 ml	15.0 s	12.1 s	19.4	4926
	25.1 s	20.1 s	20.0	2950
770 ml	47.0 s	41.1 s	12.6	5423
	62.2 s	54.4 s	12.6	5636
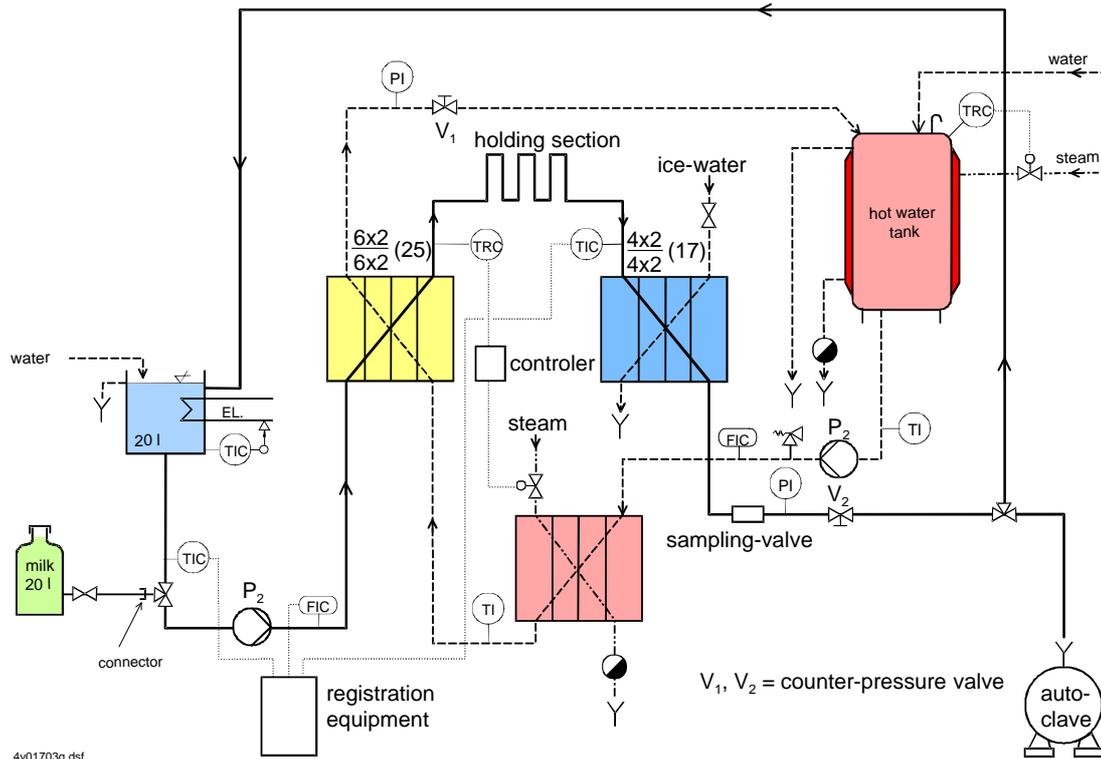
Fig. 1: Technical drawing of the pilot plant

Exclusion of plant cross-contamination

To make sure that the results were not biased by cross-contamination in the pilot plant, the following preventive measures and tests were applied:

- extended pressure at the product side (1-6 bar), at least 1 bar above the pressure at the process water side
- starting each run with the highest temperature
- within-run intermediate flushing with > 3 litre water (total volume of the unit 1.4 l). Before "counting" the water flush, the milk/water mixture was removed from the unit until the flush water was completely clear.
- cleaning and sterilization between runs (after run: 30 min alkaline at 80-90 °C, 10 min acid at 60 °C, 30 min potable water at 98 °C; before a run: 30 min potable water at 98 °C). The sample tanks were disconnected and autoclaved separately at 121 °C for 20 min.
- flush water, cooling water and process water were checked (0.2 µm filtration of 5 l water, culture, PCR and staining from filter retentates)
- leak controls of the plate apparatuses for heating and cooling were in place (7 bar overpressure, overnight)

Exclusion of cross-contamination in the laboratory

Every day an uninoculated, untreated milk sample was placed randomly among the samples from the heating experiments. It was treated just like the other samples in terms of centrifugation, decontamination, inoculation steps and incubation. In none of these samples was growth of MAP ever observed. Another hypothetical source for contamination in the laboratory is that mycobactin J (necessary as growth factor in the culture media) is usually made from MAP. The manufacturer certified that the protein mycobactin J is purified and chemically sterilized (statement of the supplier (Synbiotics Europe)).

Sample preparation

For inoculation of skim milk and cream each MAP strain was grown for 8-10 weeks in 400 ml modified Dubos medium. Bacteria were harvested from the growth medium by centrifugation for 15 min at 2200 x g, and pellets resuspended in 0.85 % (w/v) NaCl by gentle shaking and mixing. They were not homogenized prior to further use. Raw skim milk was obtained from raw milk of the Federal Research Centre for Nutrition and Food's experimental dairy. Cream was purchased from a local supplier (30% fat, heated 15-20 s at 105 °C). Samples were inoculated directly with the strain cocktail and continuously stirred with a magnetic stirrer

in the sample tank of the pilot plant during the heating run. Colony counts prior to heating were estimated by direct plating onto HEYM.

Heating experiments
The experiments performed with the different substrates are listed in Table 2. One experiment within a series is defined by a certain time-temperature combination of heating with a cocktail of MAP strains and a certain inoculum level (usually 10^3-10^5 cfu ml^{-1}). Three independent experiments were performed for each time-temperature combination.

Table 2. Heating experiments performed with skim milk and cream

Substrate	No. experiments	Treatment	Temperature (°C)	Holding time (s)
skim milk	93	HTST	67, 72, 77, 82, 87, 90	15, 30, 45, 60
cream	57	HTST	85, 90, 95, 100	15, 30, 45, 60

Detection of MAP in heat-treated milk
A modified Dubos medium was used for resuscitation and enrichment of heat-injured MAP. For direct plating HEYM agar slants was used. The exact composition for both media is given in Hammer et al. 2002.

Milk samples were collected from the outlet of the equipment. For each experiment 300 ml were collected and split into three subsamples of 100 ml which were processed independently. This splitting approach reduced the risk that the entire sample would be lost to overgrowth by nonmycobacterial organisms, important because no decontamination procedure of the heat-treated samples was performed. Raw skim milk especially may contain sporeformers amongst other bacteria that can spoil the subsequent isolation procedure of MAP. The sample temperature at collection was below 12 °C. Further cooling was not applied. Directly after collection (max. 1 h delay), samples were centrifuged at 4 °C in a refrigerated centrifuge at 14,000 x g for 10 min. The resulting pellets were suspended in 2 ml 0.85 % NaCl. For each sample 3 HEYM slant agar tubes and 3 vials of modified Dubos medium were inoculated with equal parts of the 2 ml suspension. Cultures on HEYM agar were evaluated monthly and finally after an incubation of 6-8 months at 37 °C, visible colonies of bacteria were identified as MAP by an auramin staining and an IS*900* based PCR. The Dubos vials were incubated for the same time period, pooled, centrifuged and pellets streaked onto 2 HEYM slant agar tubes. If heavy contamination of the Dubos medium was detected, decontamination with N-acetyl-L-cysteine (NALC) was applied prior to culture on HEYM agar. After 3-6 months of incubation visible colonies of bacteria were identified as described above.

PCR identification of MAP
The PCR is based on the detection of an insertion sequence (IS*900*) unique for MAP. DNA extraction was performed by boiling one colony for 10 min at 100 °C. Primers according to Moss et al. 1992 were used in a standard protocol (see Hammer et al. 2002) resulting in a 400 bp band in agarose gel electrophoresis. Primer:

Para-Tb 4: 5'-GTT CGG GGC CGT CGC TTA GG-3'
Para-Tb 5: 5'-GAG GTC GAT CGC CCA CGT GA-3'

Table 3: Inactivation of MAP in skim milk: Distribution of 54 samples showing survival of MAP after enrichment and/or by direct culture within experiments with various time-temperature combinations

Sample no. :	93		Detection via direct culture:			21
Positive:	54		Detection via enrichment:			40
Negative:	39		Detection via both:			7

| Time (s)	Temperature (°C) (Positives/total)					
	67	**72**	**77**	**82**	**87**	**90**
15	3/3	1/3	2/3	1/3	1/3	0/3
30	5/5	4/5	4/7	5/7	6/6	5/9
45	2/3	1/3	2/3	1/3	0/3	3/3
60	2/3	1/3	2/3	2/3	0/3	1/3

RESULTS

Heating of skim milk at 67-90 °C for 15-60 s
An overview of the results achieved via enrichment and/or via direct culture is given in Table 3. In Table 4 the results from direct culture are displayed separately and in detail. The results from direct culture show very low numbers of survivors. These values correspond to a 3-6 $\log_{10}$ reduction from the initial MAP concentration. It is to be noted that total volumes of 300 ml were used for quantification. Therefore, calculated numbers of survivors may result in figures < 1. The log reduction in the samples positive only after enrichment was between 5-7 $\log_{10}$ cycles from a starting concentration of between 10^3-10^5 cfu ml^{-1}.

Table 4: Inactivation of MAP in skim milk: Calculation of the log reduction in heating experiments showing survival of MAP after direct culture

Experiment-no.	Temperature (°C)	Holding time (s)	Inoculum, cfu/ml	Survivors, cfu/ml	Log reduction
240	66.5	15.6	4.9×10^5	0.90	6
246	67.0	15.1	7.0×10^5	2.80	5
252	67.0	15.4	6.0×10^3	1.80	3
262	76.9	31.9	1.9×10^4	0.02	6
263	71.9	31.8	4.0×10^4	0.08	6
265	82.4	31.4	4.0×10^4	0.02	6
266	82.3	31.4	4.0×10^4	0.04	6
270	67.0	31.6	4.4×10^4	4.00	4
281	72.2	58.8	4.4×10^4	0.02	6
282	67.2	58.2	4.4×10^4	0.06	6
283	90.0	57.7	4.2×10^4	0.08	6
363	81.9	44.1	5.8×10^3	0.04	5

The scatter plot in Fig. 2 shows the distribution in MAP survival after the heating experiments.
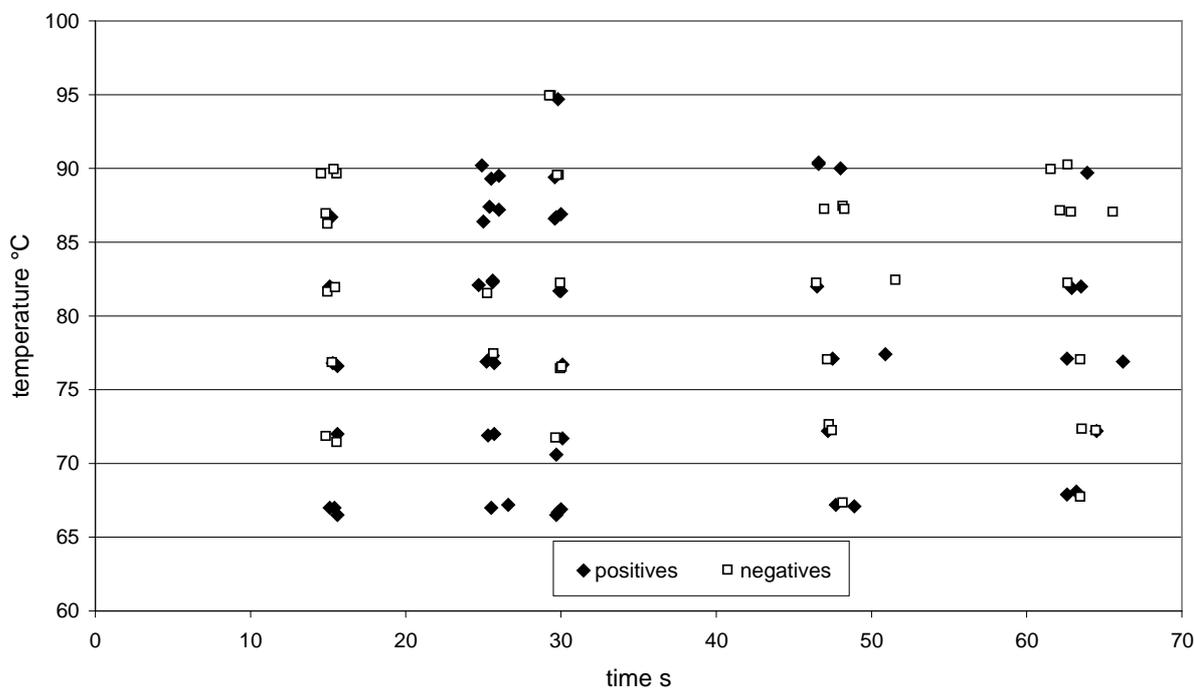
Fig. 2: Inactivation of MAP in skim milk: distribution of positive and negative results of heating experiments with MAP at various time-temperature combinations

Heating of cream at 85-100 °C for 15-60 s
An overview on the distribution of results achieved via enrichment or via direct culture is given in Table 5. From only one experiment was MAP detected via direct culture, showing a reduction of 6 $\log_{10}$ cycles during heating at 95 °C for 15 s. In samples requiring enrichment for the detection of survivors the reduction was 5-7 $\log_{10}$ cycles as was also seen with skim milk trials.

Table 5: Inactivation of MAP in cream: Distribution of 9 samples showing survival of MAP after enrichment or by direct culture within experiments with various time-temperature combinations

Sample no.:	57	Detection via direct culture:	1
Positive:	9	Detection via enrichment:	8
Negative:	48	Detection via both:	0

	Temperature (°C) (Positives/total)			
Time (s)	**85**	**90**	**95**	**100**
15	0/3	0/3	1/3	1/3
30	1/3	1/6	0/6	1/6
45	1/3	0/3	0/3	1/3
60	0/3	0/3	0/3	1/3

Statistics

A logistic regression model was applied to check the possible influence of heating temperature and holding time on the variance of the results. Due to the relatively small database for skim milk less than 20% of the variance could be explained by the model. As temperatures were elevated, the results with survivors decreased ($p < 0.05$). The same pattern prevailed for holding times ($p < 0.01$). However, it has to be noted that the model can explain only 20% of the variance, which means that it is not very precise.

For cream the model was not applicable due to the low numbers of results with surviving MAP. By evaluation of comparable time-temperature regions of heat treatment (84-92 °C) with Fishers exact left-sided test it was shown that survival in skim milk is much more likely than in cream ($p = 0.005$).

DISCUSSION

The following aspects of MAP heat resistance should be considered:

- Cross-contamination

Measures to prevent and exclude cross-contaminations in the pilot plant and during treatment of the heated samples in the laboratory are listed above. While cross-contamination can never be completely excluded, given the control measures cross- contamination is not very likely.

- Effect of clumping

MAP cultures were not homogenized prior to heat treatments. This may have affected the results for two reasons.

1) The "true number" of individual bacterial cells experiencing the heat treatment cannot be properly estimated because colony forming units, as recorded in the colony count of the initial inoculum, may be formed by few to numerous cells resulting in small to large clumps.
2) Clumps of different sizes were likely to occur throughout the process as the sample to be heated contains a mixture of small and large clumps and it is stirred during the entire heating experiment.

- Physiological properties of MAP

At present no physiological properties of MAP are known to explain its exceptional heat resistance. Hypotheses include effects of metabolic activity, dormancy or even spore-like properties. As stated in a report of an ILSI working group on "MAP and the food chain", the heat resistance of MAP cannot be explained. The final sentence on the topic in that report is: "If such mechanisms do not withstand experimental scrutiny we would be forced to reconsider the possibility that, despite the great care taken by experimentalists, there is some unknown property of MAP that prevents some cells receiving the intended heat treatment in pasteurizers or submerged ampoules". (Gould et al. 2004). In addition, in these and in earlier experiments (Hammer et al.2002), survival of a few microorganisms seems to be less dependent on heating temperature and holding time. From the visual inspection of the scatterplot in Fig. 2 one could even postulate that the experimental conditions had no influence on the survival. This also can only be explained by an "unknown property".

- Log reduction

Although small numbers of survivors were observed in many experiments, a reduction of at least 5 to 7 log cycles could be demonstrated during heating of skim milk and cream. To some extent these results are

supported by results on the investigation of retail milk in the UK (Grant et al. 2002), recently in the USA (Ellingson et al. 2005) and in the Czech Republic (Ayele et al. 2005) which showed that low numbers of surviving MAP can be occasionally be expected in pasteurised drinking milk.

The inactivation of MAP was more effective in cream than in skim milk though fat generally is considered as a factor protecting bacterial cells during heating. This observation is one more factor that can not be explained with regard to the exceptional heat resistance of MAP. It is known that MAP may also be present in the fat fraction after centrifugation of milk. Prior to the heating experiments, trials were conducted to determine whether the centrifugation step at 14,000 x g is sufficient to concentrate survivors in the pellet. After this centrifugation, no remaining MAP could be detected in the fat fraction (data not shown).

CONCLUSION

Despite all the uncertainties regarding the complete inactivation of MAP during heat treatment and the possible reasons for survival, a reduction of at least 5 to 7 log cycles could be demonstrated during heating of both skim milk and cream. Regarding the probably low numbers of MAP, which can be expected in naturally contaminated milk, heating processes according to the time-temperature combinations tested will lead to a significant, but not complete, reduction of MAP cells.

ACKNOWLEDGEMENTS

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Clumping of *Mycobacterium avium* subsp. *paratuberculosis* in milk and feces and growth activation after milk heating

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ABSTRACT

Scientific research has demonstrated the survival of MAP in milk after commercial pasteurization, but so far the reason for this phenomenon has not yet been elucidated. If this heat resistance could be explained by thermal death curves only, clumps must contain more than 10^4 individual MAP cells. This paper, however, reports that fecal MAP clumps from naturally infected cows did not contain more than 10 individual cells. The absence of large clumps in naturally infected feces and milk was confirmed by a culture experiment in which no increase in MAP numbers was observed after three different declumping methods. In recent years, an unidentified heat activation mechanism has been suggested as a possible alternative explanation for the heat resistance of MAP. Milk samples from MAP positive dairy cows were subjected to different heat treatments; considerably more positive cultures were obtained early in the incubation process after applying high heating temperatures (80-100°C). With an additional chemical treatment of the milk before heating there was a tendency towards more positive cultures early in the incubation period, even at the lower heating temperatures (60-70°C). Neither heat nor a chemical treatment increased the total MAP growth after 10-12 months of incubation. One individual cow milk sample, taken from a commercial Belgian dairy farm with a known history of Johne's disease and cultured under different conditions, revealed a mean MAP count of 6.67 ± 20.00 cfu per 100ml during a first sampling, whereas the bulk tank milk contained 10.00 ± 20.00 cfu per 100ml. During a second sampling, individual cow milk samples (n = 6) had a mean MAP count of 42.33 ± 19.67 cfu per 100 ml whereas the tank milk contained 11.67 ± 12.33 cfu per 100ml.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, milk, survival of pasteurization, clumps, heat activation

INTRODUCTION

Mycobacterium paratuberculosis (MAP) is the causative agent of Johne's disease in cattle and other ruminants. The microorganism is also suspected of playing a role in human Crohn's disease. Milk destined for human consumption can become contaminated by fecal contamination as well as direct shedding by clinically ill as well as by asymptomatic animals (Taylor et al., 1981, Sweeney et al., 1992, Streeter et al., 1995). Although various studies have indicated the frequent presence of MAP in cow's milk at the farm and in industrial collection tanks (Corti & Stephan, 2002, Grant et al., 2002a), precise cell numbers have thus far not been determined due to the lack of a suitable detection method. Culture methods can take up to 12 months and they frequently suffer from overgrowth by competitive microflora, Lethal or sub-lethal damage to MAP due to the sample decontamination step prior to incubation also affects culture results (Dundee et al., 2001). Although PCR is able to detect the specific IS*900* sequence in the MAP genome it does not distinguish between viable and dead cells, a crucial element needed when evaluating the survival of MAP after commercial milk pasteurization. Earlier scientific papers report survival of MAP under pasteurization conditions (Hammer et al., 2002, Grant et al., 2002a, Grant et al., 2002b) but the survival mechanism has not yet been elucidated. One explanation could be that the survival of MAP after pasteurization is due to the bacteria's tendency to clump, each clump being counted by the culturing method as 1 cell. If clumps would consist of more than 10^4 individual cells as suggested by Klijn et al. (2001) the actual number of MAP cells present would be underestimated, thus overstating the effectiveness of current pasteurization methods. As an alternative hypothesis, an unknown heat activation mechanism has been suggested (Hammer et al., 2002).

The present research aims to determine the actual number of MAP cells per clump in naturally contaminated milk and feces and to evaluate the activation of MAP in naturally contaminated milk under different heating conditions. In addition, the potential effect of a chemical trigger was investigated.

MATERIALS AND METHODS

Culture of MAP
Milk and fecal samples were obtained from three clinically normal but ELISA or direct fecal PCR-positive cows at a commercial Belgian dairy farm with a history of Johne's disease. MAP was isolated from milk by the method of Dundee et al. (2001). That is, 50 ml milk was centrifuged (9500 x g, 15 min) and washed with Ringer's solution (Oxoid Ltd, London, UK). The pellet was resuspended in 0.75% cetylpyridinium chloride (CPC; Sigma-Aldrich, Saint-Louis, Miss., USA) and incubated for 5 h at room temperature. The samples were then centrifuged (9500 x g, 15 min) and washed twice with Ringer's solution. Three different declumping procedures were tested: sonication at 35 W (35 s) (Labsonic U, Needle probe 40T; Sartorius, Melsungen, Germany), pulling 20 times through a sterile syringe and needle ($\varnothing$ 0.45 mm) and Turrax (high shear mixer treatment (1 min, 24,000 min-1) (Ultra-Turrax T25; Van der Heyden, Brussels, Belgium). Afterwards, the pellet was inoculated onto Herrold's Egg Yolk Medium (HEYM, Merkal, 1971) supplemented with mycobactin J (2 mg/L) (Synbiotics Europe, Lyon, France) and incubated at 37°C.

Fecal samples (2 x 4 g) were diluted with 20 ml Ringer's, vortex-mixed for 2 min and decontaminated with ½ x (0.9% CPC/Brain Heart Infusion Broth) (Oxoid Ltd) for 24 h at 37°C. After centrifugation (900 x g, 30 min) the pellet was washed, inoculated onto HEYM supplemented with mycobactin J (2 mg/L) and incubated at 37°C. When applicable, a declumping step was incorporated in the protocol just before decontamination.

For each milk or fecal sample three decimal dilutions were analyzed in triplicate. The number of MAP cells was estimated by the Most Probable Number method (de Man, 1975). Culture-positive tubes were further confirmed by IS*900* PCR. The primers were IS900R3(2) (reversed primer) (Vansnick et al., 2004) and MAP 1 (X16293, starting at position 467 – 5' gggcagcggctgctttatat 3') (forward primer). A standard 40 cycle PCR was performed with an annealing temperature of 68°C.

Microscopic determination of the clumping of MAP in cow feces
Fecal samples obtained from antibody-positive cows on Belgian farms were diluted with 30 ml commercial bleach water, allowed to stand overnight and filtered through sterile hydrophilic gauze (20 x 20 cm) (Dierengezondheid Vlaanderen, personal communication). The sample was divided and subjected to three different declumping procedures: sonication at 35 W (15 s) (Labsonic U, Needle probe 40T; Sartorius, Melsungen, Germany), pulling 20 times through a sterile syringe and needle ($\varnothing$ 0.45 mm) and Turrax (1 min, 24,000 min-1) (Ultra-Turrax T25; Van der Heyden, Brussels, Belgium). A Ziehl-Neelsen stain was applied and the results were microscopically analyzed. For each treatment 20 individual microscopic fields were evaluated and the number of clumps and individual cells was counted. Samples without the declumping procedure were evaluated similarly in parallel.

Survival of MAP in milk after heat treatment
A total of 9 milk samples (the first sampling included one individual cow milk sample and a tank sample, results presented in Table 2; the second sampling included 6 individual cow milk samples and a tank milk sample, results presented in Fig. 1) were heat-treated under lab conditions (10 min at 60, 70, 80, 90 and 100°C, respectively, and 30 min at 63°C). In a second trial with these samples, milk components were broken down by a mixture of 5% diethyl ether, 12% petroleum ether, 0.1% sodium dodecyl sulphate and 0.5% NH_3. When using this chemical milk treatment, exposure to the decontamination step in 0.75% cetylpyridinium chloride (CPC; Sigma-Aldrich) (see above) was reduced from 5 h to 10 min at room temperature. All samples were kept for 2 days at 4°C before inoculation on HEYM tubes to allow recovery of the MAP cells (Grant et al., 2003) and were subsequently incubated at 37°C. Culture-positive tubes were confirmed by IS*900*-based PCR. The number of MAP cells was estimated by MPN as described above. A non-parametric bootstrap procedure was used to determine the 95% confidence intervals on the

percentage of positives as a function of incubation period and treatment (Vose et al., 1996). When these confidence intervals do not overlap for points at the same incubation period it can be concluded that the treatment had a significant effect on MAP isolation.

RESULTS

The declumping experiments showed no increase in total number of individual cells according to microscopy when compared to samples without declumping. A declumping experiment combined with subsequent culturing was performed on MAP-containing milk and feces. The results (Table 1) demonstrate the absence of large MAP clumps in naturally contaminated milk and feces as no increase in MAP numbers was observed after the 3 declumping methods. Mean cfu's per 100 ml milk or per g feces first without declumping and then after sonication, syringe/needle treatment and Turrax were, respectively, 0.95, 0.55, 0.40 and 0.10 (milk) and 0.52, 0.35, 0.73 and 1.57 (feces).

Table 1: Number of MAP cells in naturally contaminated milk and feces before and after different declumping methods.

Sample	MAP counts (cfu per 100 ml or per g) after different declumping methods			
	No declumping	Sonication 15s	Syringe + needle	Turrax
Milk 1	1.40	0.73	0.47	0.20
Milk 2	0.73	0.73	0.73	0.10
Milk 3	0.73	0.20	0.00	0.00
Feces 1	0.15	0.15	1.05	3.50
Feces 2	1.05	0.55	1.00	1.05
Feces 3	0.35	0.35	0.15	0.15

Table 2: Mean percentages of MAP positive culture tubes obtained for one bulk tank milk sample and one individual cow milk sample incubated for 12 months and for different treatments. Each figure represents the percentage of positive tubes calculated on a total of 18 tubes (2 samples incubated in 3 times 3 decimal dilutions). Significant differences are indicated with a different letter in the exponent.

Treatment	Percentage of MAP positive culture tubes (%) after different incubation periods				
	2 months	3 months	8 months	10 months	12 months
None	0^A	0^A	0^A	11^{ABC}	61^A
CT*	11^{AB}	11^{AB}	11^{ABC}	22^{BCD}	72^A
10min/60°C	0^{AB}	0^A	0^A	11^{ABC}	72^A
CT + 10min/60°C	11^{AB}	17^{AB}	22^{BCD}	22^{BCD}	56^A
30min/63°C	0^A	0^A	6^{AB}	6^{AB}	72^A
CT + 30min/63°C	39^{BC}	39^{BC}	39^{CD}	39^{CDE}	50^A
10min/70°C	0^A	0^A	0^A	0^A	83^A
CT + 10min/70°C	22^{BC}	22^{BC}	22^{BCD}	22^{BCD}	39^A
10min/80°C	22^{BC}	39^{BC}	44^D	44^{CDE}	50^A
CT + 10min/80°C	28^{BC}	28^{BC}	28^{BCD}	28^{BCDE}	39^A
10min/90°C	22^{BC}	28^{BC}	28^{BCD}	33^{BCDE}	83^A
CT + 10min/90°C	17^{AB}	22^{BC}	39^{CD}	44^{CDE}	50^A
10min/100°C	56^C	61^C	61^D	78^E	78^A
CT+ 10min/100°C	44^{BC}	50^{BC}	50^D	61^{DE}	78^A

*CT: chemical treatment

To evaluate the effect of chemicals and heat on the growth of MAP, both a culture-positive tank milk sample as well as a culture-positive individual cow milk from the same farm were subjected to different heating conditions and a chemical treatment. Activation of MAP growth was not observed at the lower heating temperatures (60-70°C) but was significant after heating at 80°C, 90°C and 100°C for 10 min (Table 2). Chemically treated samples showed partial growth activation when subjected to no or moderate heating and a significant activation at 63°C for 30 min. No additional growth stimulation was observed at higher heating temperatures. In the absence of chemical or heat treatment, growth of MAP is delayed but the final

numbers of positive tubes at the end of the incubation period the total are comparable. The amount of MAP cells in individual cow milk samples was estimated at 6.67 cfu per 100ml (mean value for the different conditions) with a standard deviation of 20.00. For the bulk tank milk sample, the MAP cell estimate was 10.00 ± 20.00 cfu per 100ml. No decrease in MAP growth was observed even after heating the milk at 100°C for 10 min.

Fig. 1 (n = 7) displays the results of a similar experiment, in which both bulk tank milk and individual milk samples from 6 cows obtained 4 months later from the same farm were subjected to the same chemical treatment as well as heating at 80°C for 10 min. A significant growth activation was observed after heating the milk samples with a mean value of 41% of the positives already detected after 3 months of growth as compared to 6% for the samples without heat treatment. Partial growth activation was also observed in the chemically treated samples with 24% being detected as positives after 3 months. After 10 months no significant difference in total MAP growth was observed. The individual cow milk samples revealed a mean MAP contamination level of 42.33 ± 19.67 cfu per 100 ml after 10 months. For the bulk tank milk, the contamination level was 11.67 ± 12.33 cfu per 100ml.

Each result represents the percentage of positive tubes calculated on a total of 63 tubes (7 samples incubated in 3 times 3 decimal dilutions). Significant differences are indicated with a different letter.
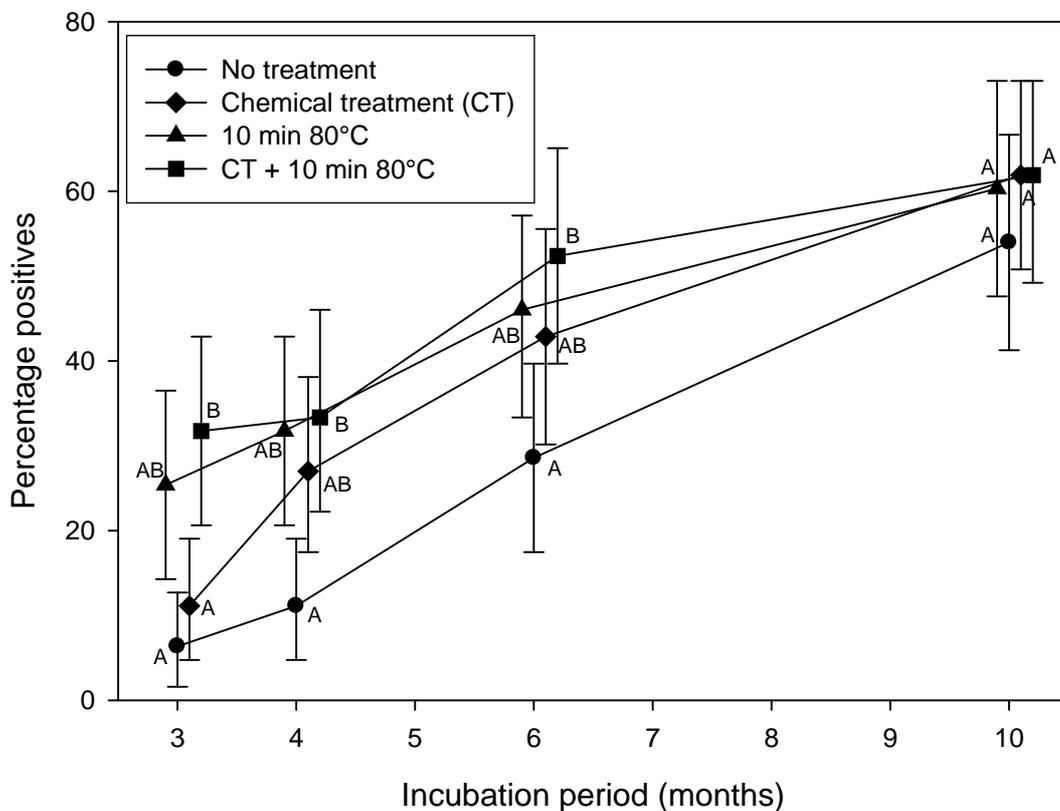
Fig. 1: Mean percentages of MAP positive culturing tubes (with 95% confidence intervals) obtained for 7 milk samples during a time period of 10 months and for different treatments.

DISCUSSION

The mechanism of MAP survival in milk after commercial pasteurization has not yet been elucidated. Some authors suggest a highly elevated number of MAP cells per clump as an unknown concentration factor that contributes to pasteurization survival (Klijn et al., 2001). R Both direct secretion and fecal contamination from asymptomatic as well as clinically affected animals can result in raw milk with MAP. Because the presence of clumps in milk appears mainly to be correlated with fecal contamination, we determined the number of MAP cells in fecal clumps originating from infected cows as an indication of the number of MAP cells per clump present in milk. Microscopic examination revealed no large MAP clumps in feces and no

concomitant increase in MAP cells was obtained after various declumping methods. Therefore no evidence was found that the number of individual cells in a clump exceeds 10 cells. These microscopy results were confirmed by culturing naturally contaminated feces and milk. Because no direct counting of MAP colonies was possible on the HEYM slopes due to the presence of opaque white milk particles, the MAP contamination was estimated by the statistical MPN approach. These results do not support the hypothesis that the survival of MAP after milk pasteurization is due to the presence of large MAP clumps that allow individual cells to evade the effects of high heat.

In addition, our findings on the survival of MAP after heating the milk at up to 100°C confirm the earlier results of Hammer et al. (2002), who were able to grow MAP after heating temperatures up to 90°C. However, it should be noted that our heating experiments were performed on a lab scale, which not necessarily entails the extrapolation of their results to real industrial conditions of milk heating. Thus far, a decisive physiological explanation for the survival of MAP to high temperatures has not yet been given. It can, however, not be ruled out that additional clumping takes place during heating and that cells within clumps undergo a physiological adaptation leading to an increase in heat resistance (Gould, 2004). Stress response genes affecting heat resistance have been described in many bacterial species (Yura et al., 2000, Hengge-Aronis, 2000) and can also be the cause of heat resistance increase. However, an increased heat resistance to the level observed for MAP has not yet been observed in any other vegetative bacterium. In the absence of a physiological explanation, another mechanism that could account for such extreme heat resistance could be a change in the physicochemical environment of the organism (Gould, 2004). Vegetative bacteria are protected from heat when present in foods that are low in moisture or high in fat, and at low water activity, and under these conditions they can resist temperatures of 100°C or higher (Oslen & Nottingham, 1980). The protection afforded to bacteria present within lipid material is due to an increased solubility of water in the lipid phase as temperatures rise and a concomitant reduction in the local water activity (Senhaji, 1977, Senhaji & Loncin, 1977). This would prevent some MAP cells in milk from receiving the intended heat treatments in experimental (and commercial) designs, probably as a consequence of their hydrophobic characteristics, i.e. the extensive presence of mycolic acids in their cell wall.

Heat activation of MAP after heating of milk to at least 74°C for 33 s was for the first time described by Hammer et al. (2002). Our results confirmed this observation with a significant heat activation at 80°C for 10 min. Chemical treatment alone of milk resulted in a partial activation of MAP growth. Heat and chemical treatment are not increasing the total MAP growth obtained after culturing the samples for 10-12 months. One possible explanation could be that heat as well as chemical treatment are capable of changing the structure of the main milk components like protein and fat molecules. Since testing 50 ml of milk is indeed causing an inoculation of milk components to the slope, this structural change could influence the nutrient availability during MAP growth on HEYM slopes.

The MAP counts in milk samples from serologically positive cows on a farm with a MAP history were estimated in two samplings to be 6.67 ± 20.00 cfu per 100ml and 42.33 ± 19.67 cfu per 100ml, respectively. For the tank milk sample the contamination level was estimated at 10.00 ± 20.00 cfu per 100ml in the first and 11.67 ± 12.33 cfu per 100ml in the second sampling. The high standard deviations are indicative for the poor reproducibility of the MAP culturing experiments. This poor reliability has previously been suggested as being the main cause of conflicting results regarding MAP survival to heat treatments (Gould et al., 1994), differences between PCR and culturing results (Giese & Ahrens, 2000, Grant et al., 2002a) and the importance of optimization the decontamination and resuscitation step of MAP culturing methods (Dundee et al., 2001, Grant & Rowe, 2004).

CONCLUSION

The results presented in this paper indicate the absence of big MAP clumps in naturally contaminated cow's milk and feces. This observation does not fit with the earlier hypothesis that the survival of MAP after milk pasteurization would be due to the presence of clumps containing more than 10^4 individual cells (Klijn et al., 2001).

Heat activation of MAP in milk has for the first time been described by Hammer et al. (2002). Our results confirmed this by demonstrating a significant heat activation effect, even at the harsh heating condition of 100°C for 10 min. Chemical treatment of the milk resulted in a partial growth activation of MAP. Neither heat nor chemical treatment did increase the total MAP growth obtained after culturing the samples during 10-12 months.

The individual cow milk samples taken at a commercial Belgian farm were found contaminated with a mean value of 6.67 ± 20.00 to 42.33 ± 19.67 cfu per 100ml, the tank milk with 10.00 ± 20.00 to 11.67 ± 12.33 cfu per 100ml.

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Mycobacterium avium subsp. *paratuberculosis* in powdered infant formula[1]

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ABSTRACT

Fifty one powdered infant formula products produced by 10 companies from seven countries available on the Czech market were tested. Milk used for these products is pasteurized prior to drying. IS*900,* the specific fragments for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were detected using PCR in 25 samples (49.0 %) and fragment f57 by real time PCR in 18 samples (35.3 %). One sample was positive by culture, but the finding was not successfully repeated. These results correspond to the epidemiological situation in Europe and are not unexpected. Paratuberculosis in cattle was almost unknown in the Czech Republic until 1990. An increase in the number of cows with paratuberculosis found in slaughterhouses and the incidence of Crohn's disease in the last decade is evident. The possible risk of killed MAP cells or bacterial structures in food is discussed in respect to an autoimmune cause Crohn's disease. The national programmes of paratuberculosis control and certification of paratuberculosis–free herds should be strongly supported to decrease the risk for children and other people under higher risk. Producers should use MAP-free milk for baby food production on a voluntary basis.

Key words: Johne's disease, paratuberculosis, control, Crohn's disease, IS*900,* f*57*, infant formula

INTRODUCTION

Paratuberculosis (Johne's disease), *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection and Crohn's disease are the focus of growing interest, with the number of research projects and published results doubling between 1994 and 2003 (Hruska, 2004). Paratuberculosis is the widely distributed infectious disease of cattle and other domestic and wild ruminants caused by MAP infection (Kennedy and Benedictus, 2001). Up to 70% of dairy herds suffer from this infection in most European countries, the United States and Canada. The financial losses were already estimated at about $1.5 billion per year in the USA in 1998 (Stabel, 1998). Paratuberculosis is a notifiable disease for the OIE, but it is not classed as zoonotic or an emergency disease or. An OIE Technical Disease Card on paratuberculosis is not yet available. Milk and meat from infected herds is not banned if the general food-handling rules are fulfilled. Diagnosis of the disease is rather difficult as infected animals don't always shed MAP in faeces or milk. Serologic methods have low sensitivity and specificity, and cultivation of the agent, although considered "the gold standard", takes several months with some MAP forms not growing in vitro at all (Pavlik et al., 1999; Machackova et al., 2004).

If the infection is not efficiently controlled it is guaranteed to spread MAP to most animals in the herd, although the genetic influences in the susceptibility of cattle to paratuberculosis have been reported (Koets et al., 2000). Subsequently, as a result of different stress factors e.g. parturition, malnutrition, transportation etc., some animals suffer from the clinical form of the disease. Massive shedding of MAP in faeces contaminates the environment and transmits the infection to other animals. The most susceptible are calves during the first weeks of their life. Evidence of the pathogen has been found not only in the intestine but also in milk, lymph nodes, and different parenchymatous organs (Pavlik et al., 2000; Ayele et al., 2004). Confirmed MAP isolates were cultured from 1.8% of the commercially pasteurized milk samples in the U.K (Grant et al., 2002). Similar data were published from the U.S.A. (Ellingson et al., 2005). In the U.K. study

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the 10 culture-positive pasteurized milk samples were from just 8 (3.3%) of the 241 dairy processing establishments that participated in the survey (Grant et al., 2002). In the same study 11.8% of samples of retail milk were MAP-positive by PCR. In Switzerland, 19.7% of bulk-tank milk samples were IS*900* PCR positive (Stephan et al., 2002). Goat and sheep bulk tank milk samples were also PCR-positive for IS*900* (23.0 and 23.8%, respectively), providing presumptive evidence for the presence of MAP in Switzerland (Muehlherr et al., 2003). MAP has been cultivated from cheese (Donaghy et al., 2004; Ikonomopoulos et al., 2005) as well.

MAP is very resistant to high temperatures and chlorination. The organism remains cultivable in lake water for 632 days and persisted for up to 841 days (Pickup et al., 2005). MAP cultivation needs up to four months for cultivation with some forms not growing *in vitro* at all. However, the concentration of MAP, quoted in colony forming units (CFU), does not convey the total number of cells present. Molecular biology techniques offer a more rapid and specific detection of MAP and its quantification in milk, cheese, and meat.

Autoimmune diseases are an important health problem for humans. The Autoimmune Diseases Coordinating Committee of the U.S. National Institute of Health reported to Congress that the prevalence of all autoimmune diseases ranged from 5 to 8 percent of the U.S. population (14.7 to 23.5 million people) in 2003. The expenditures for autoimmune diseases research reached nearly $600 million. The inflammatory bowel disease Crohn's disease, a chronic autoimmune inflammatory bowel disease with similar pathological changes to paratuberculosis (Chiodini, 1989), had the fifth highest research budget in 2003 (Anon, 2005). The American Academy of Microbiology Colloquium "Microbial Triggers of Chronic Human Illness" used Crohn's disease to illustrate a condition that does not result from infection alone but from the confluence of infection and genetic susceptibility. Susceptible individuals, who carry the NOD2 or TNFR polymorphisms, may respond to certain commensual intestinal flora, stimulating acute inflammation that leads to chronic inflammation and colitis. The Colloquium also stated that it can be extremely difficult to prove that a pathogen is the cause of a chronic disorder when the onset of disease begins some time after the exposure. Often times it is not practical or even possible to use Koch's postulates to prove the infectious nature of chronic illnesses (Carbone et al., 2005)

MAP and other agents *(Clostridium, Campylobacter jejuni, Campylobacter feacalis, Listeria monocytogenes, Brucella abortus, Yersinia pseudotuberculosis, Yersinia enterocolica, Klebsiella spp., Chlamydia spp., Eubacterium spp., Peptostreptococcus spp., Bacteroides fragilis, Enterococcus feacalis, and Escherichia coli)* have been considered possible triggers of Crohn's disease (Carbone et al., 2005. MAP cells contain peptidoglycans and heat shock proteins that are able to initiate the inflammatory changes in the intestine (El-Zaatari et al., 1995; Chamaillard et al., 2003). The highest reported prevalence of Crohn's disease to date is in north-eastern Scotland, where almost 0.15% of the population has the disease. It is not far from the truth that Crohn`s disease affects hundreds of thousands of people around the world. Based on the latest epidemiology research from the United States, the most likely conclusion is that there are 400,000 people in the United States who suffer from Crohn's disease. Since the population of the United States is 270 million people, this means that the current prevalence of Crohn's disease in the United States is 148 cases per 100,000 people. In the United States, in 1990, Crohn's disease costs between $1.0 and 1.2 billion. Other countries with a high prevalence of Crohn's disease are Canada, Sweden, Norway, Germany, United Kingdom, Netherlands, Belgium, France, Switzerland, Austria, Spain, Portugal, Greece, Italy, Ireland, Australia, New Zealand, and many countries of Eastern and Central Europe. In all these countries bovine paratuberculosis is a commonly found infection in cattle herds but the incidence of paratuberculosis in cattle is not known with precision because it is hard to diagnose and under-reported (Ayele et al., 2001). The prevalence of the disease is also unknown in sheep, goats and game ruminants. Some authors have described a parallel increase in paratuberculosis and Crohn's disease prevalence and discuss the possible links between them (Hermon-Taylor and El-Zaatari, 2005). Paratuberculosis in cattle was sporadically diagnosed until 1990 in the Czech Republic. When the import of heifers and dairy products started an increase of paratuberculosis was found in slaughtered cattle (Vecerek et al., 2003).

Dried milk baby food products (infant formula) originating from nine European countries including two new EU member states were all available on the Czech market in 2004. In all these countries paratuberculosis is present in dairy herds where milk and beef from pre-clinically affected animals can be sold on the market. The risk associated with the presence of cultivable MAP in retail dairy products has been noted by a

number of authors. The presence of the specific IS*900* was also confirmed. These findings are unsurprising as the prevalence of paratuberculosis in dairy cattle herds is high and MAP can be present in milk even in cows without the clinical form of the disease. Current milk and meat products regulations are being met by dairies and food producers.

Data on the increase of the incidence of Crohn's disease has been published from different countries. Some authors noted an increase in children with different autoimmune diseases, including Crohn's disease. In Scotland the incidence of Crohn's disease has increased in children by 30% since 1993 (Armitage et al., 2001). An increase of incidence was also reported in Denmark (Fonager et al., 1997), Israel (Shapira and Tamir, 1994), Minnesota, U.S.A. (Loftus et al., 1998) and in the region of Northern Stockholm (Askling et al., 1999; Hildebrand et al., 2003). The index of patients treated for Crohn's disease in the Czech Republic between 1995 and 2004 increased to 2.9; in the age category up to 19 years to 4.6 and in patients older than 65 to 6.6 (Fig. 1).

Milk and dairy products are important components of human nutrition. However, the autoimmune character of the Crohn's disease does not exclude a risk for genetically susceptible people when linked with bacterial triggers. This may occur even though live MAP cells are not present in food. At higher risk are children and direct relatives of Crohn's disease patients. Given the presence of MAP IS*900* in dairy products has been reported, the aim of this study was to evaluate MAP contamination in dried milk baby food (infant formula) available in the Czech Republic.
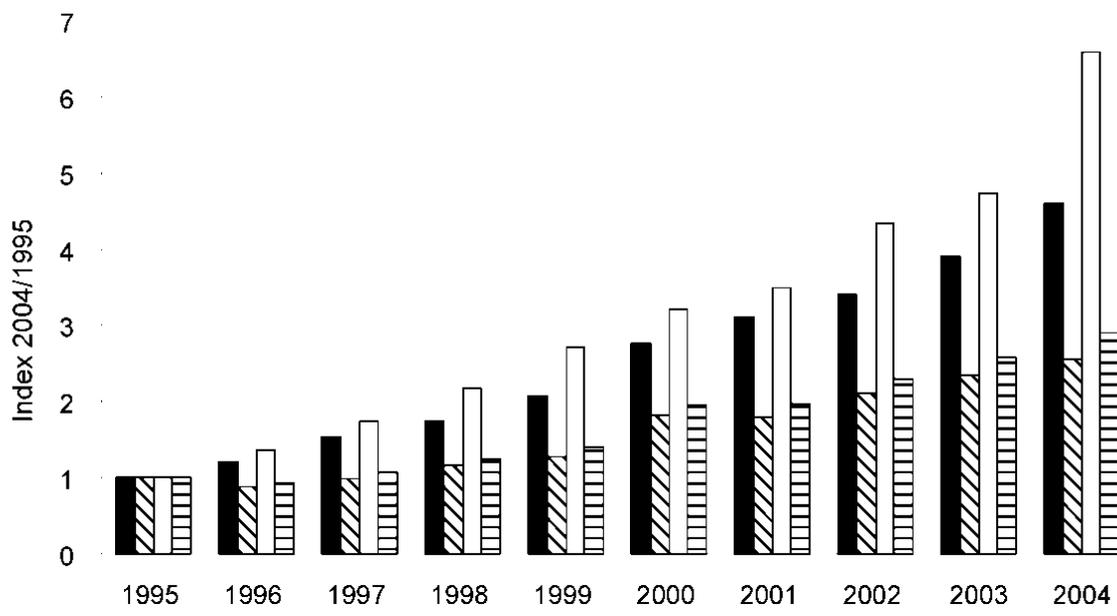
Fig. 1: Crohn's diseases in the Czech Republic in 1995 to 2004. Age in years 0-19, 20-65, 65 and older and all ages are depicted in full, hatched, open and horizontally hatched bars, respectively (Institute of Health Information and Statistics of the Czech Republic)

MATERIALS AND METHODS

Samples
Fifty one dried milk baby food products (infant formula) from 10 producers operating in seven European Union countries were tested. The milk for these formulas is pasteurized before it is dried during preparation of these products.

IS900 determination
A total 20 mg of dry milk samples were diluted in 200 µl of MAP-free water. DNA was isolated by QIAamp DNA Blood Kit (QIAGEN, Germany) according to manufacturers' instructions. From the resulting volume 200 µl a total of 4 µl of DNA was used for PCR (Ayele et al., 2005). The highly sensitive PCR (sensitivity in

tenths of specific loci per reaction) was performed with Taq PCR Master Mix Kit (QIAGEN, Germany) using primers IS*900*-P3N: 5´-GGG TGT GGC GTT TTC CTT CG-3´ and IS*900*-P4N: 5´-TCC TGG GCG CTG AGT TCC TC-3´ in a concentration of 10 µmol per reaction. The expected length of amplification product was 257 bp. An internal standard with a length of 591 bp was used to control false negatives.

Real time PCR for the specific fragment f57 was based on partial sequence described by (Poupart et al., 1993) (GenBank Acc. No. X70277; http://www.ncbi.nlm.nih.gov). BLAST analysis revealed that this fragment is located in a coding region of the hypothetical protein MAP0865 predicted according to the complete MAP genome sequence (GenBank Acc.No. AE017229). Primers and specific TaqMan probes were designed according to the above mentioned f57 sequence and synthesized in TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany. A full description of the method and results will be published separately.

MAP cultivation
Culture of the infant formula products was performed according to Ayele et al. (2004). The milk powder was reconstituted (1 g in 5 ml of distilled water) and the mixture was centrifuged at 2,500 rpm (800 x G) for 15 minutes. The supernatant was decanted and the sediment resuspended in 5 ml 0.75% HPC (hexa-decyl-pyridinium chloride: cetylpyridinium chloride, No. 102340 Merck, Darmstadt, Germany). The tubes were incubated for 4 hours at room temperature with intermittent agitation and centrifuged again for 15 minutes under the same conditions. The supernatant was decanted and the sediment resuspended in 1 ml sterile distilled water. A 200 µl aliquot of the suspension was inoculated onto three Herrold's egg yolk media with mycobactin J.

RESULTS

The IS*900* insertion sequence was found in 25 samples (49%). Products from three manufacturers with the highest numbers of samples tested, i.e. 24, 11, and 7 were PCR-positive 47.7, 45.5, and 85.7% of cases, respectively (Table 1).

Fragment f57 was found in 18 of 51 samples tested (35.3%). An isolate of MAP was made from one sample. Repeated testing of this sample was did not isolate MAP.

Table 1: *IS900* tests results

Producer	No. of products	Positive	Negative	Inhibition*	Positive (%)
1	24	10	13	1	41.7
2	11	5	6		45.5
3	7	6	1		85.7
4	3	1	1	1	33.3
6	1	1			100.0
9	1		1		0.0
10	1	1			100.0
7	1			1	0.0
5	1	1			100.0
8	1		1		0.0
total	51	25	23	3	--
percent	--	49.0	45.1	5.9	--

*If the internal standard with a length of 591 bp was negative, the result was
evaluated as inhibition or "false negative"

DISCUSSION

The results are not unexpected. Dairy products from herds not warranted as MAP-free are likely to contain IS*900* from the dead cells in varying amounts unless action is taken to control the infection and thereby reduce MAP contamination of milk.

A single isolate of MAP from one of 51 samples was obtained, but upon repeat culture no additional isolation was made. The non-uniform distribution of the organism and the small number of cells surviving milk treatment protocols used to produce infant formula could explain this finding.

Crohn's disease is a chronic inflammatory bowel disease similar to paratuberculosis in ruminants. It is classified as an autoimmune disease but its trigger mechanisms are not fully understood. The primary impulse may be the sensitisation of the innate immune system at an early age (Hermon-Taylor and Bull, 2002). The innate immune system is the most ancient and ubiquitous system of defence against microbial infection. The microbel sensing proteins involved in innate immunity recognise conserved and often structural components of microorganisms. Published data has strengthened the association of MAP with Crohn's disease (Chiodini et al., 1984; Chiodini et al., 1986; Chiodini, 1989; Hermon-Taylor et al., 2000; Autschbach et al., 2005; Sechi et al., 2005). Crohn's disease affects hundreds of thousands of people around the world. The current prevalence of Crohn's disease is 50 to 150 cases per 100,000. Paratuberculosis is a common disease in dairy and beef cattle herds in countries with a high prevalence of Crohn`s disease.

Many papers describe the presence of mycobacteria-specific DNA sequences in Crohn's disease patients. Specific probes based on the IS*900* sequence (Green et al., 1989) are usually used to detect MAP although some different specific loci were described (Poupart et al., 1993; Eriks et al., 1996; Bannantine et al., 2002; Vansnick et al., 2004). The presence of of MAP antigen and the antibody array from Crohn's disease patients indicate a unique immune response to MAP and suggest that this organism may play some role in the pathogenesis of Crohn's disease. The insertion sequence IS*900* reveals a unique protein product, p43. The anti-p43 antibody identifies p43 as a 28 kDa processed product in Western blots of protein extracts from MAP (Tizard et al., 1992). Mycobacterial 65KDa heat shock proteins (Hsp65) are among the most extensively studied mycobacterial proteins, and their immunogenic characteristics have been suggested to be the basis for autoimmunization in chronic inflammatory diseases (Elsaghier et al., 1992; Stevens et al., 1992; Szewczuk and Depew, 1992).

In humans, the strong antibody reactions of some sera from Crohn's disease patients compared with non-inflammatory bowel disease patients showed a positive correlation with mycobacterial diseases (El-Zaatari et al., 1995). Serum antibodies (IgG, IgA, and IgM) to protoplasmic antigen of MAP were quantified in patients with Crohn's disease and in control subjects using an enzyme-linked immunosorbent assay (Suenaga et al., 1999). Crohn's disease patients' antibodies were tested by immunoblotting against recombinant antigens identified from MAP genomic library (Naser et al., 2000). Immunoglobulin M (IgM)-, IgA-, and IgG1- and IgG2-isotype-specific enzyme-linked immunosorbent assays for MAP-derived antigens (heat shock proteins of 70 kDa (Hsp70) and 65 kDa (Hsp65), lipoarabinomannan, and MAP purified protein derivative (PPD) was measured by Koets (Koets et al., 2001). Peptidoglycan-polysaccharide complexes were detected intracellularly in the mucosa and submucosa of the bowel wall of Crohn's disease patients. The results show the presence of bacterial peptidoglycan in the bowel wall and the immune responsiveness, especially at the site of inflammation, to these antigens in active Crohn's disease. These results present suggestive evidence for the role of peptidoglycan in the etiology and/or pathogenesis of Crohn's disease (Klasen et al., 1994). The results of mycobacterial genomics are important for a further research (Bannantine et al., 2004).

Multiple genetic variants of NOD2/CARD15 have been associated with a susceptibility to Crohn's disease. NOD2/CARD15 recognizes muramyl dipeptide (MDP) derived from bacterial peptidoglycan (PGN), but the molecular basis of recognition remains elusive (Tanabe et al., 2004). Comprehensive reviews of experimental data supporting a genetic disposition to Crohn's disease and immunity, inflammation and allergy in the gut were published recently (MacDonald and Monteleone, 2005; Kobayashi et al., 2005; Maeda et al., 2005).

Paratuberculosis in cattle causes considerable economic losses for farmers (Mason et al., 1997; Stabel, 1998; Kennedy and Benedictus, 2001). Crohn's disease is also important, both for the pain and difficulties it causes and for the huge expenditure for treatment (Juan et al., 2003; Bassi et al., 2004; Ebinger et al., 2004). The information already available is sufficient to support the possibility of a health risk for consumers resulting not only from viable MAP, but also from inactive or dead cells and even from their structural components. The number of MAP cells present in food is very important. Intake should be

minimised in highest risk people e.g. in newborns, children and genetically susceptible persons, namely patients suffering from Crohn's disease and their direct relatives.

It is most important
- to consider the hypothesis of a possible link between MAP structural components and Crohn's disease
- to decrease the risk of MAP for consumers by introducing MAP-free dairy and beef products and to encourage producers to start this on a voluntary basis
- to support national programmes for certification of dairy and beef cattle herds free of paratuberculosis
- to support the national control programmes for paratuberculosis.

Certification and control programmes have already started in some countries. Paratuberculosis should be considered a herd disease and certification must be based on periodic culture of pooled faeces samples and PCR confirmation of specific DNA sequences from milk four times a year. Culling of shedding animals, careful evaluation of suspect clinical cases of paratuberculosis and post-mortem inspection of all culled cows is recommended to reach the status of a paratuberculosis-free herd. Closing the herd until it is possible to purchase animals from guaranteed paratuberculosis-free herds is absolutely essential. Finally, producers of baby food formulas should require milk either free of MAP or with minimal MAP contamination. Thus a reliable quantitative or semi-quantitative method for identifying MAP or its specific components in milk is necessary.

To avoid panic and misinterpretation, thorough education on this topic should be provided immediately. Beef and dairy products are an important component of human nutrition and cannot be omitted. The dairy industry is a valuable sector of agriculture and food production and should be supported in order to rapidly reach a solution of the problem.

CONCLUSION

The possible risk of *Mycobacterium avium* subsp. *paratuberculosis* dead cells or bacterial structures in food in respect to autoimmune Crohn's disease should be carefully monitored. National programmes of paratuberculosis control and certification of paratuberculosis–free herds should be strongly supported to decrease the risk of exposure for children and people under the highest risk for Crohn's disease. Producers of infant formulat should use MAP free milk on a voluntary basis.

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Heat sensitivity of *Mycobacterium avium* subsp. *paratuberculosis* in milk under pilot plant pasteurisation conditions

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ABSTRACT

Raw cow's milk inoculated with four laboratory strains (10^2-10^5 cfu/ml) of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was pasteurised in a custom designed pilot plant pasteuriser having a maximum throughput of 580 litres/h under turbulent flow conditions. Following 16 pasteuriser trials none of the MAP strains survived HTST (high temperature short time) conditions (72.5^oC x 27s). Milk was collected from two dairy herds with faecal positive cattle. The milk collected from one herd on 5 occasions in the autumn did not contain any detectable MAP organisms. Milk from the second herd, sampled on only one occasion in early winter, contained MAP at a low concentration. MAP was not detected in any of this milk following pasteurisation at 72.5^oC for 27 seconds. Two isolates of MAP inoculated into milk were likewise inactivated on pasteurisation. This study supports current evidence that a minimum 4 $\log_{10}$ reduction of MAP is occurs under turbulent flow HTST conditions employed in the dairy industry. The results also support the belief that even if MAP is present due to faecal contamination, it is likely to occur at relatively low levels in processing milk at the factory.

Key words: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), pilot plant pasteuriser, milk.

INTRODUCTION

MAP has long been recognised as an animal pathogen causing Johne's disease, a severe intestinal inflammation in ruminants. Its involvement in a similar type disease in humans (Crohn's) is controversial (Chacon, 2004). Potential sources for MAP infection in humans include milk and contaminated water supplies. The presence of the organism in raw milk from infected cows is well documented (Sweeney et al. 1992). The reported survival of MAP in laboratory pasteurisation studies is a cause for concern raising the possibility that retail supplies of pasteurised milk could contain the organism (Grant et al. 1996, Sung and Collins, 1998). The need to confirm the results of these experiments under conditions similar to the industrial process was recognised and the effectiveness of pasteurisation on inactivating MAP using pilot plant or commercial scale equipment has been studied (Pearce et al. 2001, Grant et al. 2002c, Hammer et al. 2002, Rademaker et al. 2002, McDonald et al. 2005). It is now generally agreed that turbulent effect pasteurisers such as those used in the industrial process achieve significant inactivation of MAP, though reports continue to appear suggesting that HTST pasteurisation is not reliable on every occasion and the organism can occasionally carry over in pasteurised product (Grant, et al. 2002b).

It is not certain what conditions might favour survival of MAP during HTST processing though factors such as initial numbers present, strain type, and degree of cell clumping may contribute to survival (Grant et al. 2002a, Gao et al. 2002, Klijn et al. 2001). The isolation protocol and culture conditions for the organism could also influence the detection of sub-lethally damaged cells of MAP in the heated milk (Grant and Rowe, 2004). This study augments others in describing the heat sensitivity of MAP under HTST conditions simulating the industrial process using laboratory and field isolates of the microorganism.

MATERIALS AND METHODS

Bacterial strains
MAP reference strains ATCC 19698, 43015, and NCTC 8578 as well as a bovine field isolate B4 were used in these studies. *Mycobacterium smegmatis* mc(2)155 and *Mycobacterium fortuitum* an unclassified ATCC strain (both gifts from University College Cork) were also included in heat inactivation trials. The survival of a recognised thermoresistant (i.e., thermoduric) species, *Enterococcus faecium* ATCC 49624 was also studied in the pilot plant pasteuriser.

Growth of cultures
MAP cultures (200ml) were grown statically for up to 4 months at $37^{o}C$ in Mycobacteria 7H9 broth (Difco) containing in 1 litre, 100ml OADC enrichment (Becton Dickenson), 2ml glycerol and 2mg Mycobactin J (Allied Monitor Inc. Fayette, Mo. 65248 USA). Confirmatory tests for MAP included slow growth, IS*900* PCR positive results, acid fast by Ziehl-Neelson staining and mycobactin dependency. The other mycobacterial species were grown in stirred culture without Mycobactin J. *E. faecium* was grown in brain heart infusion broth (BHI-Merck) to early stationary phase.

Addition of laboratory isolates to raw milk
Raw milk was collected directly from the milking line prior to the bulk tank in the dairy, and transported directly to the pasteuriser unit in sterile containers. As a result the milk contained only low numbers of bacteria, including few thermoresistant organisms. This was beneficial in subsequent experiments where a decontamination step was necessary to remove contaminating background microorganisms. Typically, 1 litre of the bacterial culture was added to 60–70 litres of milk in the balance tank of the pasteuriser which was fitted with a high speed agitator and lid to ensure good mixing. Both the culture and inoculated milk were sampled for bacterial numbers.

Addition of field strains to raw milk
Two herds testing culture positive for MAP in faecal samples were identified. Chilled milk from these herds (ca. 70 l) was pasteurised within 24 hours of collection. Faecal material was collected from a Johne's (faecal) positive animal and 15g added to 100 ml of milk, vigorously shaken and used to inoculate 20 l of raw milk in the pasteuriser balance tank. The heat sensitivity of the unknown number of MAP strains in the milk and faecal inoculated milk sample was determined. MAP strains were subsequently isolated from the milk and faecal samples and following laboratory culture and inoculation into raw milk, the heat sensitivity of these isolates was also determined.

Microbiological analysis of unheated samples
All raw milk samples were assayed for total bacterial count, thermoduric and spore count by plating on milk plate agar using standard procedures. For determination of MAP, milk microbial background was decontaminated by centrifuging a 50 ml sample at 3,600 x g for 15 minutes and adding 10 ml of 0.75% (w/v) hexadecylpyridinium chloride (HPC-Sigma) to the pellet. For milk spiked with laboratory isolates, the pellet with HPC was incubated at room temperature for 2 hours. For milk collected from Johne's positive herds, the combined pellet and cream from the centrifugation step was decontaminated for 3 hours. The pellet from milk inoculated with faeces was decontaminated for 5 hours in HPC. Following treatment, samples were centrifuged as above and the pellet suspended in 0.5ml sterile phosphate buffer- saline (PBS) at pH 7.0. MAP was enumerated by plating on Herrold's egg yolk medium (HEYM) supplemented with Mycobactin J as follows: twelve millilitres of the medium tempered a t $45^{o}C$ were aseptically dispensed into 60 ml culture flasks (Sarsted) and the agar surface air dried in a laminar flow hood. Five replicate 0.1ml volumes of the resuspended pellet, diluted as required, were added to the agar surface in the culture flasks which were rotated to aid distribution of the sample and incubated at $37^{o}C$ for up to 6 months The fast growing mycobacterial species were re-plated on Mycobacteria 7H 11 Agar (Difco) containing OADC supplement but no Mycobactin in standard petri dishes and colonies counted after incubation at $37^{o}C$ for 3 days. *E. faecium* was determined by plating on kanamycin aesculin agar (KAA-Merck). No decontamination or centrifugation steps were applied in the case of these bacteria.

Details of the pilot plant pasteuriser
The heating trials were carried out using a pilot scale pasteurising plant (Fig. 1) with a rated capacity of 580 litres per hour located in a Class 2 facility. The plant contained a balance tank (ca.100 l) with lid and

variable-speed agitator to ensure safe yet thorough mixing of milk inoculated with MAP. Milk was heated in a three-stage heat exchanger comprising a regeneration section, a hot water heating section and a cooling section. The sampling port was located after the cooling section. (Fig. 1). A Satline process control system (Tetra Pak) allowed precise temperature control along with full data capture and storage. A two-stage homogeniser could be connected in-line, upstream of the final heating section, though for most plant runs the homogeniser was by-passed. Residence time distribution in the pasteuriser was measured by injecting a saturated salt solution and recording the conductivity of the solution exiting the holding tubes with a conductivity meter.

1.	Balance tank
2.	Pump
3(a).	Regen. heat ex.
3(b).	Hot water heating
3(c).	Chilled water cooling
4.	Holding section
5.	Hot water circuit
6.	Sterilising tank

Temperature transmitter
Flow transmitter
Sampling point
Conductivity transmitter
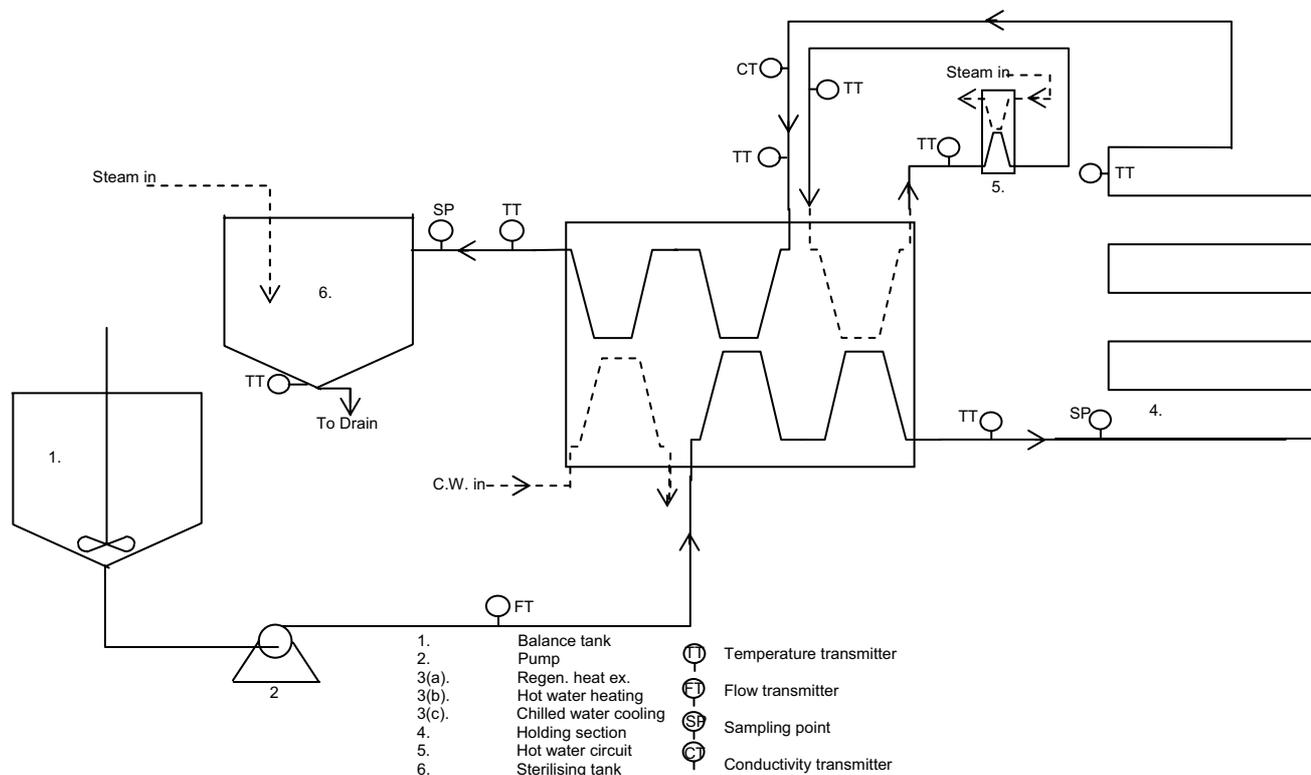
Figure 1. Pilot plant pasteuriser schematic

Heat treatment of milk
HTST conditions used for studying survival of MAP were 72.5^{o}C for 27s. On-line monitoring of physical parameters for assuring performance of the pasteuriser was accompanied by periodic assay of the milk enzymes, alkaline phosphatase (Painter and Bradley, 1997) and lactoperoxidase (Barrett et al. 1999). Partial kinetic inactivation data was derived by heating at 65°C (mycobacteria) and at 70°C (*E. faecium*) at holding times of 10-50 s.

Collection of samples after heat treatment
For small samples, milk was sampled though the rubber seal on the sampling port using a needle and 10 ml vacutainers (Becton-Dickenson). Where larger samples were required 250 ml sterile bags (Model no. S1000-4-6-1, Novaseptic Sweden) were used. Approximately 100 ml volumes of heated milk were collected aseptically from the sampling port on the pasteuriser line. These samples were stored at 4°C for 24-48 hours prior to assay and MAP numbers determined as described for the non-heated samples but without a decontamination step.

RESULTS

Physical measurements of pilot plant operation
Operation of the pilot plant at various flow rates are shown in Table 1. The predicted and measured holding times of the pasteuriser unit are compared, with the former based on the geometry of the holding tube and

the plant flow rate. The ratio of average:shortest hold time is 1:1 for turbulent flow and 1:2 for laminar flow (Pearce et al. 2001) and it is seen that values reported here fall within those specified for turbulent flow. Reynolds numbers for the flow rates measured ranged from 12,000 to 5,000, again within acceptable values for turbulent flow (Hastings et al. 2001) and results in Table 1 indicate turbulent flow conditions were in place at all flow rates in the pilot plant. All survival data for the mycobacteria are based on the average measured value of the 27s holding tube at the flow rate of 500 l/h and a holding temperature of 72.5^{o}C where a Reynolds value of 10,348 is indicated. Residual alkaline phosphatase activity of pasteurised milk should not exceed 350 mU/l (Painter and Bradley, 1997) to conform with governmental food legislation; values less than this (68 mU/l) were measured in the milk following heating at 72.5^{o}C for 27 s in the pilot plant pasteuriser. Lactoperoxidase activity was not affected by these conditions since residual enzyme activity was detectable up to 80°C.

Table 1: Flow characteristics of pilot plant pasteuriser.

Flow (litres per hour)	580	500	400	300	250
Predicted time (sec)	22.0	25.0	31.5	42.0	50.0
Measured time (sec)	24.3	27.3	32.9	43.1	50.3
Fastest particle time (sec)	21.9	24.4	27.9	35.7	39.4
Ratio average:fastest	1 : 1.1	1 : 1.1	1 : 1.2	1 : 1.2	1 : 1.3
Reynolds No.	12107	10348	8278	6209	5174

Growth characteristics of the mycobacteria

MAP cultures were dependent on Mycobactin J for growth but only following thorough washing to remove any carry over of Mycobactin J remaining from previous culture media. Substituting lactoferrin (an iron binding protein present in milk) for Mycobactin J did not support growth. MAP culture isolates were acid-fast and IS 900 positive. Growth in the MAP cultures took many weeks to appear whereas isolates appeared within 2-3 days for fast growing species.

Mycobacteria- HTST inactivation

In a total of 16 pasteuriser trials four laboratory strains of MAP and two field sources of strains (milk and faeces) were evaluated for survival at 72.5^{o}C x 27s in the pasteuriser. Results are shown in Table 2. Most culture flasks were free of any growth after 6 months incubation. In all cases where bacterial colonies were detected none were typical of MAP on the basis of colony morphology or cell staining and it was concluded that none of the MAP strains survived pasteurisation. These results indicate a 4-5 log10 kill of MAP on HTST pasteurisation. Most heat inactivation studies were carried out on non-homogenised milk. Inclusion of a homogeniser prior to heating appeared to have a minimal effect on cell numbers. Cell clump disruption resulted in at most a 0.5 log increase in numbers compared with the non-homogenised milk. *M. smegmatis* and *M. fortuitum* did not survive pasteurisation at 72.5^{o}C for 27 s at inoculation levels of 2x104 and 6 x 105 cfu/ml respectively in the raw milk.

Table 2: Effect of HTST pasteurisation on survival of MAP in milk.

Pasteuriser runs*	MAP strain	Inoculated milk-cfu/ml**	Survivors
6	19698	4.0×10^{2} to 2.4×10^{4}	None detected
2	8578	4.0×10^{3} and 6.7×10^{4}	None detected
2	B4	1.3×10^{3} and 1.5×10^{3}	None detected
2	43015	6.0×10^{4} and 2.0×10^{5}	None detected
2	Faecal isolate	1.4×10^{1} and 1.4×10^{3}	None detected
2	Milk isolate	< 1 and 1.2×10^{4}	None detected

*Number of replicate trials undertaken; **Minimum and maximum numbers detected

Field populations of MAP –HTST inactivation

Milk collected from two fecal culture positive herds was pasteurised to determine survival of any field strains of the organism present. One herd was sampled five times in the autumn and no viable MAP organisms were detected in either the raw or pasteurised milks. (Table 3) MAP was present in raw milk from the second herd sampled just once in early December but at a very low level (<1cfu/ml). No organisms were detectable following pasteurisation (Tables 2 and 3).

The field MAP strains were present at a low level in both the herd milk sample and in the milk inoculated with faeces and it was necessary to isolate and culture the organism so as to obtain realistic numbers in the milk for pasteurisation studies. On addition of these isolates to milk at relatively high numbers (10^3-10^4 cfu/ml) and following HTST pasteurisation, no organisms survived (Table 2).

Table 3: MAP in milk from two Johne's (faecal) positive herds and affect of pasteurisation.

Month	Milk collected (litres)	Thermoduric count(cfu/ml)	Presence of MAP	
			Raw	Heated
September*	45	250	ND	ND
October*	90	325	ND	ND
October*	70	100	ND	ND
October*	80	23	ND	ND
October*	90	No value	ND	ND
December**	70	215	Positive	ND

* Herd 1 : ** Herd 2; ND= None Detected

Inactivation kinetics

Partial inactivation kinetics for the mycobacterial species was established at 65°C where significant survival of these bacteria occurs and where measurable values for heat inactivation could be derived. A $D_{65°C}$ value of 20 s was obtained for MAP strain 19698 with corresponding values of 25 s and 10 s obtained for *M. smegmatis* and *M. fortuitum* respectively (Fig 2). The thermoduric species, *E. faecium* exhibited little or no inactivation at 65°C and it was necessary to increase the pasteuriser temperature to 70°C to achieve measurable inactivation rate of 11s. Survivors of *E. faecium* were readily detected on HTST pasteurisation of milk (105 cfu/ml) with counts more than doubling following a resuscitation treatment of 24 h incubation at 4°C.
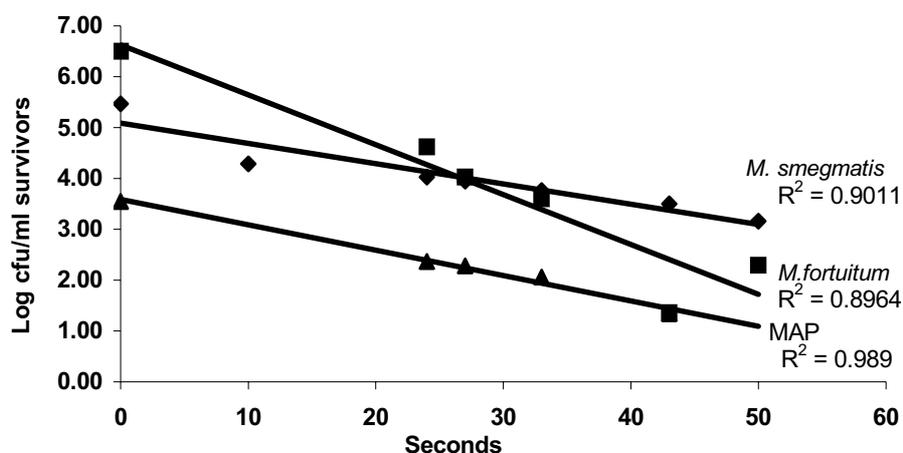
Figure 2. Inactivation of three mycobacterial species in raw milk at 65°C. (Mean of triplicate analysis)

DISCUSSION

In this study considerable attention was given to characterising the performance of the pasteuriser, as results are meaningful only when parameters such as temperature profiles, retention times, and flow characteristics are accurately measured. The process control system ensured that each run on the pilot plant was monitored via the data collection system and on-line sampling did not commence until conditions were seen to stabilise. Particular attention was also given to the source of the raw milk used in these experiments with emphasis on obtaining milk with low thermoduric counts. Milk collected on-line contained few thermoduric bacteria, a necessary condition in order to avoid a decontamination step prior to plating the pasteurised milk on HEYM agar. The concern is that following heating, sub-lethally injured MAP subjected to a further stress of decontamination with HPC may fail to grow, giving rise to false negative milk culture results. Low starting thermoduric counts, coupled with inhibition of growth of some thermoduric species on HEYM, resulted in minimum background growth on the plates. This was particularly the case for milk heated at 72.5°C, though more background contamination was encountered with milk heated at 65°C.

More background contamination from pasteurised milk was evident when the pellet fraction only was plated vs. the combined pellet and cream fractions. We have noticed significant inhibition of MAP following decontamination with HPC and this has also been observed by Grant and Rowe (2004), who concluded that chemical decontamination should not be applied to pasteurised milk in heat inactivation studies. However others have reported no inhibition of MAP by HPC treatment or antibiotic selection (Pearce et al. 2002: McDonald et al. 2002). In the present study, all heated milk samples were incubated at 4°C for 24-48h prior to plating on HEYM to allow for resuscitation of any heat-injured MAP as recommended by Grant et al. (2002c), though in a subsequent study these authors concluded that there was no clear evidence of a beneficial or detrimental effect of a refrigeration recovery step (Grant and Rowe, 2004). It is interesting to note that *E. faecium* benefited from a resuscitation step and the organism was routinely recovered following heating at 72.5^{o}C for 27s.

In the present study, none of the four laboratory strains of MAP survived pasteurisation at 72.5^{o}C for 27s in the pilot plant pasteuriser. This is in agreement with the results obtained by Pearce et al. (2001) and Rademaker et al. (2002) in their pilot plant studies. McDonald et al. (2005) studied survival of MAP inoculated into milk in an industrial pasteuriser and found no survivors following heating under HTST conditions in 17 out of 20 runs. In three of the trials a few viable organisms were found by the Bactec 12B culture method but seemingly none on culture on HEYM and these authors concluded industrial pasteurisation produces a 4-6 log10 reduction in MAP numbers. Grant et al. (2002a) isolated MAP in pasteurised milk of naturally infected cows and Hammer et al. (2002) found survival of MAP in MAP-spiked milk in a pilot plant pasteuriser over a temperature range of 68°C to 90°C and holding times of 18 to 60s, Survival was independent of initial count, bacterial strain and time-temperature conditions tested in the 68-79°C temperature range and holding time of 18s. In these experiments special attention was paid to lengthy resuscitation and incubation times which may have influenced the outcome of some of the results. It is interesting to note that the two fast growing mycobacterial species tested here did not survive HTST pasteurisation.

The D_{65^oC} value of 20 s obtained for the type strain of MAP (19698) compares with a D_{66^oC} value of 5s reported by Pearce et al. (2001) for the same strain of the organism treated in a pilot plant pasteuriser. Differences in conditions of culture growth and treatment of cells prior to inoculation of milk might explain the difference in kinetic data obtained for MAP in the two pilot plant experiments. Pearce et al. (2001) cultured MAP on a roller apparatus for up to 44 days and homogenised the cells in a sonicating water bath to reduce the degree of clumping, whereas in the present study MAP was grown statically for up to 120 days and a declumping step was not involved. Bacterial cells tend to be more sensitive to environmental stress while in the exponential growth phase with such cells reported to be more sensitive to heat than cells in stationary phase (Jay, 1992). Also, MAP cultured statically grows as a pellicle at the base of the culture vessel and exhibits a high degree of cell clumping. The significanc e of clumping in determining the heat resistance of MAP is still not resolved and it has been suggested that survival has more to do with the fraction of clumps, with large cell numbers, rather than a heat resistant cell fraction (Klijn et al. 2001). On the other hand, Hastings et al. (2001) considered the heat resistance of MAP from a food engineering perspective and concluded that the thermal process delivered during pasteurisation is relatively unaffected by particle diameter making the presence of clumps unlikely to reduce the effectiveness of HTST pasteurisation.

Most reports on the isolation of MAP from raw milk are of a qualitative rather than a quantitative nature with few if any accurate estimates available for numbers of the organism present in raw milk. The problem of accurate measurement of MAP numbers is compounded by the absence of a selective growth medium and Grant et al. (2001) have indicated that MAP might survive HTST pasteurisation when present in milk at levels >100 cfu/ml. Sweeney et al. (1992) reported a MAP count of 2-8 cfu/50 ml in milk taken from subclinically infected animals and Grant et al. (2002b) estimated counts between 4-20 cfu/50 ml in silo milk collected in their survey of UK dairy processing plants. Stabel and Lambertz (2004) reported MAP to be present at highest concentration in early colostral milk (50 cfu/ml) declining by 5 days after calving to less than 1 cfu/ml. In the present study, MAP could not be detected in milk from one infected herd sampled on 5 consecutive occasions, though it was detected at a low level (< 1 cfu/ml) in a second infected herd. These results are in line with the above findings that MAP is shed at relatively low levels in milk. In contrast, MAP is known to be shed at high levels in faeces of infected animals and faecal contamination of milk may be of more concern than direct shedding of MAP into milk (Grant et al. 2000b). However, the amount of faecal

shedding of MAP in an infected herd can vary greatly with animals described as low, moderate and heavy shedders and estimates for these groupings have been given as <300, 300-3,000 and >3,000 cfu/g MAP, respectively (Van Schaik, 2002). In the present study the faecal sample inoculated into milk was calculated to contain ca. 104 cfu/g and gross contamination of milk with this faeces resulted in a count of 14 cfu/ml milk.

The well-documented presence of MAP in raw and pasteurised milk is of continuing concern. Recently, Ayele et al. (2005) reported the presence of live MAP in 1.6% of retail pasteurised milk in the Czech Republic and concluded that the organism may occasionally survive commercial pasteurisation. A recent study of retail pasteurised milk in the United States (Ellingson et al. 2005) reported the presence of viable MAP in 2.8% of the samples tested and considered post-pasteurisation contamination as one possible reason for the presence of the organism. In the absence of evidence of pasteurising conditions applied in the processing plants however it is also possible that the positive samples were the result of inadequate pasteurisation treatment. A survey of MAP in both raw and pasteurised processor supplies was undertaken in the Republic of Ireland (O'Reilly, 2004) and based on the presence of MAP DNA in samples, similar results were obtained as for a UK survey (Grant et al. 2002b) with 12.9% and 9.8% of raw and pa steurised respectively positive for MAP. However in contrast with the UK results where 6.7% and 6.9% of the raw and pasteurised milks were culture positive, O'Reilly et al. (2004) found that only 0.3% of the raw and none of the pasteurised milk samples were culture positive for MAP and concluded that although MAP DNA is occasionally present at low levels in raw and pasteurised milk, current pasteurisation conditions are effective in the control of this suspect human pathogen.

CONCLUSIONS

Results of these experiments confirm a minimum of 4 log kill of MAP under pilot plant HTST pasteurisation conditions and also indicate that MAP shed into milk by infected animals may be present at relatively low levels. Furthermore, in view of the fact that MAP does not replicate in milk it is likely that entry via the faecal route would require gross contamination with faeces to achieve significant numbers of the organism in milk at the processing plant.

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Waterbourne transmission of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from animals to humans in South Wales

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ABSTRACT

Epidemiological research carried out in Cardiff in the mid-1970s demonstrated a highly significant increase in the incidence of Crohn's disease in 11 of the local electoral city wards. Sampling of the river Taff water above Cardiff was done twice weekly from November 2001 to November 2002 (Fig. 1, site C). MAP was detected by IS*900* PCR and culture. Thirty-one of 96 (32.3%) daily samples were IS*900* PCR positive. Colonies typical of MAP were obtained from 12 of the 18 (66%) PCR-positive Taff water samples and sediment samples. It is hypothesized that the pattern of clustering of Crohn's disease diagnoses in Cardiff may be due to the inhalation of MAP from the river Taff.

Key words: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), environment, river water, aerosol, Crohn's disease.

INTRODUCTION

In South Wales (UK), a populated coastal region lies beneath hilly pastures grazed by livestock in which MAP is endemic. The Taff is a major spate river which runs off the Brecon hills in a south easterly direction and through the city of Cardiff beside the sea. The River Taff is formed by the confluence of the Taff Fechan River from the East which rises in the Brecon hills and is controlled by Pontsticill Reservoir, and the Taff Faw River to the West, which also rises in the Beacons and is controlled by Llwyn-on Reservoir. These rivers meet at Cefn Coed just north of the town of Merthyr Tydfil to form the River Taff itself (Fig. 1). Epidemiological research carried out in Cardiff in the mid-1970s demonstrated a highly significant increase in the incidence of Crohn's disease in 11 of the local electoral city wards (Mayberry, 1989; Mayberry et al., 1978). Of these 11 high incidence wards, 8 directly bordered the River Taff, and the three that did not were immediately adjacent to the north east (Mayberry, 1989; Mayberry et al., 1978). It has been proposed that this is the direction in which aerosols containing MAP may be carried by the prevailing south westerly wind (Hermon-Taylor, 1993). This work was the first step in examining this hypothesis experimentally by testing for the presence of MAP in the catchment of the Taff (Fig.1), and by monitoring the presence of organism twice weekly in Taff river water, from November 2002 over a period of one year.

RESULTS AND DISCUSSION

We sampled Taff water above Cardiff twice weekly from November 2001 to November 2002 (Fig. 1, site C). MAP was detected by IS*900* PCR and culture. Thirty-one of 96 (32.3%) daily samples were IS*900* PCR positive (Fig. 2).
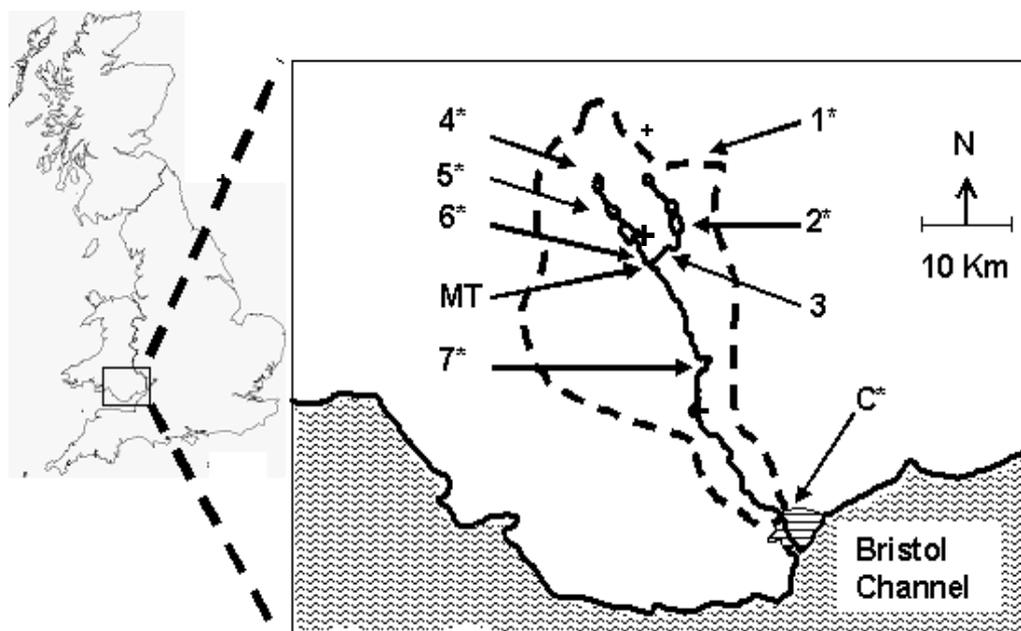
Fig. 1. Location of the study region in South Wales, UK (Pickup et al., 2005). The enlargement shows the catchment (dashed line) of the Taff which at the time of the study contained 30,435 beef and dairy cattle, and 571,429 sheep of which 304,443 were breeding ewe. The River Taff itself is a spate river which runs down from the Brecon Hills and through the city of Cardiff before entering the Bristol Channel. 1.Upper Neuadd reservoir, 2.Pontsticill reservoir, 3.Taff Fechan River, 4. Brecons reservoir, 5.Llwyn-on reservoir, 6. Taff Faw River, 7. River Taff. MT is the town of Merthyr Tydfil and C is the city of Cardiff. * are sampling sites and + denotes IS*900* positive sites, larger + denotes monitoring point.
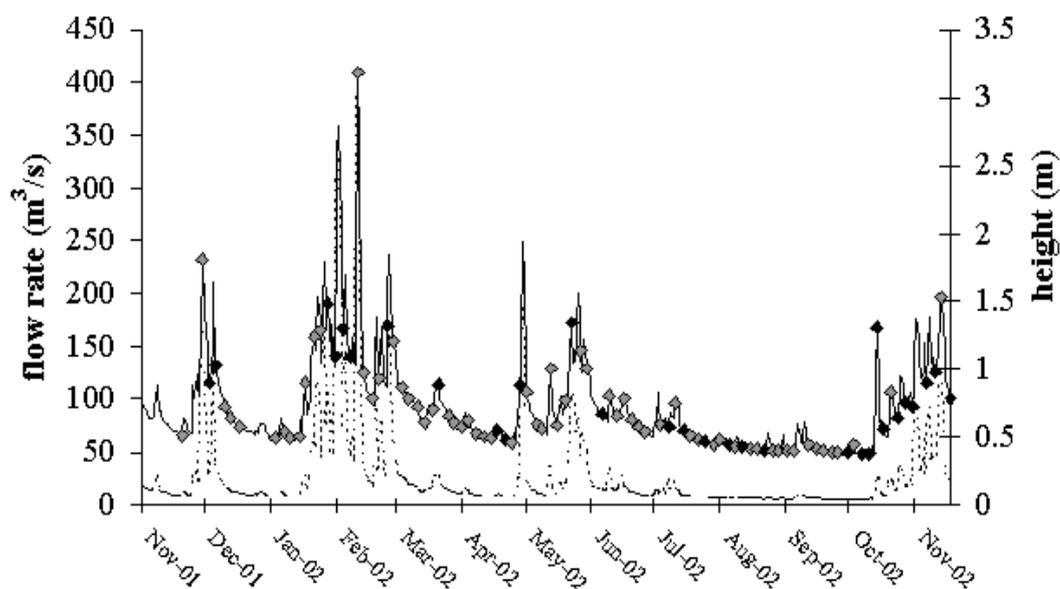
Fig. 2. Relationship between presence/absence of MAP and flow and river height of River Taff (Pickup et al., 2005). Flow rate and height of the river Taff (November 2001-November 2002). Data were obtained using the HYDROLOG Data Management System V2.61 (Hydro-Logic Ltd, UK) from Pontypridd logging station (grid reference ST07938973). Plotted data were recorded at 9:00 am each day. The full line plot represents the river height data whilst the dotted line represents the flow rate. Sample points are shown on the river height plot as grey diamonds and samples PCR positive for MAP as black diamonds.

Statistical analysis of data combining the river parameters (rainfall, river flow and height) with the twice weekly monitoring for IS*900* showed that:
1. rainfall in the catchment and river parameters (river flow, river height) were significantly associated.
2. presence of IS*900* was significantly associated with higher rainfall events on the sample day, and on each of the six preceding days.

3. presence of IS*900* could not be predicted confidently from river parameters (such as river flow) as it lay outside the 5% confidence limits but was close at 8% indicating that MAP was also entering the river through rainfall-independent events (probably point sources).

Sequence analysis of IS*900* PCR products from river water showed that 16 of 19 amplicons had a single nucleotide polymorphism at position 214. This is consistent with a different strain of MAP in the river, unculturable by the methods we used.

Sediment samples from the catchment were analysed and the highest reservoirs (Fig. 1; site 1) were negative by IS*900* but positive by culture. The result is consistent with the entry of these pathogens into the catchment at the highest level. This is supported by the positive tests on the Pontsticill and Llwyn-on reservoirs adjacent just lower down the catchment (Fig. 1; sites 2 and 5). Deposition rates for sediments based on assumptions for other lakes, indicated that MAP deposition had occurred for the last 30-50 years.

Colonies typical of MAP were obtained from 12 of the 18 (66%) PCR positive Taff water samples and sediment samples. All were cultured on solid HEYM medium; of a crumbly appearance (typical of MAP); appearing after 8 to 11 months of incubation often first appearing below the surface of the media prior to breaking through the surface; all isolates from the Taff River water and the sediments were confirmed as MAP by IS*900* PCR and amplicon sequencing; MIRU typing (Bull et al., 2003) showed the same profile as the control bovine MAP strain recently cultured from a Johne's disease cow within the study region; and all were confirmed as MAP of bovine origin. In addition, parallel studies showed that MAP remained culturable in lake water microcosms for 632 days and persisted out to 841 days.
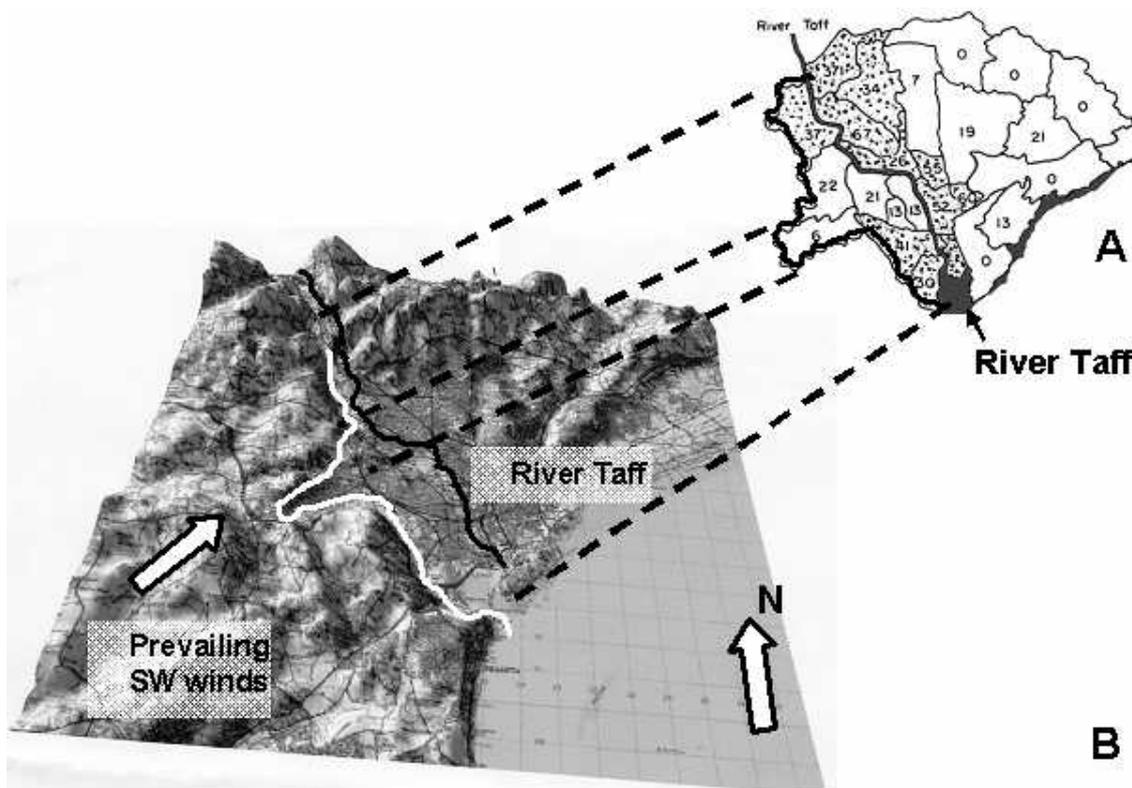
Fig. 3. Relationship between disease clusters and prevailing wind in Cardiff, UK (Pickup et al., 2005). The panel A upper right shows the distribution of the 11 electoral wards in the city of Cardiff shown previously (Mayberry, 1989; Mayberry et al., 1978) to have a highly significant (P=<0.001) increase in the incidence of Crohn's disease. The panel B lower left is a relief map of Cardiff with the course of the river Taff through the city indicated in blue. The wards with the high incidence of Crohn's disease are seen to lie along the Taff with the exception of a gap in the centre stretch of the windward right bank of the river (facing downstream). This gap directly faces a valley between hills to the north and south, which is open to the prevailing south westerly winds.

Previous epidemiological research in Cardiff demonstrated a highly significant increase of Crohn's disease in 11 districts (Mayberry, 1989; Mayberry et al., 1978). These bordered the river except for a gap on the

windward side. A topographical relief map shows that this gap is directly opposite a valley open to the prevailing south-westerly winds (Fig. 3). This would influence the distribution of aerosols carrying MAP from the river as on the leeward side 3 additional high incidence wards lie immediately adjacent to those bordering the river to the northeast. This is the direction aerosols containing MAP would be carried on the prevailing southwesterly winds (Hermon-Taylor, 1993; Pickup et al., 2005).

CONCLUSION

The following four factors buttress the hypothesis that the pattern of clustering of Crohn's disease in Cardiff may be due to the inhalation of MAP from the river Taff (Fig. 3, Pickup et al., 2005): aerosol droplets concentrate bacteria, the spread of mycobacteria in aerosols are well described (Falkinham, 2003; Primm et al., 2004; Wendt et al., 1980), inhalation is a probable route of MAP infection in cattle (Corner et al., 2004; Falkinham, 2003) and almost everyone with chronic inflammation of the gut of the Crohn's disease type is found to be infected with this chronic pathogen (Hermon-Taylor et al., 2004; Naser et al., 2004; Sechi et al., 2005).

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Effective heat inactivation of *Mycobacterium avium* subspecies *paratuberculosis* in milk

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ABSTRACT

This study was designed to evaluate the effectiveness of high-temperature short-time (HTST) pasteurization and homogenization on the inactivation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP-contaminated feces of cows with clinical symptoms of Johne's disease was used to contaminate the raw milk with MAP to final concentrations varying between 10^2 to $3.5 * 10^4$ cells per ml. Twenty two different time-temperature combinations were evaluated ranging from 60 - 90°C and holding times of 6, 10 and 15 seconds using a pilot scale turbulent-flow pasteurizer. A total of 136 observations were obtained; prior homogenization occurred in 34 of these samples. Regression analysis of 69 data points allowed for modeling of the heat inactivation of MAP. From these inactivation experiments the following conclusions can be drawn: HTST pasteurization conditions as applied in the Dutch dairy industry result in a >6 log reduction of MAP. Homogenization did not contribute significantly to the inactivation.

Key words: Pasteurization, homogenization, MAP, HTST, turbulent flow.

INTRODUCTION

Heat treatment is the most frequently applied process to ensure the quality and safety of milk and milk products. Several research groups have published conflicting data on heat inactivation of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in milk under various pasteurization conditions. Based on the knowledge of critical factors from published papers, a set of experiments was set up to determine the effectiveness of heat combined with homogenization to inactivate MAP in naturally contaminated raw milk.

MATERIALS & METHODS

A pilot plant scale turbulent flow pasteurizer was used at temperatures between 60- 90°C and holding times of 6, 10 and 15 seconds for a total of 136 tested samples. (For details of the experimental set-up see Rademaker et al. 2003). Additional homogenization of raw milk was applied prior to heating in 34 of the 136 experiments. MAP-contaminated feces of cows with clinical symptoms of Johne's disease were obtained from Dr. D Bakker (CDIC, Lelystad, The Netherlands). This manure was used to contaminate the raw milk to a final concentration of 10^2 to $3.5 * 10^4$ cells of MAP per ml. Batches of milk were processed using 22 different time-temperature combinations (Table 1). Survival of MAP was quantified by growth in MGIT media using a MPN format. Tubes were judged on visible growth during > 50 weeks of incubation at 37 °C. Initial MAP levels and positive cultures were confirmed by an IS*900*-based real time PCR (Rademaker et al., 2003).

RESULTS

MAP inactivation data were obtained from 136 observations at various time/temperature combinations (Table 1). No survival was found in 56 cases with temperatures higher than 70 °C at holding times of 6, 10

and 15 s. No significant reduction (maximum 1.5 log reduction) was found in 11 cases at temperatures between 60-66 ºC and a holding time of 6 seconds. In 69 cases partial inactivation was found.

Table 1. Decimal reduction after pasteurization at different time-temperature combinations.

Temperature	Time		
	6 s	**10 s**	**15 s**
60 ºC	0.0-0.5	0.4-0.5	0.0-1.5
62 ºC	0.5-1.1	0.0-1.1	0.0-0.7
64 ºC	0.5	0.0-1.1	0.6-1.6
66 ºC	0.0-1.5	0.5-2.1	1.0-2.8
68 ºC	0.6->5.7	3.1->7.1	4.1->7.1
70 ºC	3.1->7.1	>4.2->7.1	>4.2->7.1
72 ºC	>4.7->7.1	ND	>4.2->7.1
80 ºC	>4.2->7.1	ND	ND
90 ºC	>4.2->7.1	ND	ND

Regression analysis of these data points allowed modeling of the heat inactivation kinetics of MAP. Holding time, but not heating and cooling time, was included in the model. Fig. 1 shows the inactivation curves of MAP (1-6 log reductions) and the heat inactivation processes applied in the dairy industry such as thermization and pasteurization. (Thermization is heat treatment at moderate temperatures; holding time is in seconds.)
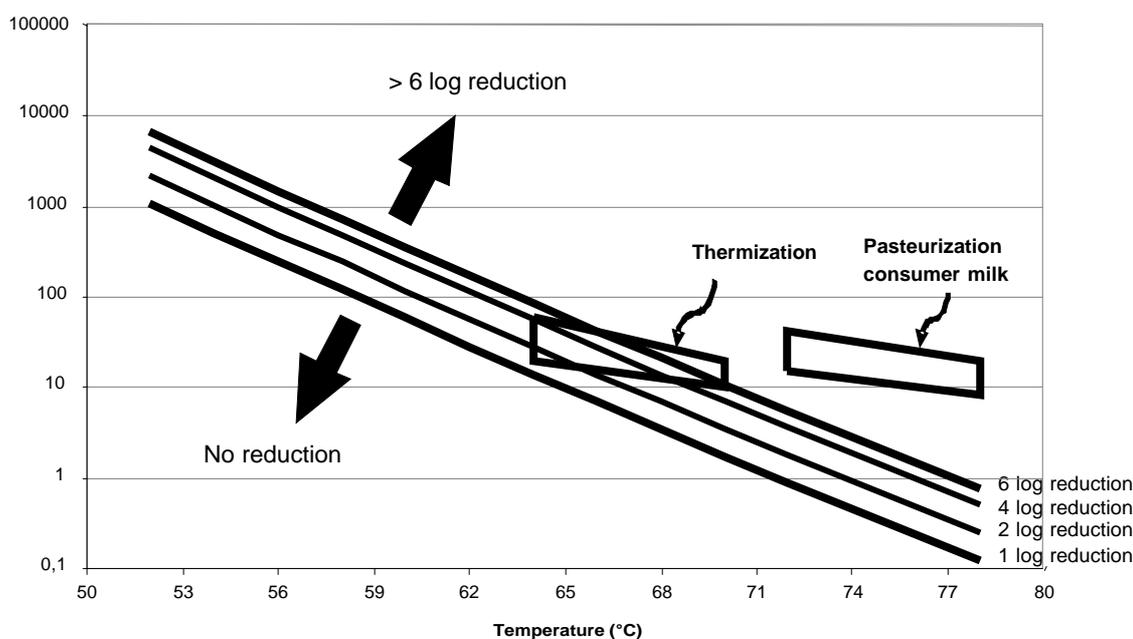
Fig. 1. Temperature - time combinations needed for inactivation of MAP and during various dairy production processes.

The model simulations showed that at the standard high-temperature and short-time pasteurization conditions used in the Dutch dairy industry a >6 log reduction of MAP is achieved.

No significant additional effect of homogenization of raw milk on MAP inactivation was observed in the model as shown by plotting the inactivation constant k vs. the temperature (Fig. 2).
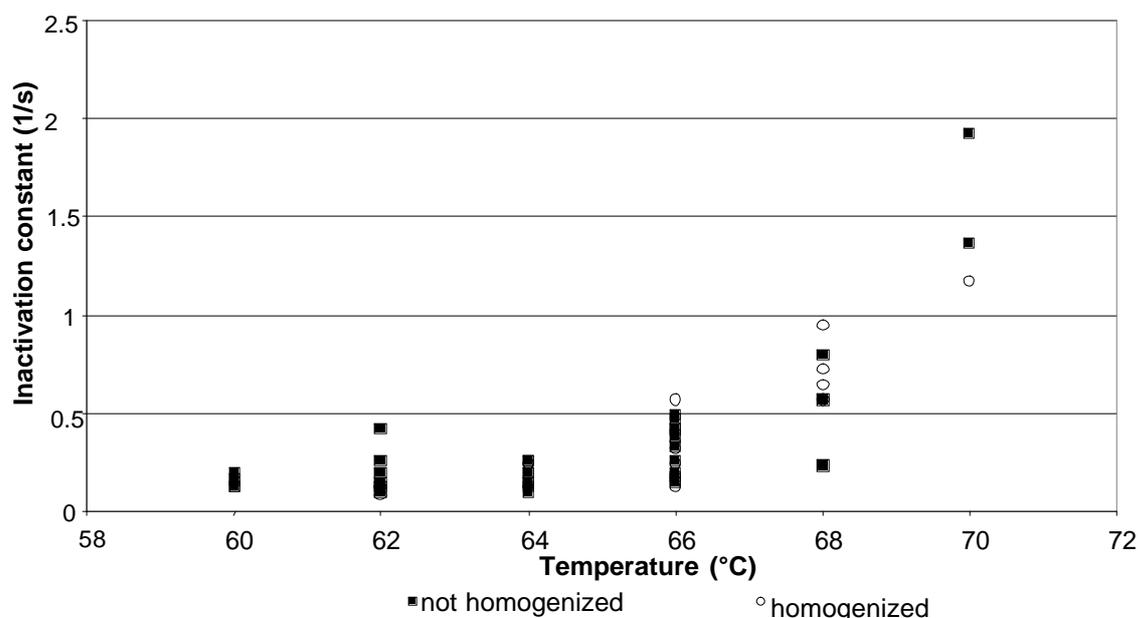
Fig. 2. The effect of homogenization on MAP inactivation.

The higher the constant k, the more inactivation occurs per second. As can be observed in Fig. 2, the results of the experiments with and without homogenization were comparable.

DISCUSSION

The current study showed significant reduction of MAP by pasteurization. In the past 5 years several studies have been published about heat inactivation of MAP in milk (Table 2).

Table 2. Turbulent flow pasteurization experiments.

Reference	This study	McDonald et al., 2005	Grant et al., 2005	Stabel et al., 2004	Hammer et al., 2003	Grant et al., 2002	Pearce et al., 2001	Pearce et al., 2001
Survival until	70 ºC, 6s	72 °C, 15 s	82.5°C, 60 s	71.7 °C, 15 s / 74.4 °C, 15 s	72 ºC, 18 s, 90 ºC, 60 s	73 ºC, 25 s	69 ºC, 15 s	69 ºC, 15 s
Decimal reduction	>4.2->7.1	>4 - > 6	4 - 5.2	5 – 7.7	Not reported	Not reported	>7	>7
Additional reduction by homogeni-zation	not at 15 s	ND	at 25 s not at 15 s	ND	ND	Is reported	ND	ND
Inoculate	faeces	lab strains	lab strains	lab strains	lab strains	"naturally"	faeces	strains
Cfu/ml after inoculation	10^2 to 3.5 x 10^4	4.7 x 10^3 to 1.4 x 10^5	10^1-10^5	10^5 / 10^8	~10^1-10^5	unknown	2-3.2 x 10^2	0.7-16 x 10^3
Heat exchanger	spiral	plate, holding section tube	plate	slugflow and lab scale pasteu-rizer	plate; holding section spiral	Combination of plate and tube	plate	

Overall, a significant reduction of MAP after pasteurization is reported in current scientific literature. Some studies (Hammer et al., 2003, Grant et al., 2005, Grant et al., 2002, McDonald et al., 2005) report survival at high temperatures (>72 ºC). However, a clear explanation for this has not been found. Results can be conflicting due to the use of collection- or field-strains and their passage history, the presence of clumps,

different heating methods during pasteurization, recovery and culturing of survivors as well as the determination of initial and post pasteurization quantity of viable MAP cells. Only limited information is available on the effect of homogenization on the survival of MAP during heat treatment.

In this study, the effectiveness of heat inactivation of MAP in naturally contaminated milk was determined. The procedure used yields MAP-contaminated milk that likely best mimics a natural situation, both in terms of history of the cells and clumps present.
From the inactivation results the following conclusions can be drawn: HTST pasteurization conditions as applied in the Dutch dairy industry resulted in a >6 log reduction of MAP. Additional homogenization did not contribute significantly to the inactivation.

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Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in raw waters in Northern Ireland

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ABSTRACT

A limited study was undertaken in Northern Ireland to test for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in raw waters entering nine water treatment works (WTWs) over a one year period. Three detection methods were employed: IMS-PCR (IS*900*) and culture on Herrold's egg yolk medium and BACTEC 12B medium. Of the 192 samples tested 15 (7.8%) tested positive by one or more of the three detection methods and because of so few positives any conclusions must only be regarded as tentative. Although the highest incidence of MAP was found during the period March to May, overall there was no statistically significant difference between the months. No significant correlation was found between numbers of coliforms or faecal coliforms and presence of MAP. In general, an increased incidence of MAP was found in those WTWs that had a high mean pH value over the sampling period. In experiments involving coculture of MAP with two protozoa species *Acanthamoeba castellanii* and *A. polyphaga* using a MAP inoculum of 10^6 cfu/ml and a protozoan population of 10^5 protozoa/ml the intracellular MAP counts (Log_{10}) were 5.15 and 4.82 respectively. On extended incubation (up to 24 days) MAP was found to survive and even apparently increase in number after 7 days. When challenged with chlorine (0.5, 1.0 and 2.0 g/ml) MAP located intracellularly was significantly more resistant than free MAP. This work shows that water may be a vehicle of spread of MAP, the possible role of protozoa as a survival strategy and the need to determine the efficacy of water treatment operations to remove or inactivate MAP.

Key words: Raw water, culture, IMS-PCR, protozoan engulfment, chlorine resistance.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is excreted in the faeces of symptomatic and asymptomatic cattle and other ruminants suffering from Johne's disease (Clarke 1997) and can survive for protracted periods in the environment (Whittington *et al.* 2004). The organism potentially can therefore contaminate water through agricultural run-off in catchment areas supplying water for domestic use as well as to the estuarine environment. Although mycobacteria in general are ubiquitous in the environment, to the authors' knowledge there are no reports of the isolation of MAP from raw or treated waters destined for domestic use. This study must be seen in the context of the United Kingdom's desire to minimise exposure of the public to this organism as part of a precautionary response (Rubery 2001).

One of the strategies adopted by MAP that may aid its survival is to interact with free-living protozoa. Such protozoa have been isolated from reservoirs used for drinking water as well as water distribution systems (Tondoleke *et al.* 2002). MAP is able to survive digestion by protozoa that are usually bacteriovores. Indeed this, it has been suggested, allows MAP to acquire a phenotype more pathogenic to humans (Hermon-Taylor 2001). Such internalisation may also afford MAP added protection against agents such as chlorine that are used in water treatment operations.

In this work the occurrence of MAP in raw waters entering nine water treatment works (WTWs) within Northern Ireland was determined using both culture and molecular methods. The WTWs were chosen to provide geophysical distance and also different types of geophysical catchment areas to represent Northern

Ireland as well as possible. In addition, the internalisation of MAP by two free-living protozoa, i.e. *Acanthamoeba castellani* and *A. polyphaga,* was studied and the comparative resistance of both internalised and free-living MAP to chlorine was investigated.

MATERIALS AND METHODS

Molecular and culture techniques for MAP
For the IMS-PCR each water sample (1 litre) was centrifuged at 2,500g for 20 min and the pellet resuspended in 10 ml of 0.01 M phosphate buffered saline (pH 7.4, Sigma, Poole, UK) supplemented with 0.4 v/v Tween 80 (PBS-T, Sigma). This was again centrifuged at 2,500g for 20 min and the pellet resuspended in 1 ml PBS-T. The samples were transferred to Eppendorf tubes and 10 μl of antibody-coated beads added and mixed for 30 min at room temperature. These polyclonal antibody-coated beads were prepared 'in-house' and have been used previously for recovering MAP from milk (Grant *et al.* 1998). The IMS procedure was performed and the beads washed three times with PBS-T with magnetic separation for 2 min between washes. After the final wash the beads were resuspended in 800 μl of lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM Tris-HCl, 0.6% sodium dodecyl sulphate, pH 8.0, Sigma) containing 20 μl proteinase K (Sigma) and incubated overnight at 37°C. The incubated samples were transferred to Hybaid Ribolyser tubes (Anachem Ltd, Luton, UK) and ribolysed at 6.5 m/s for 25 s after which they were placed on ice for 15 min to allow any foam to dissipate. The treated samples were taken to a separate location for DNA extraction and IS*900* PCR. The DNA was extracted by adding 500 μl of phenol (pH 8.0, Sigma) and vortexing for 1 min followed by centrifugation at 6,500g for 10 min. The resulting top aqueous layer was transferred to a new centrifuge tube, an approximately equal volume of chloroform:isoamylalcohol (24:1; Sigma) added and the mixture vortexed for 30s followed by centrifugation at 6,500g for 10 min. The upper layer was transferred to a fresh tube containing 0.6 volume isopropanol (400 μl; Sigma) to precipitate the DNA during a period of 30 min at -20°C. The DNA was recovered by centrifuging at 6,500g for 10 min and washed once with 70% v/v alcohol (Sigma). After the ethanol was carefully decanted, the resulting DNA was allowed to air dry before being resuspended in 50 μl of Tris-EDTA buffer (10 mM, pH 8.0, Sigma). The total reaction volume of the IS*900* PCR was 50 μl and was comprised of 31 μl sterile distilled water; 5 μl Taq buffer (x10; Invitrogen, Paisley, UK); 1.75 μl MgCl$_2$ (50 mM); 2 μl of primers P90 and P91 (100 ng/ml; Moss *et al.,* 1992); 3 μl dNTP mix; 0.25 μl Platinum Taq polymerase (5U/μl, Invitrogen) and 5 μl of template DNA. The thermal cycle used was an initial denaturation cycle of 94°C for 2 min, 62°C for 15s, 72°C for 1 min followed by 35 cycles of 94°C for 30s, 62°C for 15s, 72°C for 1 min and a final extension cycle of 94°C for 30s, 62°C for 15s and 72°C for 5 min. The amplicons were separated by gel electrophoresis and visualised by staining with ethidium bromide (1 mg/ml, Sigma). A molecular weight marker ϕX174 RF DNA *Hae* III digest (Sigma) was used and a positive and negative control were included in each run.

Culture was performed by centrifuging the water samples (1 litre) at 2,500g for 20 min. The pellets were resuspended in 10 ml of freshly prepared 0.75% w/v cetylpyridinium chloride (Sigma) and the mixtures transferred to sterile centrifuge tubes (50 ml) and incubated at room temperature for 5h with occasional shaking. The cultures were subsequently centrifuged at 2,500g for 20 min and the pellets resuspended in 1 ml PBS-T. For each sample two slopes of Herrold's egg yolk medium (HEYM) supplemented with 2 μg/ml mycobactin J (Synbiotics Europe SAS, Lyon, France) were inoculated with 200 μl of re-suspended pellet. In addition, one vial of BACTEC 12B medium (Becton Dickinson, Oxford, UK) supplemented with PANTA antibiotic supplement (Becton Dickinson) was inoculated with 200 μl of re-suspended pellet. The HEYM slopes were examined periodically for evidence of typical growth. The BACTEC vials were read weekly on a BACTEC 460 machine (Becton Dickinson). When growth was observed in either medium the presence of acid-fast cells was confirmed by carrying out a Ziehl-Neelson stain. Acid-fast isolates from HEYM or BACTEC were subjected to IS9*00* PCR to confirm MAP identity.

The soil analysis of the catchment areas was as described by Cruickshank (1997).

Ingestion capacity of protozoa for MAP
Axenic cultures of *Acanthamoeba castellanii* (CCAP 1501/1B) and *A. polyphaga* (CCAP 1501/3B) were maintained in tissue culture flasks containing 10 ml PPG medium incubated horizontally at 25°C and

passed every 4-5 days (volume of passage 1:10). The PPG medium consisted of proteose peptone (15 g/l; Oxoid Unipath, Basingstoke, UK) and glucose (18 g/l; Sigma). The PPG medium was made to volume with amoeba saline solution (ASS) instead of water. The ASS consisted of two stock solutions viz. solution 1: NaCl, 24 g/l; $MgSO_4.7H_2O$, 0.8 g/l; $CaCl_2.6H_2O$, 1.2 g/l and solution 2: Na_2 HPO_4, 28.4 g/l; KH_2PO_4, 27.2 g/l. The final ASS consisted of 5 ml of each of the stock solutions and the mixture made to volume (1 litre) using glass-distilled water. MAP (NCTC 8578) was maintained on Protect cryobeads (Technical Service Consultants, Heywood, UK) at $-80^{o}C$. A working culture of MAP was generated by inoculating a bead into Middlebrook 7H9 broth with OADC supplement (Becton Dickinson) and 2 μg/ml mycobactin and incubating for 6-7 weeks at $37^{o}C$.

The amoebae were grown in PPG at $25^{o}C$ for 4 days after which the medium was poured off. The remaining monolayers were washed with ASS once and the monolayer resuspended in fresh ASS before being incubated overnight at $25^{o}C$ to starve the protozoa. A working culture of MAP was centrifuged at 2,500g for 20 min, the pellet washed twice with ASS and finally resuspended unhomogenised in ASS to give a final cell concentration of approximately 1 x 10^7 cfu/ml. The starved *A. castellanii* and *A. polyphaga* cultures were agitated slightly and the cells counted using a haemocytometer. The respective protozoan cultures were centrifuged at 400g for 5 min and the pellets resuspended with the MAP-ASS suspensions (10 ml) at a multiplicity of infection (MOI) of 10 MAP to one *Acanthamoeba* cell and incubated at $25^{o}C$ for 180 min. The MAP-ASS suspension was decanted off (not discarded) and the monolayer washed twice with 10 ml ASS. The composite ASS washings were centrifuged at 2,500g for 20 min and the pellet resuspended in 10 ml ASS. Decimal dilutions were prepared using ASS and 0.5 ml aliquots of each inoculated into BACTEC 12B medium and read regularly on a BACTEC 460 machine. This was used to estimate the extracellular MAP population using the method of Lambrecht *et al.* (1988). The protozoan monolayers, after the final washing, were resuspended in 10 ml PBS-T and incubated at $25^{o}C$ for 10 min before being sonicated (2 X 30s at 50% intensity with 10s between bursts) to lyse the amoebae. The lysate was centrifuged at 2,500g for 20 min and the pellet resuspended in 10 ml PBS-T and decimally diluted. The dilutions were inoculated into BACTEC 12B medium incubated and counted as before. This was taken as an estimate of the intracellular MAP population. Three replicate runs were performed and the mean results of the two MAP populations compared using analysis of variance (ANOVA).

Survival of intracellular MAP
The protozoan/MAP co-cultures were prepared as before and incubated at $25^{o}C$ for up to 24 days. After 7, 14 and 21 days the PPG medium was replaced. Sub-samples (1 ml) were removed after 2h and 2, 4, 7, 14 and 21 days, centrifuged at 2,500g for 20 min and the pellet resuspended in PBS-T (10 ml). The suspension was lysed and the lysate decimally diluted, inoculated into BACTEC 12B, incubated and counted as before. The means of three replicate runs were compared by ANOVA.

Comparative resistance of intracellular and extracellular MAP to chlorine
A chlorine stock solution (1:1000 dilution) was prepared using Chloros (approx. 10% available chlorine) using distilled reverse osmosis water. This was used to prepare test chlorine solutions containing 0, 0.5, 1.0 and 2.0 μg/ml free chlorine. The free chlorine concentrations were confirmed using the N, N-diethyl-p-phenylenediamine ferrous titrimetric method (American Public Health Association, 1993). The test solutions were freshly prepared before each experimental run and were held at room temperature for no longer than 5 min before inoculation. A starved co-culture of MAP and *A. polyphaga* was prepared as before along with individual control cultures of MAP and *A. polyphaga* alone. The prepared test chlorine solutions (10 ml) were inoculated with co-culture and control cultures and incubated at $20^{o}C$ for the respective contact times of 15 and 30 min. After the contact time had elapsed the chlorine was neutralised with 1 ml of 1% w/v sodium thiosulphate. The intracellular MAP and control MAP and *A. polyphaga* cultures were prepared as before. Three replicate runs of the experiment were preformed and the results compared using ANOVA.

RESULTS

A MAP positive result from one or more of the three methods (IMS-PCR, HEYM and BACTEC culture) was obtained for 15 of the 192 raw water samples tested (Table 1). Two of the water samples tested positive by both IMS-PCR and culture (i.e. 17 positive results from 15 water samples). In these cases it was assumed

that the responses were due to the same strain of MAP. Eight MAP isolates were obtained during the study, five were cultured from HEYM only and three from BACTEC only. MAP was detected from eight out of the nine WTWs surveyed with no MAP being obtained from WTW3, however a Chi-square test showed no significant difference (5% level) in detection rates from the nine WTWs surveyed.

The highest detection rate, taking primary test results into account, was during the spring months (March to May, Fig. 1) when 11 out of 83 samples tested positive. The next highest detection rate was in the summer months (June to August, Fig. 1) when 3 out of 28 were positive. During autumn (September to November, Fig. 1) only 1 out 36 were positive while during winter (December to February) none out of 36 was positive. Again however, using the Chi-squared test no significant difference (5% level) in detection rates was found by month or season.

There was also no significant difference (5% level) between contrasting catchment features i.e. upland vs. lowland, impounded vs. non-impounded and forested vs. non-forested (data not presented). The greatest occurrence of MAP was found in WTW2 which had a catchment soil type described as surface water with subsurface clay-impeded drainage shale till, brown ranker; <40 cm mineral soil, shale; alluvium and shallow brown earth – 40-60 cm deep-shale. This may however have been due to the fact that the greatest number of samples was tested from WTW2 compared to the others (n=41). Interestingly WTW2, WTW4 and WTW6 were the only WTWs not to contain peat as one of their predominant soil types.

Table 1. Comparison of the rates of detection of *MAP* in raw water at nine water treatment works in Northern Ireland by the three methods employed.

Water Treatment Works	Number of IMS-PCR positive samples	Number of BACTEC culture positive samples	Number of HEYM culture positive samples	Number of water samples positive by any method	Mean water pH for WTW over period of study
1 (n=27)	1 (3.7%)	0	0	1	7.4
2 (n=41)	3* (7.3%)	1 (2.4%)	2* (4.9%)	5	8.1
3 (n=7)	0	0	0	0	6.7
4 (n=7)	1 (14.3%)	0	0	1	7.5
5 (n=7)	2 (28.8%)	0	0	2	8.2
6 (n=25)	1 (4%)	0	0	1	7.3
7 (n=26)	0	1 (3.9%)	1 (3.9%)	2	7.7
8 (n=27)	0	0	1 (3.7%)	1	7.4
9 (n=25)	1* (4%)	1 (4%)	1* (4%)	2	7.5
Total (n=192)	9 (4.7%)	3 (1.6%)	5 (2.6%)	15 (7.8%)	

* denotes that one HEYM culture positive and one IMS-PCR positive result were obtained from the same water sample.
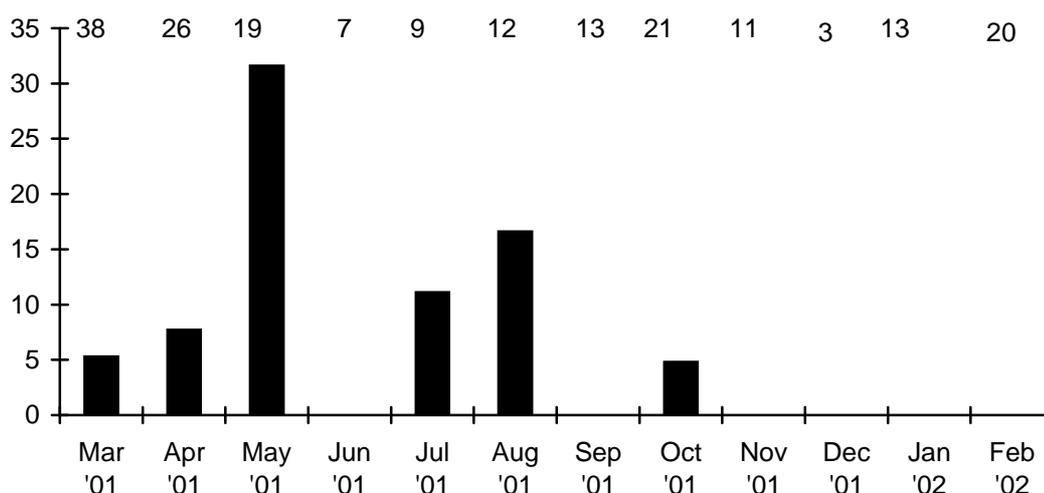
Fig. 1. Detection rates of MAP in raw waters in Northern Ireland by month, March 2001-February 2002. Water treatment works (WTW) testing positive for MAP: March, WTW 4 and 5; April, WTW 2, 5 and 8; May, WTW 1, 2, 6, 7, 8, 9; July, WTW 2; August, WTW 2 (x2); October, WTW9. The number above each column is the number of samples tested in the particular month

There appeared to be no correlation between occurrence of MAP and high coliform or faecal coliform counts (data not shown). As regards water pH there appeared to be a tendency for MAP detection when the mean water pH in the WTW was comparatively high (Table 1). It is interesting to note that the mean pH of water entering WTW3, from which no MAP was isolated, was the lowest mean water pH of all the WTWs surveyed.

When an initial inoculum of 10^6 cfu/ml of MAP was incubated with approximately 10^5 protozoa/ml of *A. castellanii* and *A. polyphaga* individually for 180 min the intracellular MAP counts (Log_{10}) were 5.15 and 4.82 respectively. There was no significant difference (P=0.457) in the ingestion capacity of the two *Acanthamoeba* species. The two intracellular MAP counts did not significantly alter (P<0.001) between *Acanthamoeba* species for at least 7 days incubation at 25^oC after which there was an apparent increase in intracellular MAP up to 24 days at which time the experiment was terminated (Fig. 2).
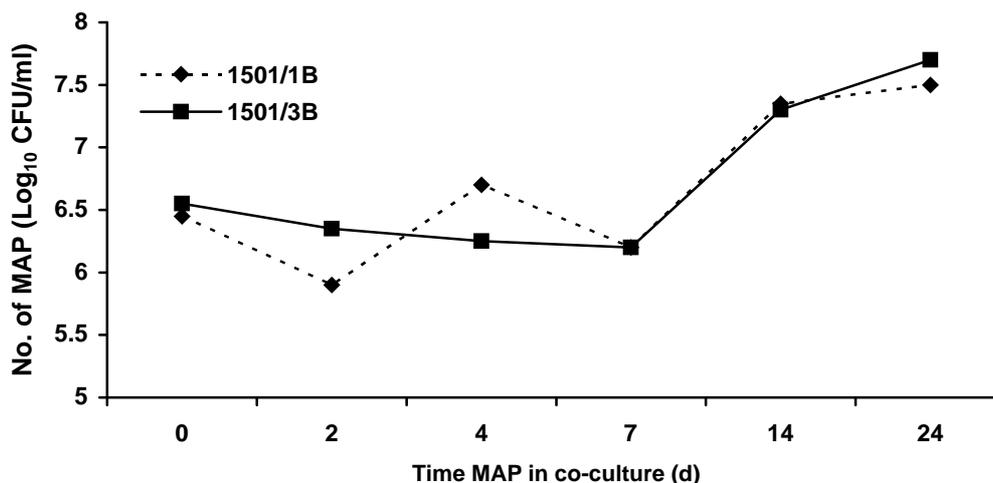
Fig. 2. Survival of MAP after co-culture with *Acanthamoeba castellanii* (1501/1B) and *A. polyphaga* (1501/3B) for up to 24 d at 25^oC.

Table 2. Impact of chlorine concentration (0.5, 1 and 2 μg/ml) and contact time (15 and 30 min) on inactivation of free and *Acanthamoeba*-internalised MAP in water.

Chlorine concentration (μg/ml)	15 min contact time		30 min contact time	
	Free MAP	**Internalised MAP**	**Free MAP**	**Internalised MAP**
	Log_{10} reduction achieved			
0.5	1.6	0.4	1.1	0.4
1	1.8	0.8	2.1	0.7
2	1.6	0.5	2.1	1.1

At all chlorine concentrations (0.5, 1.0 and 2.0 μg/ml) and both contact times (15 and 30 min) MAP that were located intracellularly within *A. polyphaga* were significantly more resistant to chlorine (P< 0.001) than MAP in the absence of the protozoan (Table 2).

DISCUSSION

To the authors' knowledge no published water survey has isolated MAP so the findings of this survey will be compared with those of other studies for isolation of mycobacteria in general. Although 192 raw water samples representative of the province were tested over a period of a year only 9 IMS-PCR positives and 8 MAP culture positives were obtained and hence any conclusions must be regarded as preliminary. It is also recognised that the PCR assay, except when performed on a colony, does not indicate viability since a positive response could be obtained from dead, viable but non-culturable MAP, or even extraneous DNA. The fact however that culture positives were obtained provides evidence that MAP can survive sufficiently

in the environment to make agricultural runoff a possible route of exposure to the public. In the present study although no significant difference was found in detection rates between months or seasons, the greatest incidence was found during the period March to May corresponding to spring and early summer in N. Ireland. This is in contrast to a number of studies on the isolation of mycobacteria that, in general, found a greater incidence during the summer and autumn months (Kirschner *et al.* 1992; Donoghue *et al.* 1997). The incidence in this study may however be explained by the fact that in N. Ireland cattle are usually put to pasture in the spring and summer and housed during autumn and winter with the slurry during that time largely being retained in pits. The organism was no more frequently isolated from lowland river catchments than upland impounded reservoirs, which was unexpected since in a comparable survey Hunter *et al.* (2001) reported more mycobacteria from lowland rivers than other sources. It is interesting to note that the catchment area that had the greatest occurrence of MAP (WTW2) did not contain peat as a predominant soil type since other reports have indicated a higher isolation rates of mycobacteria from peaty soil (Iivaanainen *et al.* 1993) This may however have been due to the fact that the greatest number of samples (n=41) were tested at WTW2.

The absence of a significant correlation between occurrence of MAP and coliform or faecal coliform counts cast doubt on the value of such indicators as predictors of the presence of MAP. Perhaps enterococci counts may have been a more useful test since their source is animal rather than human faeces but such counts are not routinely performed on raw waters in the province. In general those WTWs that had a high mean water pH over the sampling period showed a high incidence of MAP, in contrast to other workers who described a negative correlation between incidence of mycobacteria and high water pH values (Iivaanainen *et al.* 1993; Sung *et al.* 1997).

Both protozoa strains not only showed a high MAP ingestion capacity after only 180 min incubation but MAP was also able to survive for at least 24 days at 25°C (Fig. 2). The increase in intracellular MAP after 7 days is probably due to growth or multiplication of protozoa and consequent increase in gross ingestion capacity since MAP would be unable to grow because of the low incubation temperature and absence of exogenous mycobactin. The resistance of MAP to the bacteriocidal activities of the protozoa may not only be due to the impermeability of its thick lipid-rich cell wall but also the presence of biologically active compounds shown to be effective against macrophages (Tessema *et al.* 2001). Perhaps this finding is not surprising since other mycobacteria have been shown to survive protozoan ingestion (Cirillo *et al.* 1997; Steinhert *et al.* 1998). The finding of added resistance of intracellular MAP to chlorine (Table 2) has been found with other organisms (King *et al.* 1988) and adds impetus for further research to determine the efficacy of water treatment processes in general for inactivating or removing MAP from raw waters.

CONCLUSION

To the authors' knowledge this research is the first published study detailing the isolation of MAP from raw waters destined for domestic use. It also provides evidence for the possible role of protozoa as a survival strategy for MAP in the environment and protozoan mediated resistance to chlorine inactivation. Perhaps most importantly it highlights the urgent need to determine the effectiveness of water treatment processes to inactivate or remove MAP from water and hence contribute to reducing the exposure of the public to this organism.

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Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from patients with Crohn's disease, ulcerative colitis and healthy controls: preliminary results

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ABSTRACT

Crohn's disease (CD) is a gastro-intestinal inflammatory disease with uncertain origin. Autoimmune and/or infectious etiologies have been proposed as possible causes. A role for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in CD has been hypothesized. However, the role of MAP in causing this disease is subject to debate. The aim of the present study is to compare the rate of MAP isolation from patients living in an area of Northern Italy characterized by the presence of high prevalence of MAP infected cattle herds. In the region of concern, a mean annual incidence rate of 3.4 CD patients per 100,000 inhabitants has been reported. This study enrolled 186 patients: 23 patients with CD, 26 patients with ulcerative colitis (UC), 6 patients with not specific inflammatory bowel disease (IBD) and 131 healthy patients. For each patient, six biopsy tissue specimens from ileo-colon region were collected and processed for MAP isolation. Culture was performed with BacT/ALERT 3D system (bioMérieux) using a Middlebrook 7H9-based liquid medium. All samples were culture-negative for MAP after three months of incubation. An acid-fast, IS*900* negative organism was recovered from an healthy control patient. The isolate was able to grow in the absence of mycobactin J. At present, our findings do not support a role for MAP as a microbial causal agent of CD. A larger number of CD patients and an extension of incubation times of cultures are needed to make clear the pathogenic relationship between MAP and CD in this region.

Key words: Crohn's disease, biopsies, culture, paratuberculosis, PCR.

INTRODUCTION

Crohn's disease (CD) is a human gastro-intestinal inflammatory disease of uncertain origin. A role for *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causal agent of paratuberculosis in ruminants, in CD was first considered following the isolation of three strains of a MAP-like organism from 3 of 14 patients with CD (Chiodini et al., 1984). However, the involvement of MAP in the etiology of CD is still under debate. Main evidence supporting the causal link are: (a) many studies (Quirke, 2001; Grant, 2005) have indicated a higher presence of MAP in patients with CD than in controls either by culture and/or by PCR or hybridization methods using biopsies and/or on surgically resected tissue samples; (b) a therapy based on broad spectrum antibiotics believed effective against MAP such as rifabutin, clarithromycin and clofazimine, led to good results in the treatment of CD (Borody et al., 2002; Shafran et al., 2002). Moreover, the presence of MAP has been detected in blood samples from Crohn's patients (Naser et al., 2004). Nevertheless, other authors have reported the absence of a significant positive correlation between CD and the frequency of detection of MAP (Chiba et al., 1998; Kanazawa et al., 1999; Van Kruiningen, 1999). Sceptics do not accept the hypothesis of an active role of MAP in CD. There is insufficient evidence to describe the effect of antibiotic treatments against MAP specifically rather than against other enteric bacteria. The fact that CD patients' symptoms do not worsen when treated with immunosuppressive agents is not in keeping with an infectious cause of Crohns disease. Patients whose tissues are reported to contain MAP in an obligate intracellular spheroplastic form do not present a cell mediated immune response to MAP antigens. Finally, there is no evidence that humans living in contact with animals infected with MAP have a higher risk of developing the disease for (Sartor, 2005). Many infectious agents other than MAP have been suggested as causes of CD, including the measles virus, *Listeria* spp. and *Escherichia coli* (Grant, 2005). A possible commensual presence of MAP in CD patients has been also hypothesized. On the other hand, a mutation in the gene encoding nucleotide-binding oligomerization domain 2 (NOD2) has

been associated with a susceptibility to CD (Hugot et al., 2001; Ogura et al., 2001). Moreover, functional variants of organic cation transporter (OCTN) genes are associated with CD (Peltekova et al., 2004). Taken together, these findings indicate a complex polygenic nature of the disease.

A contentious debate is in progress as indicated by criticisms addressed to some of the studies proposing that MAP might be a cause of CD (Roholl et al., 2002; Gaya et al., 2004; Huggett et al., 2004). The picture shared by most scientists is that environmental risk factors add to predisposing gene variations in leading to an abnormal immune response at the intestinal level.

The aim of the study was to compare the rate of MAP isolation from human patients living in an area of Northern Italy characterized by a high prevalence of MAP-infected cattle herds.

MATERIALS AND METHODS

Population and study area
The study was carried out on 186 subjects presenting to the referral hospitals of the Provinces of Crema (Ospedale Maggiore) and Cremona (Ospedale Maggiore), Region Lombardia, Northern Italy, with symptoms compatible with an inflammatory bowel disease (IBD). Patients underwent a diagnostic work-up and were assigned to four different groups: patients with CD, patients with ulcerative colitis (UC), patients with non-specific IBD and healthy patients.

Diagnostic criteria
For each patient, clinical information was carefully considered. The diagnosis of IBD was made on the basis of clinical, endoscopic, radiological and histological criteria. In some patients the diagnosis was further confirmed by serological determination of anti-*Saccharomyces cerevisiae* antibodies (ASCA), of anti-neutrophil cytoplasmic antibodies (p-ANCA) and by videocapsule endoscopy. ASCA+pANCA (-) results are mostly associated with CD patients, whereas ASCA-pANCA (+) results are mostly associated with UC patients or with patients with an UC-like form of CD. Pathological findings mimicking IBD and with an alternative diagnosis were excluded.

Samples
At present, the study enrolled 186 patients: 23 patients with CD, 26 patients with UC, 6 patients with non-specific IBD and 131 healthy patients. For each patient, six tissue specimens from the ileo-colon region were collected and processed for MAP isolation.

Culture
The six biopsy tissue specimens were collected from the patient and immediately homogenized in 5 ml of sterile saline. The suspension was diluted to 50 ml with sterile saline and 5 ml of digestion reagent was added (0.75 N NaOH, 0.114 M sodium citrate tribasic – Biolife Italiana, Milano, Italy). After agitation the suspension was incubated at 37°C for 5 min and than for 10 min at room temperature (RT) with continuous agitation. The suspension was then added to 40 ml of phosphate buffer (0.032 M potassium phosphate monobasic, 0.031 M potassium phosphate bibasic, pH 6.8 - Biolife Italiana, Milano, Italy), mixed and centrifuged at 3000 × g for 20 min at 10°C. The pellet was resuspended with 0.5 ml of phosphate buffer and inoculated in a Middlebrook 7H9-based liquid medium, namely BacT/ALERT MP Process Bottle (bioMérieux, Durham, North Carolina), plus MB/BacT Antibiotic Supplement, BacT/ALERT MB Enrichment Fluid and 0.0002% (wt/vol) mycobactin J (Allied Monitor, Fayette, MO). The samples were incubated at 35-37°C in the BacT/ALERT 3D system for times ranging from 42 to 90 days and monitored for microbial growth. The suitability of the BacT/ALERT 3D system to support MAP growth was assessed by cultivation of the ATCC 19698 strain.

Identification of MAP
The protocol provides that culture-positive samples are identified as MAP-positive on the basis of Ziehl-Neelsen staining and confirmed on the basis of the mycobactin J-dependence of the bacterium and by IS900-PCR.

DNA extraction
DNA extraction was performed on isolated acid-fast organisms by a phenol/chloroform method. Briefly, 100 µl of cultured bacterial suspension were pelleted, resuspended with 100 µl of TE buffer (1 mM tris/HCl, pH 7.6, 0.1 mM EDTA) and added with 100 µl of phenol/chloroform. The suspension was mixed by inversion of the tube and centrifuged. One-hundred µl of the aqueous phase were recovered and transferred to a clean tube. After extraction, the DNA was precipitated at RT with one volume of isopropanol and one-tenth volume of 3 M NaOAc (pH = 4.8). The pellet was then washed once with 70% ethanol, resuspended with 20 µl of deionized sterile water and stored at –20°C until amplification.

PCR
The PCR was performed by using IS900-specific primers, namely P90 – P91 (Schwartz et al., 2000) either on the extracted DNA or directly on the cultured bacterial suspension which had been previously washed and resuspended with sterile PBS. The reactions were carried out in 50 µl containing 2 U of *Taq* DNA polymerase (Klen Taq 1, Ab Peptides, Inc – USA), 1X PCR buffer (1X PC2), 0.2 mM of each dNTP, 0.5 µM of each primer, and 5.0 µl of DNA sample. PCR was performed by 35 amplification cycles, each consisting of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and chain elongation at 72°C for 1 min. In the first cycle the samples were denatured at 95°C for 5 min and then rapidly ice-cooled before polymerase addition. In the last cycle the extension step was increased to 10 min. Detection of amplified products was performed on 2% agarose gel by ethidium bromide staining.

RESULTS

All biopsy tissues from all four diagnostic categories were culture negative for MAP. An acid-fast organism other than MAP was recovered from a healthy control patient. The isolate was negative when tested by IS900-PCR and was able to grow in the absence of mycobactin J, although less robustly than in media containing this iron chelating factor.

DISCUSSION

In the study area, a mean annual incidence rate of 3.4 CD patients and of 7.0 UC patients per 100,000 inhabitants has been reported by a study based on a four year observation period (Ranzi et al., 1996). These values are relatively high when compared to data regarding other Italian areas (Tragnone et al., 1996). Moreover, this area of Northern Italy is characterized by a high prevalence of MAP-infected cattle herds. However, no studies have been made to detect a correlation between a high prevalence of CD and a high prevalence of MAP-infected cattle herds. A possible route of MAP transmission of MAP from infected cattle to human beings may be milk, since MAP can be present in milk due to direct secretion and/or fecal contamination. Pasteurization is able to reduce the degree of milk contamination, but does not guarantee a complete elimination of MAP (Grant, 2005). Less data are reported about the persistence of MAP in milk products. Studies regarding the survival of MAP in cheeses, carried out using experimentally MAP-contaminated milk, showed an incomplete inactivation of MAP in cheddar, semihard and hard cheeses during manufacturing and ripening (Spahr and Schafroth, 2001; Donaghy et al., 2004).

Anamnestic data of the examined patients has not been considered in this phase of the study. Interesting data could also be derived from the serological analysis of collected sera from CD patients included in the study.

The difficulty in isolating MAP from tissue samples of human origin has to be taken into account in interpreting these results. One of the difficulties for the isolation of the micro-organism is the possible presence of the bacterium as wall-deficient spheroplastic form in tissues. Moreover, in this study, biopsies were utilized due to the unavailability of surgically resected tissue samples which are quantitatively and qualitatively more suitable for isolation of MAP (Schwartz et al., 2000). However, positive cultures for MAP from human microscopic biopsies have been reported by using the Becton-Dickinson mycobacterial growth

indicator tube (MGIT) system (Schwartz et al., 2000). Other attempts to grow MAP from human intestinal mucosal biopsies on MGIT have also been made (Bull et al., 2003). In the present study, multiple tissue samplings from each patient was made in order to increase the sensitivity of the test. In this way a greater amount of tissue from different mucosal areas was collected. However, this study's sampling technique does not reach the deeper layer of the mucosa and this limitation could be affect the ability to isolate MAP, as hypothesized by Schwartz and co-workers (2000).

The suitability of the BacT/ALERT 3D system to support MAP growth was assessed by cultivation of the ATCC 19698 strain. Success in MAP isolation differs depending on the culture systems that used. An example is reported by Schwartz and co-workers (2000) who found MAP-positive MGIT cultures but MAP–negative BACTEC cultures in primary isolation of MAP from the same clinical specimens. Both systems, and the BacT/ALERT 3D system as well, use media deriving from Middlebrook 7H9-broth base. Different rates of MAP isolation could be due to the presence of different media additives in the two systems. Finally, another parameter to be considered is the incubation time. At present, for this study, cultures have been incubated for 3 months and longer incubation times are needed to exclude false-negative results.

CONCLUSION

In this preliminary study, MAP was not isolated from multiple intestinal mucosal biopsies collected from patients with CD, patients with UC, with non-specific IBD and from healthy patients. At present, our findings do not support a role for MAP as microbial causal agent of CD. The samples are still incubating after three months and the study is still in progress.   Either a higher number of CD-affected patients or an extension of incubation times of cultures are needed to make clear the pathogenic relationship between MAP and CD in this region.

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Detection of Mycobacterium avium subsp. paratuberculosis in Crohn's disease patients and ruminants intestine by in situ hybridization in Mexico

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Abstract

Objective: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and specially its cell wall deficient form has been suspected to play a role in the pathogenesis of Crohn disease (CD). There are very few studies of Mexican patients with CD and none of them investigated the possible link between MAP and CD. Methods: Solitary or multiple specimens from 14 patients with CD (10 with granulomas and 13 without granulomas); 15 paratuberculosis (8 Ziehl Neelsen (ZN) positive and 4 ZN negative) as well as 1 intestinal tuberculosis were analyzed by in situ hybridization (ISH) based on the avidin biotin-labeled MAP IS*900* fragment. Samples were counterstained with light green. Results: 8/10 (80%) samples and all patients with granulomatous CD showed positive signals in macrophages, epitheliod cells and giant cells. All positive signals were observed within granulomas. 4/13 (30.76%) and 4 patients with nongranulomatous disease were MAP positive, the signal appears in few macrophages cytoplasm. In paratuberculosis samples, 8/8 (100%) ZN positive were also positive for ISH as well as 4/4 (100%) without acid fast rods detectable by ZN. Tuberculosis patient were MAP negative as expected. Conclusion: These initial results are consistent with the exhibition of the Mexican population to MAP. The demonstration of MAP DNA from CD tissues supports and confirms previous reports of its association with the granulomatous type of the disease.

Application of different methods for the detection of M. avium subsp. paratuberculosis (Map) in Argentina

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Abstract

Introduction: Paratuberculosis has a high prevalence in Argentina. Some regions of the country show seroprevalence between 2.5 to 51.5 %. The purpose of the present study was to evaluate the efficacy of different methods to detect paratuberculosis in dairy cattle herds in Argentina. Materials and methods: Fifty-two cows were selected from a dairy herd in Argentina. Sera from the animals were analyzed by indirect ELISA, using Map protoplasmatic antigen. Nine recombinant antigens were used in IFN-γ assay, 3 of Map (Lpp24, P34 and Bacterioferritin) and 6 of *M. bovis* (ESAT-6, CFP-10, 2624, 3747, Tpx, Trbb). Responses to recombinant antigens and to aviar PPD were analyzed by an *in vitro* assay for IFN-γ (Bovigam). Faeces, milk and in-line milk filters culture, following decontamination with hexadecylpyridinium (0.75%w/v) for 24 h, were done in tubes containing Herrold's culture medium supplemented with mycobactin (2mg/L) and pyruvate (4g/L). Samples were also tested by IS*900*-PCR. Results: The results showed that 45 out of 52 (86.5%) animals were positive to ELISA. Fourteen animals were culture positive in faeces (14 of 52, 27%). All the animals that excreted the bacteria through faeces were ELISA-positive. Only one of these animals was culture positive in milk. However we were able to isolate one colony from the in-line milk filter at farm level. Nine animals showed significant levels of IFN-γ with aviar PPD (9 of 52, 17%). All IFN-γ positive samples were ELISA-positives and faecal culture positives. Unfortunately, in the present study the use of recombinant antigens in IFN-γ assay did not enhance the specificity of the standard test using aviar PPD. We obtained negative PCR results in all samples. Conclusion: In a control programme, an ELISA test would be a good first step for identifying the affected herd, following of culture of faeces.

Quantifying the risk of Mycobacterium avium subsp. paratuberculosis surviving pasteurisation

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Abstract

A risk analysis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk harvested using good milking practice and treated in validated pasteurisers was carried out. We used the results from a survey of MAP in New Zealand raw milk silos in conjunction with data from our earlier kinetic study of the survival of MAP after high temperature–short time (HTST) pasteurisation, as well as data from two other published studies. Raw milk samples were collected at approximately weekly intervals over a 12-month period from raw milk silos at six dairy plants located throughout the major New Zealand dairying areas. One hundred and seventy five raw milk samples were forwarded chilled by overnight delivery to an accredited testing laboratory (AgResearch) for culturing. Over that period these samples represented a total volume of 4.6 million cubic metres of raw milk. One 50 ml aliquot was confirmed as being MAP culture-positive. A first-order kinetic heat inactivation model was developed to predict the likelihood of MAP being present in milk immediately after pasteurisation. The model took into account the expected concentration of MAP in the incoming raw milk supply (with recognition of the limitations imposed by the data set) and the variability of the lethality of the heat treatment on the five different strains reported in our earlier kinetic study. This variability was consistent with the variation between three strains reported by others from laboratory pasteurisation. The model simulations were run using @Risk software. The risk analysis demonstrated that good milking practice combined with properly operated and validated pasteurisers operating at 72°C/15 s will give an extremely low probability of any viable MAP surviving per cubic metre in pasteurised milk. As such, commercial pasteurisation can be considered to be an effective treatment step for the destruction of MAP.

Reduction of Mycobacterium avium subsp. paratuberculosis colony forming units in milk by means of High Hydrostatic Pressure held at mild temperatures

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Abstract

Reports on persistence of viable *Mycobacterium avium* subsp. *paratuberculosis* (Map) in pasteurized milk have lend support to the hypothesis that milk consumption can be a cause of human exposure to this pathogen that has been implicated in the etiology of Crohn's disease. The efficacy of high hydrostatic pressure (HHP) for inactivation of micro-organisms has been the object of intensive research, mostly with gram positive and negative cells. However, mycobacteria have never been included in those studies. Here we report an experiment where this method was used to assess the inactivation of Map in milk by moderate HHP treatments held at mild temperatures. Two strains of Map (ATCC 19698 and field isolate 3644/02) were inoculated (4.05×10^8 UFC/ml and 3.24×10^8 UFC/ml, respectively) into commercial sterilised milk and submitted to 10 minutes treatments at 300, 400 and 500 MPa at 5 and 20°C in duplicate. Dilutions of the treated milk where inoculated into duplicate mycobactin J-suplemented agarose solidified Middlebrook 7H9 OADC and Herrold's egg yolk tubes. After 16 weeks of incubation at 37 °C, colonies were counted and logarithmically transformed to calculate the rate of reduction at each level of treatment. Maximum reduction was 4.8 log at 500 MPa, but culture media and strain also accounted for significant effects. Temperature had no significant effects. HHP milk treatment can be thus considered at least as efficient as pasteurization for Map inactivation, but with the advantage of avoiding the effects of heating on milk properties.

Isolation of Mycobacterium avium subsp. paratuberculosis from human beings in India

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Abstract

Stool samples of 8 human beings were processed for isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Of these 2 were suffering from clinical bowel disorders (frequent stomach upset), weakness, weight loss and intermittent diarrhea and were positive (25.0%) for the typical MAP colonies. More than 20 colonies appeared around 26 days of incubation in one case and around 35 days in second case. Colonies were identified on the basis of morphology, media discoloration, slow growth, mycobactin J dependency and acid fastness. One person had Hb, 7.5 ESR was always more than normal and also had the history of consuming raw cow milk. Rests of the samples were negative (up to 150 days incubation), and persons were apparently healthy. Isolation of MAP bacilli from the stool of persons with clinical symptoms established the role of MAP in the pathogenesis of Crohn's disease. This study is the first report of isolation by culture of MAP from stool samples of human beings in India. However, on the basis of plate ELISA on 45 serum samples of human beings from Agra region the prevalence of Crohn's disease was 46.6% and 44.4% using Bison type and Bovine type antigens, respectively.

Recent developments in the molecular biology of *Mycobacterium avium* subsp. *paratuberculosis*

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DEFINITION OF MOLECULAR BIOLOGY

The field of molecular biology can be broadly defined to encompass any recombinant DNA technology, including DNA sequencing, cloning, DNA amplification methods, etc. Furthermore, molecular biology can claim proteomics and production of recombinant proteins in addition to DNA manipulation. In fact molecular biology is the study of the molecular mechanisms of cellular processes which includes DNA replication, transcription and translation. Therefore, this discipline is enormously complex and crosses over into other fields such as biochemistry, cell biology, genetics and genomics. For the purpose of this review and perspectives paper, I will narrow this definition to crystallize the current thrust of molecular biology research as it relates to *Mycobacterium avium* subspecies *paratuberculosis* (MAP).

This review will encompass any study that identifies a novel characteristic of MAP genomic DNA. It will also encompass extraction techniques of cellular components (either protein, RNA or DNA). In light of this focus, DNA amplification studies are excluded from consideration, especially where the objective is for detection or diagnosis of MAP. Also, gene expression studies are excluded because the vast majority, if not all, of those studies involve production of recombinant proteins and their potential as MAP antigens. However, any differential gene expression study looking at changes in MAP RNA levels under defined conditions using RT-PCR or microarray are eligible. Unfortunately, none of these studies were found in the literature as yet. They will, no doubt, be forthcoming in the near future as discussed below. Changes in host gene expression with and without exposure to MAP have been considered (Coussens *et al.*, 2004a; Coussens *et al.*, 2004b; Madsen *et al.*, 2004); however, those studies are outside the scope of this review as they evaluate how the host responds to infection.

HISTORY OF MAP MOLECULAR BIOLOGY

To gain a solid perspective on where the field is now, it is necessary to take a brief look back to where it started. The genetic manipulation of MAP has proven to be more difficult than most other bacteria. Therefore molecular genetic studies have lagged behind other bacteria and even other mycobacteria such as *M. tuberculosis*. Nonetheless, owing to the importance of Johne's disease worldwide, researchers have continued to grind out progress, albeit slowly, much like the growth pattern of the organism itself.

The proposal of a subspecies designation for MAP was based on early DNA-DNA hybridization studies (Saxegaard *et al.*, 1988; Thorel *et al.*, 1990; Yoshimura & Graham, 1988). There are no naturally occurring plasmids or extrachromosomal elements in MAP, so vectors for genetic manipulation need to come from other mycobacterial species (Labidi *et al.*, 1984) and be able to replicate autonomously in MAP. The insertion sequence IS*900* was discovered (Collins *et al.*, 1989; Green *et al.*, 1989) and its characterization was the subject of several papers (Doran *et al.*, 1997; Doran *et al.*, 1994; England *et al.*, 1991; Tizard *et al.*, 1992) because it is present uniquely in MAP. There was also cloning and sequencing of a very limited number of MAP genes prior to 1994 (Colston *et al.*, 1994; De Kesel *et al.*, 1993; Stevenson *et al.*, 1991). The PAN promoter was identified, characterized and shown to express LacZ in *M. bovis* and *M. smegmatis* (Murray *et al.*, 1992). Then in 1995, the first concerted effort to develop the genetics of MAP was

performed by Raúl Barletta's laboratory at the University of Nebraska (Foley-Thomas *et al.*, 1995). Studies in his laboratory demonstrated for the first time that MAP could be transformed with foreign DNA as well as transfected with bacteriophage DNA (Foley-Thomas *et al.*, 1995). Furthermore, they found that MAP could be productively infected with the mycobacteriophage TM4. They capitalized on this observation to develop a transposon mutant library that was delivered to MAP using a temperature sensitive derivative of TM4 (Harris *et al.*, 1999). However, these techniques have not yet reached routine use among multiple laboratories.

A SURVEY OF THE MAP LITERATURE (JANUARY 2003 – PRESENT)

To objectively examine the most recent published works in the area of molecular biology of MAP, a PubMed search of all papers containing both the keywords "paratuberculosis" and "molecular biology" either in the title or the abstract was undertaken. Amazingly, only 21 entries were returned in the entire database. Inspection of these 21 entries quickly showed that this search method did not target the right publications as only five of the 21 entries could correctly be classified as a paratuberculosis research paper in the field of molecular biology. A second PubMed search, using only the term "paratuberculosis", was performed. This time 305 publications were retrieved from the database between January 2003 and the present time (August 1, 2005). The breakdown of manuscripts published by year is shown in Fig. 1. In 2003, 106 papers were published and year 2004 had 135 papers published. Note that the volume of paratuberculosis-related literature has remained steady each year and 2005 is projected to reach 110 publications. Each of these 305 publications was manually assigned into one of 20 subject categories as shown in Fig. 2. The subject category containing the most publications was the "all other" or "not classifiable" category. Essentially this was the default category of any publication that could not readily be categorized into one of the 19 other subject categories.

Manuscripts assigned to this category included such titles as "the veterinary community" (Anonymous 2004) or "Johne's disease, PRRS projects win funding" (Kahler, 2004). Categories involving Crohn's and prevalence studies each had 32 publications, and hence were tied for second largest subject category. This finding reflects the increased concern as well as funding for public health issues surrounding MAP. These numbers do not reflect pasteurization studies or the presence of MAP in milk as those publications were assigned a category all their own (Fig. 2). Prevalence studies will always have a prominent presence in the literature because researchers and the public want to know how big the Johne's disease problem is in all regions of the world and to monitor changes in control of Johne's disease. The immunological subject category was further subdivided into two categories: MAP immunology-host response and cytokine immunology (Fig. 2). The cytokine immunology category included publications that measured cytokine changes in response to host infection with MAP. Examples of publications in this category included identification of gamma interferon inducing antigens of MAP (Nagata *et al.*, 2005), cytokine expression profiles of MAP infected dendritic cells (Langelaar *et al.*, 2005) and the use of gamma interferon as an indicator of MAP infection (Huda *et al.*, 2003; Storset *et al.*, 2005). MAP immunology-host response was the larger of the two categories with 25 total publications.

Publications by Year
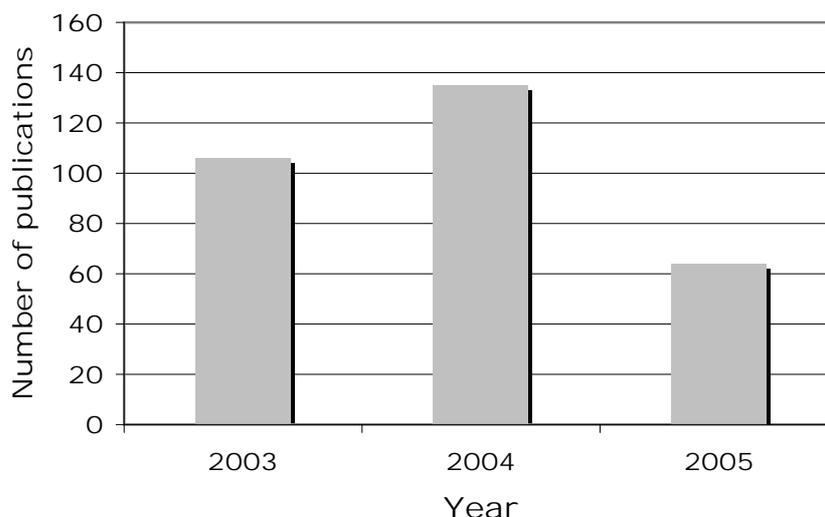
Fig. 1. A graphical representation of the volume of literature in the PubMed database retrieved when using the search term "paratuberculosis"
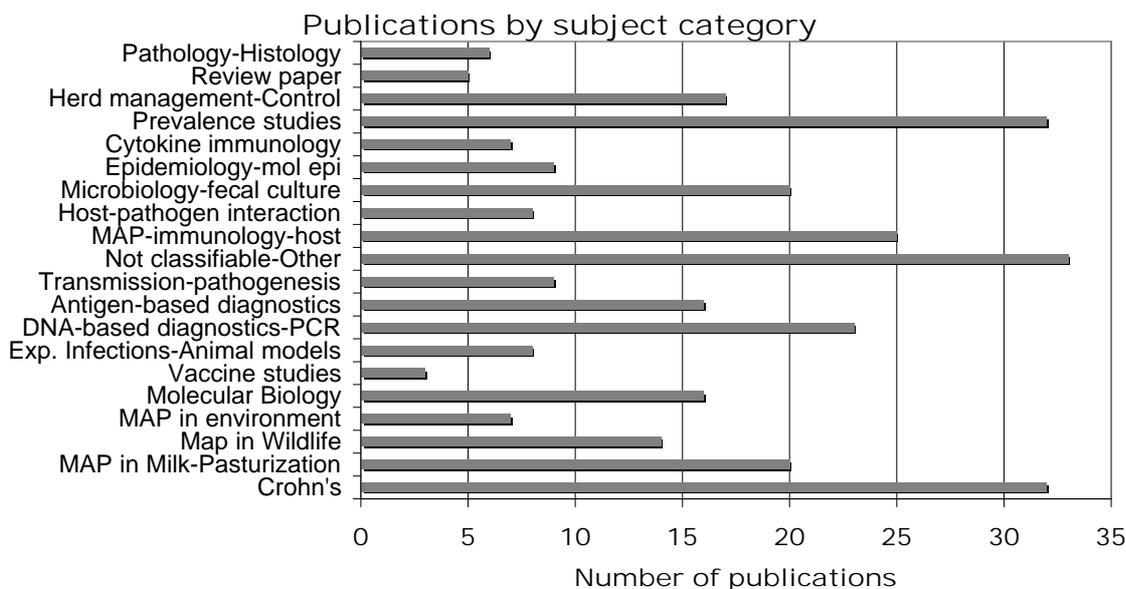
Fig. 2. The number of MAP publications arranged in subject categories from 2003 to present

The molecular biology category had 16 publications assigned to it. These could be further subdivided into extraction methods of cellular components (3 papers), IS elements (3 papers), analysis of a specific segment of the MAP genome (2 papers) and genetic diversity (8 papers). The molecular biology and epidemiology-mol epi categories may initially seem to overlap; however, if the emphasis of the manuscript was to USE the technique in the field, it was assigned to the epidemiology-mol epi category. If the emphasis was on defining genetic differences with application or benefit to molecular epidemiology, that study would be categorized in molecular biology. Fully half of the molecular biology publications were assigned to the genetic diversity subcategory.

The two most recent colloquia on paratuberculosis (held in Bilbao, Spain and Copenhagen, Denmark) were also evaluated for molecular biology-based presentations. During the 7th ICP in 2002, there were 26 presentations in the molecular biology section. At the 8th ICP in 2005, there were a total of 40 presentations in the molecular biology, microbiology and culture section. Of the 40 presentations at that meeting, 16 related exclusively to molecular biology.

THE MOLECULAR BIOLOGY OF MAP: RECENT LITERATURE

A survey of the 16 molecular biology publications retrieved from PubMed shows that the MAP field is still missing some fundamental genetic systems. For example, mutagenesis studies of MAP remain notably missing in spite of Raúl Barletta's efforts 6 years ago (Harris *et al.*, 1999). This situation should change soon as at least three investigators in the United States are actively screening transposon mutant banks of MAP. Also, MAP transformation of foreign DNA is not in routine use. Nonetheless, progress in molecular biology of MAP is being made with greater promise in the near future.

As mentioned above, genetic diversity is the predominant theme among MAP molecular biology papers from 2003 to the present. From repetitive DNA sequences (Amonsin *et al.*, 2004; Bull *et al.*, 2003) to amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) analysis (de Juan *et al.*, 2005; O'Shea *et al.*, 2004), differences on the MAP chromosome have been identified and utilized for discriminatory subtyping of isolates. Many of these studies have used the genome sequence of MAP to aid in the identification of genetic regions of variability (Amonsin *et al.*, 2004; Overduin *et al.*, 2004; Paustian *et al.*, 2005; Semret *et al.*, 2004). The genome sequence was deposited in GenBank and released to the public in January of 2004. This resource will continue to aid researchers as they seek to define additional genetic variation among MAP isolates, especially differences between the cattle and sheep isolates as discovered by Marsh and Whittington and reported on at this colloquium as well as in the published literature by Dohmann et al. (Dohmann *et al.*, 2003). Techniques that both reveal genetic diversity and can be used to discriminate among MAP isolates include short sequence repeat analysis (Amonsin *et al.*, 2004), variable number tandem repeat analysis (Bull *et al.*, 2003; Overduin *et al.*, 2004), PFGE (de Juan *et al.*, 2005), AFLP (O'Shea *et al.*, 2004), microarray hybridization (Paustian *et al.*, 2005; Semret *et al.*, 2004) and representational difference analysis (RDA) (Dohmann *et al.*, 2003). It will be interesting to see which of these techniques emerges as the most discriminatory, the easiest to perform, and most frequently put into routine use.

Two other publications classified in molecular biology give a more detailed analysis of specific segments or regions of the MAP genome (Sheridan *et al.*, 2003; Stratmann *et al.*, 2004). Sheridan and coworkers (Sheridan *et al.*, 2003) examined the GS element of MAP that was previously found using RDA and is reported absent in *M avium* subsp *avium* (Tizard *et al.*, 1998). This 6500-bp region was analyzed in silico using bioinformatics tools that predicted that coding sequences are involved in GDP-fucose biosynthesis and modification of the oligosaccharide moiety of GPL. Stratmann and coworkers (Stratmann *et al.*, 2004) also used the RDA technique to find a novel 7-kb ABC-transporter operon located within a 38-kb segment that is flanked by an insertion sequence. Also located on this 38-kb island are several gene clusters thought to be involved in iron uptake. These investigators went further by demonstrating the location and expression of two coding sequences in the ABC transporter operon to support their in silico findings. They found that both MptC and MptD were surface located on the MAP bacilli. They conclude that this is the first pathogenicity island discovered in MAP.

The genome sequence of MAP has revealed a total of 19 different IS elements in the K-10 bovine strain (Li *et al.*, 2005) and three publications discuss two of these IS elements. Olsen et al (Olsen *et al.*, 2004) discovered the ISMpa1 element and showed three copies were present in the genome. The genome project designation of ISMpa1 is IS_MAP12 and sequence data analysis confirms that it is present in 3 copies in the K-10 genome (MAP0832c, MAP1287 and MAP2050). This element was found in all MAP isolates examined and in selected porcine isolates of *M. avium* subsp *avium* (Olsen *et al.*, 2004). Another study by Johansen et al (Johansen *et al.*, 2005) examined two insertion sequences, IS1311 and IS1245, that share 85% homology in an effort to clear up discrepancies in some published studies involving these elements. The authors discovered that IS1245 could mistakenly be observed in MAP when using a long IS1245 probe; however, they designed a shorter, more specific probe to show the element is in fact not present in MAP. This discrepancy was attributed to the sequence similarity between IS1245 and IS1311, an element that is represented seven times in MAP. Lastly, a publication describing the integration sites of IS901 in the *M. avium* subsp *avium* genome (Inglis *et al.*, 2003) was included in this category, but really should have been added to the "not classifiable-other" category (Fig. 2) because MAP is not the main focus of the manuscript.

The final sub-category among the MAP molecular biology publications involves extraction methods of cellular components. Three publications were placed in this group with 2 involving extraction of DNA for amplification reactions (Chui *et al.*, 2004; Stabel *et al.*, 2004) and the other asking the question of how the MAP proteome might be affected by two different physical methods of MAP lysis (Lanigan *et al.*, 2004).

THE MAP GENOME

Molecular biology and genomics have now become synonymous in the discipline of microbiology. Microbial genome sequences are being published at a growing rate each year since the initial *Haemophilus influenzae* genome was published in 1995 (Fleischmann *et al.*, 1995). At the time of this international colloquium, there are a total of 182 microbial genome sequences completed. The MAP strain K-10 genome has now been accepted for publication in Proceedings of the National Academy of Sciences USA (Li *et al.*, 2005). This genome contains 4.829 million base pairs and encodes 4,350 genes, 45 tRNAs and one rRNA operon. Whole-genome arrays have already been produced based on the sequence and initial studies have been published (Paustian *et al.*, 2005; Semret *et al.*, 2004). Gene expression profiling using these same arrays will, no doubt, follow soon. It is anticipated that the genome sequence will help in moving molecular biological studies of MAP forward at a rapid pace.

CONCLUSION

Substantial work has been completed in this field over the past 2.5-3 years, but much remains to be accomplished. The dominant theme in this area of MAP research appears to be defining genetic diversity and using those differences in the molecular epidemiology of MAP. Because of this focus, researchers now have an excellent understanding of differences in the genomes of MAP isolates and species within the MAC complex. All of the IS elements have been identified, and some have been found to be useful in subtyping isolates. The genome sequence has now defined the complete catalog of genes that make MAP what it is. Although similarity searches and bioinformatics analyses have assigned potential function to hundreds of genes in this pathogen, the future challenge is to begin to systematically identify gene function through mutagenesis studies and biochemical approaches. Also extraction of RNA protocols would be of benefit for future transcriptional profiling experiments using a whole-genome MAP array.

Author Disclaimer
My apologies to any published study that legitimately fits within the scope of this review, but was not included. I made the attempt to be as comprehensive as possible, yet narrow enough to give reasonable focus. Also, only work published from 2003 until the present was considered even though many publications prior to this would have fit in well with this topic.

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Current culture methods for *Mycobacterium avium* subspecies *paratuberculosis*

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Key words: culture methods, mycobacteria, feces, cattle, sheep

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis or Mycobacterium paratuberculosis* (MAP) is an organism first observed by Johne & Frothingham in 1895. It causes paratuberculosis or Johne's disease, an intestinal granulomatous infection most often seen among domestic and wild ruminants and has a global distribution. In individual animals, especially from a newly infected farm, a tentative clinical diagnosis must be confirmed by laboratory tests. Confirmation of paratuberculosis depends on the finding of a) either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and b) the isolation in culture of MAP (Harris and Barletta, 2001; OIE, 2004).

DIAGNOSTIC TECHNIQUES

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: fecal smears, fecal and tissue culture, DNA probes using feces or tissues, serology, necropsy and histology (OIE, 2004).

Identification of the agent: bacteriology (microscopy)
Ziehl–Neelsen-stained smears of feces are examined microscopically. A potential diagnosis of paratuberculosis can be made if clumps (three or more organisms) of small (0.5–1.5 µm), strongly acid-fast bacilli are found, although it is not always possible to distinguish between MAP and other environmental or pathogenic mycobacteria in a fecal smear. A smear may be made from the processed fecal sediment and stained by the Ziehl–Neelsen method or with fluorochrome stain (auramine O or auramine-rhodamine). The presence of single acid-fast bacilli does not indicate a definitive diagnosis since only about one-third of cases can be confirmed on microscopic examination of a single fecal sample (OIE, 2004).

Identification of the agent: bacteriology (solid media culture)
MAP infection mainly involves the lower small intestine and adjacent cecum. The MAP organisms are vastly outnumbered by other bacteria in fecal and intestinal tissue specimens. Primary colonies of MAP may be expected to appear any time from 4 to 16 weeks after inoculation on solid media. Primary colonies on Herrold's egg yolk medium (HEY) containing mycobactin J (MJ) are very small (1 mm in diameter), colorless, translucent and hemispherical. Their margins are round and even, and their surfaces are smooth and glistening. The colonies become more opaque and increase in size (4 or 5 mm) as incubation continues. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate (OIE, 2004; Thorel, 1984).

The identification of MAP is based on its requirement for mycobactin and its pathogenicity in the host. Most mycobacteria are able to metabolize mycobactin for themselves. *Mycobacterium paratuberculosis, M. silvaticum* and some primary isolates of *M. avium* require mycobactin to grow in the laboratory. Thus, the mycobactin requirement characteristic exists to various degrees within the *M. avium* group (Thorel, 1991). For identification of MAP, small inocula of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. The uncommon, bright yellow pigmented sheep strain is difficult to grow on artificial solid media. It has been reported that unpigmented

sheep strains grow less well than cattle strains, and no cultures should be discarded as negative without prolonged incubation (up to 6 months) (OIE, 2004).

Decontamination methods
There are two basic methods in use for the culture of MAP from clinical specimens: (1) oxalic acid and NaOH for decontamination and Löwenstein–Jensen medium for growth, and (2) hexadecylpyridinium chloride (HPC) for decontamination in combination with Herrold's egg yolk medium (HEY) for growth. Both media contain mycobactin (OIE, 2004).

Media
Examples of suitable media are: Lowenstein Jensen medium with MJ, Modified Middlebrook 7H10 with MJ (Australian formula for sheep strain), Middlebrook 7H9 with M J, Becton Dickinson (BD) BACTEC™ 12B culture medium with PANTA antibiotic supplement, MJ & 50% egg yolk supplement, TREK ESP® para-JEM broth with para-JEM GS, AS, EYS and AS w/BLUE, BD BACTEC™ MGIT™ 960 medium with OACC, amphotericin B, naladixic acid, vancomycin (ANV) antibiotic supplement, MJ & 50% egg yolk enrichment, and HEY with MJ and ANV. The advantage of Middlebrook 7H9 and Middlebrook 7H10 media enhanced with MJ is that it is transparent which facilitates the early detection of colonies (Jorgensen, 1982; Merkal, 1970; Payeur et al., 2005; Wiszniewska et al., 2005).

Herrold's egg yolk medium with MJ and ANV has several ingredients which enhance the growth of MAP and decrease the contamination of other organisms. The egg in Herrold's medium contributes sufficient phospholipids to neutralize the bactericidal activity of residual HPC in the inoculum. The other media (Middlebrook 7H9 & Middlebrook 7H10) do not have this property. Other treatments can be used for sample decontamination, i.e., oxalic acid at 5%. HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B enhances the selectivity of the medium by inhibiting contaminating fungi. Nalidixic acid inhibits contaminating gram-negative organisms and vancomycin inhibits contaminating gram-positive organisms. Malachite green is included to help control contaminants and enhance the visibility of colonies. Egg yolk and glycerol provide fatty acids and other nutrients required for the metabolism of mycobacteria (Johansen et al., 2004; Merkal, 1970; OIE, 2004).

Sample preparation
Although fecal culture is technically difficult and time-consuming, it is the only test that does not produce false-positive results (100% specificity) for the live animal. It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100% if shedding (OIE, 2004).

Processing fecal specimens - Pooled fecal culture using 5 samples per pool
Because of the cost of individual fecal cultures, pooling methods are being evaluated for the detection of MAP in bovine and ovine feces. Several studies have indicated that pooling feces from herds that contain heavy shedders or have a high prevalence herds is equivalent in sensitivity to culturing individual animals. Pooling feces from herds with low or moderate shedders or in low prevalence herds may fail to detect the organism (Jensen et al., 2005; Pradenas et al., 2005; Ruzante et al., 2005; Wells et al., 2002; 2003; Whittington et al., 2000).

Homogenization by stirring
 Weigh 2 grams of each sample to be pooled and place into a sterile 50 ml conical centrifuge tube or other suitable vessel. Mix samples by stirring with a sterile wooden stick. If using a 50 ml conical tube, vortex samples vigorously for 10 to 15 seconds until the mixture appears homogeneous. Remove 2 g of the resulting mixture for processing and culture using any of the recommended methods (Payeur et al., 2005).

Homogenization by stomaching
Weigh out 2 grams of each sample to be pooled and place into a stomacher bag. Be sure to add the samples to the same corner of the bag to ensure even mixing. Stomach the samples on the highest setting for 2 min. Inspect the mixture to determine if it appears homogeneous. Additional stomaching may be required to homogenize the mixture. Remove 2 g of the resulting mixture for processing and culture using any of the recommended method (Payeur et al., 2005.

Processing fecal specimens – Individual fecal culture
Suspension and decontamination of feces
No refrigerant or chemical preservative is used. If fecal specimens can not be processed within a week upon receipt at the laboratory, they can be frozen at -70°C to -80°C. Place one centrifuge tube containing 35 ml of sterile distilled water onto a weigh scale located in the Class II Biological Safety Cabinet. Using a sterile wooden tongue depressor, transfer 2 ± 0.1 gm of the fecal specimen into the tube. Mix tube vigorously by shaking for 15 seconds to break up large clumps of fecal matter. Place on commercial shaker for a minimum of 30 minutes, then allow material to sit upright undisturbed at room temperature for a minimum of 30 minutes. With a sterile disposable pipette, transfer 5 ml of supernatant (minimizing fiber transfer) into 25 ml of 0.9% HPC in ½ X BHI broth solution contained in a separate disposable 50 ml conical centrifuge tube. If a refrigerated centrifuge is used, the temperature must be kept above 10°C, as the HPC will precipitate at 4°C. Incubate for 18 to 24 hrs at 37° ± 2°C. After overnight incubation, centrifuge the samples at 900 x g for 30 ± 2 minutes. Discard the supernatant, and resuspend the remaining pellet in 1 ml of antibiotic brew (BHI broth containing 100 µg/ml nalidixic acid, 100 µg/ml vancomycin and 50 µg/ml amphotericin B). Mix the sample by shaking or vortexing vigorously for a minimum of 15 seconds. Incubate the fecal inoculum in the antibiotic brew at 37° ± 2°C for 24 to 72 hours. After the overnight incubation of the fecal inoculum in antibiotic brew, vortex it vigorously for a minimum of 15 seconds (Payuer and Capsel, 2005; Payeur et al., 2005).

Inoculation of culture - solid media
Use Herrold's egg yolk medium (HEY) with mycobactin J (MJ) for the culture of MAP from bovine feces and use Modified Middlebrook 7H10 (MM7H10) with (MJ) for the isolation of MAP from ovine feces. After vortexing the specimens, inoculate four tubes of HEY with MJ and one tube of HEY w/o MJ for bovine specimens, or four tubes of MM7H10 with MJ and one tube of MM7H10 w/o MJ for ovine specimens by pipetting 200-250 µl of the antibiotic brew suspension onto the surface of the media. Rock the tubes to evenly distribute the inoculum. If a liquid culture media (Bactec 12B, MGIT, ESP) is also being used, eliminate one HEY with MJ slant in the setup for both bovine or sheep feces. Slant the inoculated tubes at a 30° angle, ensuring that the caps are loose, and incubate at 37°± 2° C for 5 to 7 days to allow absorption/evaporation of any remaining liquid present on the slant. After all remaining visible liquid has been absorbed, tighten down the caps on the media and continue to incubate upright at 37°± 2° C. Examine tubes for growth and contamination at 4, 8, 12 and 16 weeks post-inoculation for bovine samples and at 4, 8, 12, 16, 20 and 24 weeks for ovine samples. Use of a stereomicroscope will facilitate examination of the tubes at 4 weeks. Document all observations (Gwozdz, 2003; Payeur et al., 2005).

Interpretation of results (identification of solid media cultures positive for MAP)
Samples that exhibit bacterial growth typical of MAP on one or more tubes HEY or MM7H10 with MJ, but do not exhibit growth on HEY or MM7H10 w/o MJ at 4, 8, 12 or 16 weeks of incubation (ovine samples can require up to 24 weeks) are considered suspect positive. Typical colonial morphology for MAP is small (1 mm diameter), colorless, translucent and hemispherical, smooth and glistening. The colonies become more opaque and increase in size (ca. 4 mm) and roughness as incubation is continued. The use of a stereomicroscope is highly recommended for earlier detection. Most colonies are visible by 8 weeks after inoculation. Contamination usually increases between 8 – 16 weeks which impedes detection of MAP colonies (Gwozdz, 2003; Payeur et al., 2005).

A single bacterial colony from a HEY or an MM7H10 with MJ should be acid-fast stain tested. Typical cellular morphology of MAP is acid-fast, small (0.5 µm x 1 µm) and generally observed in clusters or clumps. If acid-fast positive, a PCR test should be performed for the presence of the IS900 insertion element. The specimen is considered positive for MAP if growth is observed on one or more tubes of HEY or MM7H10 with MJ, no growth is observed on HEY or MM7H10 w/o MJ, the acid-fast smear is positive and the PCR result is positive.

Variations in the above methods have been described (Collins et al., 1990; Merkal et al., 1968; Shin, 1989; Shin et al., 1990; Singh et al., 1991, Whitlock and Rosenberger, 1990; Whitlock et al., 1991; 2000). The sensitivity of culture may be enhanced using liquid media with centrifugation rather than sedimentation techniques. The double incubation method assists with decontamination of the inoculum (Stabel, 1997). Evaluations have been done using CB18, a zwiterrionic detergent which has been shown to increase the

recovery of MAP, but it also has a high level of contamination associated with it (Johansen and Payeur, 2004; Ruzante et al., 2005).

Liquid media culture
A more rapid technique for the isolation of MAP from bovine or ovine feces employs the use of a radiometric-based detection system, the BACTEC™ 460. The BACTEC™ 12B medium contains ^{14}C-labeled palmitic acid which is metabolized by *Mycobacterium spp*. The resulting release of radiolabeled CO_2 is measured by the BACTEC™ 460 reader, giving an indication of bacterial growth that is termed the growth index (GI). Samples are decontaminated by a recommended method and inoculated into BACTEC™ 12B medium, incubated at 37°C and read weekly on a BACTEC™460 reader for 6 weeks. Any sample with a GI>10 is subcultured or confirmed directly by acid fast staining and PCR. However, as this system is radiometrically based, it is not feasible for use in some laboratories and has been phased out in others (Gwozdz, 2003; Payuer et al., 2005; Whittington et al., 1999).

There are several non-radiometric methods available for the culture of MAP with liquid media. The BD MGIT™ Para TB liquid culture media contains a fluorescent compound (Tris 4, 7-diphenyl-1,10-phenanthroline ruthenium chloride pentahydrate) embedded in silicone in the bottom of the culture tube. This compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the amount of dissolved oxygen present in the broth quenches emissions from the compound and little fluorescence is detected. Later, actively respiring micro-organisms consume the oxygen and diminish the quenching of the fluorescence, allowing it to be detected by the BACTEC™ MGIT™ 960 instrument (Fyock et al., 2005; Payuer et al., 2005; Thomas et al., 2005).

Culture tubes entered into the BACTEC™ MGIT™ 960 instrument are incubated at 37ºC for 6 weeks and monitored hourly for increasing fluorescence. Analysis of the fluorescence emitted from each tube is used to determine whether it contains viable organisms. A specific algorithm is used to determine whether the instrument signals positive for a sample for the growth of MAP. A sample is reported as positive if it contains acid-fast bacteria and is determined to be positive for the presence of the IS*900* element using PCR (Fyock et al., 2005; Payuer et al., 2005).

Another non-radiometric system is the ESP® Culture System II (TREK Diagnostics) which is based on consumption of gases by viable mycobacteria as part of their metabolic growth cycle. This creates a negative pressure change within the headspace above the broth culture medium within the sealed ESP® bottle. The change in pressure is monitored by a computerized system that signals the user when growth is detected. When the ESP® Culture System II identifies a positive specimen, a red indicator light will be lit on both the outside of the machine and the specific location within a drawer. If the corresponding graph for the specimen indicates a typical Mycobacterium pattern, consisting of a noticeable sharply defined downward curve to the baseline (e.g. "elbow" or "knee" shaped) after approximately 4 or greater days of incubation, the specimen is considered a suspect positive. The specimen is considered positive for MAP if the acid-fast smear is positive and the PCR result is positive for the presence of the IS*900* insertion element (Kim et al., 2002; Payuer et al., 2005; Shin, 1989; Shin et al., 1990; van Maanen et al., 2005).

Severe problems were encountered during initial experiments in which these liquid culture methods were tested on fecal samples due to overgrowth by other bacteria (spore forms and fungi); however, these methods have been further developed and are now used with some success in many laboratories (Eamens et al., 2000 ;Thomas et al., 2005).

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Production and characterization of monoclonal antibodies, aptamers and single chain antibodies to *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

New technologies such as immunomagnetic separation have immediate applicability in Johne's disease research, but specific antibodies available in unlimited quantities have not been produced. Furthermore, the development of monoclonal antibodies to *M. avium* subsp. *paratuberculosis* (MAP) was identified as an unmet need at the 7th International Colloquium on Paratuberculosis in Bilbao, Spain. To fill this gap in Johne's disease research, monoclonal antibodies (mAbs) against MAP were produced from BALB/c mice immunized with sonicated MAP extracts or recombinant fusion proteins. A total of ten stable hybridomas producing monoclonal antibodies to MAP proteins ranging from 12-40 kDa were identified in immunoblot assays. Six mAbs were classified as broadly cross reactive and four showed limited cross reactivity when analyzed against a panel of whole cell protein lysates comprising seven different mycobacterial species. The mAbs were characterized for their isotype, binding specificity, nature of binding epitope, reactivity in immunoassays, etc. The identity of the epitopes that bind selected monoclonal antibodies was determined by screening a MAP-phage expression library. This approach revealed that 9G10 detects MAP1643 (isocitrate lyase), 11G4 detects MAP3840 (70-kDa HSP) and 8G2 detects MAP2121c (a membrane protein), three proteins present in high relative abundance in MAP bacilli. The epitopes for 11G4 and 8G2 were mapped to the N-terminal half of each protein whereas 9G10 binds to the C-terminal half of MAP1643. Among the panel of mAbs generated in this study, 9G10 and 14D4 appear to label MAP best by immuno-electron microscopy. Aptamers, or nucleic acids that bind specific protein sequences, were also generated against the hypothetical protein encoded by MAP0105c and tested for specificity to MAP. Finally, a phage-produced single-chain antibody (ptb9) was identified that binds to the MAP-specific protein encoded by MAP0858. These detection reagents will be beneficial in many Johne's disease research applications.

INTRODUCTION

Despite a concerted effort by researchers, diagnosis and control of Johne's disease (JD) remains a significant challenge. New approaches, such as functional genomics, are needed to solve this persistent animal health problem. With the genome sequence of MAP now completed, the next step is to characterize the entire catalogue of genes in order to obtain clues to combat and control JD. Monoclonal antibody (mAb) production is an obvious initial step in this direction. At the Seventh International Colloquium on Paratuberculosis, held in Bilbao, Spain on June 11-14, 2002, there was an urgent plea by the scientific community in attendance to develop mAb reagents to detect MAP for a variety of research applications. This plea was made because of the distinct lack of such reagents as well as their increased need to move new technologies along. More than just their obvious applications in diagnosis of JD, mAbs are critical reagents in cell biology and pathogenesis studies, including macrophage-pathogen interactions, Luminex and magnetic bead technologies and histopathology studies. With the exception of a single study (Mutharia *et al.*, 1997), the scientific literature is silent on the subject of MAP mAbs and their use in JD research. Only mAbs that detect unique MAP proteins should be incorporated into diagnostic assays for other organisms such as those already developed for *Campylobacter* (Brooks *et al.*, 2004) and *E. coli* (Kerr *et al.*, 2001).

In histopathological examination of infected tissues, typically the lamina propria of the intestine, acid-fast staining has been used to demonstrate the presence of MAP. However, this technique has low sensitivity

and specificity (Thoresen *et al.*, 1994). Acid-fast techniques can also have false-positive reactions because they detect environmental mycobacteria, *Nocardia* and *Corynebacteria* (Shinnick & Good, 1994). Immunohistochemical detection of MAP antigens in tissue sections would lead to a more specific and sensitive method. Cross-reacting mAbs and polyclonal antibodies developed from *M. bovis* have been used in diagnosis of JD in bison (Leid *et al.*, 2002) and bovine tissues (Brees *et al.*, 2000). This strategy is risky however and may result in misdiagnosis (false-positives) if the animals were exposed to *M. bovis*, or even *M. avium*. In one study, immunohistochemistry using polyclonal antisera was a better indicator of infection than acid-fast stains because it detected more infected animals (Thoresen *et al.*, 1994). However, the antibodies used in that study were made in limited quantities by individual laboratories and are unavailable for widespread use. In this study several novel mAbs were developed that detect MAP.

MATERIALS AND METHODS

Mycobacterial strains and culture conditions
The National Animal Disease Center's mycobacterial culture collection served as the source of all strains used in this study. MAP 1434 is a sheep isolate, and all others are cattle isolates, including K-10, the sequenced isolate. All mycobacteria were cultivated at 37 $^{\circ}$C in Middlebrook 7H9 media supplemented with OADC and mycobactin J.

Production and screening of monoclonal antibodies
Standard techniques approved by the National Animal Disease Center Animal Care and Use Committee were used in the immunization of Balb/c mice for production of mAbs against recombinant MAP proteins (Goding, 1996; Harlow & Lane, 1988). Balb/c mice were immunized with purified recombinant protein (MAP2121c) or a sonicated extract of MAP ATCC19698 mixed with TiterMax adjuvant (CytRx corporation) which does not contain mycobacterial components that could produce cross-reacting antibodies. Mice were boosted every 2-3 weeks with purified proteins and serum titers checked by ELISA and immunoblot assays. When antibody levels were sufficiently high, the spleen from the best responder mouse was removed and fused with the SP2/O myeloma cell line. The cells were then distributed into 96 well plates containing HAT medium supplemented with horse serum and cultured approximately 2 weeks.

The same two antigen sets used to immunize mice were also used to screen the hybridoma clones by preparative immunoblot or ELISA analysis. When screening with the purified recombinant MAP2121c protein, the maltose binding protein (MBP) affinity tag alone was also incorporated into screening assays. The supernatant from individual wells containing expanded clones were evaluated in a differential ELISA to identify mouse antibodies that react with the recombinant fusion protein but not the MBP tag used for purification of the protein. Supernatants that displayed this pattern of differential reactivity were re-screened on immunoblots containing the purified fusion protein, the MBP affinity tag and MAP whole cell sonicated antigen similar to that shown in Fig. 3. Cells secreting antibodies that react with only the fusion protein and whole cell sonicated antigen, but not MBP, were further expanded. Selected cell lines were frozen (-140 degrees Centigrade) for long-term storage.

RESULTS

MAbs against MAP whole cell homogenates cross react with other mycobacteria
To obtain mAbs against MAP proteins, six-week-old female BALB/C mice were immunized three times intraperitoneally with a sonicated protein lysate of MAP K-10 (100 mg per injection) suspended in 0.5 ml of phosphate-buffered saline (PBS; pH 7.3; GibcoBRL) at 14-day intervals. Ten stable hybridomas were identified using this immunization regimen and were analyzed together on a single preparative immunoblot (Fig. 1). These hybridomas contain mAbs that react with a variety of MAP proteins of different sizes as shown in Fig. 1.

However, when selected mAbs were evaluated against other mycobacterial whole cell lysates, including *M. avium* subspecies *avium, M. avium* subspecies *sylvaticum* and other species of mycobacteria, they were

found to cross-react with similarly sized proteins in most species of mycobacteria (Fig. 2). Interestingly, mAbs 9G10, 12C9 and 11G4 did not react with either *M. bovis* strains or with *M. phlei* (Fig 2). This cross reactivity is not surprising, given the broad genetic similarity observed for mycobacterial subspecies belonging to the MAC complex (Bannantine *et al.*, 2002). In the future, we plan to develop monoclonal antibodies that detect MAP only, not other mycobacterial species.
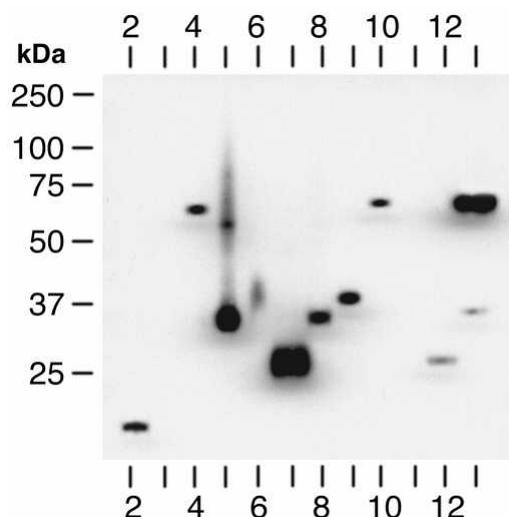
Fig. 1. Preparative immunoblot of culture supernatants containing monoclonal antibodies against MAP. Mice were immunized with a whole cell homogenate and spleen cells were collected and fused to myeloma cells. Positive hybridoma culture supernatants were analyzed in parallel on a preparative slot immunoblot containing MAP whole cell homogenates separated by SDS-PAGE. Lane assignments: #2-mAb 5F5; #3-mAb 2E8; #4-mAb 14F11; #5-mAb 13B7; #6-mAb 13E5; #7-mAb 12C9; #8-mAb 14D4; #9-mAb 4B6; #10-mAb 13A4; #11-mAb 9G10; #12-mAb 14G3; #13-mAb 14G11. Kilodalton size standards are indicated in the left margin.

Six MAP surface proteins have been identified using a proteomic approach
Not all mAbs are used for diagnostic purposes. MAbs are also important in research applications. However, it is critical to have mAbs against proteins that are located on the cell surface or cell membrane for ease of bacilli detection in downstream applications. While mAbs to cytoplasmic proteins may be important in focused studies on particular proteins, they may not be as effective at detecting bacilli in diagnostic or general research settings. Furthermore, we are interested in surface-located proteins as vaccine candidates and targets to block initial invasion of bovine intestinal epithelial cells.

Use of the MAP genome sequence, combined with proteomics, quickly identified a set of proteins that are likely surface-located due to their hydrophobic nature. Membrane proteins of MAP were enriched by phase separation in Tx114. Bacilli were solubilized in Tx114 and the detergent was heated to 37 °C. The centrifuged pellet contained the great majority of the membrane proteins. Following separation on one-dimensional SDS-PAGE, proteins were enzymatically digested and fragments analysed using a capillary-flow liquid chromatograph coupled to a state-of-the-art nano-spray ion-trap mass spectrometer. Six of eight proteins that partitioned in the detergent phase were identified from these analyses and include MAP0872, MAP2455c, MAP2683, MAP3697c, MAP3698c and MAP3972c. Furthermore, all of these proteins were previously annotated as 'hypothetical'. Therefore, this experiment has revealed new information about these uncharacterized proteins produced by MAP. The proteins encoded by these sequences are likely to be excellent candidates for mAb development.

Table 1 shows how the Tx114 factions compare with protein location as predicted using the PSORT algorithm. This is software widely used for bacterial protein subcellular localization prediction (www.psort.org). Five out of six proteins that were present in the detergent phase were predicted to be membrane localized by PSORT. The only protein that partitioned in the detergent phase and was predicted to be a cytoplasmic protein using PSORT was MAP3697c (Table 1). Closer inspection of this protein suggests that it may interact with the gene product encoded by its neighbor, MAP3698c, which also partitioned in the detergent phase and is likewise a subunit of succinate dehydrogenase and immediately downstream of MAP3697c.
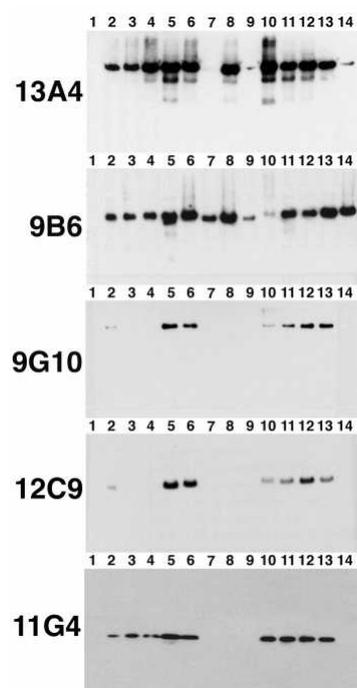
Fig. 2. Evaluation of expanded hybridoma supernatants against whole cell homogenates from several mycobacterial species. Immunoblot analysis shows reactivity with other mycobacterial species than MAP lysates. Lane assignments: lane 1: protein standards; lane 2: *M. sylvaticum*; lane 3: *M. scrofulaceum*; lane 4: *M. abcessans*; lane 5: MAP K-10; lane 6: *M. avium* (TMC702); lane 7: *M. bovis* (strain 95-1315); lane 8: *M phlei*; lane 9: *M. bovis* BCG; lane 10: MAP ATCC19698; lane 11: *M. avium* (TMC715); lane 12: MAP (Linda); lane 13: *M. intracellulare*; lane 14: *M. kansasii*.
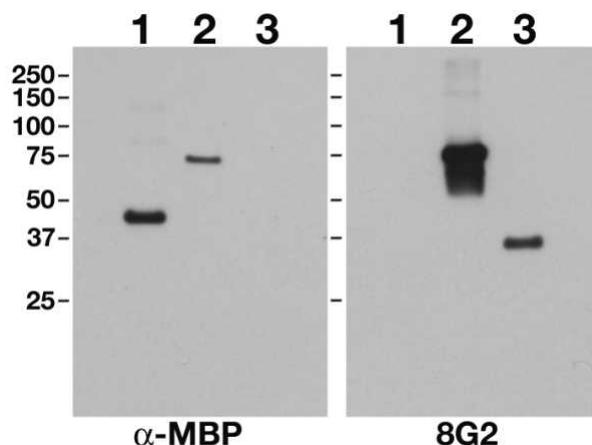
Fig. 3. Immunoblot analysis of mAb 8G2 shows reactivity with the *E. coli* expressed recombinant as well as the MAP protein. Note that the mAb developed to the MBP affinity tag does not detect any MAP protein whereas the 8G2 mAb detects only the *E. coli* expressed MBP/MAP2121c fusion and MAP2121c in MAP. Lanes: 1=purified MBP; 2=MBP/MAP2121c; 3=MAP whole cell sonicated lysate. Kilodalton size markers are shown in the left margin along with corresponding notches between the blots.

Similarly, one protein present in the aqueous phase of the Tx114 extraction (MAP3936) was predicted to be membrane located based on PSORT analysis. However, its presence may be explained by the fact that it is part of the GroEL complex of which another subunit (MAP4265) was also identified in the aqueous phase. Therefore, an interaction between these two proteins may explain the presence of MAP3936 in the aqueous phase.

Table 1. Comparison of Triton X-114 fractionated proteins with cellular location predictions using PSORT

Genome project name	Triton X-114 phase partition	Number of amino acids	Calculated mol. Wt. (Da)	PSORT prediction	Best BLAST match
MAP3698c	Detergent	647	70,724	Membrane	Succinate dehydro. (Fe-sulfur subunit)
MAP2683	Detergent	402	44,276	Membrane	probable integral membrane protein
MAP3972c	Detergent	352	37,343	Membrane	hypothetical protein (Rv0479c)
MAP0872	Detergent	369	37,193	Membrane	phospate binding protein
MAP3697c	Detergent	248	28,639	Cytoplasm	succinate dehydrogenase
MAP2455c	Detergent	177	18,810	Membrane	AtpF – ATP sythase beta chain
MAP1643	Aqueous	762	85,214	Cytoplasm	AccAB – isocitrate lyase
MAP3840	Aqueous	623	66,519	Cytoplasm	DnaK – Hsp70
MAP2453c	Aqueous	554	59,993	Cytoplasm	AtpA – ATP synthase alpha chain
MAP3936	Aqueous	541	56,643	Membrane	GroEL2 – Hsp65
MAP4265	Aqueous	538	55,793	Cytoplasm	GroEL1 – Hsp60
MAP2451c	Aqueous	402	44,255	Cytoplasm	AtpD – ATP synthase
MAP4143	Aqueous	396	43,739	Cytoplasm	Elongation factor EF-Tu
MAP3060c	Aqueous	318	32,085	Cytoplasm	FixB – electron transfer protein
MAP1339	Aqueous	147	15,437	Membrane	Hypothetical protein (Rv1636)

Importantly, these data show that PSORT can accurately predict membrane located proteins for MAP. Armed with this information, 19 of the MAP-specific proteins were evaluated by PSORT. As shown in Table 2 below, 11 MAP proteins are predicted to be present in the membrane. Of these putative membrane proteins, six have weak homology to another protein in the non-redundant sequence databases. The remaining 5 proteins that show no hit from BLASTP analysis are among the best targets for future mAb production. PSORT also predicted eight MAP-specific proteins are cytoplasmically located.

Our preliminary results using specific protein markers show that we are able to generate comparatively 'clean' preparations of mycobacterial cells (i.e., free from both cytosolic and extracellular contamination common with mature mycobacterial cultures).

Table 2. Cellular location predictions for *M. paratuberculosis*-specific proteins using PSORT.

Genome project name	Number of amino acids	Calculated mol. Wt. (Da)	PSORT prediction	Best BLAST-P match (as of 8/25/04)
MAP0105c	889	97.6	Membrane	Hyp protein SA V157
MAP1636c	157	17.0	Membrane	no hit
MAP2149c	214	22.3	Membrane	no hit
MAP2154c	191	20.7	Membrane	no hit
MAP2753	252	25.4	Membrane	no hit
MAP2756c	1636	166.4	Membrane	gp22 mycobacteriophage
MAP2963c	874	97.0	Membrane	hyp protein SA V157
MAP3436c	231	25.7	Membrane	gp55 mycobacteriophage
MAP3732c	237	24.6	Membrane	membrane protein
MAP3736c	319	34.1	Membrane	ABC transporter
MAP3817c	312	33.4	Membrane	no hit
MAP0855	314	34.0	Cytoplasm	no hit
MAP0858	182	19.8	Cytoplasm	no hit
MAP0860c	296	32.2	Cytoplasm	no hit
MAP0862	360	39.7	Cytoplasm	no hit
MAP1345	199	21.4	Cytoplasm	MAP0791c
MAP2751	193	21.1	Cytoplasm	no hit
MAP2762c	146	16.3	Cytoplasm	no hit
MAP3437c	280	30.7	Cytoplasm	no hit

Production of monoclonal antibodies against a MAP protein expressed in Escherichia coli
A MAP protein (MAP2121c) was previously shown to play a role in invasion of epithelial cells and was localized to the bacilli surface by immuno-EM (Bannantine et al., 2003). This same 35-kDa protein was also predicted to be a membrane protein by PSORT, further giving credence to using PSORT as an accurate predictor for MAP membrane proteins. The protein has already been successfully expressed in *E. coli* and purified (Bannantine et al., 2003). The purified recombinant was used to immunize mice for production of monoclonal antibodies. Resulting tissue culture supernatants from secreting hybridomas were screened by ELISA using plates pre-coated with the purified fusion protein. Positive reacting supernatants were then evaluated by immunoblot as shown in Fig. 3. In the immunoblot screening, note that the tissue culture supernatant was used to probe both the recombinant fusion protein as well as the MBP affinity tag alone. This enabled the identification of those hybridomas that were reacting specifically with MAP2121c and not MBP. Also, note in Fig. 3 that the 8G2 mAb reacts with the corresponding protein in the MAP whole cell homogenate. Four monoclonal antibodies that react with different regions of MAP2121c were identified and cloned from this single fusion.

Identification of MAP antigens that bind mAbs
As shown in Fig. 1, several mAbs were obtained from mice immunized with a whole cell homogenate of MAP. Even though these mAbs react with other mycobacterial species (Fig. 2) they still have value in research applications. However, the MAP antigens that react with the mAbs were unknown. Therefore, two of these mAbs were used to screen a MAP-lambda phage expression library that was previously described (Bannantine & Stabel, 2001). Positive plaques were obtained for both the 11G4 and 9G10 mAbs. Fig. 4 shows the primary and secondary screening results using 11G4 to probe the nitrocellulose plaque lifts. DNA sequencing of the subcloned lambda phage inserts revealed open reading frames for MAP3840 (dnaK) and MAP1643 (aceAb) that react with 11G4 and 9G10 mAbs, respectively.
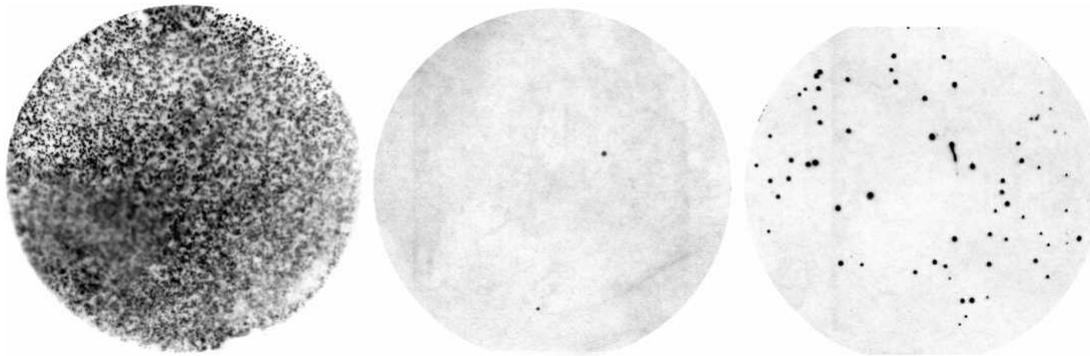
Fig. 4. Nitrocellulose lifts containing the MAP-lambda phage library probed with mAb 11G4. The circular nitrocellulose lift shown at the left was probed with rabbit sera raised against a whole-cell homogenate of MAP and serves to illustrate the density of the plaques on all lifts shown. The middle nitrocellulose filter (probed with mAb 11G4) is from a secondary screen of a picked plaque that was positive in the initial screen. Only two positive plaques were obtained from this secondary screen. The filter on the right shows the enrichment of 11G4 positive plaques following the tertiary screen. Another round of screening was necessary to obtain plaques pure enough to subclone and sequence. This same protocol was used to identify MAP1643, which reacts with mAb 9G10.

Aptamers and single chain antibodies against MAP
Selected MAP proteins that were recombinantly expressed in *E. coli* did not yield any positive or stable hybridomas. Therefore, other methods were used in these instances. Specifically, aptamers were developed against MAP0105c (Fig. 5).
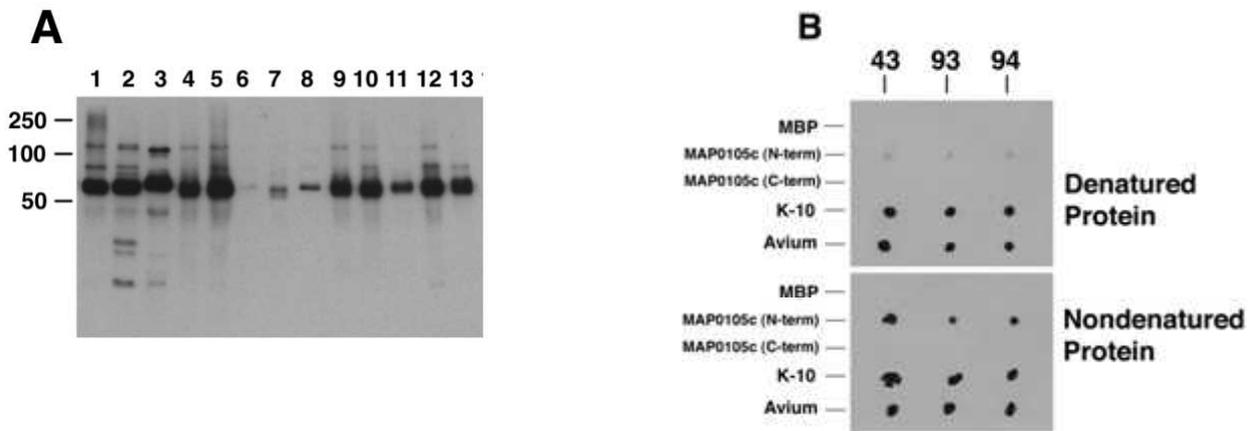
Fig. 5. Immunoblot (A) and dot blot (B) of MAP0105c aptamers. Immunoblot was expose to aptamer 94. Size standards are indicated in kilodaltons in the left margin. Lane assignments: 1: *M. avium* subspecies *sylvaticum*; 2, *M. scrofulaceum*; 3, *M. abscessans*; 4, MAP K-10; 5, *M. avium* (tmc702); 6, *M. bovis*; 7, *M. phlei*; 8, *M. bovis* BCG; 9, MAP 19698; 10, *M. avium* (tmc715); 11, MAP (Linda); 12, *M. intracellulare*; 13, *M. kansasii*. Panel B shows the dot blot exposed to the three aptamers (indicated above the blot). Proteins spotted to the membrane are indicated in the left margin and the state of the proteins is indicated in the right margin. Each of these experiments were repeated three times.

Aptamers are nucleic acid sequences that bind specific protein sequences. Therefore, aptamers have similar properties to antibodies. They can also be labeled or conjugated like antibodies. MAP0105c was considered to be specific to MAP based on similarity searches in publicly available sequence databases and PCR analysis; however, three aptamers developed against the recombinant protein encoded by MAP0105c reacted with proteins of similar size in other mycobacteria (Fig. 5A). Similarly, we have identified a single chain antibody (ptb9) from a phage library the binds to another MAP specific protein encoded by MAP0858. Data are still too preliminary to determine whether this antibody will be useful.

DISCUSSION

The predominant challenges in working with MAP – its slow growth, the lack of technology for genetic modification, and intramacrophage location during infection, combined with the complete lack of similarity of the MAP-specific proteins with any other known proteins presents challenges that require unique approaches. Antibodies developed in these studies will be invaluable for obtaining clues on the location and possible function of the novel proteins they bind. Proteomic and bioinformatics analysis has identified eleven MAP-specific proteins as surface located. MAbs against these proteins can be used in immunoelectron microscopy (immuno-EM) studies as well as Tx114 experiments to definitively pinpoint the location of these proteins within the bacilli, thus potentially confirming bioinformatic predictions. Importantly, those shown to be surface-located and immunogenic are prime vaccine candidates. Furthermore, mAbs developed in this study will be used in an attempt to block infection of cultured epithelial cells to determine if any of these novel proteins might play a role in invasion of bovine epithelial cells that line the intestine.

More practically, these novel mAbs may be used to confidently purify and concentrate MAP from environmental samples such as water or bulk milk tank samples via immunomagnetic separation technologies. Positive identification of MAP might also be obtained with these mAbs on tissues from Crohn's disease patients.   Finally, developed mAbs can confidently identify surface exposed proteins on MAP, making these gene products strong candidates for subunit vaccines.

CONCLUSION

Because of the lack of such critical reagents, novel monoclonal antibodies have been developed against MAP for research and diagnostic purposes. Furthermore, the MAP protein that reacts with four of these mAbs has been determined.

ACKNOWLEDGEMENTS

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Development of VNTR typing of *M. avium* subsp. *paratuberculosis*: comparison of results with those obtained by IS*900* RFLP

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ABSTRACT

The most widely used method to type M. avium ssp. paratuberculosis (MAP) strains is RFLP based on IS900. This method is, however, only applicable to culturable strains, is technically demanding and requires analysis of complex banding patterns. Development of alternative molecular typing methods available for other mycobacteria can provide rapid and more discriminatory procedures for studying the diversity of the MAP strains. The aim of this study was to identify new genetic markers, consisting of variable number tandem repeats (VNTRs) of genetic elements called mycobacterial interspersed repetitive units (MIRUs), to study the clonal distribution and the degree of diversity of MAP isolated from different geographic origins and from different hosts. A panel of 72 strains including bovine, caprine, ovine, deer, and human isolates from six different countries was used. Thirty-five tandem repeat (TR) loci were selected by using the Tandem Repeat Finder software and the genomic sequence of MAP K10, and by blast analysis using MIRU loci identified in M. tuberculosis. The variability of the TRs was determined by PCR amplification of the 35 loci, and sizing of the PCR products. The numbers of tandem repeat motifs in the 35 loci were then deduced from the sizes of the corresponding PCR products. The IS900-RFLP strain profiles were determined by the standard method and compared to the MIRU-VNTR types. Among the 35 TR loci, only 8 were polymorphic. Compared to the IS900 RFLP, MIRU-VNTR typing subdivided the predominant RFLP type into eleven subtypes suggesting that this approach provides an additional tool to study the genetic diversity of MAP. Although this preliminary study indicates that MIRU-VNTR typing may provide additional discrimination to distinguish different clones of MAP, it further highlights the overall genetic homogeneity of this subspecies. Therefore, a combination of minisatellite- (MIRU-VNTRs) and microsatellite- (shorter repeats) based typing might prove to be optimal for PCR-based molecular epidemiological studies of this pathogen.

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the etiological agent of a severe gastroenteritis in ruminants known since 1894 as Johne's disease (Johne and Frothinghan, 1895, pp. 438-454). Paratuberculosis is prevalent in domestic animals worldwide and has a significant impact on the economy. Recent studies have also described MAP isolation from captive wildlife (Motiwala et al., 2004, pp. 1703-1712). In addition, MAP is suggested to be involved in Crohn's disease, a chronic enteritis in humans for which the etiology remains unknown. While evidence for causal link remains controversial (Bull et al., 2000, pp. 2185-2197, Bernstein et al., 2003, pp. 4986-4990, Bernstein et al., 2004, pp. 1129-1135), concerns regarding MAP as a human and animal pathogen remain. Control of this infection require better knowledge on the causative agent, in particular its epidemiology and the subspecies biodiversity.

Study of MAP is hampered by the difficulty of manipulating it in a laboratory setting. MAP is an extremely slow-growing organism (most bovine strains require 4 to 6 months of incubation) and they depend on an iron chelator (Barclay and Ratledge, 1983, pp. 1138-1146). MAP strains are very difficult to isolate from

sheep and humans and may require years to emerge in culture. Therefore, small numbers of MAP isolates have been maintained in available collections. This situation has limited the study of the biodiversity of MAP.

Another limiting factor is the lack of convenient typing methods. The most widely used method to type MAP strains is RFLP based on IS*900* (Stevenson et al., 2002, pp. 1798-1804). This method is only applicable to cultivable strains, it is slow and technically demanding. Moreover, it requires analysis of complex banding patterns and has limited discriminatory power. Therefore, rapid and discriminatory molecular typing methods need to be assessed as alternatives for studying the diversity of the MAP strains.

Tandem repeat (TR) sequences often represent polymorphic structures in the genomes of highly monomorphic species such as *Bacillus anthracis* and *Yersinia pestis*. Variable number tandem repeats (VNTRs), in particular those of genetic elements called mycobacterial interspersed repetitive units (MIRUs), have been discovered and used for typing of various mycobacterial species, including the *M. tuberculosis* complex, *M. marinum* and *M. ulcerans* (Supply et al., 1997, pp. 991-1003, Supply et al., 2000, pp. 762-771, Roring et al., 2002, pp. 2126-2133, Ablordey et al., 2005, pp. 1546-1551, Stragier et al., 2005, pp. 1639-1647). Recently, partial genome screenings have identified a limited number of MIRU-VNTR loci in the *Mycobacterium avium intracellulare* (MAC) complex, providing very precise discrimination among MAP isolates (Bull et al., 2003, pp. 157-164, Overduin et al., 2004, pp. 5022-5028).

The aim of this study was to identify novel VNTR loci based on an exhaustive screening of TR loci in MAP genomes, and to study their variability in isolates obtained from different hosts and from different geographic origins. The discrimination provided by the novel VNTR loci was compared to that achieved by RFLP-IS*900* typing.

MATERIALS AND METHODS

Strains
MAP strains were isolated on Herrold's egg yolk medium containing mycobactin J, amphotericin B, nalidic acid (Becton Dickinson, Le Pont de Claix, France) according to the method of Whipple et al (Whipple et al., 1991, pp. 368-373.). Mycobacterial isolates were propagated in Middlebrook 7H9 broth supplemented with Middlebrook albumin-dextrose-catalase (ADC) enrichment medium (Becton Dickinson, Le Pont de Claix, France) and 2 µg/ml of mycobactin J (Institut Pourquier, Montpellier, France) when required. A panel of 74 strains was used (Table 1). The host species included cattle, sheep, red deer, fallow deer, roe deer, and humans from 6 countries. (Argentina, France, Czech Republic, the Netherlands, Sweden, and the United States). Isolates were obtained from INRA: Institut National de la Recherche Agronomique, Nouzilly, France ; AFSSA : Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort, France ; Institut Pasteur, Paris, France; NIPHE, National Institut of Public Health and the Environment, Bilthoven, The Netherlands; the University of Nebraska, Department of Veterinary and Biomedical Sciences, Lincoln, Nebraska, USA and the LVD 87, Laboratoire Vétérinaire Départemental, Limoges, France.

Preparation of mycobacterial DNA
Mycobacterial DNA was obtained according to a method modified from Baulard et al (Baulard et al., 1996, pp. 3091-3098). Mycobacteria were grown to mid-log phase in 10 ml of Middlebrook 7H9 broth and harvested by centrifugation at 6,000 x g for 15 min. The pellet was resuspended in 10% glycerol and centrifuged again at 6,000 x g for 10 min. The semidried mycobacterial pellets were resuspended in 1 ml of buffer 1 (3% sodium dodecyl sulphate, 1 mM $CaCl_2$, 10 mM Tris-HCl [pH8.0], 100 mM NaCl, 200 µg of proteinase K per ml). After the addition of 200 mg of sand (sulphuric acid wash grain size, diameter of 150 to 210 µm, Merck, Briare le Canal, France), the suspension was vigorously shaken for 10 min, incubated for 30 min at 56°C, and shaken by hand for 1 min. The suspension was centrifuged for 10 min at 14,000 x g, and the supernatant was adjusted to 2 mM final concentration EGTA (Sigma). After addition of 0.8 volume of isopropanol and 0.3 M sodium acetate (final concentration) the DNA was precipitated by centrifugation for 10 min at 14,000 x g and resuspended in 0.5 ml of TE buffer. One volume of Phenol-Chlorophorm (50/50) was then added and the solution gently mixed. The organic extraction was performed by using Phase Lock Gel (Eppendorf Hamburg, Germany). Sodium acetate (0.3 M final concentration) and 2.5

volumes of isopropanol were added to the aqueous phase and incubated at 4°C for at least 10 min. After centrifugation for 30 min at 14,000 x g, the DNA pellet was washed with 70% ethanol, then air dried and dissolved in 50 μl of TE buffer and stored at -20°C until further use.

Table1.StrainsusedandresultsforIS*900*-RFLPandVNTRtyping

Strain	Country of origin	Host origin	Source[a]	RFLP patterns	VNTR patterns
K10	USA	Bovine	DVBS	R01	32332228
ATCC19698	USA	Bovine	AFSSA	R01	32332228
1	FR	Bovine	AFSSA	R01	32332228
8	FR	Bovine	AFSSA	R01	32332228
9	FR	Bovine	AFSSA	R01	32332228
13	FR	Bovine	AFSSA	R01	32332128
14	FR	Bovine	AFSSA	R01	42332228
18	FR	Bovine	AFSSA	R01	32332228
19	FR	Bovine	AFSSA	R01	32332128
20	FR	Bovine	AFSSA	C18	32332118
21	FR	Bovine	AFSSA	C18	32332118
26	FR	Bovine	AFSSA	R01	42332228
27	FR	Bovine	AFSSA	R01	42332228
28	FR	Bovine	AFSSA	R01	42332228
37	FR	Bovine	AFSSA	R01	32332118
38	FR	Bovine	AFSSA	R01	42332228
40	FR	Bovine	AFSSA	R01	42332228
53	FR	Bovine	AFSSA	R01	42332228
54	FR	Bovine	AFSSA	R01	32332229
55	FR	Bovine	AFSSA	R01	32332229
56	FR	Bovine	AFSSA	R01	42332228
58	FR	Bovine	AFSSA	R01	42332218
60	FR	Bovine	AFSSA	C2	42332218
61	FR	Bovine	AFSSA	R01	32332228
76	FR			R01	42332228
84	FR	Bovine	AFSSA	R01	32332228
86	FR	Bovine	AFSSA	C18	32332218
87	FR	Bovine	AFSSA	R01	32332218
88	FR	Bovine	AFSSA	R01	32332218
91	FR	Bovine	AFSSA	R01	42332228
92	FR	Bovine	AFSSA	R01	42332228
94	FR	Bovine	AFSSA	R01	42332218
98	FR	Bovine	AFSSA	R01	42332228
99	FR	Bovine	AFSSA	R01	42332228
100	FR	Bovine	AFSSA	R01	32332229
101	FR	Bovine	AFSSA	R01	3233222(10)
102	FR	Bovine	AFSSA	R01	32332229
104	FR	Bovine	AFSSA	R01	32332228
106	FR	Bovine	AFSSA	R01	42332228
107	FR	Bovine	AFSSA	R01	42332228
108	FR	Bovine	AFSSA	R01	42332228
109	FR	Bovine	AFSSA	R01	32332228
110	FR	Bovine	AFSSA	R01	32332228
111	FR	Bovine	AFSSA	R01	32332228
114	FR	Bovine	AFSSA	R01	32332218
115	FR	Bovine	AFSSA	C	32332228
139	FR	Bovine	LVD87	R01	32332128
140	FR	Bovine	LVD87	R01	42332228
141	FR	Bovine	LVD87	R01	42332228
143	FR	Bovine	LVD87	R01	42332228
146	FR	Bovine	LVD87	R01	42332218
148	FR	Bovine	LVD87	R01	32332228
159	FR	Bovine	LVD87	R01	32332328
160	FR	Bovine	LVD87	R01	32332328
161	FR	Bovine	LVD87	R01	32332228
163	FR	Bovine	LVD87	R01	42332228

164	FR	Bovine	LVD87	R01	32332228
170	FR	Bovine	LVD87	R01	42332228
176	FR	Bovine	AFSSA	R01	22522228
190	FR	Caprine	AFSSA	R01	42332228
199	FR	Ovine	AFSSA	R01	32332228
200	FR	Ovine	AFSSA	R24	32332228
201	FR	Humain	IP	R01	21332228
210	NL	Bovine	NIPHE	R35	32332228
218	NL	Bovine	NIPHE	R25	32332228
220	NL	Bovine	NIPHE	R01	32332228
226	ARG	Bovine	NIPHE	R31	32332428
231	CZ	RedDeer	NIPHE	R34	42332228
234	VEN	Bovine	NIPHE	R04	32332218
7912	FR	Bovine	AFSSA	R01	21332228
186A	FR	Caprine	INRA	R01	42332228
187D	FR	Bovine	INRA	R01	42332228

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Table2. Tandem Repeat loci positions and primer sequences.

# TR	Size of PCR (pb)	Primer Forward	Primer Reverse	Tm	Buffer used 1µlDMSO	5µlTponQ
32	298	ccacagggtttttggtgaag	ggaaatccaacagcaaggac	55°C	+	-
8	255	agaagtgttgaggccgtgag	atcacgatgccgttgttctt	58°C	+	-
22	300	cttgagcagctcgtaaagcgt	gctgtatgaggaagtctattcatgg	58°C	+	-
25	350	gtcaagggatcggcgagg	tggacttgagcacggtcat	58°C	+	-
14	301	ccagctaggtgacgggtg	gggcatgcagaagcggat	58°C	+	-
13	200	cgcgctgacgatgttcct	caccacttcaccagccacag	58°C	+	-
36	205	gacggtcgacttcgcttgt	caaggcggccaagaagac	58°C	+	-
52	250	cagaacggcccgaacaac	ggttctggtcgtggtcgtag	58°C	+	-
3	208	catatctggcatggctccag	atcgtgttgaccccaaagaaat	60°C	+	-
9	231	gacgacgatcttcccgttcc	cggtagttgcggtcatcgtg	60°C	-	+
11	214	ggtcccacaacggattacaa	cgaacagctgctgggtgtagt	60°C	+	+
49	391	cggtcaccttcccatccc	gtgatctcggcctgcgtc	60°C	+	-
10	303	gacgagcagctgtccgag	gagagcgtggccatcgag	60°C	-	+
1	211	gtggtggccatggcagat	gccggtgatcagacccag	60°C	+	+
2	203	aggtcagacgtcctaacgagg	gcggagacaatccgtcat	60°C	+	-
7	203	gacaacgaaacctacctcgtc	gtgagctggcggcctaac	60°C	+	-
23	260	ctcatgcactgaggatggc	cagcacccggagagtttaag	60°C	+	-
27	371	agtacgtcaaggacattcgc	accagcacgttctgagtcgt	60°C	+	-
12	359	gaggacgtccagcaggtgat	aattcgaacaggaagcgggt	60°C	+	-
5	208	agggcggtatcacccaaaac	gtagcgacgttggtctggat	62°C	+	-
34	204	agactggaaagaccatcggg	attgtcccagaggaactcaagtc	62°C	+	-
43	267	cgtcaccaaggacgtgaact	aacaagctgctggccgagat	62°C	+	-
45	323	gagtcgaccagcgaatccag	gtgtccatcgtgttcttcgac	62°C	+	-
21	184	attcgccggcgtagtcgtag	ctgttcatcggcttcatgttc	62°C	+	-
6	405	aactgacctcgaaactgcttgt	aaagtagcggaggccaacag	62°C	+	-
4	206	gaacagcagcgtgtaccagg	gtgaaactaacttcacggtcgg	64°C	+	-
47	217	cgttgcgatttctgcgtagc	ggtgatggtcgtggtcatcc	64°C	+	-
39	205	agctgggtgacggtatcagt	gagcgtcgaactgaatcaatg	64°C	-	+
24	381	gtgaacttcctgccgaacaac	actaggttctggcggctttag	64°C	+	+
16	200	ctcctgtatcacaccaaacgc	aaggtcagatcgttgatgtcg	66°C	-	-
17	200	aaggtcagatcgttgatgtcg	ctcctgtatcacaccaaacgc	66°C	-	-
26	274	gacagccccggcaactac	ccgggatgggaaggtgac	66°C	+	-
18	375	gagcaccaccagcaccac	gtgggcgatcacgggtag			
292	265	actggacgagttggatgaggagcgccc	tacgagctgctcaagctgcggg	58°C	+	-
X3	196	aacgagaggaagaactaagccg	cccgctggccttcctgctccgtaa			

PCR analysis
All MAP and *M. avium* ssp. *avium* isolates were screened for the presence or absence of IS*900* and IS*901* insertion sequence. Synthetic oligonucleotides (Sigma) used were IS*900* forward (5' GTT CGG GGC CGT CGC TTA GG) IS*900* reverse (5' CCC ACG TGAC CTC GCC TCC A), IS*901* forward (5' CTG ATT GAG ATC TGA CGC) and IS*901* reverse (5' CAC CAC GTG GTT AGC AAT CC). PCR conditions were as follows: 1 cycle of 15 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 1 cycle of 7 min at 72°C.

IS900 RFLP typing
IS*900* RFLP typing was performed as previously described by van Soolingen et al. (van Soolingen et al., 1994, pp. 196-205), with some modifications. Lysozyme incubation was performed overnight and proteinase K-sodium dodecyl sulfate treatment was done for 20 min at 65°C. Digestion was performed with 3 µg of DNA and 7 U of *Bst*EII (Promega) at 37°C for at least 4 h. Fragments were resolved by agarose gel electrophoresis and transferred onto Immobilon-S nylon membranes (Qbiogen) by vacuum transfer with the Vacu-Gene System (Pharmacia LKB Biotechnology). Detection of DNA fragments hybridizing with the biotinylated probe was performed with the Phototope-Star Detection kit for Nucleic Acids (New England Biolabs), according to the manufacturer's instructions. A photobiotinylated mixture of *Hin*dIII-digested Lambda DNA and *Hae*III-digested X174 DNA at a concentration of 100 ng/ l (Biolabs) was used as molecular size markers.

Preparation of the IS900 probe
The IS*900* DNA probe was prepared by PCR amplification of a 707-bp fragment of the IS*900* insertion sequence specific for *MAP.* Synthetic oligonucleotides (Sigma) used were RFLP-IS*900* forward (5' ACG CCG CGG GTA GTT A) and RFLP-IS*900* reverse (5' GGG GCG TTT GAG GTT TC). PCRs were performed with 10 ng of chromosomal DNA of strain ATCC 19698 by using a Bio-Rad thermal cycler model iCycler. PCR conditions were as follows: 1 cycle of 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and 1 cycle of 4 min at 72°C. PCR products were purified using Qiaquick spin columns (QIAGEN) according to the manufacturer's instructions. The probe was biotin-labeled with the NEBlot Phototope kit (New England Biolabs), following the instructions of the manufacturer.

Analysis of the RFLP patterns
Analysis of the RFLP patterns was performed according to Overduin (Overduin et al., 2004, pp. 5022-5028). Conserved bands of 8.8, 5.2, 3.0, 2.4, 2.1, and 1.6 kb in the IS*900* RFLP patterns were used as the internal standard for the normalization of RFLP patterns.

Identification of TR loci
The published genomic sequence of MAP strain K10 (GenBank accession number NC_002944 [http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=380] was used to identify TR sequences to be tested in VNTR typing. Tandem repeats were identified by using the Tandem Repeats Finder software of the Department of Biomathematical Science of Mount Sinai of Medicine (http://c3.biomath.mssm.edu/trf.html) under the default settings of the program.

Identification of MIRU loci
The MIRU loci were identified on the MAPK10 chromosome by searching sequences homologuous to those of known MIRU loci described in *M. tuberculosis* H37Rv chromosome (Supply et al., 2000, pp. 762-771) using the BLAST 2.2.11 software at the NCBI website [http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi].

VNTR and MIRU typing
Primers designed to target flanking regions of the VNTR and MIRUs and details of the PCR amplification are listed in Table 2. PCRs were performed with 10 ng of purified chromosomal DNA by using a Bio-Rad thermal cycler model iCycler. To detect differences in repeat numbers, the PCR products were analysed on a 1,5 % agarose gels in 0.5 x Tris-borate-EDTA containing 0.5 µg/ml of ethidium bromide (Euromedex).

Calculation of the discriminatory power
The Discriminatory Index (DI) described by Hunter and Gaston (Hunter and Gaston, 1988, pp. 2465-2466) was used as a numerical index for the discriminatory power of each typing method IS*900*-RFLP and VNTR. The DI was calculated using the following formula:

$$DI = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1) \right]$$

where N is the total number of strains in the typing scheme, s is the total number of distinct patterns discriminated by each typing method and strategy, and n_j is the number of strains belonging to the jth pattern.

Table 3a. VNTR patterns

Strains(n)	Number of isolates	Number of TRs at locus 292-X3-25-47-3-7-10-32	Patterns VNTR	DI
	25	42332228	A	
	21	32332228	B	
	5	32332218	C	
	4	32332229	D	
	4	42332218	E	
	3	32332128	F	
Map (72)	3	32332118	G	0.789
	2	32332328	H	
	2	21332228	I	
	1	3233222(10)	J	
	1	32332428	K	
	1	22522228	L	

Table 3b. IS900-RFLP patterns

Strains (n)	Number of isolates	Patterns VNTR	DI
	61	R01	
	3	C18	
	1	C2	
	1	C	
Map (72)	1	R04	0.283
	1	R24	
	1	R25	
	1	R31	
	1	R34	
	1	R35	

Table 3c. Combination of methods patterns. DI: Discriminatory index

Strains (n)	Number of isolates	Combination methods patterns	DI
	24	1	
	17	2	
	4	3	
	3	4	
Map (72)	3	5	0.833
	2	6	
	2	7	
	1 x 17	8-24	

RESULTS

RFLP typing
For a valid study of MAP TR variability, a representative strain panel from diverse geographic regions and host origins was built based on a preliminary IS*900* RFLP typing analysis. All of the isolates analysed in this

study were found to be positive for IS*900* and negative for IS*901* by PCR, assigning them as MAP strains. Over 72 isolates were subjected to IS*900* RFLP typing. Eleven different RFLP types were found (Table 1 and Table 3b). The vast majority (61 isolates, 82%) was of type R01, the type C18 was found in 3 isolates (4%), whereas R04, R24, R25, R31, R34, R35, C2, C types were only found in single isolates. Thus, 64 isolates belonged to 2 clusters whereas 8 patterns were unique.

In silico identification and characterization of tandem repeats (TR) and MIRU loci
The genome sequence of MAP strain K10 was analysed for the presence of tandem repeats. Over 363 tandem repeat sequences were identified in this genome. We focused on TRs of the mini-satellite category, defined by a repeat unit size in the range of 10 to 100 bp, as their corresponding allelic differences can be easily resolved by agarose gel electrophoresis. Thirty-three TRs present in more than two copies and with 85% or more nucleotide identity among individual repeat units were selected for experimental analysis. These two criteria were used on the basis of the observation that the presence of at least two identical or nearly identical repeats is necessary and sufficient to generate TR variability in the case of *M. tuberculosis* mini-satellites (Supply et al., 2000, pp. 762-771).

In addition, two MIRU-VNTR loci were identified in MAP strain K10 by BLAST searches using as templates the sequences of the flanking genes of two highly polymorphic MIRU-VNTR loci in *M. tuberculosis*. These two MIRUs loci were called MAP SenX3-RegX3 and MAP 2920c-2921c. The repeat units of these MIRUs in MAP K10 genome have a length of 53 pb and are present with a copy number of 2 and 3 in MAP SenX3-RegX3 and MAP 2920c-2921c respectively. These MIRU loci were added to the above selection of TR loci for further experimental analysis.

Polymorphism in repeat numbers among MAP isolates
The polymorphism of the selected TR and MIRU loci was initially investigated using a sub-collection with diverse RFLP-IS*900* types, geographic and host origin (data not shown). Only the eight TR and MIRU loci that showed a polymorphism in this sub-collection were used for typing the 72 strains previously analysed by RFLP-IS*900.* Twelve different MIRU-VNTR types were found (Table 1 and 3a). Patterns A and B represented the majority of the isolates (34% and 30%, respectively) followed by C (6,8%), D, E (5,4%), F,G (4,1%) , H, I (2,7%) and J, K, and L (1,36%). In total, MIRU-VNTR grouped 69 isolates into 9 clusters, whereas 3 MIRU-VNTR patterns were unique (Table 4).

Comparison of IS900 RFLP typing, MIRU-VNTR typing and combination of the two methods
Interestingly, the major RFLP type R01 containing 61 isolates could be subdivided into 11 different MIRU-VNTR types. Similarly, the two isolates of RFLP type C18 have two different VNTR types. Conversely, the 25 isolates of VNTR type A could be divided into two RFLP types (24 R01 and 1 R34), the 21 isolates with VNTR type B could be divided into 5 RFLP type (17 R01, 1 C, 1 R24, 1 R25 and 1 R35), the 5 isolates with VNTR type C could be divided into 3 RFLP type (1C18, 3 R01 and 1 R04), the 4 isolates with VNTR type E could be divided into 2 RFLP type (3 R01 and 1 C2) and the 3 isolates with VNTR type G could be divided in 2 RFLP type (1 R01 and 2 C18).
In total, the association of the two methods distinguished 24 distinct patterns, including 7 cluster patterns comprising 55 isolates and 17 unique patterns (Table 4).

Table 4. Discriminatory power of IS900-RFLP, VNTR MIRU Typing used alone and in combination

Typing method	No. of different patterns	No. of clusters	No. of clustered isolates	No of unique size isolates	Size of cluster	Discriminatory Index
RFLP	11	2	64	8	3-61	0-283
VNTR MIRU	12	9	69	3	2-25	0-789
RFLP + VNTR	24	7	55	17	2-24	0-833

Discriminatory index
Discriminatory indices of IS*900*-RFLP and VNTR-MIRU typing alone or in combination were calculated based on the types found for the 72 isolates, according to the method of Hunter and Gaston (Hunter and Gaston, 1988, pp. 2465-2466). The discriminatory indices were 0.283 for IS*900*-RFLP alone, 0.789 for VNTR typing alone, and 0.833 for the combination of the two methods.

DISCUSSION

VNTRs of the mini-satellite class are valuable markers used for genotyping several mycobacterial species (Supply et al., 1997, pp. 991-1003, Supply et al., 2000, pp. 762-771, Roring et al., 2002, pp. 2126-2133, Bull et al., 2003, pp. 157-164, Overduin et al., 2004, pp. 5022-5028, Ablordey et al., 2005, pp. 1546-1551, Stragier et al., 2005, pp. 1639-1647). Two previous studies have identified a few VNTR loci in MAP isolates, based on partial screenings of MAP K10 genome. In this study, we have performed an exhaustive screening of potential VNTR loci in this genome, using a reference set of MAP isolates with diverse IS*900* RFLP, geographic and host origins. By this means, we have identified 8 VNTR loci, of which seven are novel; one (*senX3-regX3*) has been previously identified by Bull et al., 2003, pp. 157-164.

When used alone, this eight-locus typing system was more discriminatory when compared to IS*900*-RFLP typing. Nevertheless, the resolution could be enhanced by combining the two typing methods. Interestingly, the R01 RFLP type representing the majority of the MAP isolates found in this study and in the other studies could be successfully divided into 11 subgroups by MIRU-VNTR typing. On the other hand, 5 VNTR types could also be divided into subgroups, but each of these VNTR types was subdivided into only two RFLP subgroups. As suggested by the results of others (Bull et al., 2003, pp. 157-164, Overduin et al., 2004, pp. 5022-5028), the VNTR loci could be subjected to further DNA sequence analysis to detect possible sequence polymorphisms among repeat units in addition to the variation in the number of repeats. This potential polymorphism and the addition of the few non-redundant VNTR loci described by these authors may further improve the discriminatory index of this typing method.

In conclusion, we have described here the identification of novel MIRU-VNTR markers for more specific differentiation of MAP isolates. Our preliminary analyses suggest that this approach is more discriminatory than IS*900* RFLP method, and could therefore be a useful additional tool for typing MAP. This result should be verified with a panel of strains that contain additional IS*900*-RFLP pattern types, if possible. Moreover, as the collection of MAP strains used in this study was mainly composed by bovine isolates, it would be interesting to analyse more isolates from other hosts. Recently, a multilocus short sequence repeat sequencing approach was described for discriminatory genotyping of MAP strains (Amonsin et al., 2004, pp. 1694-1702, Ghadiali et al., 2004, pp. 5345-5348). A combination of this method with MIRU-VNTR - based typing might prove to be optimal for PCR-based molecular epidemiological studies of this pathogen.

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Isolation and purification of intact *Mycobacterium avium* subspecies *paratuberculosis* from intestinal sections of cattle and goats

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease in ruminants has been purified from infected cattle and goat tissue for the specific purpose of proteomic analysis. After removal of luminal contents, the submucosa and mucosa of ileal and jejunal sections were harvested and MAP extracted from macrophages by hypotonic lysis, sonication and differential centrifugation. This method is also suitable for DNA extraction from mycobacteria.

Key words: Extraction from tissues, Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis* (MAP)

INTRODUCTION

A characteristic feature of mycobacteria is the thick, waxy cell wall comprised of composite layers of mycolic acid and arabinogalactans covalently linked to peptidoglycan. With this highly impermeable outer surface, mycobacteria can survive in extreme environmental conditions, even in the presence of antibiotics and disinfectants (Brennan 1995, Jarlier 1994, Manning 2001, Rastogi 1991, Sung 1998). This outer surface also makes mycobacteria refractory to all but the harshest of cell lysis methods (Cheung, 1994).

Comparative proteomic analysis of *in vivo* and *in vitro* derived mycobacteria is a suitable method for identification of bacterial virulence factors that can be used as diagnostic or vaccine candidates. In order to compare *in vivo* derived bacteria with those grown in the laboratory, target bacteria must be extracted and separated from eukaryotic cellular material and co-existent microflora. The problem with isolating intact bacteria for protein analysis is that many of the harsh chemical methods used to destroy eukaryotic cells and natural flora from the tissue also destroy the target bacteria. MAP can survive extended periods in harsh conditions such as suspension in hyper- or hypotonic solutions and short bursts of sonication which can be used to destroy eukaryotic cells and other bacteria. Any method developed to purify MAP from tissue must also take into account the use of detergents which can remove membrane bound protein from the lipid cell wall and membrane, hence altering the proteome composition.

Several methods have been developed for the purification of mycobacteria including *M. leprae* from armadillo tissues (Rodde 1992) and MAP from sheep mucosa, for analysis of bacterial strain variations at the DNA level (Choy 1998), however no method has been established to date for the comparison of bacterial proteomes of mycobacteria purified from infected tissue (*in vivo* derived) and laboratory grown mycobacteria (*in vitro* derived). We report on the method for the isolation of MAP from gut tissue of animals with Johne's disease in a manner compatible with subsequent proteomic analysis.

MATERIALS AND METHODS

Bacterial strains
MAP strain CLIJ623, an Australian wild-type bovine isolate, was incubated for 28 weeks and grown as pellicles on modified Watson-Reid medium at 37°C (Vaughan 2005). Bacteria were harvested by centrifugation at 3,500 x g for 30 min and stored at −20°C until required.

Intestinal tissue collection from JD positive animals
One Jersey and one Friesian-cross female dairy cow between 6-10 years of age were acquired from regional Victoria and found to be naturally infected with a bovine isolate of MAP. Two male angora goats were experimentally infected with MAP strain CLIJ623. Animals used in this study were monitored for infection with MAP by faecal culture, weight measurement and commercially available tests for JD including Parachek™ and Bovigam™ (Billman-Jacobe 1992, Collins 1991, Cox 1991). Cattle were autopsied once clinical signs of JD were apparent after which sections from the ileum and jejunum were excised, immediately flushed with PBS at 37°C and snap frozen in liquid nitrogen prior to mycobacterial extraction.

Extraction of acid-fast bacteria from gut samples
Gut samples were thawed on ice in a refrigerator and all subsequent manipulations were performed at 4°C unless otherwise indicated. The superficial mucosa was removed and tissue scrapings were suspended in 5 volumes of 10 mM Tris-HCl pH 7.4 in sterile, screw-capped tubes and incubated on ice for 2 hours. Samples were then sonicated on ice with 10, 3 second pulses using an XL Ultrasonic Processor (Heat Systems NY, USA) with a 4 mm tapered microtip at 70% power, to lyse non-mycobacterial cells. Connective tissue and cellular debris were removed by centrifugation at 200 x g for 20 minutes. Supernatant from the previous spin was collected and further centrifuged at 2,000 x g and again at 4,000 x g for 20 minutes to isolate mycobacteria. Pellets isolated from the above centrifugation steps were combined, overlaid on a solution consisting of 30% (v/v) Percoll (Amersham Biosciences, Uppsala, Sweden) and 0.25 M sucrose, and centrifuged at 10,000 x g for 20 minutes; the resulting pellet was then washed twice in PBS. The presence of acid-fast bacilli (AFB) in ileal sections and jejunal sections were determined by Ziehl-Neelsen staining of gut samples prior to sonication and of the pellets resulting from each centrifugation step (Cruikshank 1973).

Proteome separation
Acid-fast bacilli (harvested from bovine and caprine gut tissue) were lysed and prepared for 2DE as described by Lanigan et al. *2004. Similarly, bovine and caprine gut samples from JD-negative animals were sonicated, centrifuged at 4,000 x* g *and samples prepared for 2DE. Proteins were detected by staining with silver nitrate (Heuseshoven 1988). Analysis of protein gels was performed on scanned gel images acquired using 600dpi and an ImageMaster desk top scanner (Amersham Biosciences) and Labscan software, V.3.00 (Amersham Biosciences)*

Proteome analysis
2DE gel images were analysed using ImageMaster 2D Elite (V 4.01c). Initially data-sets were generated using the in-built automated gel-spot detection, with manual editing to remove obvious artifacts and to include any spots not detected automatically. Spots in replicate gels were matched to each other and were used to generate an averaged reference map for each replicate group. Reference maps were then used to determine differences between *in vitro* MAP samples with and without the use of the purification method.

LCMS analysis of selected proteins
Proteins were digested in a semi-automated fashion using the Montage™ In-gel Digest$_{96}$ Kit (Millipore, Bedford, MA, USA). Extracted tryptic peptides were transferred to microfuge tubes and concentrated in a SpeedVac concentrator to approximately 45 µl for analysis by liquid chromatography-mass spectrometry. Reverse phase chromatography was performed on a Vydac C18 column (0.3 x 150 mm, 3 µm, 300 , Hesperia, CA) using a Surveyor HPLC system (Thermo, San Jose, CA). Mobile phase buffers were 0.2% (v/v) formic acid in water (A) and 0.2% (v/v) formic acid in acetonitrile (B), run at a flow rate of 1.3 µL/min with a linear gradient of 5-95% B over 20 min, held at 100% B for 10 min and finally 95-5% B over 2 min. The effluent from the column was connected directly on-line to the nanospray ion source of a LCQ Classic quadrupole ion-trap mass spectrometer (Thermo, San Jose, CA) with nanospray source settings as follows; spray voltage 2.25 kV, capillary temperature 150^{0}C, capillary voltage 30 V and tube lens offset 0 V. Mass spectra were acquired in the data-dependent "TOP3" mode where by the three most intense ions in each

full scan (m/z 400-2000) were automatically selected and sequentially subjected to tandem mass spectrometry (MS/MS) product-ion scans. MS/MS scans were performed with a normalised collision energy of 35%.
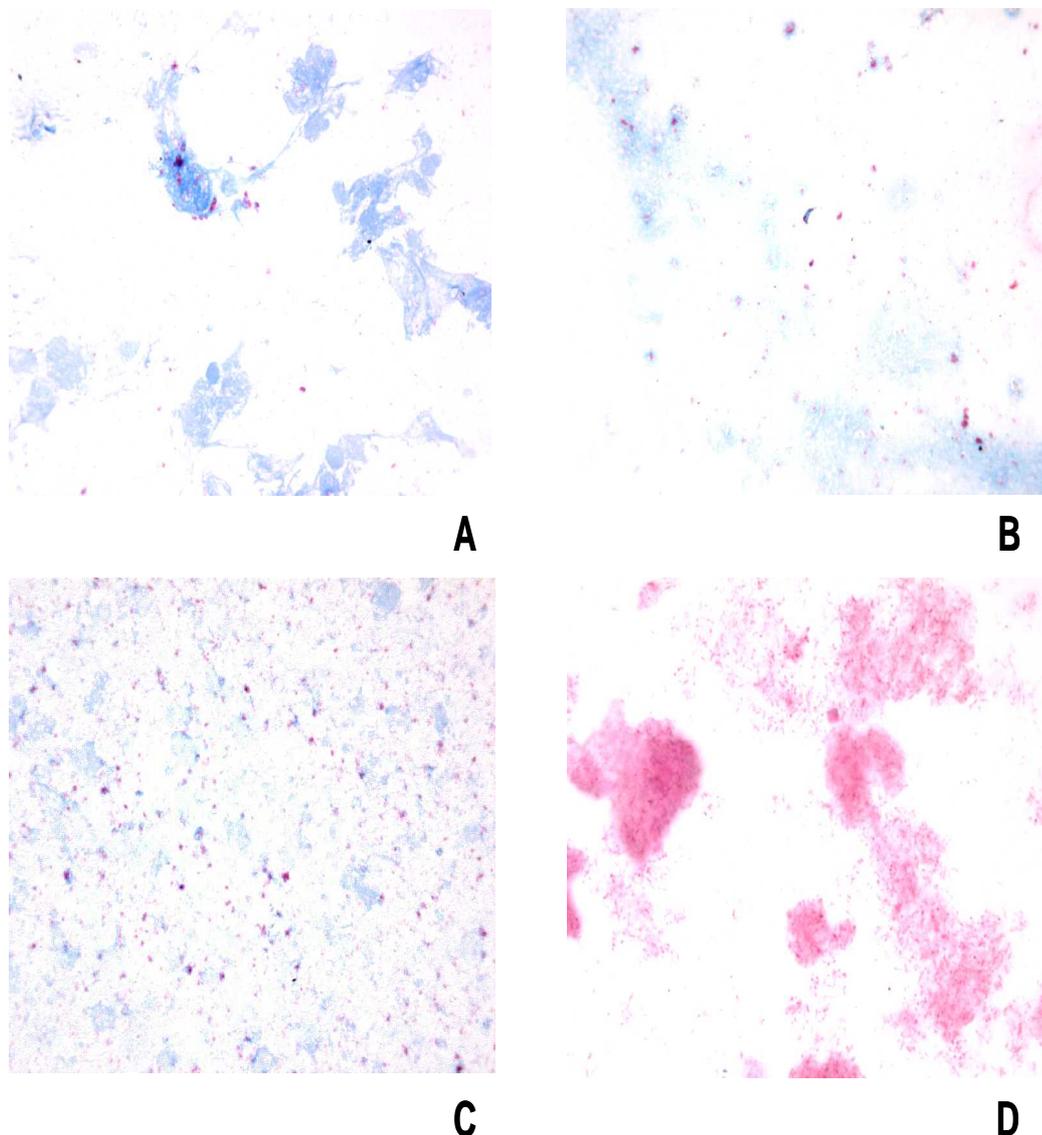
Fig. 1: Separation of *in vivo* MAP from contaminating bovine material. AFB from macerated cattle intestine (A) was sonicated to lyse eukaryotic cells and resident microflora (B). Differential centrifugation was used to concentrated the AFB (C) and the AFB was separated from non-acid fast material through a solution of 30% Percol and 0.25 M sucrose (D). Pellets were stained for AFB using the ZN stain, which indicated the majority of contaminating bovine tissue had been removed using this method.

Identification of AFB using 16s and IS900PCR analysis
DNA was extracted from acid-fast bacilli for PCR analysis according to the method by Tizard *et al.* 1998. Primers were designed to be complementary to conserved regions of 16S rRNA and the insertion sequence IS*900* as shown in Table 1. The 16S RNA primers were specifically designed to amplify a segment of non-conserved nucleic acid to differentiate between MAP and other closely related mycobacteria or microflora ordinarily found in the ruminant intestine. Reactions were performed in Easystart PCR reaction tubes (Molecular Bioproducts, San Diego, CA, USA) in a volume of 50 µl. Primers were used at a final concentration of 50 nM per reaction, with the addition of 10% DMSO (v/v) and 1 µl of Red Taq (Sigma). Template DNA was at a concentration of ~20 ng in test samples. The PCR reaction was performed using a DNA thermocycler 480 (Applied Biosciences) using an initial cycle of 94°C for 5 minutes followed by 35 cycles at 94°C for 1 minutes, 60°C for 1 minute, 72°C for 1 minute and finished with a cycle of 72°C for 10

minutes. Amplified products were sequenced on an ABI 377 automated DNA sequencer at Newcastle DNA, Biomolecular Research Facility, University of Newcastle, Australia.

Table 1: Primers used in this study for PCR analysis

| Primer	Direction	Sequence
MAP IS*900*	Fwd	ACGGTTACGGAGGTGGTTGTGG
	Rev	CTCCATCGGCCAACGTCGTCAC
MAP Conserved 16s	Fwd	CCTACGGGAGGCAGCAGT
	Rev	CGGGCCCCCGTCAAT

RESULTS

Extraction of AFB and purification process
Sections of the ileum and jejunum from JD infected cattle and goats were obtained to extract AFB, with higher yields of AFB obtained from ileal sections. Short bursts of sonication of scraped ileal sections in a hypotonic buffer resulted in the lysis of eukaryotic cells; however the AFB appeared to remain intact as determined visually by ZN stain. Differential centrifugation of removed the bulk of eukaryotic tissue however non-acid-fast material was still present.

Identification of MAP
DNA extracted from AFB-containing pellets was subjected to PCR sequencing using primers directed towards IS*900* and 16S to determine the presence of MAP.
IS*900* primers were designed to amplify the unique insertion sequence IS*900*, found only within the MAP genome (Green 1989). Direct sequencing of the amplified product indicated that all *in vivo* extracted samples were positive for MAP IS*900*. The 16S sequenced product showed 100% identity with MAP and no identity was found with Gram-positive or -negative bacteria commonly found in the gastrointestinal tract of ruminants.
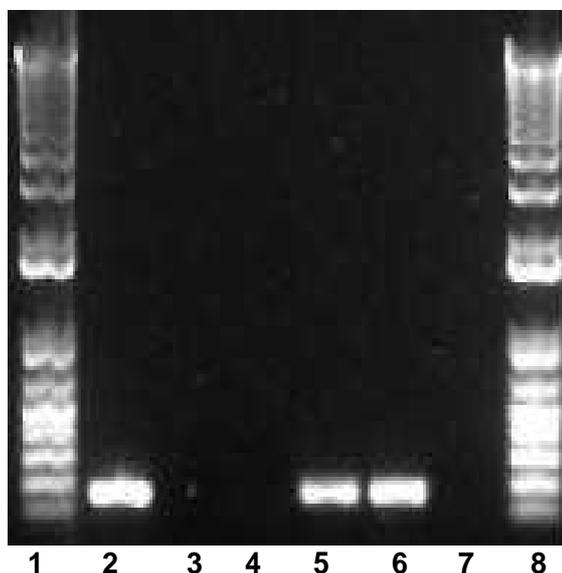
Fig. 2 IS*900* PCR results for extracted mycobacteria from JD infected cattle
DNA was extracted from ZN stained pellets, which indicated the presence of AFB. PCR was performed on the extracted DNA using primers specific for IS*900* an
insertions sequence found specifically within the MAP genome. PCR products were then separated by electrophoresis using 1% agarose in TAE; lanes 1 and 8: 1Kb+ molecular weight markers, lane 2: in vitro MAP, lane 3: *M. avium*, lane 4: bovine ileum, lane 5: in vivo MAP sample 1, lane 6: in vivo MAP sample 2, lane 7: no template DNA control.

The effect of Percoll on proteome composition

To determine the effect of Percoll on the proteome of MAP, a laboratory strain of the organism was analysed by 2DE before and after centrifugation through a Percoll gradient. Spot matching of replicate 2D gels revealed essentially identical proteomic profiles before and after Percoll centrifugation (data not shown). Random proteins were selected for MS analysis to determine if the purification method affected peptide identification by mass spectrometry. Sequence information was obtained for 9 of the 10 samples analysed.

Table 2: Comparison of mass spectrometry data from *in vitro* MAP before and after Percoll purification

% Seq. Cov		Spot Identity		% Seq. Cov
50	Hypothetical protein MAP1339	MAP1	Hypothetical protein MAP1339	15
19	Alkyl hydroperoxide reductase D (AhpD)	MAP2	Alkyl hydroperoxide reductase D (AhpD)	17
30	Alkyl hydroperoxide reductase D (AhpD)	MAP3	Alkyl hydroperoxide reductase D (AhpD)	32
7	No data	MAP4	No data	
16	Hypothetical protein MAP3567	MAP5	Hypothetical protein MAP3567	58
			Hypothetical protein MAP0792c	18
23	Heat shock protein DnaK	MAP6	Heat shock protein DnaK	25
34	Heat shock protein GroEl2	MAP7	Heat shock protein GroEl2	46
39	10 kDa chaperonin GroES	MAP8	10 kDa chaperonin GroES	39
25	Malate dehydrogenase (Mdh)	MAP8	Malate dehydrogenase (Mdh)	35
			Major membrane protein 1	26
7	Hypothetical protein MAP3035	MAP10	Hypothetical protein MAP3035	7
2	ArgJ		ArgJ	10

DISCUSSION

This report describes a simple purification method for the isolation and purification of MAP from the intestine of cattle and goats for proteomic analysis.

Intestinal tissue was taken from animals with clinical signs of Johne's disease to ensure sufficient quantities of mycobacteria were available to harvest. The amount of MAP isolated from goats was significantly less than from cattle due to the amount of tissue available for extraction. Ileal sections yielded more than 5 times the amount of bacteria than jejunal sections. This is not surprising as the major sites of infection are lymphoid tissue or Peyer's patches of the ileum (Momotani 1988, Sigurdardottir 2001).

In this study MAP was resistant to the hypotonic environment and to short bursts of sonication, resulting in intact mycobacteria as determined by ZN staining. The use of sonication in the presence of Triton X-100 has been shown to be an effective way of lysing non-mycobacterial organisms, leaving co-existent mycobacteria intact (Granger 2004). The problem with using detergents in extraction solutions is that it increases the risk of removing membrane bound or associated proteins resulting in an altered proteome of the *in vivo* bacterial sample. Our analysis indicates that the use of Percoll in our extraction procedure is sufficient to extract MAP from host tissue without adversely affecting the target organism's proteome. This provides us with a unique opportunity to study and compare *in vivo* and *in vitro* derived MAP to increase our understanding of pathogenicity, identify virulence factors and apply differentially regulated proteins to improve current diagnostic tests and vaccines with this work currently under investigation.

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Genomic and proteomic comparative study of the sheep and cattle strains of *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

In Australia and other countries the distinction between ovine Johne's disease (OJD) and bovine Johne's disease (BJD) at the microbiological level plays an important role in the development and implementation of control and evaluation programmes for both forms of this insidious disease. However, the differences between the sheep and cattle strains of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) at the DNA level that result in the different host specificities are still extremely poorly understood. To date the DNA techniques that exist to differentiate these strains provide excellent tools for epidemiological investigations and diagnosis of Johne's disease but have yielded little if any insight into the relationship between genotype and phenotype. In this study an intensive comparison was made of the sheep and cattle strains of MAP using a variety of modern DNA and protein based techniques including: representational difference analysis (RDA), polymerase chain reaction (PCR) and sequencing, microarray, two dimensional electrophoresis proteomics and surface enhanced laser desorption-ionisation (SELDI) proteomics. The majority of these techniques were used in conjunction with the recently completed MAP K10 genome sequence in order to identify those genes or proteins with unique characteristics in either strain. Using these techniques, a number of differences between the sheep and cattle strains of MAP were identified. The results from this study are discussed.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, strain differentiation, representational difference analysis, SNP, microarray, proteomics, SELDI , sheep

INTRODUCTION

After more than 100 years of research on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and Johne's disease, much still remains a mystery about this elusive organism and the disease it causes. However, what is clear is that Johne's disease presents itself differently in cattle and sheep with respect to clinical, pathological and epidemiological features (Whittington and Sergeant, 2001). Early epidemiological studies identified that cases of Johne's disease in sheep and cattle were caused by different strains of MAP (Taylor, 1945; 1951). The strains isolated from sheep were difficult to grow on primary culture (Taylor, 1945; Hole, 1958) and were readily identifiable by their orange pigmentation (Taylor, 1951). Problems associated with primary culture of sheep strains persisted (9) but have been overcome more recently (33). DNA based studies of MAP isolates from a range of hosts, using restriction fragment length polymorphism of genomic DNA, confirmed the existence of two distinct groups of MAP (9). These are now commonly refereed to as either sheep strains (S) or cattle strains (C). However, the differences observed between the S and C strains to date do not provide genotypic explanation of their altered phenotypes and divergent host specificity (Collins et al., 1990; 1997; 2002; Dohman et al., 2003. Whittington et al., 1998).

To protect disease-free flocks and herds within infected districts and the non-infected regions, control programs have been developed in Australia including the National Ovine Johne's disease Control and Evaluation Program (NOJDP). The success of control programs is dependent on the ability to make sound decisions regarding on-farm management practices and the movement of animals between districts. At

present these decisions are rely on an incomplete understanding of MAP and the disease. For example, policies regarding mixed farming of cattle and sheep have been based on current knowledge of the apparent host specificity of MAP. Similarly, de-stocking and fallow pasture polices that aim to eliminate MAP from the premises assume equivalence of strains. Consequently such polices may be flawed and the expected outcomes not achievable.

The aim of this study was to compare the S and C strains of MAP using genomic and proteomic techniques to identify differences in the host specificity and pathogenicity of this organism and provide scientific data to support the current management strategies of this disease.

MATERIALS AND METHODS

MAP isolates
MAP isolates used in this study are described in Table 1. Genomic and proteomic techniques were used to compare one S strain (Telford 9.2) and one C strain (CM00/416). A second C strain (316v) was used as a positive control when required. The S strain (Telford 9.2) and C strain (CM00/416) were grown on modified 7H10 plus mycobactin J slopes at 37°C for up to 3-4 months (Whittington et al., 1999) while C strain 316v was grown in modified Watson-Reid medium with mycobactin J (Morrison, 1965) at 37°C for 1-2 months. The cultures were harvested, washed 3 times with sterile PBS and stored at -70°C until required. DNA from the MAP K10 isolate was obtained from the National Animal Disease Centre (NADC), Ames Iowa. The remaining 32 DNA samples were from MAP isolates used in a previous epidemiological study of Johne's disease in Australia and included 16 S isolates and 16 C isolates (Whittington et al., 2000).

Table 1: MAP isolates or DNA used in this study

MAP isolate	Species of origin	Number	Strain	Isolate or DNA	IS*1311* PCR /REA	IS*900* RFLP	Ref
Telford 9.2	ovine	1	S	Isolate	S	S1	This study
CM00/416	bovine	1	C	Isolate	C	C3	This study
316v	laboratory	1	C	Isolate	C	C1	Whittington et al. 1998
K10	bovine	1	C	DNA	n/a	n/a	NADC
Field isolates	ovine	11	S	DNA	S	S1	Whittington et al., 2000
	ovine	1	S	DNA	S	SU1	Whittington et al., 2000
	ovine	1	S	DNA	S	SU1	Whittington et al., 2000
	ovine	1	S	DNA	S	SU3	Whittington et al., 2000
	caprine	2	S	DNA	S	S1	Whittington et al., 2000
	bovine	3	C	DNA	C	C1	Whittington et al., 2000
	bovine	4	C	DNA	C	C3	Whittington et al., 2000
	bovine	3	C	DNA	C	C5	Whittington et al., 2000
	bovine	2	C	DNA	C	C12	Whittington et al., 2000
	bovine	2	C	DNA	C	CU1	Whittington et al., 2000
	bovine	1	C	DNA	C	CU2	Whittington et al., 2000
	bovine	1	C	DNA	C	CU3	Whittington et al., 2000

n/a - not applicable, NADC - National Animal Disease Centre, USDA, Ames, Iowa

DNA extraction and strain confirmation of MAP strain types
DNA extraction was performed as described (Marsh and Whittington, 2005). The concentration of the DNA was calculated by spectrophotometry using the formula (μg/mL=A$_{260}$x D.F.x 50) where D.F. is the dilution factor. DNA samples were stored at 4°C. Strain identity of the Telford 9.2 and CM00/416 isolates was confirmed by IS*1311* PCR/REA (Marsh et al., 1999) and IS*900* RFLP (Choy et al., 1998).

Representational difference analysis (RDA)
An RDA protocol was developed to compare the S (Telford 9.2) and C (CM00/416) strains of MAP (Marsh and Whittington, 2005). Four rounds of RDA were undertaken using the S strain as the tester and the C strain as the driver at tester-to-driver ratios of 1:40, 1:400, 1:4000 and 1:40,000. RDA products were cloned, sequenced and used to query the MAP K10 database (formerly located at http://www.tigr.com) and *M. a. avium* 104 database (http://www.tigr.com) using the BLASTn algorithm (Altschul et al., 1997) (National Centre for Biotechnology Information, NCBI) for identification. An extensive PCR and sequencing based

strategy was used to confirm or refute the RDA sequences as divergent loci in the S and C strains (Marsh and Whittington, 2005). Confirmation of divergent regions was achieved by *Bst*E II RFLP analysis with a dig-labelled probe produced from the C strain and corresponding to the deleted region in the S strain (Marsh and Whittington, 2005). Just prior to the completion of this study the entire MAP K10 genome was made publicly available (GenBank accession, AE016958) and BLASTn and BLASTx searches of the K10 genome and GenBank were performed to identify the genes included in the S strain deletion.

Identification of single nucleotide polymorphisms (SNPs) by polymerase chain reaction (PCR) and sequencing of selected genes
Thirty loci across 29 genes (Table 2) were used in a PCR and sequencing based comparison of the S (Telford 9.2) and C (CM00/416) strains of MAP based on an experimental design used for the *M. tuberculosis* complex (Sreevatsan et al., 1997). Genes selected were either the full or partial gene sequences available for MAP in GenBank at the time this study commenced or were derived from other mycobacterial species for which homologous loci were identified in the incomplete MAP K10 genome. The *des*A1 gene in the C strain has been reported to be disrupted by a copy of IS*900* (Bull et al., 2000) and therefore was examined by PCR on its own (*des*A1) and in conjunction with IS*900* (*des*A1+IS*900*) to determine if *des*A1 in the S strain has also been disrupted by IS*900*.

Table 2: Genes analysed by PCR and sequencing for SNPs

Loci	Gene	Function	Species	GenBank Accession/Reference
1	*rpoB*	RNA polymerase beta-subunit ( )	*M. a. p.*	AF057479
2	*hsp65*	heat shock protein	*M. a. p.*	U15989
3	*hsp70*	heat shock protein	*M. a. p.*	AF254578
4	*groES*	10-kDa chaperonin	*M. a. a.*	AF071829
5	*sodA*	superoxide dismutase	*M. a. p.*	AF180816
6	*katG*	catalase peroxidase	*M. tb*	X68081
7	*85A*	fibronectin- binding antigen	*M. a. p.*	AF280067
8	*85B*	fibronectin- binding antigen	*M. a. p.*	AF219121
9	*85C*	fibronectin- binding antigen	*M. a. p.*	AF280068
10	*mce1a*	mammalian cell entry	*M.sp*	Haile et al., 2002
11	*mce2a*	mammalian cell entry	*M.sp*	Haile et al., 2002
12	*mce3a*	mammalian cell entry	*M.sp*	Haile et al., 2002
13	*mce4a*	mammalian cell entry	*M.sp*	Haile et al., 2002
14	*dnaA*	chromosomal replication initiator protein	*M. a. p.*	AF222789
15	*dnaN*	DNA polymerase III subunit	*M. a. p.*	AF222789
16	*recF*	recombination gene	*M. a. p.*	AF222789
17	*gyrB*	DNA gyrase	*M. a. p.*	AF222789
18	*ald*	L-alanine dehydrogenase	*M. a. p.*	AF322643
19	*fapP*	fibronectin-attachment protein	*M. a. p.*	AY007557
20	*inhA*	enoyl reductase	*M. a. p.*	AJ439339
21	*invA*	putative invasion protein	*M. a. p.*	AJ439337
22	*invB*	putative invasion protein	*M. a. p.*	AJ439338
23	*pks7*	polyketide synthase	*M. a. p.*	AF232752
24	*pks8*	polyketide synthase	*M. a. p.*	AF232752
25	*purH*	formyltransferase	*M. a. p.*	AF232750
26	*ahpC*	alkylhdroperxidase C	*M. a. p.*	AF334163
27	*desA1*	steroyl-ACP-desaturse	*M. a. p.*	AF305073
28	*desA1* + S*900*	steroyl-ACP-desaturse	*M. a. p.*	AF305073
29	*F57*	NO similar gene, No function	*M. a. p.*	X70277
30	*Dps*	DNA binding dormancy protein	*M. smegmatis*	AY065628

n/a – not applicable, *M. a. p.* – MAP, *M. a. a.* – *M. a. avium*   *M. tb* – *M. tuberculosis*
M. sp – *M. avium* subsp*. avium* and *M. bovis* and *M. smegmatis*

Microarray
Telford 9.2, CM00/416 and 316v were compared with the K10 isolate in Cy3 and Cy5 dye swap hybridisations, using a whole-genome array representing >95% of the MAP coding sequences (Paustian et al., 2005). Arrays were scanned using an ArrayWorx optical scanner (Applied Precision). Scanned images were adjusted with local background subtraction and LOWESS normalization (Cleveland and Devlin, 1988). The resulting data were analysed using SoftWorx Tracker image analysis software. Any spot where the

non-K10 sample was greater than 2 fold lower than the K10 sample were selected as genes absent on the non-K10 genome. Finally, open reading frames (ORFs) that were not identified by both hybridisations and that were not represented by at least 2 spot replicates were censored and not included in further analysis.

Each of the ORFs identified by microarray analysis as deleted from the S strain was evaluated by PCR to confirm its presence or absence in the S and C strains.   PCR was also performed on a number of other ORFs not identified by the microarray but residing within or immediately flanking the regions that were identified. The latter was undertaken to determine the limits of each of the S strain deletions. The amplified products from the S strain were sequenced and used in BLASTn searches of the MAP K10 genome to identify the terminal ends of each deletion. Thirty-two MAP field isolates (16 S strain and 16 C strain field isolates, Table 1) were evaluated using the S strain deletion PCR assays. Finally, the 3 DNA sequences derived from the MAP K10 genome sequence corresponding to the regions deleted in the S strains were used in BLASTn searches to identify the presence or absence of these sequences in the *M. a. avium* 104 genome (http://www.tigr.com).

Two dimensional electrophoresis proteomics
For 1D and 2D comparisons proteins were extracted using a method based on White et al. (1994).   Briefly, proteins were extracted using 0.1 μm zirconium beads in conjunction with 3x 45 sec pulses at maximum speed (6.5) in a Bio 101 Fastprep. For 1D experiments protein samples were extracted directly into sterile purified water and diluted 1:1 in standard SDS-PAGE buffer. For 2D experiments, proteins were extracted directly into standard 2D buffers. All samples were centrifuged at 103,320 *g* for 1 hr at 15°C prior to 2D electrophoresis. Protein concentrations were determined using a Bradford assay. Electrophoresis was performed in 15 cm x 16 cm (W x H) 12% polyacrylamide gels in a Hoefer SE 600 vertical slab gel electrophoresis unit for both 1D and 2D electrophoresis. Isoelectric focusing (IEF) was achieved with 11 cm IPG strips pH 4-7 passively re-hydrated. IEF was undertaken in a BioRad Protean IEF unit. Two-dimensional experiments were run in triplicate to determine the reproducibility of this protocol. Eight protein spots (2 sheep and 6 cattle) chosen based on their being either unique or demonstrating differential expression together with 3 reference proteins from each strain were analysed by mass spectrometry (QSTAR2, Bioanalytical Mass Spectrometry facility at the University of New South Wales).

Surface enhanced laser desorption-ionisation (SELDI) proteomics
Proteins were extracted directly into extraction/reducing buffer (8M urea, 1% CHAPS in PBS) using the same protocol as for the 2D experiments. S and C strain protein samples were diluted in extraction/reducing buffer and run in duplicate at two concentrations, 1.5 μg per spot and 10 μg per spot on 4 ProteinChips including: CM10 (weak cation exchange, at pH 4 and pH 7), H50 (hydrophobic), IMAC30-Cu (copper affinity) and Q10 (strong anion exchange, at pH 7 and pH 9). All ProteinChips were prepared using the manufacturers recommended protocols and scanned with a ProteinChip reader (Ciphergen Model PBS IIC) that had been pre-calibrated according to the manufacturer's recommendations. Laser intensity and deflector sensitivity were determined empirically, after which spot and chip protocols were prepared to scan each ProteinChip. The resulting data was then analysed using Ciphergen ProteinChip Software version 3.2. Spectra were examined and only those protein peaks with peak intensity equal or greater than 1 were considered.

RESULTS

Representational difference analysis
Three bands identified as theoretically divergent DNA regions were cloned and sequenced (RDA1, RDA3 and RDA4)(21). RDA1 (229 bp) contained a single G➡T base difference in the S strain relative to the C strain, producing a serine➡alanine amino acid substitution in the MAP1381 conserved hypothetical gene in the S strain. This single base difference resulted in an extra *Sau*3A I site in the C strain and this was confirmed with a *Sau*3A I restriction endonuclease digest of the PCR product. RDA4 (163 bp) was an artefact. RDA3 (206 bp) was similar to sequences in the incomplete genome sequences of MAP K10 and *M. avium* subsp. *avium* 104 which demonstrated homology with *mmpL* genes in *M. tuberculosis*. A PCR-based analysis of these regions led to the identification of a deletion within the *mmpL5* gene in the S strain. This deletion was confirmed by *Bst*E II RFLP analysis of genomic DNA from the S and C strains using (1) a

dig-labelled probe for IS*900* (Fig. 1A) and (2) a 200 bp probe for the S strain deletion (Fig. 1B).   Further PCR analysis confirmed the deletion of a 11,584 bp region that included 10 genes (MAP1734 to MAP1743c) (see Table 5, in microarray section for more detail).

Identification of single nucleotide polymorphisms (SNPs) by polymerase chain reaction (PCR) and sequencing of selected genes
Of the 30 PCR assays used in this study, 4 failed to amplify with either strain and were omitted from the study. From the remaining 26 assays, 12,117 base pairs of sequence were obtained and compared; 18 loci were found to be identical in both strains and the remaining 8 (Table 3) contained a total of 11 SNPs in the S strain compared to the C strain. Sequences from both strains were used to query the K10 genome in a BLASTn search. The C strain was identical and the 11 SNPs were confirmed in the S strain. Both *des*A1 regions (plus and minus IS*900*) were found to be identical in the S and C strains. However, a BLASTn examination of the MAP K10 genome using the DNA sequences derived from these PCR found the region flanking IS*900* at this loci to be erroneously (Bull et al., 2000) identified as the *des*A1 gene and was in fact the ORFs MAP2202c and MAP2204c. The *des*A1 gene was identified in the annotated MAP K10 genome as MAP0658c and when its amino acid sequence was compared to MAP2204c they were ~76% homologous.
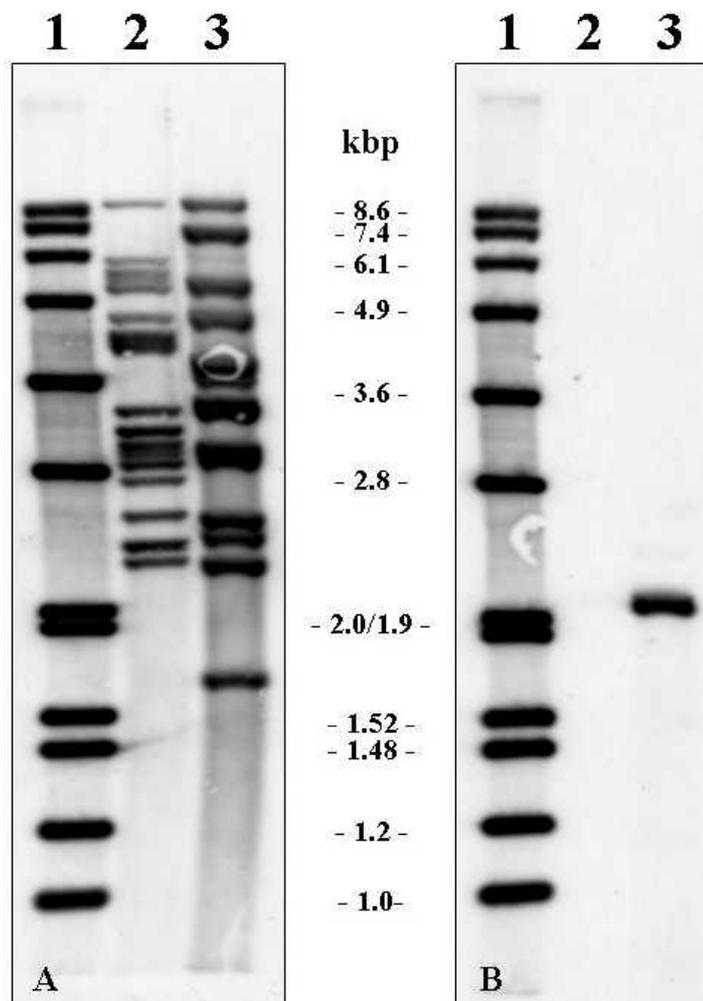
Fig. 1: RFLP probed with a 229 bp IS*900* probe (A) and then stripped and reprobed with a 200 bp probe used to confirm the S strain deletion (B). For each panel Lane 1, molecular size marker; lane 2, MAP S strain (Telford 9.2) genomic DNA and lane 3, C strain (CM00/416) genomic DNA.

The S and C strain DNA sequences were then used for *in silico* comparison with the incomplete *M. a. avium* 104 genome using 11,133 bp of sequence data (regions including IS*900* and *F57* sequence data were omitted as unique to MAP). Eighty seven SNPs (~0.8% of the sequence data compared) were identified that could be used to differentiate *M. a. avium* 104 isolate from the S and C strains of MAP (Table 4) This produced a marked difference in the ratio of synonymous to non-synonymous substitutions

compared to those differentiating the S and C strains of MAP. However, more conserved amino acid substitutions were observed when comparing *M. a. avium* with MAP than between the S and C strains of MAP (Table 4). Of the 11 SNPs that differentiate the S and C strains of MAP, 6 loci from the S strain and 5 loci from C strain were identical with the *M. a. avium* 104 genome.

Microarray
The C strain and 316v (laboratory strain) were identical to the MAP K10 strain. However, three regions including multiple ORFs were found to be deleted from the S strain including: Deletion 1 (8049 bp), Deletion 2 (19,930 bp) and Deletion 3 (1229 bp) (Table 5). In total, the 3 deletions were equivalent to 29,208 bp (~0.6%) of the MAP K10 genome and included 24 open reading frames (ORFs) (Table 5). The largest of the 3 deletions included the *mmpL5* gene, which we had identified previously using RDA. Of the remaining 24 ORFs, several have homology with genes in the *M. tuberculosis* H37Rv strain that have been shown to be associated with *in vitro* cultural requirements, intracellular survival and virulence. In a PCR-based study of 32 well-characterised MAP field isolates (16 S strains and 16 C strains), all 3 deletion regions were absent in all S strains and present in all C strains indicating conservation of the genomic differences.

Table 3: The 11 SNPs differentiating the S and C strains of MAP, the resulting amino acids for each strain and the effect of the substitution

Gene	S strain vs. C strain	S strain aa	C strain aa	Change
Hsp65	T⇒C	Thr	Thr	conserved
	A⇒G	Glu	Glu	conserved
sodA	C⇒G	Leu	Val	neutral-neutral
dnaA	T⇒C	Leu	Pro	neutral-neutral
dnaN	T⇒G	Ala	Ala	conserved
recF	C⇒T	Arg	Trp	basic-neutral
gyrB	C⇒T	Ser	Ser	conserved
	T⇒G	Ile	Met	neutral-polar
	T⇒C	Val	Ala	neutral-neutral
inhA	G⇒A	Gln	Gln	conserved
pks8	G⇒A	Pro	Pro	conserved

Table 4: The number of SNPs identified when comparing the S and C strain of MAP and *M. a. avium* including the type of substitution and the effect on the resulting amino acids.

Comparison	Number of bases compared	Total number of SNPs	Synonymous vs. non-synonymous Substitutions	Substitutions: transitions vs transversions	Conserved vs. non-conserved amino acid substitutions
S strain vs. C strain	12,117	11 (0.09%)	6/5 (55%/45%)	8/3 (73%/27%)	6/5 (55%/45%)
MAP vs. *M. a. avium*	11,133	86 (0.8%)	72/14 (84%/16%)	64/23 (74%/26%)	71/15 (83%/17%)

The genomic relationship between the S and C strains of MAP and *M. a. avium* was evaluated by using the MAP K10 sequences corresponding to each of the S strain deletions in BLASTn searches of the *M. a. avium* 104 genome. The regions corresponding to Deletions 1 and 3 were found to be complete and intact within the *M. a. avium* 104 genome but the region corresponding to Deletion 2 was incomplete. Only 17,384 bp (87.2 %) of the Deletion 2 was found in the *M. a. avium* 104 genome in 2 clusters separated by 203,000 bp and both in the opposite orientation compared to the MAP K10 genome. Therefore, ~2500 bp of this region was found to be unique to the C strain of MAP.

Two dimensional electrophoresis proteomics
The S and C strains were compared by 1D (Fig. 2, panel A) and 2D (Fig. 2, panels B and C) electrophoresis. The 1D comparison identified several bands in both strains with differential expression and at least one major unique band in the S strain. Further analysis of the protein samples by 2D electrophoresis confirmed the unique expression of this protein (Fig. 2, panel B, S1 and S2) in the sheep strain and identified several proteins in the C strain (Fig. 2, panel C, C3-C5 and C12-C14).
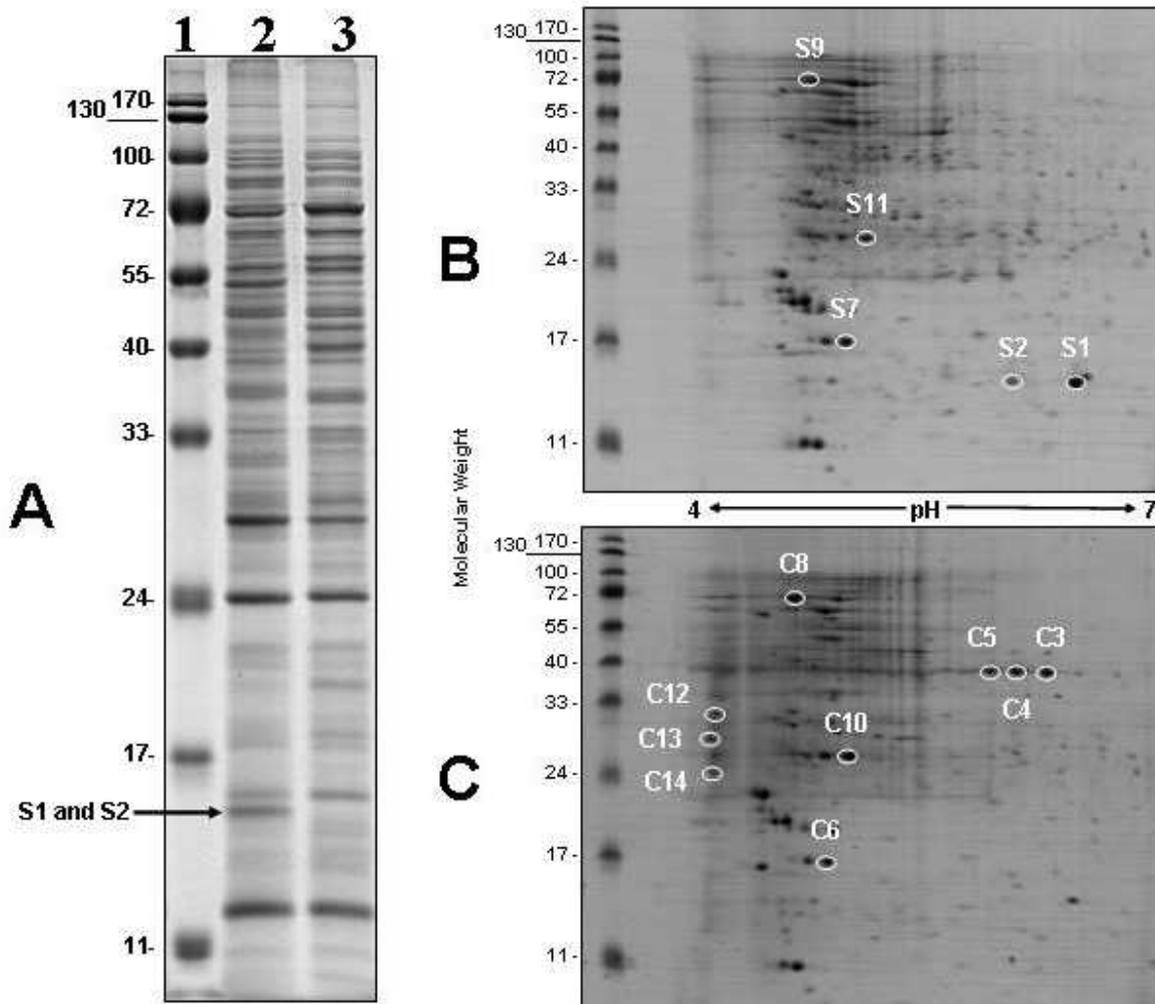
Figure 2: Proteomic comparison of the S and C strains of MAP using 1D electrophoresis (panel A; Lane 1, molecular size marker; lane 2 MAP S strain and lane 3, MAP C strain) and 2D electrophoresis (panel B, S strain and panel C, C strain). The latter shows the locations of the proteins analysed by QSTAR2 mass spectrometry.

All 8 spots were excised from the gel along with three reference spots from both strains and identified by mass spectrometry. The reference proteins were identified in both strains as: MAP1885c (C6 and S7), 70 kDa HSP (C8 and S9) and MAP3909c (C10 and S11). The strain specific proteins were identified as: MAP2821 (S1 and S2, 2 spots due to post translation modifications), fadE3_2 and MoxR (C3, C4 and C5, 3 spots due to post translation modifications), Wag31 (C12), MAP0494 (C13) and MAP3838c (C14). MAP0494 and MAP3838c were erroneously identified based on their isoelectric points (pI). At 6.24 and 10.79, respectively, these were either too high for the location of the spot or outside the pH range of the IEF strips.

Surface enhanced laser desorption-ionisation (SELDI) proteomics
SELDI spectra were obtained with all 4 ProteinChips at both 1.5 µg per spot and 10.0 µg per spot loading concentrations. However, protein peaks were only observed in the 5000 to 20,000 kDa range. Numerous peaks were observed that were common to both samples but closer examination of the spectra identified many unique peaks in the S strain and a few in the C strain (Table 6, U). Evidence was also observed for differential expression of a number of proteins (Table 6, E). Several unique protein peaks, of similar molecular weight, had been identified in the S strain with 2 or more ProteinChips including a 5.2 kDa protein peak using the CM10 and IMAC30 ProteinChips and a 13.9 kDa protein peak using the H50, IMAC30 and Q10 ProteinChips. A 9.6kDa protein peak was identified as unique in the S strain by the CM10 and Q10 ProteinChips, but as differentially expressed by the IMAC30 ProteinChip. Finally, a differentially expressed 17.7 kDa protein was identified by the IMAC30 and Q10 ProteinChips. No unique or differentially expressed protein peaks were identified in the C strain with more than 1 ProteinChip.

Table 5: S strain deletions identified by RDA and microarray including: base positions with respect to the MAP K10 genome, the partial (par) ORFs and complete ORFs. The shaded area corresponds to the region identified in the RDA study.

S strain deletion	Start of deletion, base position	ORFs included	*M.tb* equivalent	*M.ptb* Gene name	Function	End of deletion, base position
Deletion 1	1625179	par. MAP1484c	Rv3161c		putative dioxygenases	1633227
		MAP1485c	Rv0214		acyl-CoA synthase	
		MAP1486c	Rv0456c		enoyl-CoA hydratase/isomerase superfamily	
		MAP1487c	Rv2496c		pyruvate dehydro-genase E1 compo-nent [beta] subunit	
		MAP1488c	Rv2497c		pyruvate dehydro-genase E1 compo-nent [alpha] subunit	
		MAP1489c	Rv2750		putative dehydrogenase	
		MAP1490			alpha-methylacyl-coA racemase	
		par MAP1491			alpha-methylacyl-coA racemase	
Deletion 2	1888735	MAP1728c		yfnB	2-haloalkanoic acid dehalogenase	1908664
		MAP1729c	Rv2605c		thioesterase II	
		MAP1730c			putative ATP/GTP-binding protein	
		MAP1731c			hypothetical protein	
		MAP1732c	Rv0302		transcriptional regulator (TetR/AcrR family)	
		MAP1733			proline rich protein precursor	
		MAP1734	Rv2123		PPE-family protein	
		MAP1735	Rv0217c	lipW_1	probable esterase	
		MAP1736			putative tetR-family transcriptional regulator	
		MAP1737	Rv0677c	mmpS5	conserved small membrane protein	
		MAP1738	Rv0676c	mmpL5	conserved large membrane protein	
		MAP1739c	Rv2002	fabG3_1	3-oxoacyl-(25) reductase	
		MAP1740c	Rv3132c		sensor histidine kinase	
		MAP1741c	Rv2005c		conserved hypothetical protein	
		MAP1742c	Rv2026c		conserved hypothetical protein	
		MAP1743c	Rv2032		conserved hypothetical protein	
		MAP1744			hypothetical protein	
Deletion 3	2608297	MAP2325	Rv2416c		conserved hypothetical protein	2609525

Table 6: Summary of the SELDI results. The expanded spectra for each ProteinChip were used to identify protein peaks in the S and C strain of MAP as either unique (U) or representing a marked difference in expression (E). The size of each peak identified is given in kDa.

ProteinChip	1.5 µg of protein				10.0 µg of protein			
	Telford 9.2 kDa		CM00/416 kDa		Telford 9.2 kDa		CM00/416 kDa	
CM10 (pH 4)	13.9	U			5.2, 6.0, 14.4	U		
					8.7, 9.0, 9.3, 10.2, 14.7	E		
CM10 (pH 7)	5.2, 6.0, 9.6	U	8.37	E	5.2, 6.0	U		
					14.7	E		
H50	13.9	U	16.3	U	10.9, 12.9[a]	U		
					13.9[a]	E		
IMAC30 (Cu)	5.2, 5.3, 5.5[a], 13.9[a]	U			5.2, 5.3, 5.9, 13.9, 14.3	U		
	9.6, 17.7, 17.9	E			9.6, 17.7[a]	E		
Q10 (pH 7)	6.9, 13.9	U	7.36	E	9.4, 13.9	U	14.3	U
					17.7	E		
Q10 (pH 9)	8.9, 9.0, 9.6, 13.9	E			13.9	U		
	9.5[a]	U						

(a) Protein peak was < 1 in peak intensity but readily identifiable

DISCUSSION

Previously unidentified differences between the S and C strains of MAP were identified using a combination of modern DNA and protein based techniques. RDA has already been used to identify unique regions present in the S strain but absent in the C strain (Dohmann et al., 2003).   In this study two further differences were discovered in the S strain using RDA including a single base difference in MAP1381 and a large-scale deletion that included the *mmpL* 5 gene. The latter was fully characterised as Deletion 2 by microarray analysis. Deletion 2, along with two others identified by microarray, are the first large-scale genomic differences to be reported that differentiate the S and C strains of MAP. In total, the 3 deletions were equivalent to 29,208 bp (~0.6%) of the MAP K10 genome and included 24 open reading frames (ORFs) (Table 5). PCR analysis of 32 field isolates confirmed these deletions to be highly conserved in the S strain. Homologues of many of the ORFs in Deletions 1-3 have been identified in *M. tuberculosis* and shown to play important roles in pathogenicity, host cell invasion and intracellular survival as well as *in vitro* culture. The latter may be important given the marked differences in the cultural characteristics of these strains.

The *pdhABC* genes in *M. tuberculosis*, for which the ORFs MAP1487c and MAP1488c (Deletion 1) bear homology, encode subunits of pyruvate dehydrogenase, which coverts pyruvate to acetyl-coenzyme A (CoA) and carbon dioxide. They have been shown to be up-regulated during nutrient starvation (Betts et al., 2002). Similarly the ORFs MAP1740c and MAP1742c (Deletion 2) appear to be homologues of *M. tuberculosis* genes Rv3132c and Rv2026c, respectively, considered to be essential for optimal *in vitro* growth (Sassetti et al., 2003). Interestingly, homologues in *M. tuberculosis* of many of the ORFs in Deletion 2 have been studied in association with the DevR-DevS two-component regulatory system, thought to be involved in intracellular survival. This observation of *M. tuberculosis* and the clustering of MAP1740c to MAP1743c would indicate that these genes may form part of an operon involved in the intracellular response to the host environment and that the loss of this operon may explain the shift in host specificity and possibly the *in vitro* growth requirements of the S strain of MAP. Of particular interest was the absence of the *mmpS5* (MAP1737) and *mmpL5* genes (MAP1738) as part of Deletion 2 in the S strain. MmpL proteins in *M. tuberculosis* have been shown to be essential for intracellular survival and growth, are involved in pathogenicity and contribute to host-pathogen interactions (Camacho et al., 1999; Converse et al., 1999; Cox et al., 1999; Domenech et al., 2004; 2005; Lamichhane et al., 2005). Furthermore, attenuated strains of *M. bovis* that lack the *mmpL 4* gene were found to exhibit equivalent protection to *M. bovis* BCG in guinea pigs challenged with *M. tuberculosis* (Collins et al., 2005).

The smallest deletion identified in this study, Deletion 3, included only one ORF, MAP2325, which shares homology with Rv2416c in *M. tuberculosis*. Rv2416 has been described as an enhanced intracellular survival (*eis*) protein thought to be an important *M. tuberculosis* immunogen (Dahl et al., 2001) present only in pathogenic mycobacteria (Wei et al., 2000) but as yet not confirmed in MAP.

In an *in silico* analysis, DNA sequences from the C strain corresponding to the S strain deletions were used to compare MAP with *M. a. avium*. Sequences for deletions 1 and 3 were complete and intact within the *M. a. avium* 104 genome; Deletion 2 was found to be only 87.2% (17,384 bp) complete. The missing 2546 bp of this region that includes the ORFs corresponding to MAP1730 to MAP1732 are therefore unique to the C strain. This deletion is thus a very interesting target for further research on the divergence of MAP and *M. a. avium*.

PCR and sequencing revealed eleven SNPs that can be used to differentiate the S and C strains. Five of these produced non-conserved amino acid substitutions in the S strain. Further work however is required to identify what effect each of these may have on the S strain phenotype. Interestingly, a ten fold greater number of SNPs differentiating MAP from *M. a. avium* suggests that the divergence between the S and C strains of MAP is evolutionarily recent compared with the divergence between *M. a. avium* and MAP. More work is required in this area to confirm this hypothesis.

Proteomic techniques demonstrated their usefulness in identifying the protein expression characteristics of the S and C strains under routine culture conditions. The results indicated the usefulness of combining techniques given that two dimensional electrophoresis is more suited to proteins >20 kDa and SELDI is more useful for proteins <20 kDa when whole cell protein samples are examined. However, both

techniques would benefit from fractionation techniques to simplify collection of the protein samples. Interestingly, the protein identified by 2D electrophoresis in the S strain, MAP2821, was also evident in both the 1D electrophoresis (Fig. 2, panel A) and SELDI (protein peak at ~14.0 kDa) results, which demonstrates the reproducibility of these techniques.

The results from this study have clearly demonstrated that many genomic differences including deletions equivalent to ~0.6% of the genome exist between the S and C strains of MAP. These results confirm and support earlier studies that differentiate the S and C strains of MAP and can be used to reassure animal industries that rely on this strain difference for the recommended management practices used to combat Johne's disease. Furthermore, a number of important genomic loci have been identified that can be used to differentiate between the S and C strains. Differences in a number of important genes require further examination to understand what role they may play in pathogenicity, intracellular and environmental survival and specific host-pathogen interactions.

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Comparative genomic analysis of *Mycobacterium avium* ss. *paratuberculosis* isolates obtained from multiple host species

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ABSTRACT

We have designed and built a DNA microarray consisting of 70mer oligonucoeotides representing all of the ORFs identified in the MAP K10 genome sequence as well as intergenic regions in order to investigate whether the genome content of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) K10 is representative of other bovine isolates as well as isolates from other species. In addition, oligonucleotides representing coding sequences from the *Mycobacterium avium* subspecies *avium* (MAA) 104 genome that are not present in the MAP K10 genome were also included on the microarray. Genomic DNA from MAP isolates was fluorescently labeled, mixed with alternately labeled MAP K10 DNA, and competitively hybridized on the MAP microarray. ORFs were classified as present or divergent based on the relative fluorescent intensities of the experimental samples compared to MAP K10 DNA. MAP isolates cultured from cattle, bison, sheep, goat, avian, and human sources were hybridized to the MAP microarray. Three deleted regions were observed in the genomes of three MAP isolates obtained from sheep. Additionally, four clusters of ORFs homologous to sequences in the MAA 104 genome were identified in three of the sheep isolates. One of these clusters encodes glycopeptidolipid biosynthesis enzymes which have not previously been identified in MAP. Differences in hybridization across many of the isolates examined were detected for the microarray targets representing the insertion sequence IS_MAP*04*, the major membrane protein encoded by MAP2121c. Several regions encoding proteins with unknown function suggesting that there may be variation in the sequence or copy number of these regions. One cattle isolate and one sheep isolate of MAP were found to contain a different genome content compared to the other isolates examined from these species.

Key words: *Mycobacterium avium* subspecies *paratuberculosis;* microarray; comparative genomics

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic infection primarily of ruminant animals characterized by a prolonged subclinical phase leading eventually to a severe gastroenteritis that results in malnutrition and ultimately death. Restriction fragment length polymorphism (RFLP) and restriction endonuclease analyses of IS*900* and IS*1311* polymorphisms have been able to separate MAP isolates into "S" and "C" strains generally corresponding to isolates from sheep or other ruminants and cattle, respectively (Whittington *et al.*, 2000). Pulsed field gel electrophoresis (PFGE) analysis of genomic polymorphisms was also able to separate MAP isolates into two groupings (type I and type II) one of which (type I) is primarily comprised of isolates from sheep and other small ruminants (Stevenson *et al.*, 2002). Multiplex PCR of IS*900* integration loci (MIPL) and amplified fragment length polymorphism (AFLP) analyses provided similar results, with bovine MAP isolates forming a genetically conserved cluster while isolates from other species were more diverse and loosely clustered (Motiwala *et al.*, 2003). More recently, multilocus short sequence repeat (MLSSR) sequencing of polymorphic regions within the MAP genome has confirmed previous findings and provided additional resolution and subtyping of MAP isolates (Amonsin *et al.*, 2004). While the methods discussed here have yielded valuable insights into the population genetics of MAP isolates from a wide range of host species, it remains unclear how these findings relate to their actual gene content.

Previous work in our lab has utilized DNA microarrays to compare the genome content of members of the *Mycobacterium avium* complex (MAC) which includes MAP, *Mycobacterium avium* subspecies *avium* (MAA), *Mycobacterium avium* subspecies *silvaticum*, and *Mycobacterium intracellulare*. These findings revealed that non-MAP MAC isolates do not contain several large regions of genomic DNA that are present in MAP K10. Extensive genomic conservation was observed for the MAP isolates examined in the study, most of which were obtained from cattle (Paustian *et al.*, 2005). In the work presented here, we have utilized a DNA microarray constructed with oligonucleotides representing all of the predicted coding and intergenic regions from the MAP K10 genome as well as the remaining novel coding sequences from the MAA 104 genome to examine the genome content of MAP isolates obtained from a variety of host species. We hypothesize that genes found to be polymorphic among MAP isolates from different species contribute to host specificity and pathogenicity.

MATERIALS AND METHODS

Microarray design

The software program ArrayOligoSelector (Bozdech *et al.*, 2003) was used to identify 70mer oligonucleotides specific for every predicted open reading frame (ORF) in the MAP K10 genome. One 70mer was designed for each MAP K10 ORF with a total length of less than 4000 bp, while longer ORFs were split in half and one 70mer was designed for each half. One 70mer was also designed for every MAP intergenic region greater than 500 bp. Additionally, an automated annotation of the MAA 104 genome sequence (http://www.tigr.org/tdb/mdb/mdbinprogress.html) was performed using the methods described by McHardy and coworkers (2004) and one 70mer was designed for every predicted ORF that was less then 30% identical to MAP K10 sequences as determined by BLAST analysis (Altschul *et al.*, 1997). The annotated MAA 104 ORFs were numbered sequentially starting at the origin of replication, although for reference the nucleotide start and stop positions for each ORF or cluster are also reported. Oligonucleotides representing six *Arabidopsis thaliana* sequences and sheared MAP K10 genomic DNA were included on the microarray as controls. The 70mer oligonucleotides were synthesized by Illumina (San Diego, CA) and resuspended at a concentration of 60 µM in 3x SSC containing 1.5 M betaine (1x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). The oligonucleotides were arrayed in triplicate onto homemade poly-L-lysine coated glass slides with a MicroGrid II Compact robot (Genomic Solutions, Ann Arbor, MI) along with control samples that included spotting buffer alone and Arabidopsis thaliana sequences. This resulted in an array containing over 17,000 spots with an average diameter of 150 µM.

Comparative genomic hybridizations

Mycobacteria were cultured and DNA extracted as previously described (Motiwala *et al.*, 2003) or as follows. Bacteria were grown in Middlebrook 7H9 broth (pH 6.0) supplemented with oleic acid-albumin-dextrose-catalase (Becton Dickinson Microbiology, Sparks, MD), and 0.05% Tween 80. Cultures of MAP were further supplemented with ferric mycobactin J (2 mg/liter; Allied Monitor Inc., Fayette, MO). Genomic DNA was extracted from MAP K10 and 15 mycobacterial isolates (Table 1) with Genomic-tip 100/G anion-exchange columns (QIAGEN, Valencia, CA) as previously described (Bannantine *et al.*, 2002) with the following modification: D-cycloserine was not added as part of the extraction procedure.

Purified genomic DNA was then randomly sheared by nebulization on ice at 10 psi for 1.5 min, resulting in an average fragment size of 800 bp. Aliquots of sheared genomic DNA (4 µg) were labeled with Alexa Fluor 555 or 647 using the BioPrime Plus Arracy CGH Genomic Labeling System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Labeled cDNA from experimental mycobacterial isolates was then purified and mixed with alternately labeled MAP K10 cDNA in a final volume of 55 µL containing 3x SSC, 0.22% SDS, and 34 µg salmon sperm DNA (Invitrogen). This hybridization solution was incubated at 100°C for 2 minutes, applied to the MAP K10 microarray, and allowed to hybridize overnight at 65°C. The arrays were washed sequentially for 3 minutes at room temperature in 300 mL volumes of 0.5x SSC/0.01% SDS, 0.5x SSC, 0.1x SSC, and 0.01x SSC, then dried by centrifugation and scanned with a ScanArray 4000 confocal laser scanner (PerkinElmer, Boston, MA). Each mycobacterial isolate was hybridized against MAP K10 at least twice in a dye-flip experimental design.

Microarray data analysis

Raw intensity measurements for each spot on the microarray were extracted from scanned images using ScanArray Express software (PerkinElmer) and adjusted with local background subtraction and LOWESS normalization (Cleveland & Devlin, 1988). Poorly detected spots were removed by filtering out those in which more than 50% of the pixels from both samples were within two standard deviations of the local background. At this point, any ORFs not represented by at least three acceptable spots from at least two independent hybridizations were discarded from further analyses. The median intensity of the remaining spots was determined for each ORF, then ratios of the spot intensities for experimental and control MAP K10 DNA samples were calculated and log transformed for analysis. The software program Cluster 3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm) was used to filter out ORFs that were lacking values for greater than 80% of the MAP isolates examined, median center the results for each isolate, and cluster the isolates according to genome content as measured by microarray hybridization. Clustering results were visualized with Java TreeView (Saldanha, 2004). Putative deletions were identified by a visual examination of the hybridization results.

RESULTS

MAP isolates from a variety of host species were examined for genome content polymorphisms via competitive hybridizations with MAP K10 on the MAP K10 oligonucleotide microarray (Table 1).

Table 1. MAP isolates used in this study

Isolate	Host	Isolate	Host
397	Sheep	7876	Cat
467	Sheep	7887	Starling
1018	Cattle	7889	Starling
4026	Goat	7890	Starling
4054	Goat	7893	Starling
4137	Goat	7926	Armadillo
5001	Sheep	7935	Cattle
7301	Cattle	MAP4	Human
7560	Bison	MAP6	Human
7565	Sheep	--	--

Table 2. MAA 104 ORFs present in MAP sheep isolates 397, 467, and 7565

Name	Description	Start	Stop
MAA0315	Transcriptional regulator	331451	330795
MAA1834	No hits	1965913	1965746
MAA1835	Putative oxidoreductase	1965891	1966742
MAA1836	Putative tetR-family transcriptional regulator	1966739	1967332
MAA1837	Putative intracellular protease/amidase	1968632	1967946
MAA1838	No hits	1969578	1969486
MAA1839	GreA	1970060	1969593
MAA1840	Putative transcription elongation factor	1970590	1970057
MAA1841	Conserved transmembrane protein	1971576	1970944
MAA1842	Regulator of polyketide synthase expression	1973249	1971729
MAA1843	No hits	1973477	1973584
MAA1845	18 kDa antigen-like protein	1974981	1975316
MAA1846	No hits	1975906	1976097
MAA1847	Hypothetical protein MAP1697	1976294	1976668
MAA1848	No hits	1976688	1976798
MAA1849	FOG: GGDEF domain	1976948	1978027
MAA1850	FdxD	1978369	1978575
MAA1851	PPE	1980299	1978968
MAA1852	Hypothetical protein Rv1490	1982309	1981053
MAA1853	No hits	1982467	1982309

MAA1855	DrrC	1986395	1984749
MAA1856	PapA2	1987820	1986429
MAA1857	PPE	1988873	1990213
MAA1858	3-deoxy-D-arabino-heptulosonate synthase	1990597	1991691
MAA2772	Peptidoglycan biosynthesis	3014473	3014264
MAA2773	No hits	3015831	3014560
MAA2774	Putative oxidoreductase	3016612	3015851
MAA2775	Hypothetical protein MAP0708	3018981	3016609
MAA2776	Putative enoyl-CoA hydratase/isomerase	3019969	3019001
MAA2777	Hypothetical protein MAP0336c	3022044	3020842
MAA2778	Hypothetical protein MAP3331	3022942	3022268
MAA2779	No hits	3022954	3023685
MAA2780	Predicted dehydrogenases and related proteins	3024721	3023720
MAA2781	Putative D-glucose O-methyltransferase	3024864	3025529
MAA2782	Arylsulfatase	3027400	3025550
MAA2783	Conserved hypothetical protein	3027258	3028277
MAA2784	Polyketide synthesis O-methyltransferase	3029193	3028282
MAA2785	Hypothetical protein Rv0678	3029627	3029100
MAA2786	Hypothetical protein MAP0722	3030986	3029718
MAA2787	Probable aldolase	3031890	3031117
MAA2788	Pathogenicity factor-like protein	3033502	3031877
MAA2789	Hypothetical protein Rv0219	3034108	3033569
MAA2790	Hypothetical protein	3035357	3034080
MAA2791	No hits	3035367	3035483
MAA3041	GtfB	3399659	3400915
MAA3042	Dehydrogenase DhgA	3401701	3400922
MAA3043	Methyltransferase MtfD	3402593	3401775
MAA3044	Methyltransferase MtfC	3403499	3402693
MAA3045	Rhamnosyltransferase RtfA	3404882	3403596
MAA3048	Putative glycosyltransferase	3407914	3406637
MAA3049	Methyltransferase MtfB	3408843	3408037
MAA3050	MtfA	3410252	3409125
MAA3051	GepiA	3411577	3410516
MAA3052	Hypothetical protein	3412722	3411697
MAA3859	Hypothetical protein	4255362	4255898
MAA4893	Hypothetical protein	5379667	5378903
MAA4894	Hypothetical protein Rv1645c	5380772	5379819
MAA4895	Putative TetR-family transcriptional regulator	5380884	5381477
MAA4896	Lignin peroxidase LipJ	5381519	5382904
MAA4897	SAM-dependent methyltransferases	5383030	5383776
MAA4898	Hypothetical protein	5384250	5383792
MAA4899	No hits	5384428	5384255
MAA4900	3-alpha-hydroxysteroid dehydrogenase	5386038	5385193
MAA4901	Hypothetical protein Rv0090	5386807	5386043
MAA4902	Hypothetical protein MAP2775	5387718	5386861
MAA4903	Zinc-type alcohol dehydrogenase-like protein	5387843	5388895
MAA4904	Hypothetical protein	5388892	5389308
MAA4905	Oxidoreductase	5389305	5390123
MAA4906	Hypothetical protein	5390366	5391649
MAA4907	Putative oxidoreductase	5391511	5392521
MAA4908	Hypothetical protein MAP3594	5394171	5392576
MAA4909	No hits	5394269	5394823
MAA4910	Hypothetical protein Rv0088	5395294	5394824

A similar genomic profile was observed for MAP sheep isolates 397, 467, and 7565. These sheep isolates were distinguished from the other MAP isolates examined in this study by the presence of four clusters of ORFs homologous to sequences found in the MAA 104 genome but absent in MAP K10 and all other MAP

sequences that have been previously described (Table 2). Notably, the proteins encoded by the ORFs MAA3041-3052 are involved in glycopeptidolipid biosynthesis (Eckstein *et al.*, 2003). The other gene clusters containing MAA 104 ORF homologues encode a variety of proteins; however, a majority of the ORFs are conserved hypothetical proteins or are not homologous to any sequences currently deposited in Genbank. MAA315 encodes a putative transcriptional regulator and was also found to be present in the MAP sheep isolates.

Several clusters of ORFs present in the MAP K10 genome were found to be deleted in MAP sheep isolates 397, 467, and 7565 (Table 3). The MAP1485c-1491 ORF clusters as well as several ORFs in the MAP1432-1438c and MAP1728c-MAP1744 clusters encode proteins involved in lipid metabolism. Additionally, the intergenic region between MAP1484c and MAP1485c also appears to be at least partially deleted. A closer examination of this region revealed that although it was not annotated as an ORF in the MAP K10 genome it has homology to acyl-CoA synthetase enzymes. Other proteins encoded by the deleted regions include transcriptional regulators, putative membrane proteins, a PPE-family member, and several of unknown function. In addition to the deleted clusters of ORFs, MAP2656 was also identified as absent in the MAP 397, 467, and 7565 genomes. Notably, this ORF encodes an acyl-CoA dehydrogenase that, like many of the other deleted ORFs, is involved in lipid metabolism.

The genome profile of MAP sheep isolate 5001 was significantly different from the other sheep isolates examined in this study. Similar to the other sheep isolates, the MAP 5001 genome did contain the MAA3041-3052 and MAA4893-4910 clusters; however, it did not possess the MAA1834-1858 or MAA2772-2791 clusters of MAA 104 ORFs. Additional clusters of MAA 104 ORFs spanning MAA4657-4665 (5122587-5131447) and MAA4704-4709 (5173587-5179816) were also identified in the MAP 5001 genome.

Table 3. MAP K10 ORFs absent in MAP sheep isolates 397, 467, and 7565

Name	Description
MAP1432	REP-family protein
MAP1433c	3-oxosteroid 1-dehydrogenase
MAP1434	Putative phthalate oxygenase
MAP1435	Short chain dehydrogenase
MAP1436c	Putative oxidoreductase
MAP1437c	Hypothetical protein
MAP1438c	Probable lipase
MAP1485c	Acyl-CoA synthase
MAP1486c	Enoyl-CoA hydratase/isomerase superfamily
MAP1487c	Pyruvate dehydrogenase E1 component
MAP1488c	Pyruvate dehydrogenase E1 component
MAP1489c	Putative dehydrogenase
MAP1490	Alpha-methylacyl-coA racemase
MAP1491	Alpha-methylacyl-coA racemase
MAP1728c	2-haloalkanoic acid dehalogenase
MAP1729c	Thioesterase II
MAP1730c	Putative ATP/GTP-binding protein
MAP1731c	Hypothetical protein
MAP1732c	TetR-family transcriptional regulator
MAP1733	Proline rich protein precursor
MAP1734	PPE-family protein
MAP1735	Probable esterase
MAP1736	Putative TetR-family transcriptional regulator
MAP1737	Conserved small membrane protein
MAP1738	Conserved large membrane protein
MAP1739c	3-oxoacyl-ACP reductase
MAP1740c	Sensor histidine kinase
MAP1741c	Conserved hypothetical protein
MAP1742c	Conserved hypothetical protein
MAP1743c	Conserved hypothetical protein
MAP1744	Hypothetical protein
MAP2656	Acyl-CoA dehydrogenase

Several clusters of MAP K10 ORFs were divergent or deleted in isolate 5001. Notably, most of these ORFs were previously identified as absent from the genomes of non-MAP MAC isolates (Paustian *et al.*, 2005) and included MAP0093-0106c, MAP0851-0866, MAP2755-2769c, and MAP3731-3761. Many of the ORFs included in these clusters encode proteins of unknown function, some of which have been identified only in MAP isolates (Bannantine *et al.*, 2004; Paustian *et al.*, 2005). Notably, these divergent regions were shared with MAP cattle isolate 7301 that was observed to have a genomic profile dissimilar to the other cattle isolates examined in this study (Table 4). MAP isolates 5001 and 7301 shared several other features that distinguished them from the other isolates examined. Both isolates appear to have changes in the copy number or presence of several insertion sequence (IS) elements, including IS_MAP*04*, IS_MAP*05*, IS_MAP*07*, IS_MAP*12*, IS*900*, and IS*1311*.

Several regions of genomic DNA were observed to have variable amounts of hybridization across multiple MAP isolates. These polymorphic areas included MAP0282c-0284c, MAP0852-0866, MAP1232-1235, MAP2149c-2158, MAP2121c, and IS_MAP*04*. Many of the ORFs included in these regions encode proteins of unknown function, while MAP2121c encodes a major membrane protein.

Table 4. MAP K10 ORFs deleted in both MAP 5001 and MAP 7301

Name	Description
IG1617	Region between MAP2107c and MAP2108
IS_MAP*04*	Insertion sequence
IS_MAP*07*	Insertion sequence
MAP0100	GPTase-activating protein I
MAP0283c	Hypothetical protein
MAP0852	No hits
MAP0861	No hits
MAP0862	No hits
MAP0864	No hits
MAP1064	Hypothetical protein
MAP1821c	Hypothetical protein
MAP1822c	Hypothetical protein
MAP2153	No hits
MAP2158	No hits
MAP2963c	No hits
MAP3731c	Putative ABC transporter ATP-binding subunit
MAP3733c	Hypothetical protein
MAP3736c	Heavy metal tolerance protein
MAP3756c	Possible mono-oxygenase
MAP3771	50s ribosomal protein L31
MAP3773c	Ferric uptake regulatory protein
MAP4267	No hits

DISCUSSION

The use of specific genetic markers has allowed MAP isolates to be separated into two general populations: a relatively homogenous group comprised of primarily bovine isolates and a more heterogeneous group that includes isolates from small ruminants and other mammals. The goal of the present study was to identify variations in genome content between MAP isolates from several different host species in order to determine which genes may contribute to host specificity and pathogenesis. The isolates obtained from goat (n = 3), bison (n = 1), bird (n = 4), armadillo (n = 1), cat (n = 1), and human (n = 2) hosts did not contain any large polymorphic regions when compared with the MAP K10 cattle isolate. Three of the four sheep isolates examined (397, 467, and 7565) shared large polymorphisms that included the loss of ORFs present in the MAP K10 genome as well as the acquisition of ORFs that are present in the MAA 104 genome. The ORFs included in the deleted regions (n = 32) are predicted to encode proteins involved in variety of functions including lipid and energy metabolism, virulence, and transcriptional regulation. The insertions containing homologues to ORFs in the MAA 104 genome (n = 73) encoded proteins involved in

glycopeptidolipid biosynthesis, transcriptional regulation, virulence, and metabolism. These polymorphic regions also contained a number of proteins with unknown function.

MAP isolates 5001 and 7301 shared many genome features and were noticeably different when compared with the other sheep and cattle isolates, respectively. MAP 5001 lacked several of the MAA 104 ORF clusters that were present in the other sheep isolates, while MAP 7301 appears to contain some MAA 104 ORFs that were not found in the other cattle isolates. The genomes of MAP 5001 and MAP 7301 both contained multiple divergent regions including putative deletions that corresponded to segments of the MAP K10 genome that were found to be deleted in non-MAP MAC isolates, some of which include ORFs that have only been found in MAP. The identification of genomic regions that are variable across all of the isolates examined may be indicative of areas under significant selective pressure or with locally high frequency of recombination. Many of these shared polymorphic regions encode proteins of unknown function, making it is difficult to establish a common trend, although it is intriguing that MAP2121c encodes a major membrane protein and was identified as polymorphic – an observation that is being followed up by sequencing this ORF from multiple isolates.

This study has identified several polymorphic regions within the genomes of MAP isolates obtained from sheep and cattle. The presence of ORFs homologous to MAA 104 sequences in MAP sheep isolates indicates that the evolutionary history of MAP may not follow a linear path. The divergent genome profiles of MAP 5001 and MAP 7301 suggest that additional variability may be present among MAP isolates, although the extent to which this may be true remains to be determined.

ACKNOWLEDGEMENTS

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Evaluation of different organism based methods for the detection and identification of *Mycobacterium avium* subspecies *paratuberculosis* from bovine feces

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ABSTRACT

United States Department of Agriculture (USDA) regulations have stated that an organism-based assay (culture/PCR) is the official test for determining the infectious status of an animal for Johne's disease. Recent method evaluation tests performed for laboratory approval for the Voluntary Bovine Johne's Disease Control Program (VBJDCP) indicate multiple culture methods were being used in the United States. The yearly evaluations have indicated that there were a wide range of sensitivities associated with the different culture methods. The National Veterinary Services Laboratories (NVSL) have been requested to establish a standardized protocol for detecting *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in fecal samples that is reproducible and has a known sensitivity.

The NVSL have also been requested to establish the criteria for well-characterized bovine fecal panels for use in organism-based detection procedures and methods evaluation. These panels will be used to validate different diagnostic procedures, including serological assays and USDA licensed diagnostic kits used for Johne's disease detection.

Based on the results of the last six years of proficiency tests for detecting MAP, several methods have been chosen for further evaluation. These methods included different decontamination methods involving sedimentation or centrifugation and different media including solid and liquid which have been used by multiple laboratories. Preliminary evaluation based on proficiency test results indicate that centrifugation methods are more sensitive than sedimentation decontamination methods and liquid media methods are faster than methods using Herrold's Egg Yolk media with mycobactin J (HEYM). More PCR methods are being introduced and evaluated by different laboratories each year.

Varied growth performances in the solid media used with different culture methods were also noted during the last six check tests. There are now two commercial sources of HEYM available in the United States which will be evaluated along with in-house media for growth performance.

Key words: culture methods, feces, cattle, proficiency tests, mycobacteria

INTRODUCTION

United States Department of Agriculture (USDA) regulations have stated that the official test for determining the infective status of an animal for Johne's disease is organism-based (culture/PCR). Recent method evaluation tests performed for laboratory approval for the Voluntary Bovine Johne's Disease Control Program (VBJDCP) indicate multiple culture methods were being used in the United States. The yearly evaluations have indicated that there were a wide range of sensitivities associated with the different culture methods. The National Veterinary Services Laboratories (NVSL) was been requested to establish a standardized protocol for detecting *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in fecal samples which is reproducible and has a known sensitivity.

The NVSL have also been requested to establish the criteria for well-characterized bovine fecal panels for use in organism-based detection procedures and methods evaluation. These panels will be used to validate different diagnostic procedures, including serological assays and USDA licensed diagnostic kits used for Johne's disease detection.

The goals of the NVSL Laboratory approval program for Johne's disease include the following: (1) standardize the methods for antibody and organism based tests; (2) provide information to the laboratory on their own testing performance in relation to other labs; (3) maintain a list of approved laboratories for state programs and producers who participate in the VBJDCP; and (4) establish minimum standards for diagnostic testing and interpretation of Johne's disease test results in different species of animals. The Johne's Disease Laboratory Approval Program is voluntary. Failure on the check tests does not prevent testing by the participating laboratories. The VBJDCP requires states to use NVSL approved laboratories for testing samples. Annual participation is required to maintain approved status.

MATERIALS AND METHODS

Laboratory participation has increased every year since the program started in 1996. Laboratories from the United States, Canada, The Netherlands, Denmark and Sweden have participated during the last 8 years (Table 1). The fecal test panel consisted of approximately 25% negative (autoclaved) samples, 25% TNTC (too numerous to count) and the remaining 50% with different levels of organisms that were less than 100 CFU per sample (Table 2).

Table 1. Check test participants

	1997	1998	1999	2000	2001	2002	2003	2004
No. labs	33	41	48	48	52	60	64	72
U.S.	32	40	44	42	48	55	56	64
Canada	1	1	3	5	4	4	6	5
Netherlands	0	0	1	0	0	1	0	0
Denmark	0	0	0	1	0	0	2	2
Sweden	0	0	0	0	0	0	0	1

Table 2. Fecal panel contents

No. cfu	1997	1998	1999	2000	2001	2002	2003	2004
Negative	10	4	7	6	6	6	7	7
Positive	20	16	18	19	19	19	18	18
> 50	7	7	5	6	10	6	8	8
11-49	7	3	8	4	4	3	5	6
1-10	6	6	5	9	5	10	5	4

The criteria for laboratory approval included the following: (1) a laboratory must identify 100% of the negative samples – no false positives; (2) a laboratory must identify 100% of the TNTC (too numerous to count) positive samples – no false negatives; (3) a laboratory must identify 70% of the remaining positive samples which had 1-50 organisms; (4) the laboratory should have a minimum score of 85%; (5) a valid sample is one in which 70% of the laboratories had a consensus of agreement.

Each laboratory submitted 12 tubes of HEYM for evaluation. Each tube of media was evaluated for pH, sterility and growth performance. The recommended pH was 7.0 to 7.5. Sterility was checked at 24 and 48 hours and weekly for 6 weeks. Growth performance was checked weekly for 6 weeks. HEYM was inoculated with 0.5 McFarland standard cultures of *Mycobacterium paratuberculosis* ATCC 19698, *M. avium* ATCC 25291 and *M. intracellulare* ATCC 13950. The following criteria were used to grade the growth on each tube: P +1 = 1-10 cfu, P+2 = 11-30 cfu, P+3 = 31-50 cfu, P+4 = 51 to TNTC cfu.

RESULTS

Preliminary evaluation based on proficiency test results indicate that centrifugation methods are more sensitive than sedimentation methods based on a comparison of colony counts on each sample. Liquid culture methods are faster than either sedimentation or centrifugation methods using HEYM since their incubation times are 6 weeks as compared to 16 weeks, respectively. (Table 3 and Table 4). Each year

more laboratories are submitting results based on PCR direct on feces and the number of approvals has increased each year (Table 5).

Table 3. Laboratory approval for organism based tests – fecal culture

	1997	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**
No. Labs approved	26	16	33	35	45	50	47	56
No. Labs Not approved	2	12	11	6	2	12	5	8

Table 4. Fecal culture by methods

	1999	**2000**	**2001**	**2002**	**2003**	**2004**
Methods approved	33	35	45	50	57	69
Centrifugation	21	29	36	40	40	42
Sedimentation	11	4	6	4	5	4
BACTEC 460	1	2	2	1	4	3
TREK ESP	0	0	1	5	7	15
MGIT 960	0	0	0	0	1	5
Methods not approved	11	6	2	12	5	8
Centrifugation	5	5	2	10	4	7
Sedimentation	4	1	0	1	0	0
TREK ESP	0	0	0	0	0	0
BACTEC 460	2	0	0	1	1	1
MGIT 960	0	0	0	0	0	0

Table 5. Laboratory approval for organism based tests – PCR direct on feces

	1997	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**
Labs approved	1	0	2	4	8	8	7	15
Labs not approved	5	4	1	2	2	8	6	5

Varied growth performances in the solid media used with different culture methods were also noted during the last 6 check tests. Media which was made by the laboratory (In-house) varied in the color, pH, and growth performance compared to the commercially prepared media. There are now 2 commercial sources of HEYM available in the United States which is evaluated along with in-house media for growth performance. Laboratories that used media produced by Becton Dickinson (BD) only had to submit the lot numbers that were used because the NVSL obtained the quality control data for each lot from BD. The number of laboratories that participate in the media evaluation has decreased over the last 3 years because the majority of U.S. laboratoriesuse BD HEYM or have switched to a liquid media system (Table 6).

Table 6. Media evaluation of non commercially made media (in-house)

	1999	**2000**	**2001**	**2002**	**2003**	**2004**
No. labs	12	20	21	8	8	5
Sterility	12 OK	20 OK	21 OK	8 OK	8 OK	5 OK
pH test	7-7.5	6.85-7.57	6.62-7.19	6.8-7.3	6.9-7.2	7.0
Growth performance	P+2 to P+4	P+2 to P+4	P+2 to P+4	P+4	P+1 to P+4	P+1 to P+4

The list of NVSL approved laboratories is published each year in the United States Animal Health Association Proceedings – Johne's Committee report and posted on the NVSL web site: http://www.aphis.usda.gov/vs/nvsl/labcertification/johnesculture.htm

CONCLUSIONS

Based on the results of the last three years of proficiency tests, several methods have been chosen for further evaluation. These methods include sedimentation, centrifugation, and liquid culture procedures (BACTEC 460, TREK ESP, and BACTEC MGIT 960) which have been used by multiple laboratories. The NVSL are currently evaluating inoculum volume, centrifugation speeds, and HEYM tubes versus HEYM

flasks, specimen storage time, antibiotic solutions, decontamination methods, CO_2 and *Map* strain differentiation.

Based on the media evaluation of the last six years, HEYM (produced by 2 commercial companies, Remel & BD) are used by the majority of the participating laboratories. BD HEYM appears to isolate more MAP organisms (>100 cfu / tube) than Remel HEYM in comparison testing by several laboratories and on the media performance test. HEYM produced in-house by other laboratories has more varied results with fewer organisms being isolated (10-100 cfu).

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SYBR Green and TaqMan assays for sensitive detection and differentiation of *Mycobacterium avium* subsp. *paratuberculosis* from other mycobacteria

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ABSTRACT

Sensitive real-time sequence detection methods based on two different chemistries were developed for *Mycobacterium avium* subsp. *paratuberculosis* (MAP). One is based on the detection of SYBR Green bound to PCR products and the second method is more specific, detecting the cleavage of a fluorogenic (TaqMan) probe bound to a target sequence during primer extension phase. Novel primers and probes that amplify small fragments (<80 bp) of the MAP specific insertion sequence, IS*900* were designed. Both the SYBR green and TaqMan assays are sensitive, able to detect 3 to 4 fg of DNA extracted from MAP strain ATCC19698. This amount of DNA corresponds to the detection of 0.6 to 0.8 cells per assay. MAP cells were quantified directly from 7H9 broth using the SYBR Green assay and compared to dilutions of DNA extracted from an equivalent number of cells. The SYBR Green assay of 7H9 broth resulted in a minimum detectable limit of 0.07 cells (equivalent to 0.33 fg of DNA). Media ingredients were not observed to interfere with the assay. Since no extraction step was necessary in the direct cell measurements, direct detection was ten fold more sensitive than detection of extracted DNA. Both SYBR Green and TaqMan assays are highly specific for the detection of MAP. They did not detect any closely related members of the avium complex, other species of mycobacteria, or related genera that are likely to be present in environmental samples. No reporter signal was detected during TaqMan assays performed with 100 pg of template DNA from the non-MAP organisms. As extractions are not required, direct cell assays can be easily adopted for environmental monitoring either with or with out pre-enrichments. Sensitive detection might alleviate some of the problems related to PCR inhibitors by direct-dilution and yet detect low levels of MAP from environmental samples.

Key words: real time PCR, TaqMan, quantitation,detection, SYBR Green; *Mycobacterium avium* subsp. *paratuberculosis*

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease in cattle, is responsible for a $250 million annual loss to the US dairy industry (Ott et al., 1999). Such losses can be minimized by early detection and culling of infected cattle and by preventing the oral-fecal routes of transmission, as the pathogen survives in feces for longer than a year (Whittington et al., 2004). Early detection of infection is difficult since the fecal culture methods, the current gold standard, can take as long as 4 to 6 months to confirm the presence of pathogens.

MAP is known to be associated with Crohn's disease in humans and the evidence to support the possible causation of Crohn's by MAP is growing rapidly (Bull et al., 2003; Chamberlin et al., 2001; Greenstein, 2003; Hulten et al., 2001; Naser et al., 2004; Ryan et al., 2002; Sechi et al., 2004). Recently, MAP has been cultured from the blood of Crohn's patients (Naser et al., 2004) and its presence in paraffin embedded Crohn's tissues was confirmed by IS*900* in situ hybridization technique (Sechi et al., 2004). Although alternate etiologies for causation of Crohn's have been proposed, one cannot ignore the association of

MAP with Crohn's disease. A suspected route of infection may be through drinking raw or insufficiently pasteurized milk. However, MAP is ubiquitous in the environment and its presence in municipal water supplies has been documented (Mishina et al., 1996). Thus, potable water could be an alternate source of infection.

MAP was isolated from two-thirds of manure lagoons from herds known to be culture positive (Raizman et al., 2004) and persists in bovine feces as long as 11 months (Vishnevskii et al., 1940). Irrigation of crops with manure wastewater and fertilization with manure increases the risk of MAP transmission through the consumption of contaminated fruits and vegetables.

The wide host diversity (cow, sheep, goat, deer, rabbit, mouse) of MAP (Amonsin et al., 2004) also increases the likelihood of environmental contamination. MAP persists in the environment and is known to survive for longer than a year in inoculated grass pastures (Whittington et al., 2004). Lack of rapid and sensitive methods hitherto hindered the efforts to monitor the environmental fate and transfer of MAP from animal manure to crops.

Molecular detection methods based on sequence detection of IS*900*, a 1451 bp repetitive DNA element (Bull et al., 2000), have been developed for the detection of MAP. Many of these methods are based on the detection of amplification by end-point PCR (Jayarao et al., 2004; Marsh and Whittington, 2001; O'Mahony and Hill, 2002; Pillai and Jayarao, 2002). More sensitive real-time quantitative PCR (qPCR) methods that detect amplification during the exponential phase of amplification (Kim et al., 2002; O'Mahony and Hill, 2002) also have been reported. These methods are based on the most widely used primers designed to amplify a 229 bp (Vary et al., 1990) or a 413 bp (Millar et al., 1996) fragment of IS*900*. Quantitative PCR methods are based on the fluorescence detection of SYBR Green bound double stranded DNA that is formed during amplification (O'Mahony and Hill, 2002), the detection of bound fluorescent probes (molecular beacons) (Christopher-Hennings et al., 2003; Fang et al., 2002), or detection of the 5' nuclease cleavage of a bound fluorogenic (TaqMan) probe (Kim et al., 2002) by *Taq* DNA polymerase. Molecular beacons and TaqMan probes are highly specific for the detection of target sequences.

The presence of 12 to 20 copies of IS*900* in the MAP genome makes this unique genetic element a better target for sensitive detection as compared to the detection of single copy genes (Chui et al., 2004) like *hspX* (Stabel et al., 2004) and F57 (Coetsier et al., 2000). However, the two most popular primer sets (Millar et al., 1995; Vary et al., 1990) used to detect the IS*900* region overlap regions of high homology (IS1626) with *M. avium ss. avium* (Harris and Barletta, 2001). This overlap often requires additional culture steps or detection of MAP specific sequences to confirm the presence of MAP.

Application of molecular methods for detection from fecal samples often requires clean template DNA (Fus et al., 2003; Khare et al., 2004; O'Mahony and Hill, 2002; Ozbek et al., 2003; Stabel et al., 2004) necessitating elaborate DNA extraction and purification steps during which the precious template material could be easily lost. The capsular structure of MAP was suspected to be inhibitory for the direct detection of cells by PCR (Fus et al., 2003) and DNA was routinely purified prior to detection by PCR. Sensitive methods that minimize loss of template DNA during processing are required to detect the low levels of MAP anticipated in environmental samples.

This study focuses on developing sensitive real-time quantitative PCR methods to detect MAP by using SYBR green and TaqMan assays and to selectively differentiate MAP from other closely related mycobacteria that may be present in environmental samples. Novel primers and probes targeting the amplification of short segments from different regions of IS*900* were evaluated. The wide range of primers chosen allowed us to find a selective target for improved sensitivity of detection of MAP. Dilutions of culture media were evaluated for the direct detection of MAP cells, minimizing the loss of template DNA during extraction and cleanup, and reducing the processing time for PCR.

METHODS

Mycobacterial cultures
Mycobacterium species and other related organisms were grown in 50 mL of 7H9 broth supplemented with 10% (vol/vol) Middlebrook OADC (Becton Dickinson, Franklin Lakes, NJ), 0.5% Tween 80 and 0.0002% (wt/vol) mycobactin J (Allied Monitor Inc., Fayette, MO) (Sung and Collins, 2000). The growth medium was supplemented with 100 μg/mL each of nalidixic acid and vancomycin, and 50 μg/mL of amphotericin B to minimize the contaminant growth during the 2 to 4 month long incubations. The cultures were incubated at 37°C on a shaker at 200 rpm. MAP cultures were routinely checked for purity by staining with Ziehl-Neelsen's acid fast stain or auramine-rhodamine fluorescent stain.

Extraction and quantitation of DNA
The template DNA used for real-time detection of target sequences was extracted with InstaGene^{TM} matrix according to the manufacturer's (Bio-Rad Labs, Hercules, CA) protocol. Briefly, a 1-mL culture was centrifuged, the pellet was resuspended in 200 L InstaGene matrix, vortexed, and incubated at 56°C for 30 min. The samples were vortexed again, heated at 100°C for 8 min, and then centrifuged to pellet the matrix. Aliquots of supernatants were used as template DNA without any further purification. The extracted DNA was quantitated using the PicoGreen$^®$ dsDNA quantitation kit (Molecular Probes, Inc., Eugene, OR) and by following the manufacturer's protocol. A DNA standard curve for 0 to 40 ng/200 L assay volume was prepared using the lambda DNA standard provided with the PicoGreen$^®$ kit. Assays were performed in 96 well microplates and the fluorescence was measured with a SpectraMax$^®$ Gemini microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

SYBR Green assay
PCR reactions were carried out in 50 μL volumes containing 300 nM of each primer, 100 pg of template DNA, and 15.75 μL of 10X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) resulting in 3mM MgCl$_2$, 200 μM each of dATP, dCTP and dGTP, 400 μM dUTP, 1.25 U AmpliTaq Gold$^®$ DNA polymerase, and 0.5 U AmpErase$^®$ uracil-N-glycosylase (UNG). PCR reaction mixture was prepared according to the manufacturer's protocol. An ABI PRISM$^®$ 5700 Sequence Detection System (Applied Biosystems) was used for real time detection of amplified dsDNA with SYBR Green. Thermal cycling parameters were according to manufacturer's instructions. Briefly, AmpErase UNG incubation (50°C for 2 min), followed by 95°C for 10 min to activate AmpliTaq Gold, and 40 cycles of: 95°C for 15 sec and 60°C for 1 min. Specific amplification of target DNA was monitored by comparing the normalized reporter signal (SYBR Green) for a fast threshold cycle (C_T) and the signal obtained for controls without template DNA (NTC). The determination of C_T values was according to the sequence detection system manufacturer's instructions.

Primers for SYBR Green assay
Primers targeting 50 to 60 bp fragments from different regions of IS*900* were designed using the Primer Express software (v2.0; Applied Biosystems). Twenty-six sets of primers (Integrated DNA Technologies, Inc., Coralville, IA) with T_m 58-60°C were evaluated at *900*nM in a 50 μL PCR mixture containing 100 pg of template DNA from MAP strain ATCC 19698. Each primer set was evaluated in two replicates and the C_T values obtained were compared with C_T's for NTC. Optimum concentrations of selected primers were determined with combinations of 50, 300 and *900* nM of each primer in 50 μL reaction volumes containing 100 pg of template DNA from MAP strain 19698.

Direct detection of MAP cells as compared to extracted DNA
Ten-fold serial dilutions of cells from a 6-week old culture of MAP strain 19698 were assayed directly by the SYBR Green assay. Triplicate samples were diluted in sterile PBS (0.01 M phosphate, 0.14 M NaCl) supplemented with 0.25% Tween 80. The cell numbers used for qPCR assays were determined by direct microscopy and by plate counting. The plate counts were obtained using deep (50 mL) plates of 7H10 agar (Becton Dickinson) supplemented with or without antibiotics. The 7H10 agar contained 10% OADC and 0.0002% mycobactin J. The antibiotic supplements of 50 μg/mL each of amphotericin B, nalidixic acid, and

vancomycin were added to minimize the non-mycobacterial contaminants during the 5 month long incubations at 37°C. The plates were sealed with parafilm to minimize the drying of agar during the incubations. Purity of cells was checked by acid-fast staining. Triplicate 1-mL portions of the growth were used for extraction of template DNA with InstaGene matrix. Ten fold serial dilutions of the InstaGene extracts were prepared in sterile ultrapure water. Five μl portions of each of the serial dilutions of template DNA and the serial dilutions of cells were used for qPCR detection. C_T values and corresponding number of cells or the quantity of template DNA were plotted. Minimum detectable limits for template DNA or number of cells were calculated from the regression equation for the C_T value of NTC subtracted with two standard deviations.

TaqMan assays
Primers and probes were designed with Primer Express. Probes tagged with 6-carboxyfluorescein (FAM) as the 5'-reporter dye and quenched with 6-carboxytetramethylrhodamine (TAMRA) at the 3' end, and corresponding primers were obtained from Integrated DNA Technologies. Thermal cycling conditions for primer and probe optimizations were the same as those used for the SYBR Green assays. Probes were optimized at 25 nM increments from 25 to 225 nM. The primers were held a constant concentration of 300 nM during probe optimizations. In addition to the primers and probe, the 50 μL of PCR reaction mixture also contained 25 μL of TaqMan® Universal Master mix (Applied Biosystems) and 100 pg of template DNA from MAP strain 19698. Probes at 75 nM and primers at 300nM were used in assays to quantify the dilutions of template DNA and to determine the specificity of the IS*900* TaqMan assay to MAP strains. One hundred pg of template DNA from different species of mycobacteria was used in the specificity assays.

Table 1. Detection of extracted DNA from MAP strain 19698 using specific primers to amplify short fragments from IS*900* by SYBR Green assay

Primers[a]	Forward/reverse sequences (5' → 3')	Ampli-con[c]	C_T[b]
SF56/SR109	GGACGTCGGGTATGGCTTT/AATCTCCTTCGGCCATCCA	54	19.0±0.1
SF57/SR109	GACGTCGGGTATGGCTTTCAT/AATCTCCTTCGGCCATCCA	53	18.8±0.4
SF58/SR109	ACGTCGGGTATGGCTTTCAT/AATCTCCTTCGGCCATCCA	52	18.5±0.2
SF59/SR109	CGTCGGGTATGGCTTTCATG/AATCTCCTTCGGCCATCCA	51	19.0±0.1
SF187/SR239	TGGTCGTCTGCTGGGTTGA/GCCACAACCACCTCCGTAAC	53	18.6±0.0
SF187/SR240	TGGTCGTCTGCTGGGTTGA/TGCCACAACCACCTCCGTA	54	18.6±0.0
SF188/SR239	GGTCGTCTGCTGGGTTGATC/GCCACAACCACCTCCGTAAC	52	18.6±0.1
SF188/SR240	GGTCGTCTGCTGGGTTGATC/TGCCACAACCACCTCCGTA	53	18.8±0.0
SF189/SR239	GTCGTCTGCTGGGTTGATCTG/GCCACAACCACCTCCGTAAC	51	18.5±0.1
SF189/SR240	GTCGTCTGCTGGGTTGATCTG/TGCCACAACCACCTCCGTA	52	18.5±0.0
SF190/SR240	TCGTCTGCTGGGTTGATCTG/TGCCACAACCACCTCCGTA	51	18.8±0.0
SF199/SR252	ATGATCGAGGAGCGATTTCG/CCAGACAGGTTGTGCCACAA	54	19.1±0.0
SF200/SR252	GGTTGATCTGGACAATGACGG/CCAGACAGGTTGTGCCACAA	53	19.1±0.1
SF729/SR783	GGAACGCGCCTTCGACTAC/GCCAGTAAGCAGGATCAGCG	55	19.1±0.2
SF730/SR783	GAACGCGCCTTCGACTACAA/GCCAGTAAGCAGGATCAGCG	54	19.2±0.3
SF731/SR783	AACGCGCCTTCGACTACAAC/GCCAGTAAGCAGGATCAGCG	53	19.0±0.1
SF741/SR797	CGACTACAACAAGAGCCGTGC/TCGGGAGTTTGGTAGCCAGTA	57	18.1±0.1
SF741/SR798	CGACTACAACAAGAGCCGTGC/GTCGGGAGTTTGGTAGCCAG	58	18.7±0.6
SF742/SR797	GACTACAACAAGAGCCGTGCC/TCGGGAGTTTGGTAGCCAGTA	56	18.7±0.0
SF742/SR798	GACTACAACAAGAGCCGTGCC/GTCGGGAGTTTGGTAGCCAG	57	18.7±0.1
SF745/SR797	TACAACAAGAGCCGTGCCG/TCGGGAGTTTGGTAGCCAGTA	53	19.2±0.2
SF745/SR798	TACAACAAGAGCCGTGCCG/GTCGGGAGTTTGGTAGCCAG	54	19.2±0.0
SF965/SR1025	AGGTGATGGCCCTCGACAC/CGAAATCGCTCCTCGATCAT	61	21.3±0.3
SF1003/SR1057	GCGATGATCGAGGAGCGAT/TGCTCAGGATGATTTCGGC	55	22.7±0.4
SF1006/SR1057	ATGATCGAGGAGCGATTTCG/TGCTCAGGATGATTTCGGC	52	19.6±0.6
SF1280/SR1334	CCTCCTCGCGCACCTACTAC/TGGGTGTGGCGTTTTCCTT	55	19.0±0.1

[a] Forward and reverse primers are numbered based on GenBank accession number AJ250017 GI:8919133 (Bull et al., 2000). The repeat region from 492 to 1942 bp (IS*900*) of the 5' end is numbered as 1-1451 and used in the primer notations. Primer sets with low penalty scores were chosen with Primer Express. Primers evaluated at *900*nM each. [b] Average of two replicates. One hundred picograms of template DNA from MAP 19698 was assayed. C_T value for NTC's ranged between 30 and 38. [c] Length, bp

RESULTS

Primers for IS900 detection by SYBR Green method
As the real time PCR detection methods are highly sensitive and consistent for shorter amplicons than conventional PCR coupled with detection on gels, 26 primer sets that target 50 to 60 bp fragments of IS*900* (Table 1) were evaluated for the detection of DNA from MAP strain 19698. Except for two primer sets SF965/SR1025 and SF1003/SR1057, all primers were similarly sensitive in detecting DNA from MAP strain 19698. Although they all had similar C_T values for template detection, C_T value for NTC's ranged from 30 to 38. Nearly half of the primer sets had C_T's for NTC ranged between 35 and 38. Two sets of primers from this batch, SF58/SR109 and SF187/SR239, were chosen for further evaluation.

Specificity of detection of MAP by IS900-SYBR Green method
MAP strains were differentiated from the other members of the avium complex, other species of *Mycobacterium*, and related genera (*Nocardia, Corynebacterium, Rhodococcus*) by both primer sets (Table 2). The C_T values obtained for non-MAP organisms were indistinguishable or within 2 to 4 cycles of NTC, whereas MAP strains were detected approximately 20 cycles sooner. However, variability in detection of amplified DNA was observed with different strains of MAP with both primer sets evaluated. C_T values for different strains ranged between 15 to 28 for SF58/SR109 and 13 to 19 with SF187/SR239. These results suggest that primer set SF187/SR239 readily detects all of the MAP strains tested, whereas primer set SF58/SR109 poorly detected strain 1040.

Table 2. Specificity of SYBR Green assay for *Mycobacterium avium* subsp. *paratuberculosis*

	ATCC[#a]	Origin	Normalized threshold cycle, C_T[d]	
			SF58/SR109	SF187/SR239
MAP	1038[b]	Bovine	14.8 $\pm$ 0.2	15.2 $\pm$ 0.0
MAP	5016[b]	Bovine	15.3 $\pm$ 0.1	13.4 $\pm$ 0.0
MAP	1040[b]	Bovine	27.8 $\pm$ 0.3	14.4 $\pm$ 0.0
MAP	19698	Bovine	14.9 $\pm$ 0.1	19.1 $\pm$ 0.1
MAP	KAY[b]	Bovine	18.8 $\pm$ 0.0	18.1 $\pm$ 0.1
MAP	43015	Human	14.9 $\pm$ 0.3	17.1 $\pm$ 0.3
M. avium subsp. *avium*	25291	Chicken	33.4 $\pm$ 0.3	33.6 $\pm$ 0.7
M. avium subsp. *silvaticum*	49884	Wood pigeon	34.8 $\pm$ 0.8	34.0 $\pm$ 0.4
M. senegalense	35796	Bovine	34.9 $\pm$ 0.1	35.5 $\pm$ 0.2
M. bovis	19210	Bovine	34.4 $\pm$ 0.9	32.1 $\pm$ 0.1
M. vaccae	15483	Bovine	35.3 $\pm$ 0.2	35.1 $\pm$ 0.5
M. farcinogenes	35753	Bovine	NT[c]	35.0 $\pm$ 0.4
M kansasii	12478	Human	34.1 $\pm$ 0.7	36.8 $\pm$ 0.1
M. fortuitum subsp. *fortuitum*	6841	Human	33.2 $\pm$ 0.1	35.4 $\pm$ 0.2
M. smegmatis	19420	Human	34.1 $\pm$ 0.5	35.2 $\pm$ 0.1
M. intracellulare	13950	Human	34.1 $\pm$ 0.3	35.3 $\pm$ 0.3
M. tuberculosis	27294	Human	33.7 $\pm$ 0.4	34.9 $\pm$ 0.5
M. scrofulaceum	19981	Human	32.7 $\pm$ 0.3	31.7 $\pm$ 0.1
M. porcinum	33776	Swine	34.7 $\pm$ 1.2	36.5 $\pm$ 1.4
M. phlei	11758	Hay/grass	36.0 $\pm$ 1.1	35.3 $\pm$ 0.8
M. diernhoferi	19340	Dairy water trough	35.8 $\pm$ 0.4	35.3 $\pm$ 0.1
M. fallax	35219	Water	NT[c]	34.8 $\pm$ 0.1
M. chlorophenolicum	49826	Lake sediment	32.3 $\pm$ 0.0	33.2 $\pm$ 0.4
M. thermoresistibile	19527	Soil	32.0 $\pm$ 0.3	35.1 $\pm$ 0.2
M. aurum	23366	Soil	34.6 $\pm$ 1.2	38.2 $\pm$ 0.3
M. nonchromogenicum	19530	Soil	35.6 $\pm$ 0.8	34.7 $\pm$ 0.7
M. austroafricanum	33464	Soil	34.8 $\pm$ 0.5	35.2 $\pm$ 1.0
M. neoaurum	25795	Soil	33.6 $\pm$ 0.2	NT[c]
Corynebacterium bovis	7715	Bovine	37.9 $\pm$ 1.5	36.7 $\pm$ 0.5
Nocardia asteroides	19247	Human/soil	35.3 $\pm$ 1.0	34.0 $\pm$ 0.3
Rhodococcus fascians	12974	Plants	NT[c]	33.1 $\pm$ 0.3

[a] Except for the 4 MAP strains from NADC[b], all other strains are ATCC type strains obtained from American Type culture Collection (Manassas, VA); [b] NADC strains are from National Animal Disease Center, Ames, IA.
[c] NT- not tested; [d] C_T values are averages of triplicates. The reaction mixture contained 100 pg of template DNA and 300nM of each of the primers. C_T values of NTC's for primers SF58-SR109 and SF187-SR239 were 35 $\pm$ 1 and 37 $\pm$ 3, respectively.

*Sensitivity of detection of MAP by IS900-SYBR Green qPCR assay*
Tenfold serial dilutions of template DNA from MAP strain 19698 were assayed to determine the sensitivity of detection by these two different primer sets (Fig.1). Minimum detectable limits of 3.1 and 4.5 fg of DNA were obtained for SF58/SR109 and SF187/SR239, respectively. C_T values of NTC minus 2 standard deviations used in these calculations were 33.8 and 37.4 for primers

SF58/SR109 and SF187/SR239, respectively (Fig. 1). Amplicons generated with SF58/SR109 were detected ~ 4 cycles sooner than SF187/SR239. Thus, 100 fg of template DNA was detected in 28.5 and 32.7 cycles with primers SF58/SR109 and SF187/SR239, respectively.

SYBR Green detection of cells as compared to extracted DNA.
Cells and template DNA used for the assay
Six-week-old cultures of MAP strain 19698 in 7H9 broth were used for the direct detection of cells by SYBR Green qPCR assay using primers SF187/SR239. Template DNA extracted from the same batch of cells was assayed to compare with the cell assays. Clumping of cells was minimized by including 0.5% Tween 80 in the growth medium and also by rapidly mixing the cultures during incubations. The cell growth was monitored at the end of incubations by two methods. Direct plating on deep 7H10 agar plates with and without antibiotics resulted in $1.7\underline{+}0.7 \times 10^5$ and $2.2\underline{+}1.0 \times 10^8$ CFU/mL. The template DNA extracted with InstaGene matrix from one mL of cell culture was $1120 \underline{+} 6$ ng, corresponds to 5.1 fg of DNA extracted per cell. Direct plate counts from 7H10 agar without antibiotics were used for calculating the amount of DNA extracted per cell. Antibiotics in the plating medium resulted in a 3-log reduction of cell count and these counts were not used in the calculations to determine the amount of extracted DNA per cell.
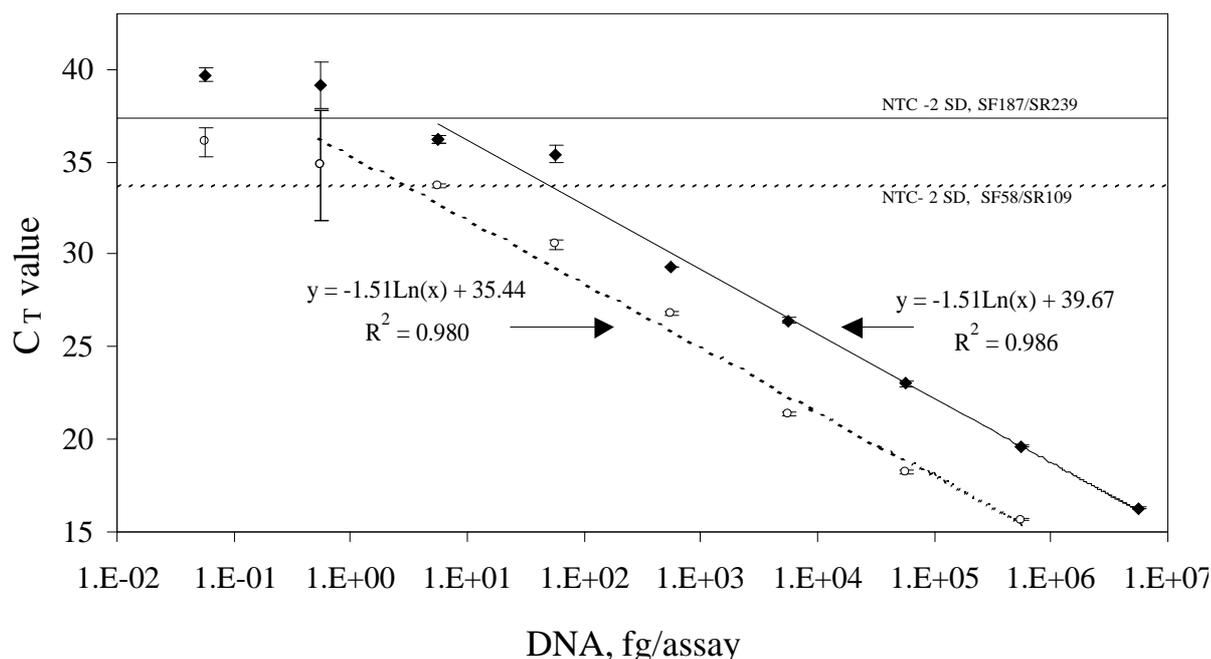
Fig. 1. SYBR Green assay of serial dilutions of DNA extracted from MAP strain 19698. C_T values are plotted against fg of template DNA per reaction using primers SF187/SR239 (◆) and SF58/SR109 (○). Standard deviations are for triplicate assays. Lines are drawn across at C_T values 37.4 and 33.8 to show the limits of detection for primers SF187/SR239 (solid line) and SF58/SR109 (dashed line).

Comparison of detection of cells and extracted DNA
Ten-fold serial dilutions of cells versus template DNA from MAP strain 19698 were assayed by the SYBR Green method using primer set SF187/SR239 (Fig. 2). This primer set was chosen for its low reporter signal obtained for NTC (Fig. 1; C_T value for NTC = 39.4). In the cell assay method, 5 μL of serial dilutions of cell culture in PBS-Tween 80 containing 1.1×10^{-2} to 1.1×10^6 cells was used. Serial dilutions containing less than a cell were assayed to detect the release of template DNA from cells into the medium. Serial dilutions of extracted DNA ranging from 5.6×10^{-2} to 5.6×10^6 fg were tested in parallel. The extracted DNA dilutions correspond to the equivalent number of cells in the direct cell assay. An inverse relationship was

observed between C_T values and log cell number ($R^2 = 0.982$) and log amount of template DNA ($R^2 = 0.988$) assayed. The minimum detectable limit of the direct cell assay calculated from the regression equation was 0.07 cells. This equals 0.34 fg of extracted DNA. Detection of less than a cell was possible as the dilutions of the growth medium contained both intact and broken cells. The minimum detectable limit for the extracted DNA assays was 3.4 fg, which is calculated to equal 0.7 cell. A value of 5.1 fg of DNA extracted per cell was used in these calculations. Controls without cells or template DNA had a C_T of 39.4 ± 1.0.

qPCR detection by TaqMan method
Primers and probes
Three sets of primers and probes were designed and evaluated for the detection of IS*900* from extracted DNA (Table 3). All three sets were equally sensitive for the detection of 100 pg of DNA from MAP strain 19698. The primer/probe/primer set SF214/PR265/SR289 was chosen for subsequent assays as the C_T for NTC was >40 cycles and it yielded a lower C_T (21.1 ± 0.8) for the detection of MAP DNA. All three probes were equally effective at all concentrations between 25 to 225 nM. Probe concentration of 75 nM was chosen for all subsequent assays to accommodate detection of high levels of template DNA.
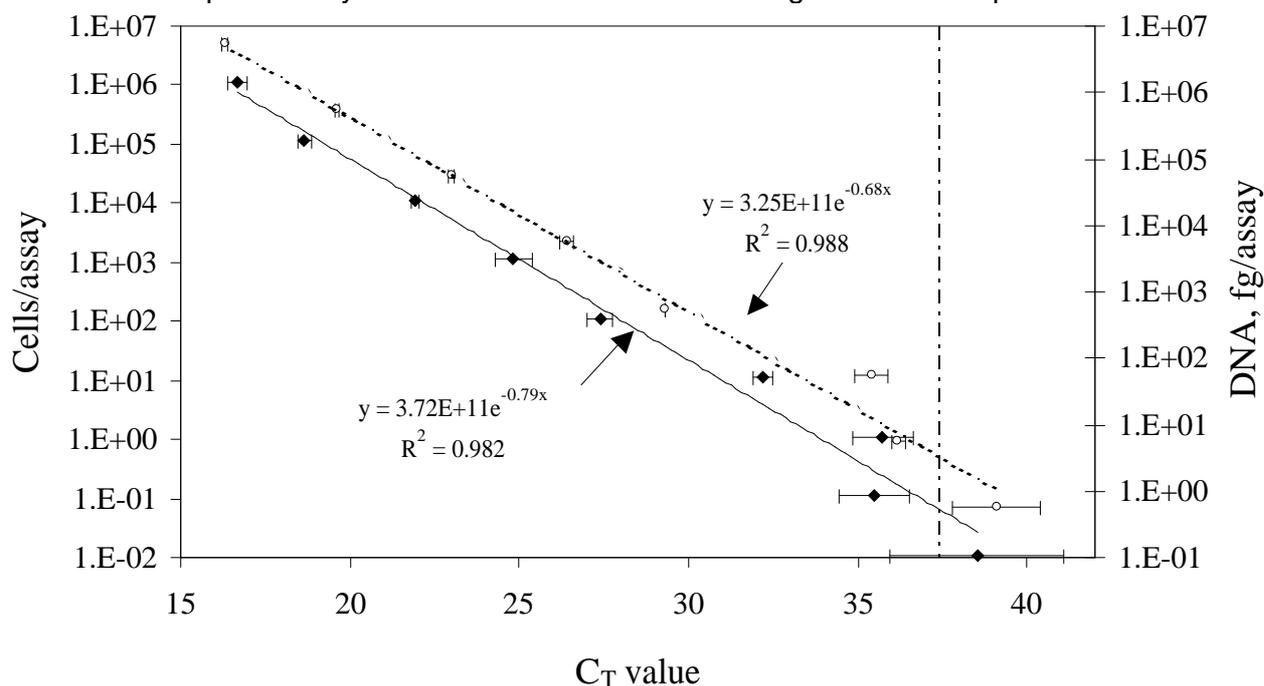
Fig. 2. Detection of cells of MAP strain 19698 as compared with DNA extracted from an equivalent batch of cells by SYBR green assay. C_T values are plotted against number of cells (♦) and fg template DNA (○). A primer concentration of 300 nM each of SF187 and SR239 were used in both cell and DNA assays. A dashed line at C_T value 37.4 (NTC minus 2 standard deviations) represents the limit of detection for both cell and DNA assays.

Table 3. Primer/probe sets used in TaqMan assays

Primer/[a]Probe	Forward/probe/reverse(5' → 3')	Length, bp	C_T[b]	C_T- NTC[c]
SF30	CGTCGCTTAGGCTTCGAATT	80	22.5 ± 0.4	38.9 ± 0.9
PR72	AGCCATACCCGACGTCCCTGGG			
SR109	AATCTCCTTCGGCCATCCA			
SF53	CAGGGACGTCGGGTATGG	67	21.6 ± 0.6	37.4 ± 1.6
PR97	CCATCCAACACAGCAACCACATGAA			
SR119	GCGGGCGGCCAATC			
SF214	ATGACGGTTACGGAGGTGGTT	76	21.1 ± 0.8	>40[d]
PR265	CGACCACGCCCGCCCAGA			
SR289	TGCAGTAATGGTCGGCCTTAC			

[a] Numbering of primers and probes were based on GenBank accession AJ250017, GI:8919133 (see Table 1). Probes designed for the reverse strand.; [b] C_T values for template DNA. Probes evaluated in triplicates of 6 concentrations ranging from 25 to 225nM. Average of 18 assays; [c] C_T values for NTC. Triplicate NTC's tested for each concentration of probe. Average C_T values for 18 NTC's; [d] No signal for NTC during the 40 cycles of amplification.

Specificity of TaqMan assay

TaqMan assays with primer/probe/primer SF214/PR265/SR289 were specific to MAP and not to any other species of mycobacteria or related genera (Table 4). DNA from other organisms gave no signal during 40 amplification cycles. Variability in C_T values was observed for the detection of same amount of template DNA from different strains of MAP. It ranged between 17 to 30 cycles to detect 100 pg of template. DNA from MAP strain KAY required most number of amplification cycles to detect.

Table 4. Selectivity of TaqMan assays for the detection of MAP and related organisms.

	C_T [a]
MAP 19698	21.7 ± 0.1
MAP 5016	17.4 ± 0.2
MAP 1040	22.4 ± 0.1
MAP 1038	24.9 ± 0.2
MAP KAY	30.1 ± 0.1
MAP 43015	19.9 ± 0.2
M. avium subsp. *silvaticum*	>40
M. avium subsp. *avium*	>40
M. intracellulare	>40
M. bovis	>40
M. tuberculosis	>40
M kansasii	>40
M. aurum	>40
M. austroafricanum	>40
M. phlei	>40
M. vaccae	>40
M. diernhoferi	>40
M. smegmatis	>40
M. thermoresistibile	>40
M. senegalense	>40
M. neoaurum	>40
M. porcinum	>40
M. fortuitum subsp. *fortuitum*	>40
M. nonchromogenicum	>40
Nocardia asteroides	>40
Corynebacterium bovis	>40

[a] Assayed with primer/probe set SF214/PR265/SR289. Reaction mixture contained 100 pg template DNA, 300 nM of each primer and 75 nM of probe. No signal detected for NTC during 40 cycles.
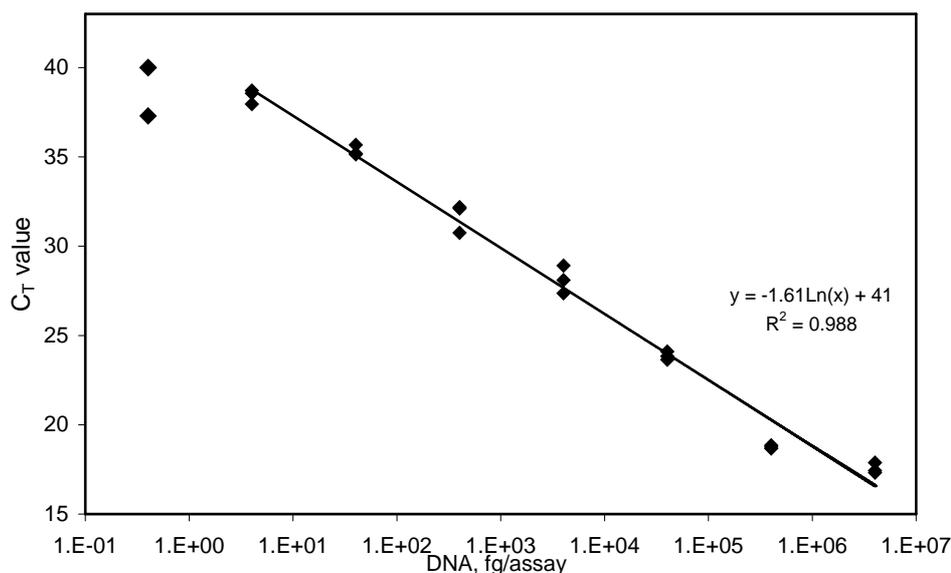
Fig. 3. Detection of template DNA from MAP strain 19698 by TaqMan (◆) assay. C_T values are plotted against fg template DNA. The assays are with SF214/PR265/SR289 primer/probe set. The reaction mixtures contain 300 nM each of primers and 75 nM of the probe.

Sensitivity of detection of MAP by the IS900-TaqMan assay
Dilutions of extracted DNA from MAP strain 19698 were assayed using primer/probe/primer SF214/PR265/SR289 and the resultant C_T's were plotted against the amount of template DNA (Fig.3). A linear relationship was observed between C_T values and log template DNA (R^2 = 0.988) concentration. As signal was not detected for NTC, the minimum detectable limits were based on actual measurements. The lowest amount of detected DNA from the slope of regression curve was chosen as the limit. The detection limit was 4 fg of DNA and is equivalent to 0.8 cells.

DISCUSSION

With the increasing number of reports of association of MAP with Crohn's patients, it is essential to determine the possible routes of transmission to susceptible individuals. One of the possible routes of transmission is through the consumption of contaminated fruits and vegetables fertilized with contaminated manure or irrigated with manure wastewater. Contamination through aerosol transport of MAP to crops grown in close proximity to dairies also is a distinct possibility. Monitoring the fate, re-growth and transport of low cell numbers of MAP from environmental samples requires more sensitive methods than are possible with the traditional culture methods that require 4 to 6 months to enumerate. In addition, the decontamination steps necessary for the gold standard culture methods are inhibitory to the growth of MAP and further reduce their usefulness as methods for monitoring the environmental fate of low numbers of MAP. It was our intention to develop rapid and sensitive detection methods that do not require cell culture or extensive cleanup and decontamination procedures.

Two different real-time qPCR methods based on the detection of short fragments of IS*900* were developed. One of them is a general-purpose method that detects any and all SYBR green bound amplified DNA. The second method is specific and detects the 5' nuclease cleavage by Taq DNA polymerase of a bound fluorogenic probe during the extension cycle of amplification.

We designed 26 new primer sets that amplify short fragments (<61 bp) from different regions of IS*900* for use in the SYBR Green assays. Although MAP strain 19698 was detected equally well by a majority of the primer sets, different strains of MAP were not detected at the same level of sensitivity by a single primer set. For example, an equivalent quantity of template DNA from strain NADC 1040 is not detected at the same level of sensitivity as other MAP strains by primer set SF58/SR109. However, this strain is detected with good sensitivity by SF187/SR239. It appears from this study, multiple sets of primers may be necessary to detect the range of MAP strains anticipated from environmental samples. Clearly, it is necessary to evaluate the relative sensitivity of detection of several environmental strains with a range of primer sets in order to identify the optimum assay conditions and primer sets. In addition, each fragment amplified by the 9 primer sets targeting the region of SF729 to SR798 (Table 1) has 8 to 9 bp mismatches as compared (CLUSTAL V) (Higgins et al., 1992) to the sequence of a new *Mycobacterium* sp strain 2333, isolated from a healthy cow (Englund et al., 2002). The genetic element of strain 2333 has 94% sequence identity to IS*900*. Because of the high number of mismatches, it is most unlikely that these targets would amplify. The ability of these 9 primers to differentiate MAP from strain 2333 is promising and needs further scrutiny especially since the widely used Millar and Vary primers (Millar et al., 1995; Vary et al., 1990) can not distinguish MAP from strain 2333 (Englund et al., 2002). Only one base pair mismatch, which is not in the primer region, is noted between the 229 bp fragment targeted by the Vary primers and the IS*900*-like sequence of strain 2333. Three mismatches were found in the 412 bp fragment amplified by Millar primers (P90/P91).

Three new primer-probe sets (Table 3) targeting amplification of short fragments (< 80 bp) were identified for specific detection of MAP by the IS*900*-TaqMan assay. The 3 primer-probe sets are equally sensitive for detecting MAP strain 19698 but the sensitivity of primer-probe set SF214/PR265/SR289 resulted in different sensitivities for other strains of MAP (Table 4). For example, equivalent amounts of template DNA from MAP strain Kay required 8 additional amplification cycles as compared to DNA from MAP strain 19698. Such variability in C_T values with strains of MAP is curious and may reflect intra-species variability within the IS*900* element.

The TaqMan method detects as low as 4 fg of MAP specific DNA per assay and has the potential to detect even smaller quantities by increasing the amplification cycles. Increasing amplification cycles is possible as no signal for cleavage of probe is detected from controls containing only TaqMan reagents, primers, and probe. A similar detection level of 5 fg of template DNA was observed in a recently developed TaqMan assay (Khare et al., 2004) using primers that amplify a 84 bp fragment of IS*900*.

The IS*900*-SYBR Green assay is equally sensitive as the Taqman method, able to detect 3.1 to 4.5 fg of template DNA from MAP strain 19698 using two different primers tested. This genetic material is equivalent to the detection of 0.6 to 0.9 cells. In contrast, 50 fg of DNA was necessary for detection with a SYBR Green assay developed earlier (O'Mahony and Hill, 2002) using the classic primers that amplify a 421 bp fragment (Millar et al., 1995). The higher level of sensitivity in the current study is attributed to the use of primers targeting short amplicons (<61 bp). The sensitivity of detection also depends on differentiating true amplification signal from that of SYBR Green bound to primer-dimers. Primer concentrations can be optimized to minimize the signal due to primer-dimers.

The SYBR green assay also quantifies cells directly from MAP cultures without any extractions or processing. Unlike the earlier observation of inhibition of PCR by media ingredients (O'Mahony and Hill, 2002), we found that the ingredients from 7H9 broth are not inhibitory. The method described here is highly sensitive, detecting 0.07 cell per assay (equals 0.34 fg of DNA). In contrast, earlier real-time assays resulted in detection of 1 to 25 MAP cells in pure or broth cultures (Khare et al., 2004; Kim et al., 2002; O'Mahony and Hill, 2002). Using molecular beacons, detection of 1 to 8 CFU by culture methods was related with the real-time detection of 0.17 fg of DNA (Fang et al., 2002). We report here for the first time a side-by-side comparison for detection of MAP cells and detection of DNA extracted from equivalent numbers of cells. Cell counts from 7H10 agar plates without antibiotics were used to calculate the extracted amount of DNA per cell. We calculated an amount of 5.1 fg DNA per cell. Sanderson et al. reported 5 fg DNA per cell based on genome copy number(Sanderson et al., 1992). Based on a MAP genome of 5,867,714 bp (Bannantine et al., 2002), each MAP cell theoretically contains 6.8 fg of DNA. Since no extraction related losses were observed, the direct cell assay appears to be ten times more sensitive than detection using the extracted DNA approach. The limit of detection for assays using extracted DNA and primers SF187/SR239 is 3.4 fg which equals 0.7 cell. The direct cell assay also resulted in a more sensitive method than the assays using extracted DNA or with the TaqMan methods developed here and elsewhere (Kim et al., 2002).

Both methods described here are specific for the detection of MAP and do not detect closely related members of the *M. avium* complex or other species of mycobacteria. Importantly, bovine, aquatic and soil mycobacteria that are common to dairy environments are well differentiated from MAP. Mycobacteria of human origin (*M. intracellulare, M. kansasii, M. tuberculosis, M. smegmatis, M. scrofulaceum, and M. fortuitum* subsp. *fortuitum*) were also differentiated from MAP.

The reporter signal observed for 100 pg of template DNA from non-MAP organisms within 3 C_T's of the controls for the SYBR green assay was suspected to make it difficult to detect low levels of MAP from environmental samples. However, a comparable signal was detected with only 6.8 fg of MAP DNA (value from Fig. 2 for NTC-C_T – 3) which equals only 1.3 cell. As the amount of non-MAP DNA assayed is equivalent to 2×10^4 cells of MAP strain 19698, it is unlikely that a high number of non-MAP mycobacterial cells occur in environmental samples to make a difference in the detection of low numbers of MAP cells. In addition, the TaqMan assays have no amplification signal with DNA from non-MAP organisms and can conclusively differentiate trace amounts of MAP DNA from that of non-MAP. Both the assays described here are simpler to use for rapid diagnostic purposes as compared to the conventional PCR panel assays developed to differentiate MAP from related species (Ellingson et al., 2000).

In summary, we developed sensitive qPCR methods for detection and differentiation of MAP from other mycobacteria. In addition, the direct cell assays can be easily adopted for environmental monitoring either with or without pre-enrichment and decontamination steps. Sensitive detection might alleviate some of the problems related to PCR inhibitors by direct-dilution (Ozbek et al., 2003) and yet detect low levels of MAP from environmental samples. Immunomagnetic separations (Grant et al., 2000) followed by real-time sequence detection hold promise in sensitive detection of MAP from environmental samples. However, these methods require validation with environmental strains of MAP and real-world matrices. Although the

methods are sensitive for MAP specific DNA, further confirmation of viability of cells by real time nucleic acid sequence-based amplification (NASBA) techniques (Rodriguez-Lazaro et al., 2004) is essential to monitor the fate, re-growth and transport of MAP in agricultural environments.

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Development of luminescent *M. avium* subsp. *paratuberculosis* for rapid screening of vaccine candidates in mice

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ABSTRACT

M. avium subsp. *paratuberculosis* (MAP*)* is a slowly growing mycobacterial species requiring 6-8 weeks of culture before colonies can be counted visually. This seriously hampers diagnosis and experimental work with MAP*.* Snewin *et al.* (1999) previously reported on a luminescent *M. tuberculosis* H37Rv isolate expressing the *luxAB* genes of *Vibrio harveyi*. With this luminescent isolate, fastidious and expensive enumeration of CFU by plating on agar can be replaced by easy and inexpensive luminometry. Here, we report on the construction of two luminescent isolates of MAP, i.e. the reference ATCC 19698 strain and clinical isolate S-23 of bovine origin. BALB.B10 mice were infected intravenously with 10^6 CFU of luminescent MAP and monitored for bacterial replication in the spleen by determining the number of Relative Light Units (RLU) at 5, 10 and 15 weeks post infection. Results show that luminescent MAP may be a valuable tool for the rapid screening of experimental paratuberculosis vaccines *in vivo.*

Key words: luminescence, mouse, plasmid, BALB.B, *Vibrio harveyi* .

INTRODUCTION

The current vaccines against *Mycobacterium avium* subsp. *paratuberculosis* (MAP*)* are based on killed or live attenuated whole bacterial preparations. Although these vaccines are partially effective thorugh reducing fecal shedding and the number of clinically affected animals in a herd, they do not protect against infection. An efficient subunit vaccine that would not interfere with bovine tuberculosis diagnosis would be very valuable in the management of paratuberculosis, but requires the characterization of immunodominant and protective antigens. A number of immunogenic MAP proteins have been described (4), but they have mostly been analyzed for diagnostic purposes (Bannantine et al., 2002) and little is known on their vaccine potential. Experimental MAP infection and vaccination in mice is seriously hampered by technical problems. Indeed, MAP is a slow growing mycobacterial species, requiring 6-8 weeks of culture before colonies can be counted visually. Determining the number of colony forming units (CFU) in organ homogenates has so far consisted of labor-intensive plating on expensive mycobactin-supplemented Middlebrook agar. Snewin *et al.* previously reported on the construction of a luminescent *M. tuberculosis* H37Rv isolate that expressed the *luxAB* genes of *Vibrio harveyi* introduced by transformation with the shuttle plasmid pSMT1 (Snewin et al., 1999). With this luminescent isolate, fastidious enumeration of CFU can be replaced by easy and inexpensive luminometry. We have used this luminescent *M. tuberculosis* isolate successfully for the screening of plasmid based DNA vaccines against tuberculosis and have shown that reductions in $\log_{10}$ mRLU values correlate exactly with reductions in $\log_{10}$ CFU counts (D'Souza et al., 2002; Tanghe et al., 2001).

The same pSMT1 plasmid was used to transform two isolates of MAP i.e. the reference ATCC 19698 strain and a clinical bovine isolate S-23 of origin. These two isolates were compared for their replication in the spleen of BALB.B10 mice upon intravenous infection.

MATERIALS AND METHODS

Electroporation of M.avium ssp paratuberculosis
MAP strains ATCC 19698 (Merkal, 1979) and S-23 (Foley-Thomas et al., 1995) grown on Middlebrook 7H9 medium supplemented with OADC and mycobactin J were transformed with plasmid pSMT1 (10) (kindly given by D. Young, Imperial College London) using a previously described procedure (3). pSMT1 DNA was prepared from *E. coli* strains either by an alkaline lysis miniprep method (9) or with the Wizard® Miniprep Kit (Promega, Madison, WI, USA). Transformants were grown at 37°C for 5 weeks on Middlebrook 7H9 agar supplemented OADC, mycobactin J and 50 μg/ml hygromycin. This is the first report on the use of this drug marker to select MAP transformants.

Infection of mice
Female BALB.B10 mice were obtained from the animal facilities of the Pasteur Institute. They were offspring from breeding couples originally received from the Netherlands Cancer Institute. Mice were infected intravenously in a lateral tail vein with re 360 Relative Light Units RLU of ATCC 19698 strain or 415 RLU of S-23 strain grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J and hygromycin, to an optical density of 0.6.
The number of bioluminescent bacteria in spleen homogenates was determined using a bioluminescence assay with a Turner Design 20/20 luminometer and 1% n-decyl-aldehyde (Sigma) in ethanol as substrate. For statistical analysis (Student's t test), mRLU values were converted to $\log_{10}$ values per organ per mouse. A comparison of mRLU and CFU values of an axenic MAP culture indicated that one milliRLU unit corresponded to 2.5 CFU units (unpublished data).

RESULTS

Bacterial replication of luminenscent MAP ATCC 19698 and S-23 strain following intravenous infection of BALB.B10 mice
As shown in Fig. 1, a sustained bacterial presence of the two luminescent MAP strains could be detected by luminometry in the spleen from infected mice. Mean $\log_{10}$ mRLU number of clinical MAP isolate S-23 increased in the mouse spleen between week 5 and 10 after infection, and subsequently mRLU values stabilized at week 15. The luminescent ATCC 19698 strain was slightly less virulent and mRLU values slowly declined between 5 and 15 weeks of infection.
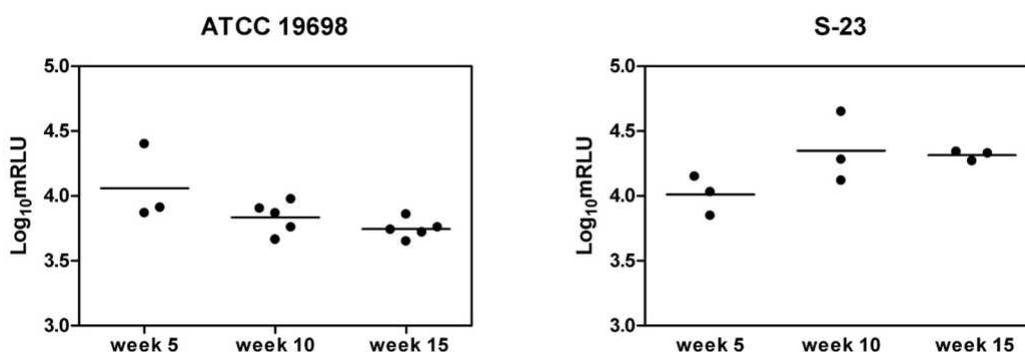
Fig. 1. Enumeration of two luminescent MAP isolates administered to naïve BALB.B10 mice, as tested by luminometry in the spleen at 5, 10 and 15 weeks post-infection.

DISCUSSION

MAP causes Johne's disease, a severe gastroenteritis in ruminants, with a significant impact on the agricultural economy, particularly the dairy industry. Vaccines consisting of whole killed or attenuated live MAP bacilli can provide partial protection by delaying fecal shedding and reducing the number of clinically

affected animals, but they do not protect against infection. Animals immunized with these paratuberculosis vaccines develop positive reactions in the tuberculin skin test (the reference bovine tuberculosis detection method) and therefore paratuberculosis vaccination is subject to approval by the veterinary services. It is clear that the development of an efficient paratuberculosis sub-unit vaccine that would not interfere with bovine tuberculosis detection would offer a solution. However, few protective antigens against paratuberculosis have been characterized in detail.

Experimental MAP infection and vaccination studies are seriously hampered by technical problems. As a slow growing mycobacterial species, determining the number of MAP colony forming units (CFU) in organ homogenates is labor-intensive plating and expensive. The use of luminescent MAP isolates as reported here will be of great benefit for future experimental MAP studies. Besides enabling an easy *in vivo* screening of vaccine candidates, these luminescent bacteria could also be used for *in vitro* drug testing (Williams et al., 1999) and *in vitro* monitoring of bacterial replication in cell lines such as bovine epithelial cells.

The clinical S23 strain (isolated in 1995) was somewhat more virulent than the ATCC 19698 strain (dating back to 1979 and subjected to extensive *in vitro* passages); it replicated slightly in the spleen of BALB.B10 mice between week 5 and 10 after infection. The BALB.B10 mouse is a MHC congenic mouse with H-2^b haplotype from the C57BL/10 strain on a BALB/c background. This mouse strain expresses the *bcg^s* allele (encoded by *nramp*, known to confer susceptibility to intravenous infection with BCG and *M. avium).* Using the luminescent MAP isolate, we demonstrated that the BALB.B10 mouse strain also expresses the susceptible allele with respect to infection with *M. paratuberculosis.* Analysis of various mouse strains expressing either the resistant or the susceptible allele of the *bcg* gene confirmed this finding (Rosseels, unpublished data).

CONCLUSION

These results show that luminescent MAP transformed with pSMT1 plasmid encoding the *luxAB* genes of *Vibrio harveyi* may be a valuable tool for the rapid screening of paratuberculosis vaccines in a murine experimental infection model*.*

ACKNOWLEDGEMENTS

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The *Mycobacterium avium* subspecies *paratuberculosis* specific peptide aMptD and its potential as a diagnostic tool

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ABSTRACT

Using a phage display library expressing random 12-mer peptides, we isolated a peptide specific for the MptD protein of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The peptide had been synthesized without further modifications and designated as aMptD (Stratmann et al. 2004). For further investigation of the specificity and a potential diagnostic applicability of aMptD, paramagnetic beads were coated with aMptD. Milk samples were spiked with MAP and incubated overnight with the beads. After repeated washing the beads were resuspended in water and boiled. A MAP-specific PCR based on the ISMav2 fragment was performed directly on the supernatant. Using this method, it was possible to reproducibly detect 10^2 MAP per ml milk. The specificity of the aMptD-coated beads was confirmed by performing a competitive capture, using a 10^3-fold excess of various mycobacterial species. Capture was followed by PCR using primers specific for the genus *Mycobacteria*. Via restriction enzyme digests of the resulting fragments, it was confirmed that only MAP had been captured. In order to show binding of aMptD to different isolates, a capture of various MAP isolates (Type I and II) out of spiked milk was performed. The protocol was shown to be able to detect MAP in individual and bulk milk samples.

Key words: MAP-specific peptide aMptD, peptide-mediated capture PCR, plasmid

INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic and incurable granulomatous enteritis of ruminants caused by MAP infection (Kreeger 1991). The disease occurs worldwide with increasing frequency (Manning and Collins 2001) and has a considerable economic impact on the livestock industry. An economically feasible alternative to eradication programs would be a control program aimed at the early identification and removal of high shedders. With this approach, environmental contamination and the infectious pressure on the herd would be reduced.

Heavy shedders are more likely to secrete MAP in milk (Taylor et al. 1981) and fecal contamination of milk is more likely to occur in herds with heavy shedders (Clarke 1997). Milk might therefore be a suitable diagnostic substrate for this control approach. The most feasible methods for the detection of MAP DNA in milk is enrichment via immunomagnetic separation (Khare et al. 2004) or peptide-mediated capture (Stratmann et al. 2002) followed by a PCR. However, a routine laboratory method incorporating an enrichment step of MAP should be based on a defined receptor-ligand interaction with highly specific and standardizable reagents, and it should be applicable to high-throughput automation.

In the study presented here we followed this approach using the phage display-derived peptide aMptD (Stratmann et al. 2004) for the capture of MAP in milk samples. We elucidated the cross-strain and species specificity of aMptD for MAP by performing competitive capture assays. Finally we applied the peptide aMptD-mediated capture to bulk milk samples followed by PCR analysis incorporating an internal amplification control (IAC).

MATERIAL AND METHODS

Bacterial strains, plasmids, primers and growth conditions.
The bacterial strains, plasmids and primers used in this study are listed in Table 1. Mycobacteria were grown on BBL[®] Herrold's egg yolk Agar slants (HEYM, Becton Dickinson, Sparks, MD, USA) or on Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich., USA) supplemented with 10% of oleic acid-albumin-dextrose-catalase enrichment (OADC; 100 ml contain sodium chloride [145 mM], bovine serum albumin [fraction V; 0.5 g], dextrose [1.1 M], catalase [3 mg], and oleic acid [60 µl]), glycerol (0.2%), and mycobactin J (2 µg l^{-1}; Synbiotics, Lyon, France). For further use in the capture assays mycobacteria were harvested from the Middlebrook 7H10 agar by careful removal from the agar and resuspended in phosphate-buffered saline (PBS [pH 7.2]; NaCl [150 mM], KH_2PO_4 [1.5 mM], Na_2HPO_4 [9 mM], KCl [2.5 mM]). Homogenization of the suspension was done by vortexing for five minutes with glass beads (30 beads of 3 mm diameter per 5 ml of bacterial suspension in a polypropylene tube). *E. coli* strains were grown in Luria-Bertani medium supplemented with ampicillin (100 µg ml^{-1}).

Manipulation and analysis of DNA
Agarose gel electrophoresis, plasmid preparation, PCR, DNA cloning and transformation of *E. coli* were done by standard procedures. DNA modifying enzymes were purchased from New England Biolabs (Frankfurt, Germany) and used according to the manufacturer's instructions. Taq polymerase and primers were purchased from Invitrogen (Groningen, The Netherlands). Sequencing reactions were done by SeqLab GmbH (Goettingen, Germany) and sequencing data analyses were performed with the Husar 5.0 program (DKFZ, Heidelberg, Germany).

Construction of an internal control for the capture-PCR
An internal amplification control designed to be amplified under the same PCR conditions as those described for the IS*Mav2*- derived primer set was constructed. In the first step a 402 bp fragment of the MAP *mptF* gene was amplified using 40-mer primers (oMAPC long) consisting of 20 bases specific for the *mptF* gene plus 20 bases that do not result in an amplification product when used with MAP DNA. The complete fragment was then cloned into the pCR[®]2.1-TOPO vector (Invitrogen, Groningen, The Netherlands) and transformed in *E. coli* Top10F' cells according to the manufacturer's instructions and selected using blue-white selection. White colonies were screened for the presence of an insert by PCR using primer oMAPC (Table 1). Plasmid DNA of a PCR-positive clone was extracted with the NucleoBond[®] AX Kit (Machery Nagel, Dueren, Germany), confirmed by nucleotide sequence analyses and designated as pMAPC. The optimal concentration of internal control plasmid pMAPC and primer oMAPC was determined by adding various quantities to PCR reactions containing defined serial dilutions of MAP DNA. This resulted in a PCR mastermix containing 5 pmol oMAPC, 25 pmol of each, oISMav1 and oISMav2, and 0,1 pg pMAPC per 50µl PCR reaction.

Peptide aMptD-mediated capture PCR
Peptide aMptD (GKNHHHQHHRPQ) was synthesized (Fa. Affina Immuntech, Berlin, Germany) and coupled directly (i.e. without a biotin-streptavidin bridge) to paramagnetic beads using the carbodiimide method (Fa. Chemicell, Berlin, Germany). Carbodiimides react with the terminal carboxylate groups on the magnetic beads resulting in highly reactive O-acylisurea derivatives which then form a covalent bond with the free amino group of the lysine of peptide aMptD. Peptide-mediated capture PCR and spiking of pasteurized milk was performed essentially as described previously (Stratmann et al. 2002), (Stratmann et al. 2004) but with some modifications allowing for automated processing. Briefly, bulk milk samples (1 ml) were distributed in deep well plates (DWP; Abgene, Epsom, Great Britain); 5 µg of peptide aMptD-coupled paramagnetic beads (suspended in 10 µl of destilled water) were added to each well and the DWP was incubated overnight at 4°C with light agitation. Beads were sedimented using the MagnaBot[®] 96 magnetic separation device (Promega, Madison, WI, USA) and the milk was removed. The,beads were transferred to U-bottom microtiter plates in 200 µl of 0.1 tris buffered saline buffer (TBS, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 0.05% Tween 20. In this plate beads were magnetically sedimented, washed 10 times with 0.1 X TBS Tween (200 µl) using an Nunc-Immuno[®] washer (Nunc, Roskilde, Denmark). After the final wash washing buffer was completely removed, the beads were resuspended in 50 µl 0.1 TE buffer (1x TE buffer is 10 mM Tris-HCl [pH 8.0] and 1 mM ethylene diamine tetraacetic acid; EDTA) and boiled in a microwave oven for 15 min at 180 W. After sedimenting the beads again, the supernatant was transferred into a new microtiter plate, and 5 µl of the supernatant was used as template in a PCR using IS*Mav2*-

derived primers. The specificity of the PCR was confirmed by restriction enzyme digestion or by sequencing of the products. The bulk milk samples tested in this study were kindly provided anonymously by several dairy plants in eastern and northern Germany.

Table 1. Strains, plasmids, primers and peptides

Strain, plasmid, primer or peptide	Characteristics	Reference or source
Strains		
MAP	Strain 6783 (DSM 44135)	Laboratory reference strain (clinical isolate)
M. avium subsp. *avium*	DSM 44156 / ATCC 25291	German Collection of micro-organisms and Cell Cultures (DSMZ)
6 *MAP* field isolates	Two type I and four type II strains	Kindly provided by K. Stevenson, Moredun Research Institute, Penicuik, Scotland
M. fortuitum	DSM 46621	
M. diernhoferi	DSM 43524	Kindly provided by I. Moser,
M. vaccae	Field isolate	Friedrich-Loeffler-Institute, Jena,
M. flavescens	DSM 43991	Germany
M. gastri	Field isolate	
M. gordonae	DSM 43212	
M. malmoense	DSM 44163	
E. coli TOP10 F'	F' *mcrA (mrr -hsdRMS-mcrBC)* Φ*80lacZ M15 lacX74 recA1 deoR araD139 (araleu)7697 galU galK rpsL* (Str^r) *endA1 nupG*	TOPO TA Cloning, Invitrogen, Groningen, The Netherlands
Plasmids		
pCR$^®$ 2.1 TOPO	Topoisomerase I "enhanced" E. coli cloning vector carrying ampicillin and kanamycin resistance determinants as well as the *lacZ* gene for blue-white selection	TOPO TA Cloning, Invitrogen, Groningen, The Netherlands
pMAPC	pCR® 2.1 TOPO containing a 402 bp fragment of the *M. avium* subsp. *paratuberculosis mptF* gene flanked by specific 20-mers	This work
Primers		
ISMav1	5'-GTA TCA GGC CGT GAT GGC GG-3'	(Stratmann et al. 2002)
ISMav2	5'-CCG CAC CAG CGC TCG ATA CA-3'	(Stratmann et al. 2002)
oMAPC long1	5'-ACA CTT GCG ATC TGG GCC TCT TGA GGT AAG CAA GTT CGC GCC-3'	This work
oMAPC long2	5'-ATC GCG CTA CCC ATT CGG TGA TCA TGG CTG AGG TAG TCG GC-3'	This work
oMAPC1	5'-ACA CTT GCG ATC TGG GCC TCT-3'	This work
oMAPC2	5'-ATC GCG CTA CCC ATT CGG TGA-3'	This work
oABC5	5'-TAG CTC GAC GAC CCA GTC CA-3' (*mptD* derived forward primer)	This work
oABC6	5'-TCA GCC CGA ATA CCA CCG TG-3' (*mptD* derived reverse primer)	This work
MK5	5'-TTC TTG AAG GGT GTT CGG GGC C-3' (IS*900* derived forward primer)	(Doran et al. 1994)
MK6	5'-GCG ATG ATC GCA GCG TCT TTG G-3' (IS*900* derived reverse primer)	(Doran et al. 1994)
MK7	5'-GTC TGG GAT TGG ATG TCC TG-3' (IS*901* derived forward primer)	(Kunze et al. 1991)
MK8	5'- CAC CAC GTG GTT AGC AAT CC-3'(IS*901* derived reverse primer)	(Kunze et al. 1991)
P264	5'-TGC ACA CAG GCC ACA AGG GA-3' (mycobacterial 16S RNA gene derived forward primer)	(Bottger 1994)
P283	5'-GAG TTT GAT CCT GGC TCA GGA-3' (mycobacterial 16S RNA gene derived reverse primer)	(Bottger 1994)
Peptides		
aMptD	GKNHHHQHHRPQ	(Stratmann et al. 2004)

A

B
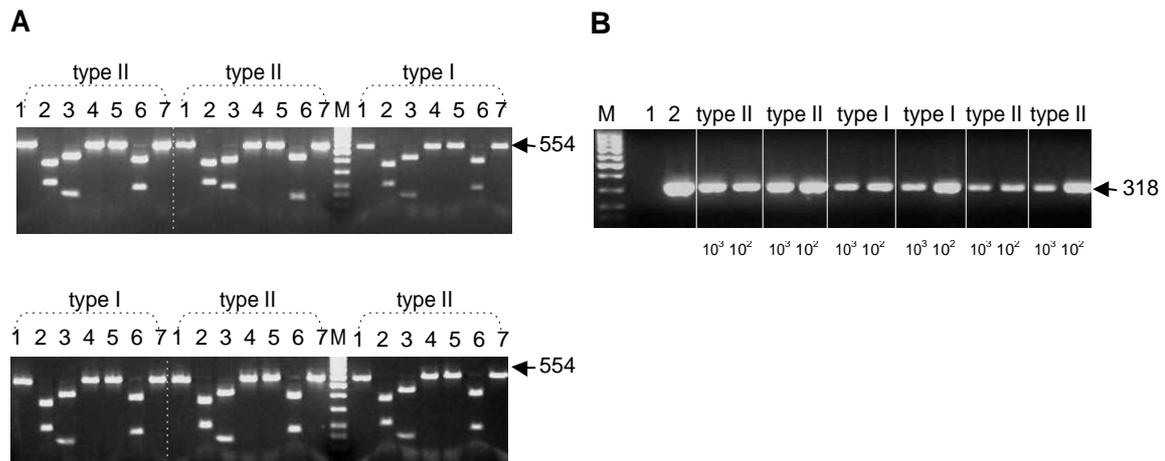
FIG. 1. Restriction enzyme analyses and peptide aMptD-mediated capture PCR of different MAP strains. (A) Restriction enzyme digest of the PCR amplified *mptD* gene. Lanes 1 contain the undigested PCR product; lanes 2, digest with Ava I; lanes 3, digest with BsrB I; lanes 4, digest with Hinf I; lanes 5, digest with Nar I; lanes 6 digest with NgoM IV; lanes 7, digest with Mse I; lanes M, 100 bp DNA marker; the arrow to the right indicates the expected position of the undigested PCR product in base pairs. (B) Peptide aMptD-mediated capture PCR of different MAP strains from artificially contaminated milk

RESULTS

Cross-strain reactivity of peptide aMptD for different MAP strains
Peptide aMptD has been shown to bind to the surface-exposed protein MptD of MAP strain 6783. In order to analyze whether peptide aMptD would have the potential to bind MptD proteins of different MAP isolates, the *mptD* genes of four type II and two type I strains were PCR-amplified and the products were analyzed by restriction enzyme digests. Digests of the PCR products with AvaI, BsrBI, HinfI, NarI, NgoMIV and MseI resulted in identical restriction endonuclease profiles for all strains, thereby indicating that the *mptD* genes in MAP type I and II strains are identical (Fig. 1a). To confirm binding of peptide aMptD to different MAP strains, it was coupled to paramagnetic beads and incubated with milk spiked with 10^2 and 10^3 CFU ml^{-1} of these strains. The following capture PCR based on IS*Mav2*-derived primers demonstrated a capture of the different strains with peptide aMptD. This finding showed that the MptD-protein is highly conserved among different MAP isolates and thereby confirmed that peptide aMptD is suitable for use as ligand in a diagnostic test (Fig. 1B).

Specificity of peptide aMptD for MAP
To confirm the specificity of peptide aMptD for MAP, a competitive peptide capture was performed in milk spiked simultaneously with 10^3 CFU ml^{-1} MAP and 10^5 to 10^1 CFU ml^{-1} *M. avium* subsp. *avium*. After overnight capture only MAP was detected by PCR analyses using IS*Mav2*-derived primers; using IS*901*-derived primers no *M. avium* subsp. *avium* could be detected (Fig. 2). In addition, competitive capture assays with seven other mycobacterial species (*M. fortuitum*, *M. diernhoferi*, *M. vaccae*, *M. flavescens*, *M. gastri*, *M. gordonae*, *M. malmoense*) were performed to demonstrate the specificity of peptide aMptD for MAP. In order to distinguish whether MAP or the competing mycobacteria had been captured, the PCR-assay was based on the amplification of the 16S-23S rRNA spacer fragment followed by digests with the restriction endonucleases AvaI, NciI and Tsp451 (Fig. 3a). For the competitive capture assay milk was spiked simultaneously with 10^2 CFU ml^{-1} MAP and a 100- and 1000-fold excess of one of the seven different mycobacterial species. The overnight capture followed by PCR and restriction endonuclease analyses clearly demonstrated that solely MAP had been captured (Fig. 3b) and thereby confirmed the species-specificity of peptide aMptD.
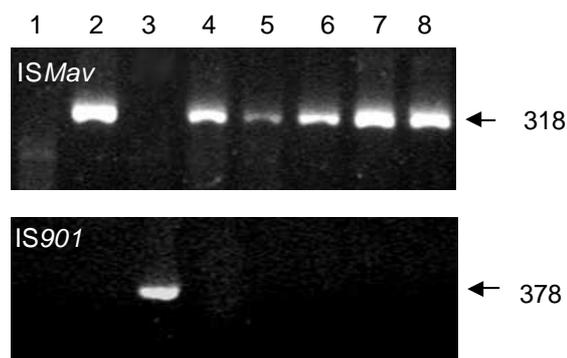
FIG. 2. Competetive capture PCR from milk artificially contaminated with a mixture of *M. avium* subsp. *avium* and MAP. Lanes 1, negative control; lanes 2, MAP DNA; lanes 3, *M. avium* subsp. *avium* DNA; lanes 4 to 8 capture out of milk spiked with 10^5 to 10^1 *M. avium* subsp. *avium*, each sample containing 10^3 MAP. The arrows to the right indicate the expected position of the PCR product obtained with IS*Mav2*-derived primers (top) or IS*901*-derived primers (bottom)
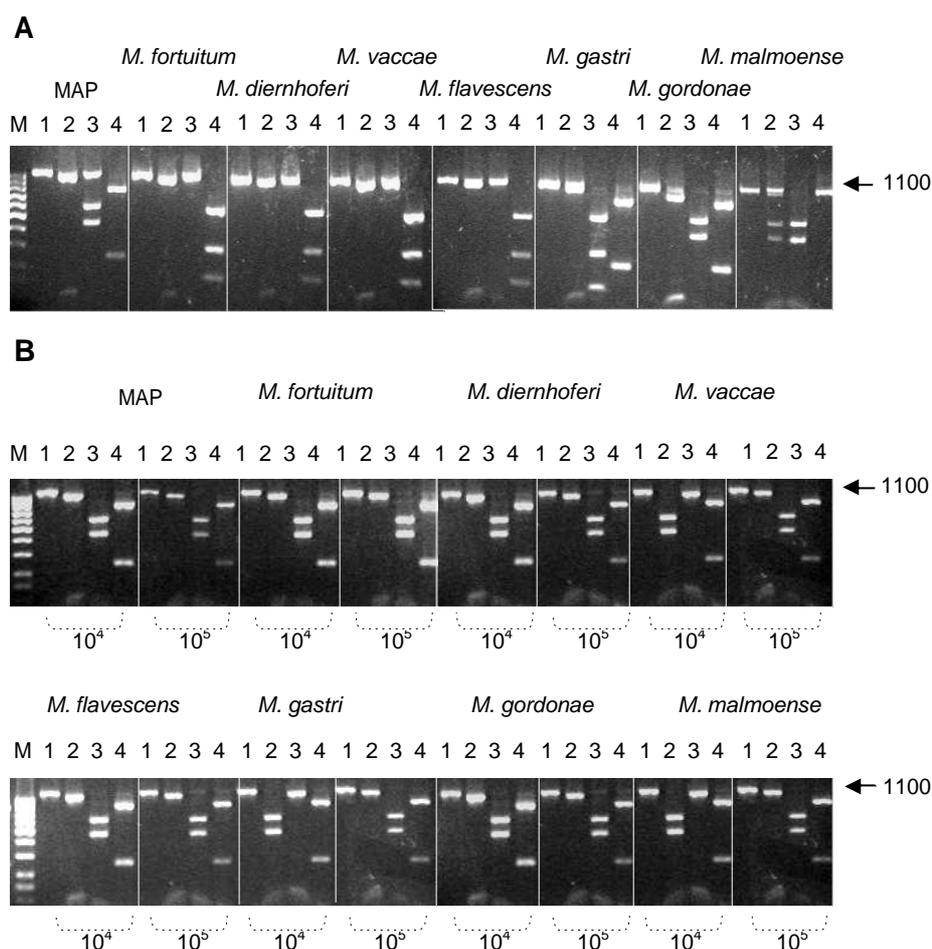
FIG. 3. Restriction enzyme analysis of the PCR-amplified 16S RNA spacer of different mycobacterial strains and competetive capture PCR from milk artificially contaminated with a mixture of MAP and seven different mycobacterial species. (A) Restriction enzyme digest of the PCR-amplified 16S RNA spacer of the mycobacterial species indicated on top. Lanes 1 contain the undigested PCR product; lanes 2, digest with Ava I; lanes 3, digest with Nci I, lanes 4, digest with Tsp 45I; lane M, 100 bp DNA marker; the arrow to the right indicates the expected position of the PCR product in base pairs. (B) Competetive capture PCR from milk artificially contaminated with a mixture of MAP and the seven different mycobacterial species followed by restriction enzyme digests. Lanes 1, undigested PCR product; lanes 2, digest with Ava I; lanes 3, digest with Nci I; lanes 4, digest with Tsp 45I ; 10^4 and 10^5 indicate the number of CFU ml^{-1} of the different strains used for spiking of milk; each sample containing 10^2 CFU ml^{-1} MAP; lane M, 100 bp DNA marker; the arrow to the right indicates the expected position of the PCR product in base pairs.

Peptide aMptD-mediated capture PCR for the diagnosis of MAP in milk samples Pasteurized milk was spiked with MAP at concentrations of 10^5 to 10^0 CFU ml^{-1}. When a peptide-based capture assay with aMptD-coated paramagnetic beads was performed, MAP could be detected in a PCR with IS*Mav2* primers at a concentration of 10^1 CFU ml^{-1} (Fig. 5a). For the detection of MAP in milk samples, an IAC was designed in order to avoid false negative results. In testing 423 bulk milk samples obtained from different herds throughout Germany, 23 (5.5 %) reacted positive and 29 (6.8%) of the PCR reactions were negative (i.e. no PCR fragment from the IAC was obtained). An example of a diagnostic PCR with IAC is shown in Figure 5b. In order to confirm the identity of the IS*Mav2* PCR-derived DNA-fragments five amplicons obtained from positive bulk milk samples were confirmed by nucleotide sequence analyses.

A

B
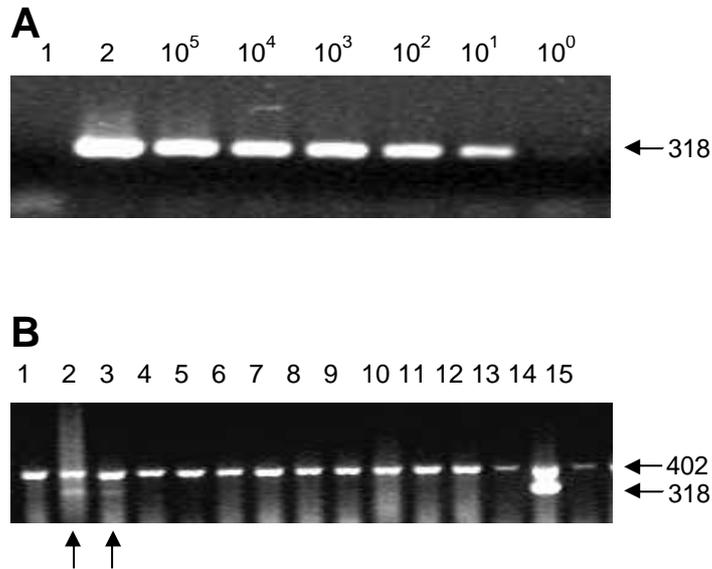
FIG. 5. Peptide aMptD-mediated capture PCR. (A) Peptide-mediated capture PCR of artificially contaminated milk using IS*Mav2*-derived primers. Lane 1, negative control; lane 2, positive amplification control using MAP DNA as template; 10^5 to 10^0 indicate MAP CFU ml^{-1}; the arrow to the right indicates the expected position of the PCR product in base pairs. (B) Peptide- mediated capture PCR from bulk milk samples using IS*Mav2*-derived primers and incorporated IAC. Lanes 1-13, peptide-mediated PCR from bulk milk samples; lane 14 positive control (milk spiked with 10^2 ml^{-1} CFU MAP); lane 15, negative control; the arrows to the right indicate the expected position of the PCR products in base pairs; the arrows on the bottom indicate the position of positive bulk milk samples.

DISCUSSION

In this paper we present the development and validation of a peptide-mediated capture PCR for the detection of MAP in bulk milk samples. A significant improvement of the method described previously (Stratmann et al. 2002) was achieved by using peptide aMptD as a ligand (Stratmann et al. 2004). This protocol permitted the capture of the organism on a defined receptor-ligand interaction. Furthermore, we coupled the peptide directly without a biotin-streptavidin linker to the paramagnetic beads, extensively validated the specificity and potency of the capture reaction and incorporated an IAC into the subsequent PCR analysis. Finally, we tested the optimized protocol under field conditions by applying it to 423 bulk milk samples.

Currently, mandatory paratuberculosis control programs in dairy cattle are rare (Benedictus and Kalis 2003); this is likely due to a lack of reliable and reasonably priced diagnostic methods. For other infectious diseases, bulk milk has been developed as the diagnostic substrate (Bitsch and Ronsholt 1995). The milk-based programs in place rely on an antibody ELISA of individual milk samples (Hendrick et al. 2005). However, despite specificities of up to 99% (Reichel et al. 1999, Jark et al. 1997) false positive reactions are bound to occur particularly in low prevalence herds leading to minimal farmer acceptance of detect-and-

cull strategies. In addition, not all shedders can be detected based on an antibody response (Stabel et al. 2002). The situation could be improved considerably if a bulk milk-based and highly specific diagnostic test, relying on direct detection of the pathogen itself, were available. Various studies have shown that this goal, in principle, can be achieved effectively based on immunomagnetic (Khare et al. 2004) or peptide-mediated (Stratmann et al. 2002) enrichment of the pathogen from milk followed by subsequent PCR analysis.

Peptide aMptD had been shown previously to bind to the surface-exposed MptD protein of MAP strain 6783 (Stratmann et al. 2004). However, it was shown that the expression profile of proteins can vary between different MAP and *M.avium* subsp. *avium* strains (Birkness et al. 1999). Therefore, in order to investigate the suitability of peptide aMptD as ligand for diagnostic purposes, the presence of the *mptD* gene in six different MAP type I and II strains was tested by PCR analysis. Subsequent restriction endonuclease digests confirmed that the gene was highly conserved among the different isolates tested. The consistent expression of the MptD protein and the cross-strain reactivity of peptide aMptD was then tested by capturing two MAP type I and four type II strains. It was shown that, independent of the strain, 10^2 ml^{-1} organisms of MAP could be reliably detected using the aMptD-based capture PCR.

Besides cross-strain reactivity, species-specificity is of major concern for diagnostic reagents. Previous studies had merely demonstrated the absence of the *mptD* gene in a closely related *M. avium* subsp. *avium* reference strain. Therefore, in order to ensure that no cross-reactive receptors are present in *M. avium* subsp. *avium* or other mycobacteria, competitive capture assays were performed. The finding that 10^2 MAP were consistently detected whereas no other mycobacteria were captured even in the presence of a two to three log-fold excess clearly demonstrated the species-specificity of the MptD protein receptor.

After having shown that peptide aMptD is specific and suitable for diagnostic purposes we tested its applicability as a ligand for the capture of MAP from bulk milk samples. Using artificially contaminated milk we found that captured bacteria remained viable. Further, initial tests of a capture PCR revealed a diagnostic sensitivity (10^2 CFU ml^{-1}) comparable to the IMS results in previously reported studies (Grant et al. 2000). The PCR was based on primers directed against the specific IS*Mav2* insertion element (Strommenger et al. 2001); this element has been shown to be specific (Shin et al. 2004) whereas for more frequently used PCRs based on the IS*900* element (Marsh et al. 2000) false positive reactions have been reported (Englund et al. 2002). In addition, an IAC was included in order to prevent the occurrence of false negative results and thereby improve the significance of the PCR results obtained. Finally, as the capture PCR should be utilized for the screening of large numbers of bulk milk samples, it needed to be adapted to routine laboratory automation. Therefore, the protocol had to be as simple and straightforward as possible. In contrast to previously described IMS protocols (Grant et al. 1998) inhibitory substances could be removed by repeated washing of the beads after capture without decreasing the diagnostic sensitivity, thereby allowing the adaptation of the test to an automated high-throughput platform.

Contamination of bulk milk samples with MAP occurs in herds with animals shedding the organism (Stabel et al. 2002), and so the applicability of the peptide-mediated capture PCR was finally evaluated by testing 423 bulk milk samples for MAP. The finding of 5% of the herds reacting PCR-positive is in line with results of other studies using different methods. That is, paratuberculosis herd prevalence in Germany is estimated to be 15% (BMVEL 2003), and slightly higher prevalence data have been reported for other countries in Western Europe (Haine et al. 2004, Muskens et al. 2000, Nielsen et al. 2000). As only 1/3 of clinically (Taylor et al. 1981) and 1/10 of subclinically infected animals (Sweeney et al. 1992) are reported to shed MAP in milk, a detection of the organism in 5% of the samples is within the range expected.

CONCLUSIONS

We developed and validated a peptide-mediated capture PCR for the detection of MAP in bulk milk samples. As this method relies on a single defined receptor-ligand interaction and is adaptable to standard laboratory automation, it might present a powerful tool for a routine paratuberculosis survey and control program aimed at identifying infected herds with high shedders.

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An IS*900*-like sequence in *Mycobacterium porcinum* strains isolated from bovine bulk milk: implications for diagnosis

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ABSTRACT

The insertion sequence IS*900* was considered highly specific for *Mycobacterium avium* subsp. p*aratuberculosis* (MAP) until the discovery of IS*900*-like elements in mycobacteria other than MAP. Nevertheless, IS*900* PCR still remains widely used as a diagnostic tool because this event is considered extremely rare. 631 bulk milk samples from different dairy herds were analysed by culture over a 7 month period (August 2004 – February 2005); suspect colonies were confirmed by IS*900* PCR and mycobactin dependency. All of 56 suspect isolates chosen on the basis of the colony morphology were positive for IS*900* by PCR. Of these, 4 (7.1%) were confirmed as MAP through mycobactin dependency and 52 (92.9%) were mycobactin-independent mycobacteria not identified as MAP by restriction endonuclease analysis of the amplified products. Sequencing analysis of the amplified fragment obtained from six of these mycobactin-independent isolates revealed a 74.4% homology with the MAP IS*900* element. Twenty-one isolates subjected to further investigation using PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene were identified as *Mycobacterium porcinum*. Six of these strains underwent 16S rRNA gene sequencing by MicroSeq 500 16S ribosomal DNA (rDNA)-based bacterial identification system (Applied Biosystems); sequence analysis by MicroSeq database system confirmed the identification of *M. porcinum* with 100% homology.
Based on these results, IS*900*-like elements could be more widespread than was previously thought. An IS*900* PCR positive result is not sufficient to identify MAP. Particularly when analysing milk, isolate identification should always include other microbiological (mycobactin dependency, slow growth) or molecular (enzymatic digestion of amplified products, amplification of other genomic target) methods.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*, diagnosis, PCR, IS*900*, *Mycobacterium porcinum*.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of paratuberculosis, commonly known as Johne's disease (JD), a chronic, progressive, granulomatous enteritis of ruminants. The current diagnostic standard method is isolation of the organism by culture, requiring several months. MAP detection by PCR represents a rapid, alternative diagnostic tool. This technique has been routinely used both to directly determine the presence of MAP from different types of specimens (faeces, milk, tissues, semen), and to confirm the identification of isolates obtained through culture.

The discovery of insertion sequence IS*900* in the MAP genome provided an ideal diagnostic PCR-target: IS*900* has been considered highly specific for MAP, where it is present in multiple copies (14 to 20), thereby also making it a very sensitive detection marker. The specificity of IS*900* diagnostic PCR however has been questioned since some reports indicated the presence of IS*900*-like sequences in mycobacterial species other than MAP. Cousins et al. (1999), reporting the amplification of IS*900*-like elements from environmental mycobacteria closely related to *M. scrofulaceum,* demonstrated for the first time that false positive results could be obtained from strains other than MAP. Following this, Naser et al. (1999) found an

IS*900*-like element in *M. avium* subsp. *avium* strains isolated from HIV infected patients, while Englund et al. (2002) isolated from bovine faeces a mycobacterium named *Mycobacterium* sp. 2333, related to *M. cookii* harbouring one copy of a sequence with 94% identity to IS*900* at the nucleic acid level. During the last two years, Motiwala et al. (2004) reported the presence of IS*900*-like elements in six isolates from wild animal faeces and tissues, and Rayeev et al. (2005) reported the isolation of one non-MAP mycobacterium strain positive for IS*900* PCR, which has not been typed.

In this study, the isolation of IS*900* PCR positive mycobacteria other than MAP from bovine bulk milk is described.

MATERIALS AND METHODS

Culture method
631 bulk milk samples collected over a 7 month period (August 2004 – February 2005) from 631 cattle dairy herds in Northern Italy were tested for the presence of MAP by culture as recommended by Dundee et al. (2001). Fifty ml milk samples were centrifuged (15 min at 2500 g), and the pellet resuspended in 25 ml 0,75% (wt/vol) cetylpyridinium chloride (HPC). Following incubation at room temperature (21°C) for 5 hours and a further centrifugation (15 min at 2500 g), the pellet was resuspended in 0,8 ml of PBS-Tween20 (0,05%). Four Herrold's egg yolk medium (HEYM) slants containing 2 μg of mycobactin J/ml, two supplemented with cloramphenicol and two with nalidixic acid and vancomicin, were each inoculated with 200 μl of the resuspended pellet. The media were incubated at 37°C and weekly examined for up to 16 weeks. When suspect colonies were observed, three identity confirmation tests were carried out: Ziehl-Neelsen staining, mycobactin dependence and IS*900* PCR.

IS900 PCR analysis
DNA extraction: one colony was resuspended in 100 μl distilled water and boiled (100°C) for 20 min.
    DNA amplifications: the PCR mixture consisted of 1X *Taq* Buffer (Eppendorf), 1.0 mM MgCl$_2$, 200 μM of each of the four dNTPs, 0,3 μM each of oligonucleotide primers (Millar et al., 1995) p90 (5'-GAAGGGTGTCGGGGCCGTCGCTTAGG-3') and p91 (5'-GGCGTTGAGGTCGATCGCCCACGTGA-3'), and 0,5 U of *Taq* DNA polymerase (Eppendorf) and 5 μl of the boiled lysate as DNA template (25 μl total volume). Amplification parameters were: 30 cycles of primer annealing for 1 min at 62°C, elongation for 1 min at 72°C and denaturation for 1min at 95°C, followed by a 10 min extension at 72°C in Mastercycler gradient (Eppendorf). PCR products were analysed by electrophoresis on a 2% agarose gel in 1X TBE buffer. The gel was stained for 60 min in ethidium bromide (0,5 μg ml^{-1}), rinsed in distilled water and visualised by UV light transillumination.

IS*900* amplified products consistent with MAP were subjected to restriction endonuclease analysis (Cousin et al.,1999) with *Alw*I and *Mse*I. Aliquots (10 μl) of PCR products were digested in a final volume of 20 μl with 2 U of one of the restriction enzymes (New England Biolabs) according to the supplier's instructions. The PCR product obtained from MAP ATCC19698 was included as a positive control. Digested products were analysed by electrophoresis on a 1,5% agarose gel in 1X TBE buffer. The gel was stained with ethidium bromide and the size of the digested DNA fragments was compared using the appropriate size marker (marker VIII, Roche).

Sequencing analysis of the IS900 amplified products
PCR products from six selected strains were sequenced using the ABI Prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacture's instructions. Reactions were analysed using ABI Prism 310 DNA genetic analyser.

Molecular typing of isolated strains
▪   *16S rRNA gene sequencing*
The 500 bp informative portion of 16S rRNA gene of 6 mycobacteria isolates was amplified and sequenced by MicroSeq 500 16S rDNA Bacterial Sequencing Kit (Applied Biosystems, Foster City, Calif.).
▪   *hsp65 PRA*

Genomic DNA from 21 strains was subjected to amplification of the 441-bp Telenti sequence of *hsp65* gene (Telenti et al., 1993; Steingrube et al., 1995) using 1 U *Taq* DNA Polymerase (Eppendorf), 1X *Taq* Buffer (Eppendorf), 1.5 mM $MgCl_2$, 200 μM of each of the four dNTPs, 0,6 μM each of primer, i.e. TB11 (5'-ACCAACGATGGTGTGTCCAT–3') and TB12 (5'-TTGTCGAACCGCATACCCT-3') and 5 μl of the boiled lysate as DNA template (50 μl total volume). Amplification parameters were: 45 cycles of 1 min at 94, 1 min at 60 and 1 min at 72°C followed by 7 min extension at 72°C.

PCR products were digested with *Bst*Ell*, Hae*III and *Bsa*HI (New England Biolabs), following manufacturer's recommendations (Wallace et al., 2004). The DNA restriction fragments were electrophoresed on a 4% agarose gel, the size of the digested DNA fragments was compared using an appropriate size marker (marker VIII, Roche).

Phylogenetic analysis
A phylogenetic tree was constructed on 16S rRNA sequences to evaluate the phylogenetic relations between MAP and non-MAP mycobacteria harbouring IS*900*-like sequences up until now reported.

  Mycobacterial 16S rRNA sequences were retrieved from GenBank. Multiple sequence alignments were determined using the CLUSTALW algorithm (Chenna et al., 2003).   Homologous positions were brought into correspondence by aligning 16S rRNA genes from the following mycobacteria:

- *M. avium* subsp. *paratuberculosis* (GeneID: 3370905)
- *M. avium* subsp *avium* (GenBank accession NO. AJ536037)
- *M. scrofulaceum* (GenBank accession NO. AJ536034). We included *M. scrofulaceum* in the analysis since this species resulted the most closely related to the non-MAP IS*900* positive strains isolated by Cousins et al. (1999)
- *Mycobacterium* sp., 2333 (GenBank accession NO. AY065649)
- *M. porcinum* (GenBank accession NO. AY012581)

The final alignment included 1445 homologous nucleotide positions common to all the genes selected for analysis.

RESULTS

Detection of MAP by culture
Culture results are summarized in Table 1. A total of 56 suspect isolates, on the basis of the colony morphology, were subjected to confirmation: they were all acid-fast rods bacteria, IS*900* PCR positive, but only 4 of them were mycobactin-dependent mycobacteria.

Table. 1. Culture and confirmation test results of 56 suspected strains isolated from 631 bulk milk samples

Milk samples	Suspect Isolates	No. (%) of isolates					
		Acid fast staining		Mycobactin dependency		IS*900* PCR	
		positive	negative	positive	negative	positive	negative
631	56 (8.9%)	56 (100%)	0 (0%)	4 (7.1%)	52 (92.9%)	56 (100%)	0 (0%)

The mycobactin-dependent were rough, not pigmented colonies that grew in primary culture in 1-4 months. The mycobactin-independent colonies were rough or smooth, negative for early and late pigmentation, and all grew in a 5-7 day period on Herrold's egg yolk medium.

A single colony grew on HEYM slants for the mycobactin-dependent PCR-positive milk samples whereas for the IS*900* PCR-positive mycobacteria that did not require mycobactin, the number of colonies was generally higher (5-50).

Only the four mycobactin-dependent mycobacteria yielded fragments of the predicted size after restriction digestion (*Alw*I: 258, 155 bp; *Mse*I: 283, 130 bp), effectively confirming that they were MAP. In the remaining 52 cases the amplified products remained undigested (Fig. 1).

Fig. 1. Restriction endonuclease analysis of IS*900* PCR products: Lane 1-4: Undigested IS*900* PCR products; Lane 5: MAP isolate; Lane 6: MAP ATCC19698.

Sequence data of the IS*900*-like element from 6 selected strains was obtained from a fragment corresponding to the 41-400 portion of the IS*900* nucleotide sequence (GenBank accession No.X16293). The PCR products were 410 bp long and the sequences were all identical (Fig. 2). There were 91 nucleotide differences between the MAP IS*900* sequence and the six IS*900*-like sequences analysed, giving a homology of 74,4% at nucleic acid level. One base difference in the *Alw*I restriction site and two bases difference in the *Mse*I restriction site explained the absence of IS*900*-like PCR product digestion.

```
IS900     TCCTTACCTTTCTTGAAGGGTGTTCGGGGCCGTCGCTTAGGCTTCGAATTGCCCAGGGAC 60
IS900like -------------------------------------CTTCGATTCACCCAGGGAC 46
                                               ****** *  ********

IS900     GTCGGGTATGGCTTTCATGTGGTTGCTGTGTTGGATGGCCGAAGGAGATTGGCCGCCCGC 120
IS900like AGCGGGTATGGCTTT-AT-TGGTCAGCCCGT--GATGGCCGAGGAGGATTGGCCGCCCGC 102
           ************* ** ****      **  ********* * **************

IS900     GGTCCCGCGACGACTCGACCGCTAATTGAGAGATGCGATTGGATC-GCTGTGTAAGGACA 179
IS900like TGTCCCGCGACGACTCGACCGCTAATTGAGAGATGCGATTCGATCCGCTGTGTAAGGACA 162
           ************************************************ **** **************

IS900     CGTCGGCGTGGTCGTCTGCTGGGTTGATCTGGACAATGACGGTTACGGAGGTGGTTGTGG 239
IS900like CGACGGCGTGGTCGTCTGGCGGGCTGGTGGAAATGGTGATGAGCACGGAGGCGGTAGTGA 222
           ** ************** *** ** *    *    *** *   ******* *** ***

IS900     CACAACCTGTCTGGGCGGGCGTGGACGCCGGTAAGGCCGACCATTACTGCATGGTTATTA 299
IS900like CACAAGCTGTTTGGGCGGGTATCGATGCTGGGAAATCGGACCACTTCTGCGTGGTCATCG 282
           ***** **** ********   *  ** ** ** **   * ***** * **** **** **

IS900     ACGACGACGCGCAGCGATTGCTCTCGCAGCGGGTGGCCAACGACGAGGCCGCGCTGCTGG 359
IS900like ATGCGCAGGGAAAGCGGCTGCTGTCGCAGCGGGTAGCCAACGACGAAGCGACCCTGTTGA 342
           * *   * *   ****   **** ********** ************  **  * *** **

IS900     AGTTGATTGCGGCGGTGACGACGTTGGCCGATGGAGGCGAGGTCACGTGGGCGATCGACC 419
IS900like AGTTGATCAGCTCTGTCGCAACCATGGCTGACGGCGGTGAA-------------------- 402
           *******    * **  * **  **** ** ** ** ** ** **

IS900     TCAACGCCGGCGGCGCCGCGTTGCTGATCGCCTTGCTCATCGCTGCCGGGCAGCGGCTGC 479
IS900like ------------------------------------------------------------ 410
```

Fig. 2. Sequence alignment of IS*900* from MAP and the sequence obtained from 6 IS*900* PCR positive strains described in this paper in the region amplified. Primer sequences (p90 and p91) are underlined and restriction sites are shown in bold.

Molecular typing of isolated strains
16S rRNA gene sequencing
The 6 strains selected for further investigations underwent 16S rRNA gene sequencing over the first 500 bp. The MicroSeq 500 16S ribosomal DNA (rDNA)-based bacterial identification system (Applied Biosystems Division, Foster City, Calif.) was able to identify *Mycobacterium porcinum* with 100% homology in all six cases.

hsp65 PRA
Since the 500 bp informative portion of 16S rRNA gene of *M. porcinum* shows 100% identity with the recently described species *M. neworleansense* (Schinsky et al., 2004), 21 strains were subjected to PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene with *Bst*EII, *Hae*III and *Bsa*HI

as described by Wallace et al. (2004) (Fig. 3). In all cases the products obtained were consistent with the PRA pattern of *M. porcinum* (Tab. 2).


Fig. 3. *hsp65* PRA patterns. Lane 1-13: *M. porcinum* isolates.

Table. 2. PRA patterns for *M. porcinum* and *M. neworleansense*

| | **Restriction fragments lenght** | | |
	***Bst*EII**	***Hae*III**	***Bsa*HI**
M. porcinum	235/210	140/125/100	260/100/80
M. neworleansense	235/115/85	140/125	/

The phylogenetic tree (Fig. 4) clearly showed that *M. porcinum* is the less closely MAP-related species, with phylogenetic distances varying from 0.00208 (*M. avium* subsp. *avium*) to 0.02148 (*M. porcinum*).
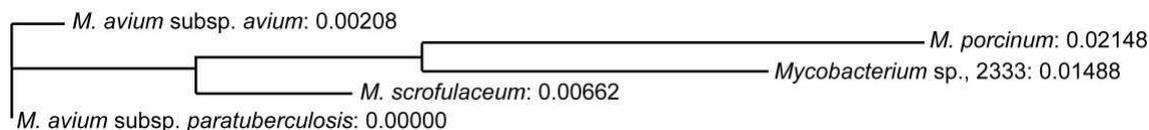
Fig. 4. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic distances between MAP and non-MAP mycobacteria harbouring IS*900*-like sequences.

DISCUSSION

In this study 52 mycobactin-independent strains were isolated that, when amplified with Millar primers for the 5' end of the insertion sequence IS*900*, resulted in amplicons of the same size as PCR products from MAP. Sequences similar to the nucleotide sequence of the IS*900* and other sequences have been reported previously. In particular, IS*900* is known to share sequence similarity with some members of the IS*110* family to which it belongs: IS*901* (*M. avium* subsp. *avium*), IS*902* (*M. avium* subsp. *sylvaticum*), IS*1110* (*M. avium* subsp. *avium*), IS*110* (*Streptomyces coelicolor*), IS*116* (*Streptomyces clavuligerus*), show 47-60% identity with IS*900*. IS*1626* (*M. avium* subsp. *avium*) is the most closely related to IS*900* (82% homology), with the greatest variability observed at 5' end of the nucleotide sequence.

The sequence similarity between IS*900* and IS*900*-like sequences reported by other authors was 71%-79% (Cousin et al., 1999) or 94% (Englund et al., 2002); in our report the sequence similarity over the 359 bp sequence (not including the primer sequences) at the 5' end of IS*900*, was 74.4%, confirming the high homology in the amplified region.

All the mycobacteria subjected to molecular typing were identified as *Mycobacterium porcinum*. The rapidly growing mycobacterial species *M. porcinum* was described in 1983 by Tsukamura et al. as a causative agent of submandibular lymphadenitis in swine. *M. porcinum* was recently isolated from environmental (tap water) and human clinical samples (respiratory infections, wound infections, catheter related infections)

(Schinsky et al., 2004; Wallace et al., 2004). While its pathogenic role in post-traumatic or post-surgical wound infections, osteomyelitis, and catheter-related infections appears clear, its role in causing chronic lung infection has not so far been established (Wallace et al., 2004).

The high rate of *M. porcinum* isolation from bovine bulk milk reported in this paper suggests that the micro-organism could be widely disseminated in cattle dairy herds of Northern Italy. This is the first IS*900*-like sequence reported in rapidly growing mycobacteria. Analysis of the rRNA genes of mycobacteria has resulted in the division of the genus into two separate clusters that correspond to the fast-growing and slow-growing mycobacteria (Stahl and Urbane, 1990). As confirmed by this paper's 16S rDNA phylogenetic analysis, *M. porcinum* is less closely related to MAP than are other mycobacteria harbouring IS*900*-like sequences.

During this study MAP and *M. porcinum* were never isolated from the same culture slant. In the MAP positive milk samples, only one colony grew in HEYM tubes while the number of *M. porcinum* colonies was generally higher. As a consequence, since *M. porcinum* colonies grow faster, they could almost completely cover the media surface, obscuring an eventual appearance of MAP. Moreover, since it has been reported that fast growing mycobacteria may inhibit MAP growth (Richards W.D., 1988), we can not exclude the hypothesis that *M. porcinum* is a MAP-inhibiting contaminant.

CONCLUSIONS

Since IS*900*-like elements have been described in rarely encountered mycobacteria, IS*900* PCR still remains widely used as a diagnostic tool. We isolated 52 IS*900* PCR-positive mycobacteria from 631 (8,2%) bovine bulk milk samples tested. These isolates were not identified as MAP by restriction endonuclease analysis of the amplified products. This data further substantiates the data reported by Cousins et al. (1999) and Englund et al. (2002). Based on these data, the following is concluded:

a) IS*900*-like elements could be more widespread than has been previously thought and may occur in mycobacteria not closely related to MAP;

b) IS*900* PCR is not sufficient to identify MAP. In order to reduce the risk of misidentification, in particular when analysing milk, IS*900* PCR should always be coupled with other microbiological (mycobactin dependency, slow growth) or molecular (enzymatic digestion of amplified products, amplification of other genome targets) methods.

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Identification and subspeciation of *Mycobacterium avium* complex isolates by PCR amplification of a fibronectin-attachment protein gene element

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ABSTRACT

The insertion sequence IS*900* has long been regarded as a genetic marker specific for *Mycobacterium avium* subsp. *paratuberculosis* (MAP). However, recent studies have found that IS*900* or closely related sequences may also be present in *M. avium* subsp. *avium* (MAA). The objective of the present study was to exploit nucleotide sequences of the fibronectin-attachment protein (FAP) gene *fap* in *M. avium* subspecies to allow MAP to be distinguished from other subspecies of *M. avium*. A pair of oligonucleotide primers was designed to flank a region near the C-terminus of *fap* from MAP and MAA. These primers were used to amplify DNA extracted from laboratory and field strains of MAP, MAA, and *M. avium* subsp. *silvaticum*. Amplicons were sequenced from selected *M. avium* isolates. Electrophoretic migration patterns were compared following restriction digestion of MAP amplicons. All MAP strains tested yielded a 151 bp product that produced identical migration patterns when digested with either *Asc*I or *Apa*I. MAA isolates produced 178 bp, 166 bp, or 118 bp PCR products, the latter two of which were apparent truncations of the 178 bp amplicon. The 178 bp PCR product obtained from *M.a. silvaticum* shared 98% identity with the 178 bp amplicon from MAA. The results show that PCR targeting *fap* can reliably distinguish MAP from other *M. avium* subspecies, thus indicating its utility as a diagnostic tool.

Key words: *Mycobacterium avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium avium* subsp. *avium,* fibronectin-attachment protein (FAP), *fap,* PCR

INTRODUCTION

Johne's disease causes a chronic granulomatous enteritis and mesenteric lymphadenitis of domestic and wild ruminants. The disease is initiated by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), an acid-fast intracellular bacillus. This disease is a major concern of the cattle industry, causing it to lose as much as $1.5 billion annually (Jones, 1989). Animals are most often infected within the first few weeks of life, however the diarrhea and wasting due to granulomatous enteritis that characterize the clinical phase of this disease are usually not seen until several years post-infection. Nevertheless, infected cattle can shed large numbers of MAP in feces before the onset of clinical disease and thereby pose a significant threat to herdmates and their offspring (Whitlock et al., 1996).

It is imperative that diagnostic procedures be developed that permit the rapid and reliable detection of MAP to eliminate Johne's disease from affected herds. To this end, numerous PCR assays have been developed to detect MAP. Almost all of these have been designed to amplify elements of IS*900* (Secott et al., 1999; Bull et al., 2000). This insertion sequence has long been regarded as unique to MAP. However, recent studies have found that IS*900* or closely related sequences may be present in *M. avium* subsp. *avium* (MAA) (Naser et al., 1999). Thus, IS*900* PCR may not be specific for MAP.

Despite the very high degree of homology between the genomes of MAP and MAA, significant differences are present in at least some genes common to both organisms. A fibronectin-attachment protein (FAP) encoded by *fap* has been identified in several *Mycobacterium* species. The purpose of the present study was to determine whether sequences differences in *fap* were sufficient to permit MAP to be distinguished from other *M. avium* subspecies by PCR.

MATERIALS AND METHODS

PCR primers were designed to amplify a region near the C-terminal coding region of *fap* from MAP and MAA. Within this region, there are gaps of 21 and 6 nucleotides in the GenBank MAP *fap* reference sequence when aligned with that of MAA. Therefore, MAP DNA was predicted to yield a 151 bp PCR product, whereas a 178 bp amplicon was expected from MAA DNA when amplified with the same primer pair.

Type cultures of MAP, MAA, *M. avium silvaticum*, and *M. phlei* were obtained from American Type Culture Collection (Manassas, VA). Field isolates of MAA were provided by V. Kapur. Field isolates of MAP and formalin-fixed cultures of *M. bovis* and *M. tuberculosis* were obtained from the Purdue University Animal Disease Diagnostic Laboratory. DNA was extracted from each bacterial strain using the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN).

One microliter of DNA extract was added to a reaction mixture containing 50mM KCl, 10mM Tris-HCl (pH 9), 0.1% Triton X-100, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 5% DMSO, 2.5U Taq polymerase and 1μM of forward primer and reverse primer. After denaturation for 5 min at 95°C, DNA was amplified for 30 cycles (30 sec each at 95°C, 62.2°C, and 72°C), followed by a final 10 min extension step at 72°C. Products were analyzed by electrophoresis through 4% MetaPhor agarose (FMC, Rockland, ME).

The 151 bp products obtained from PCR testing of DNA from all MAP field isolates were digested separately with ApaI and AscI restriction enzymes (New England Biolabs). Digestion products were analyzed by electrophoresis through 4% MetaPhor agarose (FMC).

DNA amplicons obtained from selected MAP and MAA strains were TOPO-TA cloned into pCRII (Invitrogen, Carlsbad, CA) and sequenced at the Purdue University Genomic Center. Sequences were aligned using the ClustalW algorithm in BioEdit software (Hall, 1999).

RESULTS

Thirty-one of thirty-two strains of *M. avium* subspecies yielded a product from the PCR assay designed for this study. No amplicons were obtained from the *M. phlei*, *M. bovis*, or *M. tuberculosis* strains examined (Table 1).
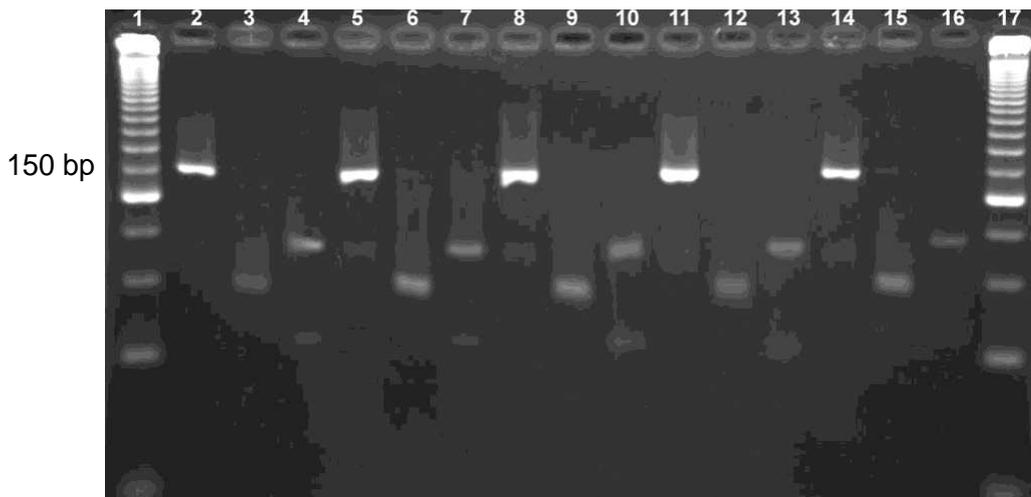
Fig. 1 – *Mycobacterium avium* subsp. *paratuberculosis* (MAP) strains yield identical *fap* PCR products of 151 base pairs (bp). Amplicons from MAP strains 2065-2 (lanes 2-4), 2062 (lanes 5-7), 2023 (lanes 8-10), 6951 (lanes 11-13), and 5781 (lanes 14-16) were loaded directly into agarose gels (lanes 2, 5, 8, 11, and 14) or digested with either *Apa*I (lanes, 3, 6, 9, 12, and 15) or *Asc*I (lanes 4, 7, 10, 13, and 16) prior to gel electrophoresis. Faint bands corresponding to primer oligomers can be seen in lanes with undigested PCR product. Lanes 1 and 17, 25 bp marker.

All strains of MAP produced a single PCR product of 151 bp. Amplicons from each strain produced a 76/75 bp doublet band following digestion with *Apa*I, and 97 bp and 54 bp bands when digested with *Asc*I (Fig. 1). Products sequenced from two selected strains were identical to the corresponding region of the GenBank reference sequence for MAP *fap* (data not shown).

Table 1. List of mycobacterial strains assayed by Polymerase Chain Reaction for the selected fibronectin-attachment protein gene element.

Organism	Strain	Host	Result	Size
MAP	5781	Bovine	+	151 bp
	4509	Bovine	+	151 bp
	5549	Bovine	+	151 bp
	3806	Bovine	+	151 bp
	4301-1	Bovine	+	151 bp
	2513-2	Bovine	+	151 bp
	7925	Goat	+	151 bp
	2700	Goat	+	151 bp
	2213	Bovine	+	151 bp
	2065-2	Bovine	+	151 bp
	2062	Bovine	+	151 bp
	2023	Bovine	+	151 bp
	6951	Bovine	+	151 bp
	7715-5	Goat	+	151 bp
	6298-1	Bovine	+	151 bp
	8080	Bovine	+	151 bp
	7948-1	Bovine	+	151 bp
	8314	Bovine	+	151 bp
	ATCC 19698	Bovine	+	151 bp
MAA	MAC 101	Human	+	178 bp
	ATCC 25291	Chicken	+	178 bp
	1012	Bovine	-	NA
	1028	Porcine	+	118 bp
	1199	Human	+	118 bp
	1224	Human	+	118 bp
	1367	Avian	+	178 bp
	1033	Porcine	+	178 bp
	1035	Avian	+	118 bp
	1182	Human	+	118 bp
	1188	Human	+	166 bp
	1346	Avian	+	178 bp
Mycobacterium avium ssp. *silvaticum*	ATCC 49884	Wood pigeon	+	178 bp
Mycobacterium bovis	E-11	Bovine	-	NA
Mycobacterium tuberculosis	327	Human	-	NA
Mycobacterium phlei	ATCC 11758	-	-	NA

PCR products of 118 bp, 166 bp, and 178 bp were obtained when DNA from strains of MAA was used as a template (5, 1, and 5, respectively, of 12 MAA strains examined) (Fig. 2). Both the MAA type strain DNA and *fap* gene plasmid DNA from the GenBank MAA *fap* reference strain yielded the predicted 178 bp amplicon. Sequencing of the smaller PCR products from MAA DNA revealed a 60 nucleotide deletion in the 118 bp amplicon and two 6 nucleotide deletions in the 166 bp amplicon relative to the GenBank reference sequences (data not shown). The 166 bp or 118 bp PCR products were truncations of the 178 bp amplicon. The size of these "atypical" amplicons remained the same when gradient PCR was used to modify the primer annealing temperature.

A 178 bp product was obtained from *M. a. silvaticum* DNA. This PCR product shared 98% identity with that of MAA. The sequence of this amplicon differed from that of the MAA GenBank reference sequence at 4 nucleotide positions (data not shown).
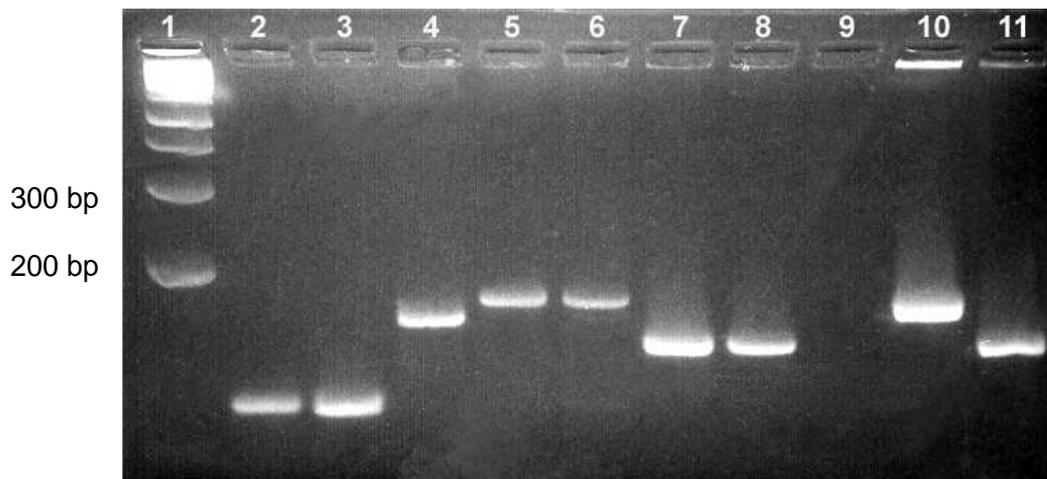
Fig. 2 – The *fap* PCR products from MAP differ in size from those of other *M. avium* subspecies. Lane 1, 100 bp marker; lane 2, MAA 1028 (118 bp); lane 3, MAA 1182 (118 bp); lane 4, MAA 1188, (166 bp); lane 5, MAA ATCC 25291 (178 bp); lane 6, *M. a.* ss. *silvaticum* ATCC 49884 (178 bp); lane 7, MAP 5549 (151 bp); lane 8, MAP 4301-1 (151 bp); lane 9, negative control; lane 10, MAA MAC 101 (GenBank reference sequence strain) (178 bp); lane 11, MAP 5781 (GenBank reference sequence strain) (151 bp).

DISCUSSION

One of the major advantages of using IS*900* as a target for diagnostic PCR is that this 10-20 copies of the sequence are present in the MAP genome (Green et al., 1989). This would be expected to improve the sensitivity of PCR-based detection of MAP. However, direct detection of MAP in feces by IS*900* PCR is considerably less sensitive than culture. The IS*900* PCR assay is currently applied to accelerating culture turn-around time (Secott et al., 1999) but the sensitivity of IS*900* PCR may not have a clear advantage over *fap* PCR for the detection of MAP.

Although the number of MAA isolates examined was low, the frequency of MAA strains yielding different *fap* amplicons was remarkable. No association between host species or geographical region and *fap* amplicon size was noted. All field strains were clinical isolates, and *fap* expression has been demonstrated to be required for M cell invasion by MAP (Secott et al., 2003; Secott et al., 2004). There appears therefore that a strong sequence conservation of *fap* is present among MAP. Preferential invasion of M cells by MAA has not been documented (Sangari et al., 2001), which may explain why sequence variation in *fap* can be tolerated in MAA.

All MAP field strains investigated were from Indiana herds. Additional work is under way to determine whether these findings extend to those strains recovered from animals in other regions of the U.S.

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Development of an internal control plasmid used for mycobacterial detection by PCR

B J Brey, R P Radcliff, D L Clark Jr, Jay L.E. Ellingson

Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the etiological agent of Johne's disease, a chronic enteritis of the gastrointestinal tract of ruminants. The insertion sequence IS*900* and the hspX gene can be targeted for detection of MAP using PCR. Generally, each set of PCR reactions contains a positive and a negative control tube. Unfortunately, it is possible for a reaction to fail while the controls do not. Thus, a single positive and negative control tube cannot determine if all PCR reactions in a set worked properly. Our objective was to construct a plasmid to serve as a universal internal control in PCR reactions that test for *Mycobacterium*, including MAP and *Mycobacterium bovis / tuberculosis*-complex organisms. A previously constructed plasmid containing an insert of *M. bovis*-hspX-*M. bovis* DNA was enzymatically digested with BSG I and Sac II to remove 71 bp of the hspX portion (including the sequence we use for the reverse hspX primer). The remaining insert was blunt-ended and ligated back together. The new plasmid was transformed into competent cells and grown on LB/Amp/X-Gal/IPTG plates for screening. Both Eco RI digestion and sequencing confirmed the desired plasmid product. A reverse primer was designed to anneal to the *M. bovis* portion of the plasmid insert located farthest from the hspX forward primer. We performed PCR reactions, using various amounts of plasmid (1 ng to 100 ag) and MAP genomic (10 ng to 100 fg) DNA (ATCC 19698), to determine the optimal amount of plasmid to be used in both IS*900* and hspX PCR reactions. The optimal amount of plasmid for IS*900* and hspX detection was 1 fg and 10 fg, respectively. In conclusion, the plasmid construct can be included in MAP PCR reactions to confirm if a PCR reaction is successful and identify true positives and true negatives within each individual reaction tube.

Prime boost vaccination with pSG2 plasmid and MVA each recombinant for a MAP fusion protein attenuates pre-existing MAP infection in C57/BL6 mice

Tim Bull, S Sidi-Boumedine, Roberto A Cervantes, H Thangaraj, A Skull, M Mura, S Gilbert, John Hermon-Taylor

Abstract

To develop a therapeutic vaccine for use in animals and humans infected with MAP, we performed a targeted bioinformatic analysis of the MAP genome and selected two secreted and two membrane bound components each related to the pathogenic phenotype. A fusion construct including these four MAP antigens was assembled from overlapping 40mer oligonucleotides incorporating a sequence adjusted for optimal mammalian codon usage. Functional domains including potential cross-reacting human epitopes and hydrophobic transmembrane regions were excluded. A monoclonal antibody recognition peptide was added to the C-terminus and a short human ubiquitin leader sequence to the N-terminus. The construct was cloned into the pSG2 expression vector and inserted by homologous recombination into Modified Vaccinia Ankara (MVA). Expression of the 95 kDa polyprotein in cell culture was confirmed. Prime boost vaccination using single dose pSG2.rec (i.m.) followed by single dose MVA.rec (i.v.) resulted in significant antigen-specific IFN-γ T-cell responses in ELISPOT assays compared with control immunization using wild type vector or buffer only. No adverse effects occurred in the hyper-immune mice. In two subsequent studies, a modified protocol incorporating two priming doses of pSG2.rec (i.m.) followed by a single boost of MVA.rec (i.v.) was tested for its ability to attenuate pre-existing MAP infection and to protect against subsequent MAP infection compared with control groups. The burden of MAP infection was measured using IS*900*-specific quantitative real-time PCR on liver/spleen DNA extracts and by culture on solid medium for future determination of cfu. Prime boost vaccination four weeks after MAP infection resulted in a significant attenuation of infective load (qRT-PCR) in spleen and liver after 26 weeks compared with controls. Vaccination prior to MAP challenge showed a protective effect in a subgroup of animals. These early results provide a promising basis for further vaccination studies in larger animals.

Proteomic identification of immunogenic Mycobacterium paratuberculosis proteins

Dong Hee Cho, N Sung, Michael T. Collins

Abstract

A comparison was made of *Mycobacterium paratuberculosis* (*M. ptb.*; strain JTC303) protein expression and immunogenic antigens resulting from Watson-Reid culture filtrate (CF) vs. cellular extract (CE) preparations. Most proteins from CF had pI values of 4.0 to 5.5 whereas pI values for CE proteins had a broader range (4.0 to 7.0). Immunogenic proteins were more numerous from CF than CE preparations according to SDS-PAGE analysis with 31 sera collected from culture-confirmed cases of bovine paratuberculosis. This difference was even more evident after absorption of the sera with *M. phlei* antigens before immunoblotting. The most immunogenic CF proteins had molecular weights (MW) of 23 to 30 kDa. Twenty-four protein spots with the greatest staining intensity and different pIs and MWs were selected for analysis by MALDI-MS. Among these, five spots were successfully identified as ModD, PepA, ArgJ, CobT, antigen 85c complex and 9 additional hypothetical proteins were found. N-terminal peptide analysis of PepA, Antigen 85c, ModD, MAP1693c, MAP2168c, and MAP1022c revealed that each has 27 to 39 amino acids that may function as a signal sequence, supporting their being secreted through a Sec-dependent pathway. These results indicate that 14 secreted protein antigens found in a CF preparation of *M. ptb.* are strong candidates for use in improved serodiagnostic tests for paratuberculosis.

Isolation of M. a. paratuberculosis strains belonging to the sheep type in cattle and goats in Spain

L de Juan, J Álvarez, B Romero, J Bezos, A Aranaz, F Lozano, C Lozano, A Mateos, L Domínguez

Abstract

M. a. paratuberculosis strains are divided in two main types, the cattle and the sheep group. The sheep group, characterised by an extremely slow growth, has been described mainly in ovine strains from Scotland, England, Denmark, Australia, Iceland, South Africa, New Zealand, and Canada This type corresponds with the sheep and intermediate group described by IS*900*-RFLP, and the Type I and III described by PFGE. The objective of this study is to demonstrate that *M. a. paratuberculosis* strains belonging to the sheep type are common in domestic ruminants in Spain, and probably in the rest of Europe. One hundred and sixty four M*. a. paratuberculosis* isolates from domestic ruminants (83 goats, 78 cows and 3 sheep) from different Spanish regions were typed with specific PCRs (Whittington et al. 1998; Collins et al. 2002) to distinguish between cattle and sheep types. Surprisingly, 31.7% of the strains belonged to the sheep type (29 cows, 20 goats and 3 sheep) and were characterised by: 1) long incubation period, 46.2% of the strains took more than 5 months to grow; 2) preference for the Löwenstein-Jensen media (55.8%); and 3) difficult to be subcultured and therefore to be characterised by IS*900*-RFLP and PFGE. Despite this fact, both protocols were applied in some *M. a. paratuberculosis* strains and results confirm their classification into the sheep group since they were typed as Type III and Intermediate group by PFGE and IS*900*-RFLP, respectively. These results highlight the fact that it is essential to identify the type of *M. a. paratuberculosis* strains to guarantee that the diagnosis protocol is appropriate. We recommend the implementation of these PCRs as a routine protocol in paratuberculosis diagnosis, since the presence of sheep type *M. a. paratuberculosis* strains is a fact that has to be taken into account in control programs.

Identification of differentially expressed genes in uninfected and Mycobacterium paratuberculosis (M. ptb.) infected sheep

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Abstract

Mycobacterium paratuberculosis causes a chronic progressive wasting disease in sheep. Most of the data on the molecular pathogenesis of the disease has come from studies in other species in the advanced stages of the disease or from macrophage infection models. Molecular techniques used in these studies include microarray analysis, selective capture of transcribed sequences (SCOTS) and RNA arbitrarily primed-PCR (RAP-PCR). The objective of this study was to examine host gene expression during the initial stages of *M. ptb.* infection and during the clinical stage of infection to identify known or novel genes regulating the response to infection, and to define genes that can be used to identify *M. ptb.* infected animals earlier than currently possible. Long distance differential display PCR (DD-PCR) was employed to identify differentially expressed genes in lymph node and ileal tissues collected from uninfected, early stage infected or clinically infected Merino sheep. The advantages of this technique include the ability to look at regulated transcripts using small amounts of starting material. RNA was extracted using the Qiagen mini RNA extraction kit. RNA was DNase treated and reverse transcribed to cDNA using oligo(dT). DD-PCR was performed according to Clontech's Delta Differential Display Kit with some modifications. Preliminary DD-PCR studies using two different primer sets revealed amplification of subsets of genes, although none appeared to be differentially expressed between uninfected and early stage infected animals. Additional primer combinations are being evaluated.

Development of cell disruption and 2-Dimensional electrophoretic methods for the proteomic analysis of Mycobacterium avium subsp. paratuberculosis

J A Donaghy, N L Totton, Michael T. Rowe

Abstract

Mycobacterium avium subsp. *paratuberculosis* (Map), an extremely hardy organism, due mainly to the chemical composition of its thick, waxy outer cell wall has been shown to survive thermal treatments such as pasteurisation. Investigation of this thermal resistance at a proteomic level may give an insight into the stress tolerance of this bacterium and may help develop better diagnostics for the detection of this organism for animal disease control. This study assessed the efficacy of a number of lysis/extraction techniques to release intracellular proteins from Map strains for proteomic analysis by 2 dimensional electrophoresis (2DE). Map cells were harvested, washed and lysed/disrupted using each of the following methods: Bugbuster (Novagen); Fast Protein Blue; Fast Protein Red (Qbiogene); B-Per, Y-Per - (Perbio Sciences), all chemical treatments, and a sonication procedure including glass beads and CHAPS detergent. Map lysates were prepared for 2DE by dilution in isoelectric focusing (IEF) rehydration solution on pH 3-10 IPG strips and focused on the Multiphor 11 apparatus. Second dimension electrophoresis was performed on precast SDS- polyacrylamide gels. Silver stained proteins were analysed using Phoretix 2D software. Comparison of the methods using strain NCTC 8578 indicated that sonication with glass beads and CHAPS detergent yielded the greatest quantity of protein of all methods tested. Lysis of Map with Bugbuster was the least efficient of the methods employed. A sonication method incorporating glass beads and the detergent CHAPS was a more effective lysis protocol than a number of commercially available chemical-based methods for the release of the Map proteome. The effective extraction of the Map proteome, through the developed method, will assist the investigation of the thermal tolerance of this organism at the proteomic level. Also, the availability of a more complete proteome will be critical to developing better diagnostics and /or vaccines for disease detection, treatment and control.

The in vitro antagonistic activities of lactic acid bacteria against Mycobacterium avium subsp. paratuberculosis

J A Donaghy, N L Totton, Michael T. Rowe

Abstract

Dairy products such as cheese and yogurt are manufactured from pasteurised milk while in some cases sub-pasteurisation temperatures are employed for the former. Recent studies have shown increased survival of *Mycobacterium avium* subsp. *paratuberculosis* (Map) under such conditions. Therefore, the inactivation of this bacterium may rely on the manufacturing process or the product's intrinsic properties. Lactic acid bacteria (LAB) are known for their potential as inhibitors of food pathogens, a property exploited in their commercialisation as 'probiotic' products. The objective of this study was to investigate the *in vitro* inhibition of Map by probiotic strains and cheese LAB isolates. Supernatant from LAB cultured in de Man, Rogosa Sharpe broth was added to BACTEC 12B medium and inoculated with Map cells (approx. 10^{4-5} cfu/ml). Growth index values were recorded regularly throughout a 10 wk incubation period. Sterile milk was co-inoculated with Map cells and 'probiotic' lactobacilli strains and incubated (35°C, 72h). The recovery of Map was monitored through the BACTEC radiometric method. Dilute supernatants from isolates identified as *Lactobacillus casei* (Danone) and the probiotic strain *Lb. rhamnosus* GG and a small number of cheese isolates prevented any increase in growth index values typical of Map metabolic activity. Map growth was inhibited (delayed) when supplemented with supernatants from a number of *Lb. paracasei* isolates. When co-inoculated with probiotic strains in sterile milk for 48 h (pH < 4.5) Map could not be detected by radiometric culture up to 50 days. The results of this study suggest the *in vitro* inhibitory effect of some lactobacilli on Map growth may be due to factors other than acid production. Irrespective of the mechanism of inhibition, the possibility exists for the inclusion of such strains in dairy products for the in situ inhibition of Map or furthermore their use as biotherapeutic agents in the gastro-intestinal tract.

Isolation of Mycobacterium avium subsp. paratuberculosis from bovine colostrum by immunomagnetic separation

M N Ebert, S A Kraft, Irene R. Grant, J A Donaghy, Michael T. Rowe, C Seyboldt

Abstract

Newborn calves are most susceptible to infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the cause of paratuberculosis. Colostrum is an important source of this organism due to direct excretion of MAP within the milk gland or indirect contamination with MAP-infected faeces. The aim of this study was to develop a rapid method based on an immunomagnetic separation (IMS) technique for the sensitive detection of MAP in bovine colostrum. The IMS was performed with mouse monoclonal antibodies (IgG) against lipoarabinomannan (LAM), a component of the cell wall of mycobacteria, secondary coated onto DynabeadsÒ Pan Mouse IgG. The hybridoma cell line used for the production of the anti-LAM IgG was kindly provided from John T. Belisle, National Institute of Health, Colorado State University, USA. IMS was carried out by adding 10 µl anti-LAM IgG coated beads (ca. 3 x 10^6 beads) to the centrifuged pellet of 1 ml MAP-spiked colostrum resuspended in *900* µl phosphate buffered saline and incubating for 45 to 60 min at room temperature with gentle rotation. After magnetic separation the cell-bead complexes were resuspended in lysis buffer, transferred to tubes containing glass beads and mechanically disrupted in a Hybaid ribolyser for 3 x 15 s at 6.5 m/s and then incubated with proteinase K for 30 min at 65°C. Following DNA extraction with NucleoSpin Food KitÒ (Macherey-Nagel), IS*900* PCR was applied for the specific detection of MAP. Experiments showed that IMS in conjunction with IS*900* PCR recovered MAP from artificially inoculated colostrum containing between 10 and 100 CFU/ml. This developed method offers a sensitive rapid detection system to screen reserves of colostrum for the presence of MAP as part of a herd-level paratuberculosis control programme.

Lipidomics of Mycobacterium avium subsp. paratuberculosis in comparison to other members of the M. avium complex

T M Eckstein, S L Duskin, S Chandrasekaran, S N Manzer, B D Acres, J T Belisle, J M Inamine

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease in cattle and other ruminants, and it is also a possible cause of Crohn's disease in humans. We have initiated studies to elucidate the biochemical differences that allow MAP to maintain a specific biological niche that is not shared by the very closely related *Mycobacterium avium* subsp. *avium* (MAA), an opportunistic human pathogen that causes systemic infections in immunocompromised individuals, respiratory diseases (e.g. "hot tub lung") in the general population, and unilateral lymphadenitis in young children. Here we report the comparative lipidomics of MAP vs. other members of the *M. avium* complex (MAC). These studies were initiated through a global lipidomic analysis of MAP strain K-10 and MAA strain 2151 (serovar 2). Total lipids of lyophilized whole cells and of lyophilized culture filtrate were extracted with chloroform/methanol (2:1) and the non-lipid components were removed by Folch wash. Lipids were separated by two-dimensional thin layer chromatography using five different solvent systems and visualized by different staining reagents to deduce the chemical nature of these lipids. Nine lipids have been identified in MAP strain K-10 that are absent from MAA strain 2151. A more comprehensive analysis with strains representing all 28 serovars of the *M. avium* complex demonstrated that four of the lipids were truly MAP-specific; two polar lipids, one apolar phospholipd, and one apolar lipopeptide complex. These four lipids were only associated with whole cells of MAP and did not appear to be present in the culture filtrate.

Comparative proteome analyses of Mycobacterium avium subspecies paratuberculosis Type I, Type II and Type III

A Garcia-Sanchez, Valerie Hughes, S Smith, Karen Stevenson

Abstract

Strains of *Mycobacterium avium* subspecies *paratuberculosis* have been subdivided into three groups, Type I, II and III, by pulsed-field gel electrophoresis (PFGE). Type I strains comprise pigmented, slow growing strains that appear to have a host preference for sheep; Type II strains comprise non-pigmented strains with a very broad host range; Type III strains comprise non-pigmented strains that have PFGE profiles intermediate between Type I and II and exhibit biological characteristics from both groups. It is proposed that Type I strains are an evolutionary intermediate between *Mycobacterium avium* subsp. *avium* and Type III strains. We have used 2-D gel electrophoresis to characterize the proteomes of the three strain types to investigate the molecular basis for the biological differences observed. Type I and II strains were found to express Type-specific proteins. No Type-specific strains were identified for Type III strains. Type-specific proteins were identified by Matrix Assisted Laser Desorption Ionisation Mass Spectometry (MALDI MS). The relevance of these proteins to phenotype and evolutionary perspectives will be discussed.

Lpp24, a novel putative lipoprotein from Mycobacterium avium subsp. paratuberculosis (Map)

A Gioffré, M J Zumárraga, V Meikle, C Morsella, F Bigi, A Alito, K Caimi, P Santángelo, Fernando A. Paolicchi, M I Romano, A Cataldi

Abstract

A Map expression library in lambda ZAP was screened with mice and cattle sera to identify novel antigens. One clone was selected, sequenced and further characterized. The sequence analysis of the putative ORF predicts a protein of 20.8 kDa with a probable signal sequence compatible with Cys-acylation at Cys24, characteristic of lipoproteins. In consequence the protein was termed lpp24. We observed an important difference between the predicted (20.8 kDa) and the apparent molecular weight, either native form in Map (28 kDa) or recombinant protein in Escherichia coli (34 kDa). Upstream of lpp24 there is a sigF homologous gene. Downstream of lpp24 there is an ORF with unknown function and transcribed in the opposite sense, followed by a tetR like regulator gene. The protein was further localized in the membrane fraction of Map and extracted in the detergent phase of Triton X-114. A strong crossreaction with a protein from *E. coli* that has a higher size was observed. No crossreaction was observed in *M. tuberculosis* extract proteins. The presence of the gene in other mycobacteria was evaluated. It was observed the protein in *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* and was absent in *M. smegmatis*, *M. bovis*, *M. tuberculosis*, *M. chitae*, *M. africanum*, *M. vaccae*, *M. fortuitum*, *M. aurum*, *M. terrae*, *M. leprae*, *M. phlei* and *M. pinipedii*. Humoral reactivity using bovine sera demonstrated that this protein is widely recognised by the infected and non infected animals as well, due in part to the conserved sequence in close related environmental bacteria as *M. avium* subsp. *avium* and to the presence of a conserved epitope in other bacteria as *E. coli*. In conclusion, these findings show that Lpp24 is a membrane protein and a putative lipoprotein present in *M. avium* complex and absent in *M. tuberculosis* complex.

Definitive identification of single versus mixed mycobacterial infection(s) in red deer (Cervus elaphus) by combined duplex upstream-p34:f57 amplification and Hpy188I enzymatic restriction of duplex amplicons

Jacques Godfroid, C Delcorps, L Irenge, K Walravens, S Marché, J - Gala

Abstract

Severe emaciation and mortalities, suggestive of mycobacterial infections, were recently reported in both adult and young wild red deer (*Cervus elaphus*) in the south-eastern part of Belgium. In deer, tuberculous lesions are not pathognomonic of *Mycobacterium bovis* (Mbo), due to gross and microscopic similarities with lesions caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map) or *Mycobacterium avium* subspecies *avium* (Maa). The aim of this study was to improve the molecular species-specific identification of Mbo, Maa, and Map in deer mycobacterial infections. DNA banding patterns were assessed prior and after Hpy188I restriction of f57:usptream (us)-p34 duplex amplicons. As reported, the duplex f57:us-p34 PCR duplex differentiated Mbo from Map and Maa infections, whereas the restriction step differentiated single Map and Maa from mixed Map/Maa infections. The endonuclease Hpy188I cleaves DNA between nucleotides N and G in the unique TCNGA sequence. This restriction site was found at position 138 upstream the us-p34 initiation codon in all Maa strains tested, regardless of their origin and the IS*901* PCR results. In contrast, the restriction site was abrogated in all Map strains tested, regardless of their origin, the Mycobactin J dependency and the IS*900* PCR results. Consequently, the two-step strategy, i.e. duplex us-p34:f57 PCR and Hpy188I restriction, allowed to exclude Mbo infection and to identify single (Map and Maa) or mixed (Map/Maa) infections in wild red deer in Belgium. Accordingly, we propose to integrate, in a functional molecular definition of Map, the absence of the Hpy188I restriction site in the us-p34 amplicon.

Survival of Mycobacterium avium subsp. paratuberculosis isolates from different host species in bovine monocyte-derived macrophages from naturally infected cows in different stages of Johne's disease

N S Gollnick, R M Mitchell, Ynte H Schukken

Abstract

Previous studies have reported that peripheral blood mononuclear cells (PBMCs) from cows with a different Johne's disease status challenged with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) show a difference in gene expression profiles. These studies have not evaluated if PBMCs show a difference in MAP killing ability as well. Other *in vitro* studies looked at gene expression patterns of macrophages from Johne's disease-negative animals when exposed to MAP and *Mycobacterium avium* subsp. *avium*. Neither of the studies looked at differences in bacterial survival and propagation after infecting PBMCs or macrophages with strains of MAP from different host species. In this study we investigated the ability of bovine monocyte-derived macrophages from naturally infected cows in different stages of Johne's disease to kill MAP isolates from different host species. We tested the hypotheses that infection history of cows does not affect macrophage killing ability and that killing capacity of bovine macrophages is not dependent on the MAP strain. PBMCs were obtained from low-shedding and high-shedding Johne's disease-positive (n = 3 per group) and Johne's disease-negative (n = 3) multiparious cows. Following differentiation, macrophages where challenged *in vitro* with MAP strains of different host specificity (bovine, sheep, bison and human). Macrophages were harvested at 2 hours, 4 days, 7 days and 10 days following infection. For each time point ingestion and intracellular survival of MAP strains were determined by fluorescent staining of the bacteria with carboxyfluorescein diacetate. Furthermore, the approximate number of surviving bacteria was estimated by quantification of the MAP hsp65 gene by real-time PCR. Results of this study will advance the understanding of the bovine immune system as it pertains to infections with MAP, the possibility of inherent susceptibility of cattle to Johne's disease and the bovine species as a reservoir for MAP strains found in other species, including humans.

Evaluation of three methods of DNA extraction for the detection of Mycobacterium paratuberculosis by polymerase chain reaction in milk

Jacak Gwozdz, R Bowles, M Carajias

Abstract

Objectives: To evaluate three methods of DNA extraction from milk for the IS*900* polymerase chain reaction (PCR) and compare analytical sensitivities of the PCR and modified double incubation radiometric mycobacterial culture (RMC) method. Experimental design: The comparative evaluation of the three DNA extraction methods (Beadbeater, InstaGene and Qiagene) and determination of the detection limits of the RMC and PCR were carried out on triplicate samples of milk inoculated with serial ten-fold dilutions of *Mycobacterium paratuberculosis*. Results: Among the three protocols of DNA extraction from milk, the Beadbeater method was the most efficient procedure for the preparation of *M. paratuberculosis* DNA template for the IS*900* PCR. The average detection limit of the Beadbeater PCR system was about 70 viable *M. paratuberculosis* cells/50 ml sample. The InstaGene and Qiagene (QIAamp DNA Stool Kit) methods produced average detection limits by PCR of 600 and 700 cells/50 ml sample, respectively. The analytical sensitivity of the RMC was about 700 viable cells/50 ml sample. Conclusions: The analytical sensitivity of the Beadbeater PCR system is sufficient for this test to be used for the detection of low levels of *M. paratuberculosis* contamination in milk. Further evaluation of this test on diagnostic samples is warranted.

Comparative evaluation of two decontamination methods for the isolation of Mycobacterium paratuberculosis from effluent

Jacak Gwozdz

Abstract

Objective: To compare the effect of decontamination of slurry and sewage at 37ºC and 42ºC on rates of isolation of *M. paratuberculosis* and culture contamination. Design: Animal slurry and raw sewage were sampled over 6 months at approximately weekly intervals and decontaminated before radiometric culture (RMC) using the double incubation method. One set of triplicate 50 mL samples of slurry and sewage was decontaminated at 37ºC and the other set was decontaminated at 42ºC. Cultures showing positive growth were subcultured onto solid media to evaluate mycobactin dependency and tested for IS*900* by the polymerase chain reaction (PCR). Results: *M. paratuberculosis* or its DNA was detected in 7 of 45 (15.5%) cultures of slurry decontaminated at 37ºC and in 14 of 39 (35.9%) cultures of slurry decontaminated at 42ºC. The contamination rates in cultures of slurry processed at 37ºC and 42ºC were 88.2% and 69.2%, respectively. *M. paratuberculosis* DNA was detected in one of 45 (2.2%) cultures of sewage decontaminated at 42ºC. No *M. paratuberculosis* or its DNA was detected in 45 cultures of sewage decontaminated at 37ºC. The contamination rates in samples of sewage processed at 37ºC and 42ºC were 84.4% and 4.4%, respectively. Conclusions: The method of double incubation at 42ºC was more selective and sensitive than the standard procedure at 37ºC. This warrants further studies to evaluate the usefulness of the former method for the decontamination of faeces, tissues and milk.

Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis isolates recovered from dairy cattle throughout the United States

N B Harris, S Sreevatsan, J B Payeur

Abstract

The multilocus short sequence repeat (MLSSR) method is a newly described approach for genotyping *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) that has been used to differentiate strains previously indistinguishable by other methods. This study used four of the most divergent MLSSR loci (L1, L2, L8 and L9) to survey a total of 149 different strains of *M. paratuberculosis* from dairy cattle across the United States. Loci were sequenced multiple times to ensure consistency. Both animal (n = 138) and environmental (n = 23) sources from 29 dairy herds in 17 states were represented in this collection. The Simpson's index of diversity was 0.73, 0.80, 0.30, and 0.34 for each of the four loci, respectively. Temporal stability studies of these loci are currently underway. Overall, the 149 *M. paratuberculosis* strains could be differentiated into 53 different genotypes, but no geographic correlation within the subtypes was observed. Multiple genotypes were recovered from 24 of the 29 herds represented by these strains. The same genotype was recovered from two other herds, and the final three herds were represented by a single isolate. Of the 13 herds from which both animal and environmental isolates were obtained, 6 had at least one animal and environmental isolate with the same genotype. To determine if multiple strains could be recovered from the same animal, two dairy cows naturally infected with *M. paratuberculosis* and housed in Biosafety Level III containment at NVSL were used. Six isolates apiece were recovered from these animals over the course of ten months. For both animals, five different genotypes of *M. paratuberculosis* were recovered. These results taken together suggest that multiple strains of *M. paratuberculosis* observed within a herd may be the result of individual animals being infected with more than one strain of this pathogen.

Distribution of ISMpa1, IS1245 and IS1311 in Mycobacterium avium subsp. paratuberculosis and M. avium subsp. avium

T B Johansen, Ingrid Olsen, M R Jensen, S Nilsen, Berit Djønne

Abstract

Mycobacterium avium subsp. *paratuberculosis* and *M. avium* subsp. *avium* are closely related, but cause different disease complexes. Distribution of various IS elements have been used for diagnosis and strain typing of these species. Our objective was to investigate the distribution of IS*Mpa1*, IS*1245* and IS*1311* in the two subspecies by PCR and Restriction Fragment Length Polymorphism (RFLP). PCR analysis showed that all examined strains of *M. a. paratuberculosis* harboured IS*Mpa1*, and an identical pattern was demonstrated inn all isolates with IS*Mpa1*-RFLP. Among the *M. a. avium* strains, only a few isolates harboured IS*Mpa1*, and the IS*Mpa1*-RFLP revealed a different pattern from the *M. a. paratuberculosis* isolates. These findings suggest that *M. a. avium* have acquired the element at a later evolutionary stage. IS*1245* and IS*1311* share a 85 % DNA sequence homology. IS*1245*-RFLP of *M. a. avium* has been performed by a standardised method, where weak and inconsistent bands have made analysis difficult. There has also been some discrepancy in the literature about the presence of IS*1245* in *M. a. paratuberculosis*. Cross hybridisation is a possible explanation for these findings. We designed shorter and more specific probes for the two elements. Performing IS*1245*-RFLP on *M. a. paratuberculosis* isolates gave no hybridisation signals, but with IS*1311*-RFLP an identical seven-banded pattern was revealed in all strains. These results show that IS*1245* is not a part of the genome of *M. a. paratuberculosis*. Isolates of *M. a. avium* from humans and swine showed multibanded patterns with both probes, with easier interpretable patterns than with the standardised IS*1245*-RFLP. It is well recognised that *M. a. avium* isolates from birds show an identical three banded pattern described as the "bird-type" pattern with the standardised IS*1245*-RFLP. It was demonstrated that this pattern actually consisted of two copies of IS*1311* and one copy of IS*1245*.

In vitro survival of Mycobacterium avium subsp. paratuberculosis strains with differing host specificity in Balb/C bone-marrow-derived macrophages: invasion, propagation and cytotoxicity

R M Mitchell, S Sreevatsan, Ynte H Schukken, D G Russell

Abstract

In this study *in vitro* survival assays of multiple strains of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were used to examine whether species-specific MAP strains have different survival rates and cytotoxicity to macrophages. Six strains of MAP were used to infect mouse Balb/C bone marrow derived macrophages at a multiplicity of infection of 10:1. The strains of MAP included several bovine strains, bison, sheep and one ovine strain. A no-infection negative control and a *M. avium* complex (Mac) positive control were utilized. Macrophages were harvested at 4 timepoints following infection – 2 hours, 4 days, 7 days and 10 days. MAP cytotoxicity to macrophages was evaluated by counting multiple visual fields and comparing density of attached macrophages to the non-infected control population. Live/dead staining of MAP with carboxyfluorescein diacetate (CFDA) and counterstaining with Evan's Blue stained viable bacteria while quenching background macrophage fluorescence. Presence of MAP hsp65 DNA relative to macrophage GAPDH DNA isolated from infected macrophages allowed a more quantitative determination of bacterial copies per macrophage. Our null hypothesis would indicate that all strains of MAP are equally viable in the mouse macrophage system, with no difference in number of bacteria successfully invading macrophages or pattern of propagation of bacteria once invasion occurs. Our working alternative hypothesis was that we would find multiple survival patterns among strains of MAP, with differences in both levels of successful invasion (percent macrophages staining positive for MAP) and propagation once within the cells. Infections with the two bovine strains of MAP were expected to have results more similar to each other than to infections with other strains tested.

Sequence analysis of 15 loci of variable numbers of tandem repeats on Mycobacterium avium

Kei Nishimori, K Tanaka, R Ishihara, T Nishimori, E Hata, H Kobayashi, M Eguchi, I Uchida

Abstract

In order to develop an easy and rapid molecular typing method of *Mycobacterium avium*, 15 loci of variable numbers of tandem repeats (VNTR) were investigated. Phylogenic analysis, which was estimated from Manhattan distance matrix data of allele profiles of VNTR by Fitch-Margoliash criterion and some related least squares criteria without an evolutionary clock using PHYLIP (ver. 3.57c), divided the reference strains of *M. avium* into 4 major clusters. *M. avium* subsp. *paratuberculosis* strains formed one cluster and *M. avium* strains with IS*901* also formed one cluster. Sequence analysis of the representative PCR products of each cluster in 15 loci revealed the presence of single nucleotide polymorphism and supported the clustering of the phylogenic analysis of VNTR.

Adaptation of Mycobacterium avium subspecies paratuberculosis to growth in bovine macrophages

T Partridge, Lucy M. Mutharia

Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) primarily infects subepithelial macrophages in the ileum of susceptible animals. It was hypothesized that like other mycobacterial species, this organism avoids macrophage killing and is able to persist within a modified phagosome. Furthermore, it was hypothesized that survival in the phagosome environment is dependent on the expression of specific virulence factors that allow MAP to prevent phagosome acidification and the arrest of endocytic processing. In this study various techniques were used to assess MAP gene expression during growth in normal laboratory culture conditions and during growth within bovine macrophages. MAP may be required to adapt to limited oxygen availability within the phagosome and also during periods of persistence or dormancy, therefore gene expression following adaptation to hypoxia was also examined. Initially, differential display – reverse transcription PCR (RT-PCR) and specific RT-PCR techniques were used to identify differences in gene expression. The expression of specific targets including, katG, furA, hspX, and an ideR homolog, were examined in all three MAP growth conditions. Subsequently results were confirmed by subjecting RNA samples to Real Time PCR (qPCR) analysis. RNA samples were also screened by DNA microarray hybridization using a MAP specific array.

Variable number of tandem repeats (VNTRs) and mycobacterial interspersed repetitive units (MIRU) in the genome of members of Mycobacterium avium complex (MAC)

M I Romano, A Amadio, F Bigi, L Klepp, I Etchechoury, C Morsella, Fernando A. Paolicchi, I Pavlik, M Bartos, S C Leão, A Cataldi

Abstract

Member of MAC are serious pathogens for humans and animals. The aim of this study was to look for VNTR-MIRU loci in the genome of MAC to type these isolates. In the present study, we identified 26 VNTR-MIRU loci by using Tandem Repeat software: eight of them possessed a structure similar to MIRU, 18 of them were tandem repeat without MIRU structure and designated as VNTR. Most VNTR loci were located within predicted coding regions. Most MIRU loci were intercistronic with their extremities overlapping the termination and initiation codons of their flanking genes. Some of these VNTR-MIRU loci exhibited polymorphism among MAC isolates due to insertion or deletion of whole repeats and/or of nucleotide sequence degeneration. We determined the variability of six VNTR-MIRU loci in 21 *M. avium* subsp. *hominissuis* (MAH) and 26 *M. avium* subsp. *paratuberculosis* (MAP). The results of VNTR-MIRU typing were compared with those obtained by using RFLP and PRA. The analysis identified 15 different alleles with the combination of six VNTR-MIRU loci in the 21 MAH with 16 different IS*1245*-RFLP and four different PRA profiles. However, neither with the six VNTR-MIRU loci nor with PRA could MAP with 5 different IS*900*-RFLP profiles be distinguished. In conclusion, some of the VNTR-MIRU loci identified were useful to differentiate MAH isolates but not the MAP isolates here included. However, there were polymorphism in VNTR-MIRU loci between MAH and MAP genomes, which could be important in the understanding of obvious differences in the pathogenic effect of these mycobacteria.

New culture protocol for isolation of Mycobacterium avium subsp. paratuberculosis in raw milk

Juliana Ruzante, W L Smith, Ian Gardner, C Thornton, J S Cullor

Abstract

A novel and more rapid culture protocol for isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from bovine milk was developed and compared with the conventional agar slant method. Bulk tank milk from a herd repeatedly tested negative for Johne's disease was spiked with different concentration of MAP, from 10^6 to 10 CFU/ml. Five milliliters of milk were incubated for 3 hours with a solution containing CB18, a zwiterrionic detergent, known to concentrate MAP. Samples were centrifuged and decontaminated with an enzyme solution and plated into Middlebrook 7H10 agar containing mycobactin J and PANTA. Plates were screened weekly under a microscope (at 40X) and the time of detection was recorded. The assay was repeated 8 times and compared with results obtained from 50ml of milk spiked with the same MAP concentrations but decontaminated with 0.75% HPC solution for 5 hours and inoculated into Herrold's egg yolk medium (HEYM) and conventionally screened for bacterial growth. The presence of MAP in spiked milk samples could be detected between 14 and 45 days (n=42, mean=22.7) using the CB18 and microscopic screening method and between 21 and 63 days (n=41, mean=31) using the HEYM conventional culture method. The time to detection also differed with the MAP concentration with the samples containing higher concentrations detected earlier than the samples with lower concentrations in both methods. The modified culture method has reduced the time to detect MAP in raw milk, therefore representing a potential tool for the improvement of MAP control programs.

Comparative genomics of M. avium subsp. paratuberculosis strains isolated from different host species

M Semret, S Sreevatsan, D M Collins, M A Behr

Abstract

M. avium subsp. *paratuberculosis* (MAP) is an emerging pathogen of livestock and other mammals, in which it causes inflammatory bowel disease. Transmission from farmed animals, usually from cows, to wildlife or other "unusual" hosts has been well documented through the use of genotyping methods. However some strains seem to cluster with specific hosts. For instance sheep (S) strains, and some strains infecting bison (B), appear to be distinct from classical cattle (C) strains both phenotypically (more difficult to culture) and by molecular typing methods (IS*1311*-based). The basis for these genotypic and phenotypic differences is poorly understood. From whole genome DNA microarray-based comparative genomic studies, we have determined that there is extensive genomic diversity among the subspecies of the *M. avium* complex, with large sequence polymorphisms (LSPs) differentiating between MAP and other members of the complex. Since analysis of a restricted set of strains has revealed that MAP strains form a relatively homogeneous group, we hypothesised that LSPs may be a source of genetic diversity among phenotypically and genotypically distinct strains. To that end, we performed DNA comparisons of 3 S strains, 3 C strains, and one bison isolate, using *M. avium* subsp. *avium* (MAA, strain 104) as the referent. In addition to previously described LSPs, we found that a 16kb sequence, present in MAA 104 and in S strains, appears to be missing from the C strains and the bison isolate tested. Conversely, a 7kb sequence present in MAA 104 and C strains appears to be missing from the S strains tested. These results suggest that some LSPs may discriminate between different MAP strains. Confirmation and testing across a larger number of isolates is underway.

Molecular typing of Mycobacterium avium subsp. paratuberculosis strains from different hosts and regions

I Sevilla, Shri N. Singh, Joseba Garrido, G Aduriz, S Rodríguez, Marivi Geijo, Richard Whittington, V Saunders, Robert H. Whitlock, Ramon A. Juste

Abstract

Genotypic differences have been used to characterise isolates of *Mycobacterium avium* subsp. *paratuberculosis* (Map), the agent of paratuberculosis in ruminants. The IS*1311* Polymerase Chain Reaction-Restriction Endonuclease Analysis (PCR-REA) is a genetic typing technique that distinguishes between C (cattle), S (sheep) and B (bison) type strains of paratuberculosis. This method was used to detect genetic differences between 381 Map isolates from cattle, sheep, goat and bison, coming from distinct regions of Spain, India and United States. In Spain, all 346 bovine isolates, 4 of 10 caprine isolates (40%) and 1 of 12 ovine isolates (8.3%) were of the C type, whereas the other 11 ovine isolates (91.7%) and 6 caprine isolates (60%) were of the S type. All 5 ovine isolates and 6 caprine isolates from India, as well as all 3 isolates from bison (*Bison bison*) from the USA, were of the B type. This is the first report on the incidence of B type strains in species other than bison, although multiplex Pulsed Field Gel Electrophoresis (PFGE) and Restricted Fragment Length Polymorphism analysis results indicate that they are not the same strain. These results suggest that there are genetic differences between Map isolates related to geographic and host factors that can have a potential for the epidemiological tracing of new paratuberculosis isolates

Mycobacterium avium subsp. paratuberculosis (bison type) genotype infecting goat population in India

Shri N. Singh, I Sevilla, Ramon A. Juste, Richard Whittington, V Kumar, V K Gupta, A K Bhatiya, A V Singh

Abstract

Genotypic differences have been used to characterize isolates of *Mycobacterium avium* subsp. *paratuberculosis* (Map), the agent of paratuberculosis in ruminants. These differences were up to 2 groups: Cattle type strains and sheep type strains. Polymerase chain reaction - restriction endonuclease analysis (PCR-REA) based on polymorphisms in IS*1311* an insertion sequence present in Map and in *Mycobacterium avium* subsp. avium (Maa) in 7-10 copies. Techniques can be used as an easy and rapid method to distinguish between Maa and both cattle and sheep paratuberculosis strains. In the present study this IS*1311* – PCR-REA typing method on the several of mycobacterial isolates from goats in India. *Mycobacterium avium* subsp. *paratuberculosis* isolated from India were identified as Bison type strains regardless of origin. These results led to the finding that strain different from sheep could be involved in these cases (cattle type) strain. However, molecular methods used in this study proved that they were B type strains only described before (*Bison bison*) from Montana, USA. Pending more extensive typing in other regions the results reported here establish an un-expected link between American Bison and Indian small ruminants. This is the first report of involvement of Bison type MAP strains in goat herds endemic for Johne's disease in India. This study also provides the genetic key to contradictory reports on the isolation of sheep strains and underlines the importance of using different strains for the diagnosis of Johne's disease

Quantitative Real-Time PCR for the detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples and liquid culture samples

Raymond W. Sweeney, B L Mangold, S McAdams, T Calvin, Robert H. Whitlock

Abstract

The objectives of this study were: to evaluate a novel fecal DNA extraction method, combined with a quantitative real-time PCR (QRTPCR) amplification method (VetAlert™, Tetracore, Inc.) for the detection of MAP in bovine fecal samples from naturally infected cows, and 2) evaluate the use of the QRTPCR for confirmation of liquid culture of MAP. Fecal samples from multiple cattle shedding MAP at various levels were tested. The samples were processed with a DNA extraction and purification method which employed bead-beating and chaotropic solid phase extraction. Fecal DNA samples were then subjected to the QRTPCR amplification procedure, which is a real-time fluorescent probe hydrolysis assay. Shedding status was classified as negative, light, medium or heavy based on HEYM culture results. All of the heavy and moderate shedders were positive on QRTPCR. Approximately 30% of the samples from light shedders were positive. All of the culture-negative samples were negative on QRTPCR. The number of PCR cycles to reach positive detection threshold, which is related to the amount of DNA in the test sample, was significantly correlated to the number of colonies obtained on culture. Various DNA extraction procedures for MAP in liquid culture media were tested. Egg yolk added to liquid culture media appeared to inhibit the QRTPCR, and thus boiling or chaotropic solid phase extraction provided best results. All samples that flagged positive in the automated liquid culture machine were also detected as positive by QRTPCR. The DNA extraction procedure and quantitative real-time PCR assay show good sensitivity and specificity for the detection and quantification of MAP in bovine fecal samples and liquid culture samples.

Diagnostic performance of a liquid culture method, the TREK ESP Culture System II and para-JEM Broth, for detection of Mycobacterium avium subsp. paratuberculosis in fecal samples

C van Maanen, M G Koene, V Oosterhuis, T von Banniseht, S E Allen, S u NM

Abstract

Recently we evaluated the performance of a new liquid culture method, the TREK ESP para-JEM Culture System II, as compared with our standard method. The ESP system performed well with a strong reduction in time to detection (TTD). The LJ system, however, demonstrated a higher sensitivity for detection of low shedders. We also observed that weekly shaking of liquid cultures enhanced growth. To optimize the diagnostic performance of the ESP system, we evaluated three decontamination methods, combined with either stationary incubation or weekly shaking, on fresh bovine fecal samples from - predominantly light - shedders and from culture negative cattle. These samples were also cultured on LJ agar slants. We confirmed in this study the previously observed reduction in TTD by weekly shaking using the prescribed decontamination method. No reduction, however, was observed for the in-house and Stabel decontamination method. Mean TTD was lowest for the in-house decontamination method. With the latter method, however, we found surprisingly high numbers of false-positives. In another experiment, fresh fecal samples (n=116) originating from four infected herds were cultured in parallel on LJ agar slants and in ESP para-JEM culture bottles after decontamination as described by Stabel et al. From one infected herd also pooled samples were investigated. Both culture systems scored 40 samples positive with 32 samples in common. The mean TTD for the ESP system (39.1 days) was significantly lower than for the LJ system (75.6 days). For pooled samples from one infected herd a good agreement was found between both culture systems and individual LJ culture results. In conclusion, the ESP System yielded equivalent or slightly better results than the standard LJ system. The Stabel decontamination method combined the advantages of low numbers of false-positives with a relatively low TTD after stationary incubation

Proteomic analysis of iron deficiency in mycobacteria

M L Wilkins, J A Vaughan, M D Lanigan, G Beddome, B J Shiell, R K Luke, Mark Tizard, Woytek P. Michalski

Abstract

Within the host, free iron is limited and its acquisition by infecting mycobacteria is thought to play a major role in the development of pathogenesis. Moreover, iron is an essential element for most organisms and functions as a prosthetic group in key metabolic pathways such as electron transport, oxidative stress and transmembrane transport. Mycobacteria have developed effective acquisition systems that involve the production of the iron-chelating molecules (siderophores) mycobactin, exochelin and carboxymycobactin. The aim was to develop iron deficient mycobacterial cultures and utilise both genomic and proteomic approaches to study the role of iron in mycobacterial cell metabolism. Conditions for iron deficient cultures were optimised for the fast growing *M. smegmatis* and applied to slow growing *M. avium* ssp. *avium* and *M. avium* ssp. *paratuberculosis*. Iron deficiency in these cultures was confirmed by Reverse Transcription Quantitative Real Time PCR and an exochelin bioassay. Proteomic profiles of iron deficient cultures have been generated using two-dimensional electrophoresis and compared with those grown under normal culture conditions. Differentially expressed proteins were analysed using mass spectrometry and N-terminal protein sequencing. Their role in cell metabolism and their potential role in pathogenicity will be discussed.

Comparison of Mycobacterium avium subsp. paratuberculosis growth on three microbiological media

A Wiszniewska, J Szteyn, M M Fus, A A Ruszczyńska

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP), the etiological factor in Johne's disease of cattle, is also mentioned as one of the factors that could cause the Crohn's disease – a chronic, ulcerating distal ileitis in people. Despite a significant progress of studies on isolation of MAP, culturing of microorganisms on microbiological media is so far the only method allowing confirmation of presence of live bacterial cells in the examined samples. MAP is difficult to culture in vitro. The time required for obtaining colonies is long as at least 4 weeks are required (most frequently 12-14 weeks). Culturing on media supplemented with mycobactin J is an important factor allowing differentiation of MAP from other microorganisms of *Mycobacterium avium* complex. Herrold's medium (HEYM), Middlebrook 7H9 medium and Löwenstein-Jensen medium, all supplemented with mycobactin J are the media recommended by O.I.E for isolation and culturing of MAP. This study aimed at a comparison of speed and intensity of MAP growth on the listed media without and with a supplement of antibiotics (vancomycin and naladixic acid at 20 mg per 1 liter of medium). The cultures were incubated at 37°C. To confirm a dependence of MAP growth on the presence of an iron chelating agent, each sample was inoculated on 2 HEYM slants, 2 Middlebrook 7H9 slants and 2 Löwenstein-Jensen slants without the supplement of mycobactin J. Growth observation started from the fourth week of incubation. The moment of appearance of the first colonies, their looks and rate of growth were recorded. The fastest MAP growth (after 5 weeks of incubation) was obtained on HEYM medium, a slightly slower growth on Löwenstein-Jensen medium (6-9 weeks). Incubation for 16 weeks did not allow obtaining MAP growth on Middlebrook 7H9 medium supplemented with antibiotics. *Supported by WAMADAIREC project OLK1-CT-2002-30401*

Paratuberculosis diagnostics: the cost versus sensitivity trade-off

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INTRODUCTION

There is a saying in the U.S: "There's no such thing as a free lunch". It means that while the cost of some things may not be obvious, there is *always* a cost. This applies to paratuberculosis diagnostics as well. This article briefly reviews the state-of-the-art in paratuberculosis diagnostics and describes the challenges for improving tests. (Of course, the opinions expressed are my own and should not be construed to be those of the International Association for Paratuberculosis, the University of Wisconsin or any other organization with which I am affiliated.

Culture of M. paratuberculosis
Diagnosis of paratuberculosis by culture was the first, and remains one of the best methods of diagnosing paratuberculosis. The availability of commercially prepared media has improved *M. paratuberculosis* isolation rates on solid agar media (Payeur, 2005). Proficiency testing of laboratories also has significantly improved the capability and reliability of many laboratories based on experience in the U.S. (Payeur and Capsel, 2005). Radiometric culture methods using the BACTEC 460 system enhanced culture sensitivity and shortened incubation times. Many labs are moving to the next generation of liquid culture platforms such as the BACTEC MGIT 960 system and Trek ESP II system. While the culture media and instrumentation required by these non-radiometric systems are more expensive, they compensate with cost savings through automation. For herd-level detection of *M. paratuberculosis* infections sampling of feces from areas of the farm where cattle congregate, so called environmental sampling, or pooling of feces collected directly from cows (normally pools of feces from 5 animals) are two very effective ways of testing whole herds at a fraction of the cost of testing all individuals.

A larger challenge for culture-based diagnostics is specimen processing. The technical aspects of fecal sample processing is nicely reviewed in these Proceedings by Payeur (Payeur, 2005). Most specialists will agree, I think, that processing methods to isolate *M. paratuberculosis* from clinical samples remain laborious and non-standardized resulting in a widely varying proficiency across laboratories (Payeur and Capsel, 2005). Failure of existing procedures to completely and reliably kill or remove non-mycobacterial microflora in fecal samples leads to a high rate of culture contamination, e.g. >10%. Cultures lost to contamination are a cost that must be considered. Methods to concentrate the typically low number of *M. paratuberculosis* in clinical samples by centrifugation, sedimentation, or filtration are non-selective: the contaminants are concentrated along with the target pathogen. Efforts to lower contamination rates by using less feces in culture procedures lowers contamination rates but also lowers the sensitivity of the assay. Immunomagnetic separation (IMS) and similar such technologies to selectively concentrate *M. paratuberculosis* from samples are essential to improvement of *M. paratuberculosis* detection assays, whether by culture of genetic-based systems (Grant et al., 1998).

Genetic detection of M. paratuberculosis
Genetic detection based assays, hereafter referred to broadly as PCR, became possible with the discovery of IS*900* (Collins et al., 1989; Green et al., 1989). Other equally specific genetic targets and amplification systems have since become available. However, even 16 years after the IS*900* discovery was reported, the promise of a fast, affordable, specific and sensitive PCR for application to clinical samples remains an elusive goal, at least as a standardized commercial assay. Admittedly, several laboratories have "in-house" PCR assays that perform sufficiently well to match culture methods on proficiency tests (Payeur and Capsel, 2005). Such assays, however, are typically not very robust when transplanted to other locations and the unsubsidized per assay cost remains significant. These assays are often touted for their speed and some say this makes it worth the cost. While speed may be nice for the laboratorian, it rarely is of

importance to the end user of the test result, the farmer. In my experience, producers frequently do not act promptly on paratuberculosis test results, making me wonder why I worked so hard to get the results to them quickly. We must accept that speed is a relative term when dealing with this chronic infection in food producing animals. Given the choice, my clients would prefer a lower cost test to a faster one; an impression that colors the message of this article.

With the increasing adoption of liquid culture systems, there is a need for rapid, simple, low-cost PCR assays to confirm if acid-fast bacteria detected by liquid culture are, or are not, *M. paratuberculosis*. In this application there are abundant target organisms, as compared to clinical samples. Consequently DNA extraction protocols do not have to be as efficient, potentially making them simpler, faster and cheaper. A PCR with a short DNA extraction step and simple visual read out would be a welcome addition to the products offered to diagnostic laboratories using liquid culture systems for *M. paratuberculosis*.

Immunological diagnosis - assays for CMI
Assays for cellular immunity (CMI) have long held promise as the best form of test for early diagnosis of *M. paratuberculosis*-infected animals, a logical assumption given the success of CMI tests for bovine tuberculosis. Adoption of CMI assays for paratuberculosis remain hampered by the lack defined antigens of high specificity for use in skin tests or gamma interferon assays. An even bigger obstacle is the difficulty of proving the diagnostic sensitivity and specificity of such tests. Validating the accuracy of CMI assay requires long-term follow up on significant numbers of CMI test-positive and CMI test-negative animals to determine their true *M. paratuberculosis* infection status. Without such studies we do not have answers to questions such as:
1) are all CMI-positive cattle truly *M. paratuberculosis*-infected?
2) do all *M. paratuberculosis*-infected cattle progress to disease
3) are CMI-positive cattle those controlling the infection or even genetically more resistant to infection than their herdmates?
For all of these reasons, I think CMI assay development will progress slowly.

Immunological tests - assays for antibody
Thanks to the pioneering work of Yuichi Yokomizo, working in the laboratory of Richard Merkal in the early 1980s, we have ELISAs for detection of antibodies to *M. paratuberculosis* with very high specificity (Yokomizo et al., 1985). The technique of absorbing serum samples with extracts of *Mycobacterium phlei* before testing the sample for antibodies to *M. paratuberculosis* truly revolutionized paratuberculosis diagnostics. Yokimozo's technique is the basis of most commercial ELISA kits on the market today. ELISAs on serum samples have become the most widely used diagnostic method for bovine paratuberculosis. They are the fastest, lowest cost, high-specificity assay available. However, as with most diagnostic tests, there is room for improvement.

The sensitivity of ELISAs for serum antibodies to *M. paratuberculosis* is roughly 30% (compared to fecal culture) (Collins et al., 2005). Many experts are discouraged by this high rate of false-negative ELISA results. The good news is that both the rate of detecting animals shedding *M. paratuberculosis* in feces (sensitivity relative to culture) and the magnitude of ELISA results (S/P or optical density readings) are directly related to the probability that the tested animal is infected and shedding the organism in feces (Collins et al., 2005). In short, ELISAs detect the most infectious animals in a herd quite well. Whether this is sufficient or not to control the infection in herds, when coupled with changes in herd management, remains to be determined.

Another important finding about ELISAs is that in detecting cows in advanced stages of paratuberculosis they also predict which cows are likely suffering from significant suppression of milk production due to paratuberculosis (Lombard et al., 2005). Rightly or wrongly, these are the cows that our dairy farmer clients are most concerned about.

The newest diagnostic challenge is application of the same antibody detection technology used on serum samples to milk samples. Many recent studies indicate that comparable sensitivity and specificity can be achieved by testing milk as compared to testing serum using ELISA technology. The primary caveat to this idea is that one must avoid milk sampling when the cow is at the peak milk production stage of lactation,

particularly in high-producing cows, as this obviously dilutes out the analyte, i.e., antibody to *M. paratuberculosis*. There are multiple good reasons to test milk samples:

1) ELISA sensitivity and specificity are basically the same as for serum samples.

2) Milk samples are collected from most cows regularly anyway, hence there are no extra cost for sample collection.

3) Laboratories doing milk quality tests have the automation and instrumentation to handle large numbers of samples.

4) Laboratories that do milk quality testing have systems to report results directly to dairy producers electronically making results reporting fast and low-cost.

The people who do not like paratuberculosis testing by milk quality laboratories using milk samples are primarily those concerned about knowing exactly who is testing for paratuberculosis and how many positive results are found, i.e., government veterinarians charged with operating paratuberculosis control programs. And this brings me to the conclusion of this Review and Perspectives article.

If paratuberculosis is a zoonotic disease the diagnostic agenda is far different from what must be done if it is not zoonotic. In the zoonotic situation, paratuberculosis becomes a regulated disease, testing and test results are controlled by veterinary officials in the government, and in most countries public financial support can be expected to underwrite the costs of testing and control measures by farmers. In this scenario, diagnostic testing costs to producers will be artificially low (the balance of costs paid by the government) and even expensive tests may seem affordable to producers (already the situation in some states of the U.S.).

If paratuberculosis is not zoonotic on the other hand society, through the government, has limited vested interest in managing this disease problem. Diagnostics will be used by animal agriculture only to the extent that they can improve the profitability of the operation. As such, only low-cost tests will be used. While some may be concerned about the zoonotic potential of *M. paratuberculosis*, today no medical scientific body has clearly stated that it <u>is</u> zoonotic. Hence, for now we must promote paratuberculosis diagnostics that can be justified as improving the producer's bottom-line.

A test not used is not a useful test. Producers in animal agriculture are very price sensitive. Resources certainly should be invested to make paratuberculosis diagnostics more accurate, however, resources should also be invested to make them more affordable. When diagnostic tests are commercialized and market forces allowed to prevail this happens automatically.

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Paratuberculosis diagnostics: their use to support preventive management

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ABSTRACT

Diagnostics have been used in the Netherlands in paratuberculosis voluntary control programs since the foundation of the Animal Health Service in Fryslân in 1919. Programs were based on the intradermal skin test and the Ziehl-Neelsen test. After 1952 the complement fixation test was added. All programs were terminated after approximately 10 years because since they failed to control the disease. A failing program was subsequently followed by a new program with different rules. Failure was attributed to the diagnostics but the question arises whether the diagnostics were really to blame. Therefore diagnostic assays used in the past were compared with their new equivalents. The comparison displayed the effectiveness of these tests from the past; they performed much better than expected. Failure of control programs is thus less likely to be caused by the performance quality of the tests than by their improper use. The skin test is not suitable to support preventive management because skin testing detects early infections and effective resistance against shedding. To support management, tests should only be used to find faecal shedders so they may be removed. Only tests detecting serum antibodies and tests detecting MAP in faeces should be used. However farmers should be encouraged to cull test-positives immediately. Therefore specificity and predictive positive value of tests should be as high as possible. Faecal culture is the preferred test but is considered too expensive for control programs. Although tests detecting serum antibodies are adequate to detect high shedders, the specificity of most commercially available is too low and only a few ELISAs reach a specificity of >99.5%. Pooled faecal cultures offer a cheap and reliable alternative, especially to certify herds as free from paratuberculosis. Certified-free herds are necessary to validate new ELISA tests.

INTRODUCTION

During the last century control programs for paratuberculosis were developed in many countries. Nevertheless paratuberculosis has not been eradicated nor even controlled in most countries with large dairy industries. The failure of these programs is often blamed on a lack of accuracy of the diagnostic tests. This seems strange because a broad array of diagnostic approaches were and are available. Diagnostics are available for detection of cell mediated immunity, serum antibodies and the organisms themselves. Diagnostics used in the early programs were a skin test for cell mediated immunity, Complement Fixation Test (CFT) for serology and Ziehl-Neelsen (ZN) test for detection of organisms. The numerous excellent researchers publishing about the diagnosis of paratuberculosis in the last century makes it hard to believe that diagnostics are responsible for the failure of control programs (Chiodini, 1993).

Infectious diseases such as brucellosis and tuberculosis were not only controlled but even eradicated within the same countries, over the same period and with the same generation of diagnostics. The CFT for brucellosis was also not perfect (Gall et al., 2004), producing both false positive and false negative test results (Emmerzaal et al., 2002). Neither were the skin test and ZN test perfect when used for tuberculosis eradication (Monaghan et al., 1994).
Paratuberculosis was eradicated from the dairy industry in Sweden, implying that, with strict measurements and available diagnostics, control of paratuberculosis elsewhere is also possible (Bölske et al,. 1999). In most countries, including the Netherlands, veterinarians did not consider test-positive animals as infected unless another diagnostic test was also positive (Larsen et al., 1963; Benedictus 1985). The tests used for confirmation were however not suitable for this purpose because they focused on a different stage of infection. For example the skin test that detects early infection was confirmed by CFT which is most effective in late infection.

Early paratuberculosis programs were based on the skin test (delayed type hypersensitivity test). Control programs in the Netherlands began with the foundation of the Animal Health Service in 1919 in the province of Fryslân. Skin test-positives were confirmed by the ZN test of fecal samples (Veenbaas et al., 1932; Houthuis, 1932). After 1952 CFT was included in the diagnostic procedure, and used in combination with skin test (Reinders 1986; Benedictus, 1985; Dinkla, 1988). However a small study with faecal culture showed that only 9% of the animals shedding MAP in their faeces were positive on both skin test and CFT (Muiser 1985). After the disappointments with control programs in the Netherlands based on immunological tests, and the withdrawal of vaccines from the market, programs based on faecal culture only were successfully introduced in 1996 (Kalis et al., 1999; Benedictus and Kalis, 2004). However, in spite of success, this approach was considered too expensive for routine application in dairy industry.

This review compares old diagnostic assays with the new generation assays to investigate whether these new diagnostics can prevent control programs from failing. Skin test was compared with Gamma Interferon Test (GIT), CFT was compared with ELISA, and individual faecal culture was compared with pooled faecal culture.

MATERIALS AND METHODS

Animals and herds
The Dutch paratuberculosis program offered the opportunity to evaluate old and new diagnostics in known infection-free herds and in known infected cattle and herds. The certified free herds were tested twice a year with culture of strategically pooled faecal samples (Kalis et al., 2000; Kalis et al., 2004). The faecal culture positive animals from the eradication program were sampled for serum antibody detection as soon as culture results were available. The effect of simultaneously used diagnostics was studied in a rapid eradication study (Kalis et al., 2002). In this study all animals >6 months of age were tested twice in one year and all positives were culled. Results were evaluated after 2 and 4 years had passed.

Diagnostics
Detection of serum antibodies
CFT was compared with two new serological tests: an absorbed ELISA delivered by IDEXX and manufactured from components of the Central Serum Laboratory (CSL) in Australia, and an absorbed ELISA, also delivered by IDEXX and manufactured by Ubitech in Sweden. Results were interpreted according to manufacturer's instructions (Kalis et al., 2002).

Detection of cell mediated immunity
The intradermal skin test was compared with the gamma interferon assay (GIT) provided by IDEXX and CSL. The skin test was interpreted by measuring the thickening of the skin 3 days after the intradermal injection of the antigen, a purified protein derivate (PPD) from MAP. An increase equal to or greater than 4mm served as the cut-off value in accordance with international standards for tuberculosis skin test interpretation. The GIT was interpreted according to manufacturer's instructions. Gamma interferon production was also evaluated with a newly developed algorithm (Fig. 1) because results obtained from recommended algorithms were considered inadequate for diagnostic purposes (Kalis et al., 2003).
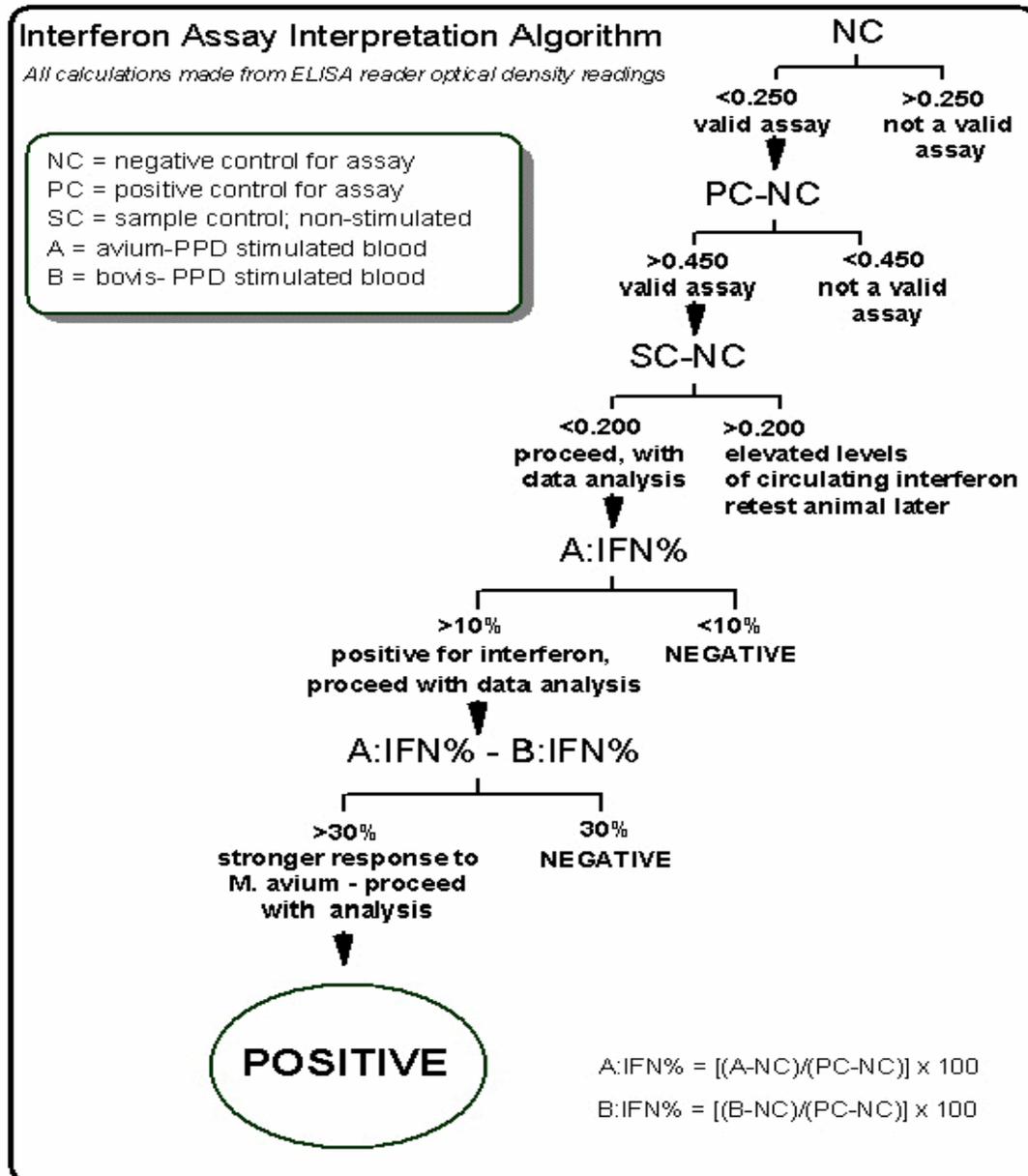
Fig. 1. Interferon assay interpretation scheme

Detection of organism
The results of individual faecal culture were compared with cultures of age-dependent pooled faecal samples. The culture method was a modified Jørgensen method, including 3 centrifugation steps to concentrate the samples and a prolonged incubation period of 16 weeks (Kalis et al., 1999).

RESULTS

The sensitivity and specificity of CFT was equal to the sensitivity and specificity of the IDEXX-UBITEC ELISA. The IDEXX-CSL ELISA had lower diagnostic sensitivity and higher diagnostic specificity (Table 1).

Table 1. Relative sensitivity and specificity of 2 absorbed ELISAs and a Complement Fixation Test (CFT) for the diagnosis of paratuberculosis in culture-positive cows (n = 198) from 53 infected herds, cows (n = 811) from 41 herds without clinical paratuberculosis and with $\geq$1 negative herd cultures, and cows (n = 346) from 19 herds without clinical paratuberculosis and with 9 negative herd cultures (Kalis et al., 2002).

	Infected herds		**Non-suspect herds**			
			$\geq$1 negative cultures		**9 negative cultures**	
	Test-positive cows	**Detection rate (%)**	**Test-positive cows**	**Specificity (%)**	**Test-pos. cows**	**Specificity (%)**
ELISA-CSL	59	29.8[*,†]	809	99.8[*,¶,#]	345	99.7[*,¶,#]
ELISA-Ubitech	78	39.4[¶]	754	93.4[¶]	322	93.1[¶]
CFT	70	35.4	771	95.1	323	93.3

[*]Different (P < 0.005) from results of ELISA-B; [†]Tendency for difference (0.05 < P < 0.10) from results of CFT; [¶] Different (P < 0.05) from results of ELISA-B, double cut-off value; [#]Different (P < 0.05) from results of CFT.

The diagnostic sensitivity of all 3 tests depended on the level of shedding, ranging from 12% in low shedders (IDEXX-CSL-ELISA) to 79% in high shedders (IDEXX-UBITEC-ELISA) (Table 2). The specificity of the tests was equal in herds declared free of paratuberculosis whether in one or in 9 herd investigations with negative faecal cultures.

Table 2. Detection rate of 2 absorbed ELISAs and a complement fixation test (CFT) for detection of antibodies against MAP related to the level of bacterial shedding.[*] (Kalis et al., 2002).

	1-10 CFUs		**10-100 CFUs**		**>100 CFUs**	
	No. Pos.	**%**	**No. Pos.**	**%**	**No. Pos.**	**%**
ELISA-CSL	11	12[¥,#]	18	40[#]	19	68
ELISA-UBITEC	22	24[¥,#]	22	50[#]	22	79
CFT	16	19[¥,#]	21	52[#]	21	75
All	7	8[¥,#]	13	30[#]	18	64
None	62	69[¥,#]	16	46	5	18

[*]CFU was not determined for all samples; [¥]Different (P < 0.01) from 10-100 CFUs; [#]Different (P < 0.05) from >100 CFUs.

Table 3: Specificity of the skin test and the gamma interferon assay for the early diagnosis of paratuberculosis. The results of the gamma interferon assay are interpreted according the test documentation provided with the kits from the two manufacturers as well as a new interpretation scheme (Kalis et al., 2003).

	Specificity
Skin test (4 mm = positive)	93.5%
Gamma Interferon Assay ("CSL" algorithm)	66.1%
Gamma Interferon Assay ("IDEXX" algorithm)	67.0%
Gamma Interferon Assay (New algorithm)	93.6%

If the rules for interpretation provided with the kits by the two companies selling the GIT kits were used, the specificity of GIT was much lower than the specificity of the intradermal skin test using a cut-off level of 4 mm. After developing a new algorithm for interpretation, the same specificity was obtained as was found with the skin test (Kalis et al., 2003; Table 3).

The high sensitivity of these tests is demonstrated in a rapid eradication program using 3 tests simultaneously: faecal culture and GIT in animals <24 months, faecal culture and ELISA in animals >24 months (Fig. 2) (Kalis et al., 2002).
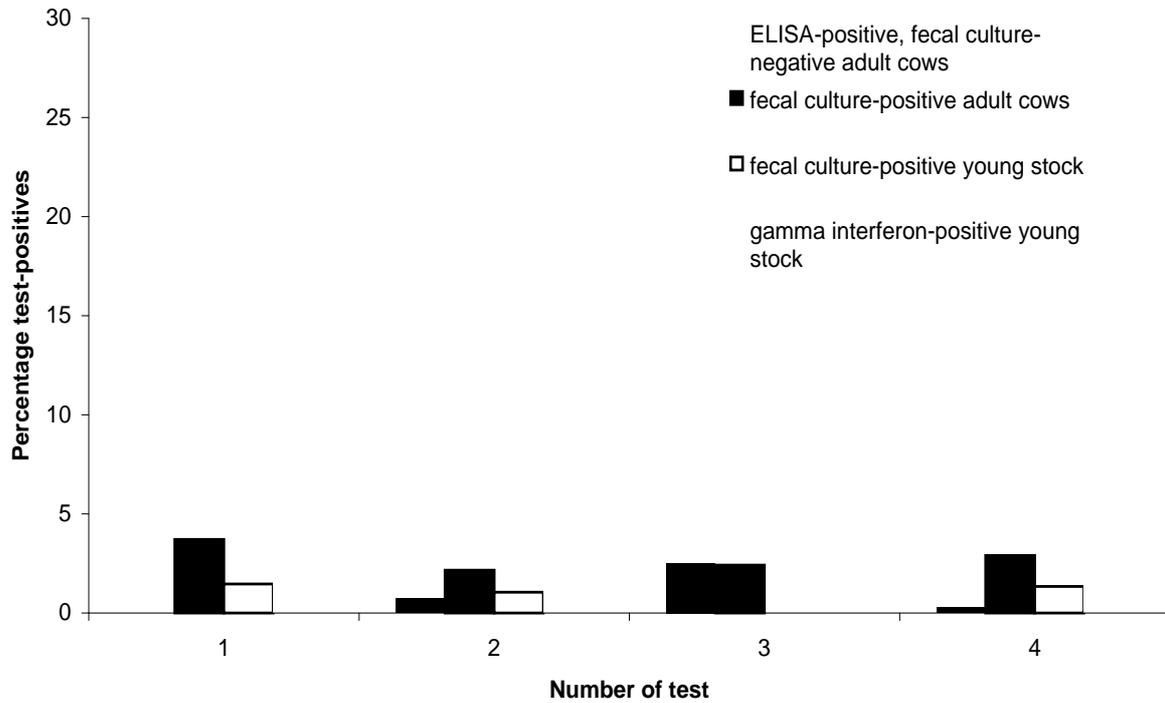
Fig. 2. Percentage of test-positive animals for 3 tests used simultaneously in 12 dairy herds in an attempt to eradicate paratuberculosis in a one-year period, evaluated after 2 and 4 years.

Table 4. Comparison of number of MAP colonies in individual and matched pooled faecal samples.

	No. pools (n=145)	Culture-positive individual faecal samples per pool	Mean total colonies per pool (range)	Mean total colonies from individual faecal samples (range)
Pool neg.[#] + individual neg.	111	0	0	0
Pool pos.[#] + individual neg.	2	0	5 (1-10)	0
Pool neg. + 1 individual pos.	6	1	0	7 (1-20)
Pool pos. + 1 individual pos.	17	1	21 (5-150)	33 (1-400)
Pool pos. + 2 individuals pos.	7	2	15 (2-30)	36 (5-116)
Pool pos. + 3 individuals pos.	2	3	28 (2-54)	244 (9-480)
Average no. in 'positive' [##] pools (n=34)		1.3	15.8	31.4

[#]Neg. = MAP-negative culture , pos. = MAP-positive culture; [##]Pools with MAP-positive culture (including positive pools with only culture-negative individuals) and pools with MAP-negative culture, but containing culture-positive individuals.

The culture of pooled faecal samples was as sensitive as the culture of individual faecal samples (Table 4). Pooled faecal culture detected 87% of culture positive individuals and 73% of infected herds. Individual faecal culture detected 94% of individual culture positive animals and 64% of infected herds (Kalis et al., 2000).

DISCUSSION

The results clearly demonstrate that the bad reputation of the old diagnostics is undeserved. As a consequence failure of the earlier control programs can not be attributed to the performance of the diagnostics. This finding emphasises that current and future control programs may be expected to fail if the diagnostics are used in the same way: focused on detection of infection instead of shedding.

A new attitude is needed for successful control. The infectious level of a herd should be lowered in combination with improved management. Management is an effective tool to block contact between adult shedders and young susceptible animals. However, faecal shedders transfer the infection at calving to the

young population, for example via the colostrum, and within the young population this transferred infection spreads horizontally due to temporary shedding by calves (Kalis et al., 1999). Therefore it is necessary to support preventive management by removing heavy shedders before calving. The only tests detecting these shedders are serum antibody assays and tests detecting organisms in faeces. However, only a few commercially available ELISAs were really as specific as published earlier by Collins et al.(1991), who described the CSL-based ELISA tested in this paper with comparable results. Many of the other commercially available ELISAs had specificities ranging from 57% to 99.8%, thus often lower than the UBITECH ELISA used for this comparison (Van Maanen et al. 2002). The importance of specificity to obtain a sufficient predictive positive value is demonstrated in Table 5.

Table 5. Predictive positive value of IDEXX-UBITECH-ELISA, with detection rate 40% and specificity 93% in a herd with 10% paratuberculosis infected cows

	Infected	Uninfected
Pos.	2	6
Neg.	8	84

As can be learned from this table, the predictive positive value is only 25%. This is partly due to the low sensitivity estimate of 20% instead of generally accepted 40%. This is because the detection rate was 40% but the sensitivity of faecal culture is generally considered to be 50%, leading to a sensitivity of ELISA of 0.4x0.5=20%. The most important factors involved in low predictive positive value are prevalence and specificity. Realising that a farmer only perceives the percentage of true positives as the important test criterion and may have a low prevalence in his herd, specificity should be as high as possible. This means that the only test characteristic to select an ELISA is specificity. Sensitivity is a far less important criterion of ELISA, because the heavy shedders are detected by all ELISAs.

Low shedders are best detected by a sensitive faecal culture method. Better standardisation and improvement of faecal culture methods in laboratories worldwide would be beneficial, as Whitlock demonstrated in the conference in Bilbao (2002). Pooled faecal culture is a specific and sensitive test available for herd certification.

The newest test for detecting organisms, the polymerase chain reaction (PCR) is not sensitive enough to replace faecal culture. The best application of this test is to replace the ZN test and to confirm the identity of colonies grown on faecal cultures as MAP.

CONCLUSION

The reason earlier control programs failed is not the quality of diagnostics; their good diagnostic quality was demonstrated in this study. The approach taken by decision makers with these diagnostics should be changed. The only purpose should be to find and remove faecal shedders. Nationwide programs could be based on absorbed ELISA with at least 99.5% specificity. For individual herd owners who want to eradicate paratuberculosis, (pooled) faecal cultures should be made available. Testing to detect cell mediated immunity should be avoided.

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Validation of sequential *Mycobacterium avium* ss. *paratuberculosis* serology (SMAPS)

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ABSTRACT

Sequential *Mycobacterium avium subspecies paratuberculosis* Serology (SMAPS) was recently proposed by Böttcher and Gangl (2004). Briefly, sera are screened in a test of higher sensitivity (Svanovir, Uppsala). Subsequently, ELISA-positive sera are tested by two tests of higher specificity (IDEXX, Wörrstadt; Pourquier, Montpellier). Individual sera are interpreted as "+++" to "neg" based on agreement of results. The highest serological score per herd defines the herd classification level (e.g. "+++"➔A; "neg"➔E). The objective of the present study was to validate SMAPS. A total of 10,656 blood samples and 1,767 faecal samples (949 animals) were collected from 137 herds. Up to three successive faecal/blood samples were collected per cow in order to increase the sensitivity of bacterial culture and to analyse serological differences over time. MAP was isolated in 33 (3.5%) of 949 tested animals and positive bacterial culture was significantly associated with "+++" seroreactivity (41.2%). MAP was isolated in "++"-, "+"-, "(+)"- and "neg"- ELISA sample animals as well, but to a lesser extent. Isolation of MAP was made in five herds classified at least one time as category A. In only one "non-A" herd was a positive culture result obtained. While a significant number (41.8%) of +++ results were missed by SMAPS vs. IDEXX/Pourquier testing in the more heavily infected control herd, fecal culture positive results were highly associated with +++ results. SMAPS is a valuable tool for detection of high risk herds ("A"). SMAPS pinpoints additional infected animals in those high risk herds. The stability of serological results over time remains to be analysed.

Key words: serology, bacterial culture, paratuberculosis, longitudinal study

INTRODUCTION

Several serologic tests have been developed and evaluated for diagnosis of paratuberculosis in cattle (Harris and Barletta, 2001). Tests with various sensitivity or specificity levels are available commercially, however high sensitivity tests result in false positives and high specificity tests fail to detect some *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infected animals. Since these serologic problems won't be resolved in the near future, a sequential serologic testing strategy combining tests of different sensitivities and specificities was proposed by Böttcher and Gangl (2004). The principles of Sequential MAP Serology (SMAPS) are summarized in Fig. 1. Briefly, sera are tested in a test of higher sensitivity; negative and suspect sera are defined as "negative". Test positive sera are then re-tested in two tests of higher specificity (IDEXX, Wörrstadt; Pourquier, Montpellier). According to the reactivity pattern in these assays, assay interpretation range from "+++", "++", "+" to "(+)" reflecting the decreasing likelihood of MAP infection. The highest serologic score obtained establishes the herd status: "+++" results in "A", "++" in "B", "+" in "C", "(+)" in "D" and "negative" results in "E". The aim of the present study was to evaluate SMAPS, that is, determine if serial screening results in loss of diagnostic information and if individual animal classification reflects the true likelihood of MAP infection.
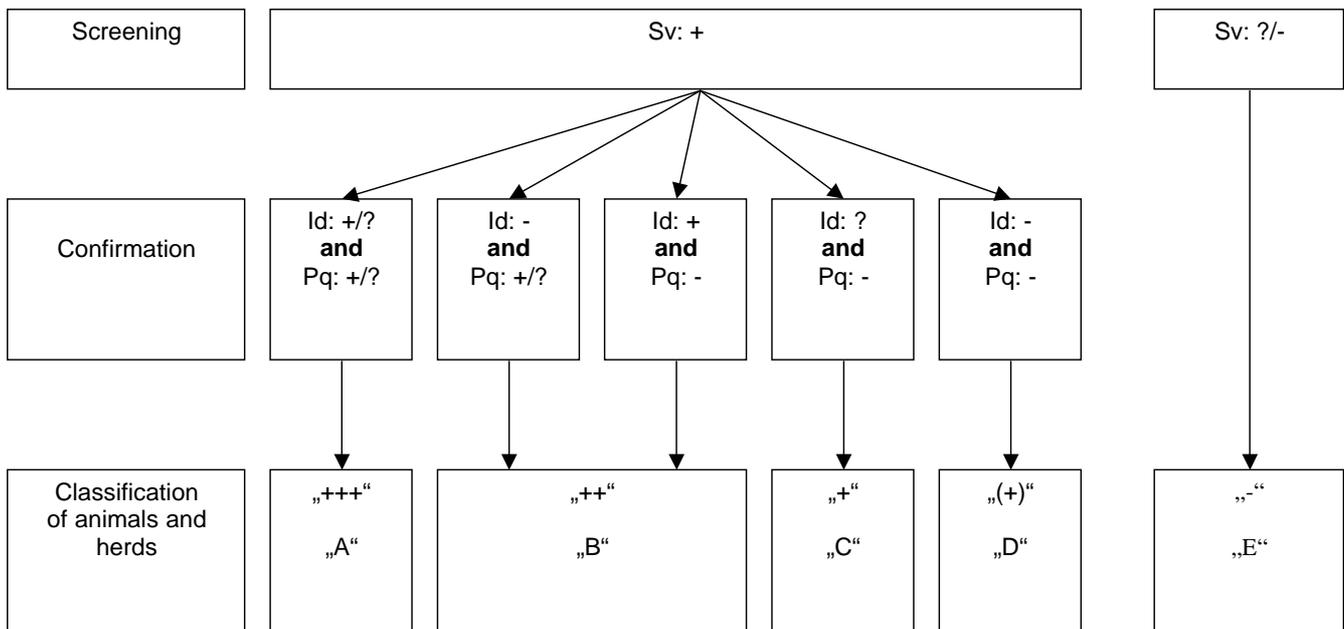
Fig. 1: Sequential MAP Serology (SMAPS, Böttcher and Gangl, 2004)
ELISA: Sv – Svanovir, Id – IDEXX, Pq – Pourquier

MATERIALS AND METHODS

The number of analysed samples (sera, faeces), and the number of dairies included in the study are listed in Table 1. Data from 2003 and on three occasions in 2004 (2004-I, -II, III) were available. Seventy-eight dairies were monitored in 2003 and 86 were included on each occasion in 2004. All herds were located in Bavaria with the exception of one herd (BB). BB was a dairy with paratuberculosis (clinically affected cows/culture positive) and served as a control.

Table 1: Numbers of sera, faecal samples and herds

	Sera	Faeces	Herds
2003	2738	0	118
2004-I	2881	425	106
2004-II	2478	671	103
2004-III	2001	661	88
BB	558	10	1
Total	10656	1767	137*

* different herds

Serologic tests
The following tests were used: SVANOVIR^TM Paratuberkulose ELISA (Para-TB-Antikörper-Test (Sv), Svanova Uppsala, *Mycobacterium paratuberculosis* Antibody Test Kit IDEXX Scandinavia – distributed by IDEXX, Wörrstadt (Id) and ELISA Paratuberculosis Pourquier (Pq), Montpellier. Tests were performed according to manufacturer's instructions. Samples were analysed in a routine diagnostic laboratory. Each serum was tested in all three tests.

Interpretation of ELISA results for SMAPS is summarized in Fig. 1. The efficiency of SMAPS is mainly influenced by the sensitivity of the screening test, thus SMAPS was compared to a testing scheme without screening based solely on Id and Pq results. These alternative interpretations are indicated by an asterisk (*) as follows: +++*: Id equivocal/positive and Pq equivocal /positive, ++*: Id positive or Pq equivocal/positive, +*: Id equivocal, neg: Id and Pq negative.

Bacterial culture
Faecal samples were decontaminated with 0.75% hexadecylpyridinium chloride (HPC). Thirty ml of HPC-solution was added to a 50-ml tube containing three grams of the faecal sample and vortexed for 15sec. After sedimentation for a short period 20ml of the supernatant was decanted in another tube. After gentle mixing for 30min samples were incubated for 24h at room temperature. The clear supernatant was discarded except 1ml of the sediment. Portions (0.15ml) of the decontaminated specimen were inoculated on Herrold's Egg Yolk with mycobactin (Becton Dickenson, Heidelberg) and Herrold's Egg Yolk without mycobactin (Becton Dickenson, Heidelberg). The media were incubated horizontally for 1 week and then for 15 weeks upright at 37°C under aerobe conditions. The test tubes were inspected weekly for contamination and growth of mycobacteria. Ziehl-Neelsen stain was used to confirm suspect colonies.

Sampling scheme
Cattle older than 18 months were included in the study. In A, B and C herds only animals with serologic results of +* to +++* (interpretation without screening) were tested by faecal culture. The "(+)" animals in D herds and every animal in E herds was tested by faecal culture. Blood and faecal samples were collected in the spring (2004-I), summer (2004-II) and autumn (2004-III). Consequently up to three faecal samples were available per animal in order to increase the likelihood of detecting MAP-shedding animals. If the serologic score of the animal increased, faecal culture was completed; if it decreased the animal remained classified in the group requiring faecal culture. In a few herds, additional faecal samples were submitted for analysis as requested by the owner.

Statistics
The two sided CHI^2-Test and the multiple testing correction according to Bonferroni were used (Sachs, 2002).

RESULTS

Data on serology are summarized in Table 2. Strong positive results (+++) were found in 0.6% to 1.1% of animals per sampling period in Bavarian dairies; in contrast strong positives represented 8.2% of tested animals in the infected control herd. Between 75.9% and 79.2% of animals were ELISA-negative and a similar result (81%) was found in the control herd. Between 20.8% and 24.1% of sera were positive in Sv, the screening test.

Table 2. Distribution (%) of ELISA interpretation for SMAPS with the number of samples positive after screening (Sv pos %).

	2003	**2004-I**	**2004-II**	**2004-III**	**BB**
+++	0.6	0.7	1.1	0.7	8.2
++	1.1	2.2	3.5	2.3	2.2
+	2.1	3.1	5.2	3.5	2.7
(+)	20.3	16.4	12.0	14.2	5.9
Neg	75.9	77.6	78.3	79.2	81.0
Sv pos (%)	24.1	22.4	21.7	20.8	19.0
Samples (n)	2,738	2,881	2,478	2,001	558

Table 3. Number (%) of samples <u>missed</u> by SMAPS due to negative results in Svanovir-ELISA vs. parallel testing with IDEXX- and Pourquier-ELISA

	2003	**2004-I**	**2004-II**	**2004-III**	**BB**
+++	0.0	16.7	25.0	11.8	41.8
++	32.6	28.1	25.2	31.9	29.4
+	12.1	26.8	31.6	35.2	44.4

In a second approach using results of Pq- and Id-ELISAs only, the number of positive samples without initial Sv screening were counted. Table 3 shows the numbers (%) of samples missed by SMAPS as compared to the testing scheme without screening. In Bavarian dairies up to 25% of +++ sera were missed in 2004-II, whereas 16.7% and 11.8% were missed during 2004-I and –III, respectively. In contrast 41.8% of +++ results were missed in the more heavily infected control herd. Even higher percentages in the ++ and +

interpretation category were missed in Bavarian dairies, however differences between Bavarian dairies and the control herd were not observed. The difference in +++ results between both testing schemes was not significant for Bavarian herds; in contrast this difference was highly significant ($p < 0.01$) for the control herd. Differences for ++ and + interpretations between SMAPS and the testing scheme without screening were significant ($p < 0.05$) for each time period.

Table 4. Faecal culture (fc) result per cow (fc^- and fc^+).

Number of cows	Number of cultures/cow	fc^- (n)	fc^+ (n)
403	1	384	19
380	2	369	11
199	3	196	3

Table 4 shows the distribution of faecal culture results over the numbers of cultures per cow. Only 33 animals were positive by faecal culture at any time. Of the 14 culture-positive cows tested more than once, three were positive on two occasions, and eleven were test-positive twice. Efficiency of screening was analysed for faecal culture positive samples. Interestingly, for +++, ++ and + results differences were not significant ($p > 0.05$) as compared to alternative testing with IDEXX- and Pourquier-ELISA.

Table 5. Comparison of SMAPS and faecal culture. Positive predictive values with faecal culture as reference (fc^+ %), 95% confidence interval (CI 95%) and number (n) of analysed animals are shown per SMAPS-result. An animal was scored fc^+ if at least one sample was positive in 2004.

	2003			2004-I			2004-II			2004-III		
IP)*	fc^+ (%)	CI 95%	n	fc^+ (%)	CI 95%	n	fc^+ (%)	CI 95%	n	fc^+ (%)	CI 95%	n
+++	28.5	0-62.0	7	42.1	19.9-64.3	19	27.0	9.9-50.0	26	56.5	36.3-76.8	23
++	0	0	21	5.4	0-11.3	56	3.9	0-8.2	77	0	0	43
+	6.7	0-15.6	30	3.6	0-7.6	83	0	0	122	1.6	0-4.7	63
(+)	0.7	0-1.6	296	0.6	0-1.4	332	0	0	196	0	0	194
neg	0.7	0.2-3.1	300	1.4	0-2.5	432	2.0	0.7-3.3	454	2.2	0.8-3.6	411

)* IP interpretation

Table 5 compares SMAPS and faecal culture. 27% to 56.5% of +++ animals on which faecal culture was done were faecal culture positive at least once. However the effect of a time lag of one year or even more between serological results for 2003 and faecal culture positive results should be considered. Faecal culture positive results of +++ animals are highly significant ($p<0.01$) as compared to other classes (neg to ++). A mean positive predictive value of 41.2% was calculated on the basis of data from 2004. Although data of Table 5 illustrate fewer faecal culture positive animals from +++ to + categories, these differences were not significant ($p>0.05$). No difference between (+) and negative results was observed, however the latter may be biased due to the sampling scheme, since (+) animals were not regularly included for faecal culture in A to C herds.

Differences of positive predictive values among (+), + and ++ reactors were not statistically significant. A comparison of results for cattle in herds classified a status "A" vs. those herds with any other classification ("Non-A"). For classification purposes the highest test result value over the testing period was used. While positive predictive values for ++ were not different between A- and Non-A-herds, a difference was found for +, (+) and ELISA-negative animals (Table 6).

Table 6. Positive predictive values with faecal culture as reference (fc^+%) were determined for serological results according to SMAPS in A- and Non-A-herds.

	A-herds			Non-A-herds			
Interp.	fc^+%	CI 95%	N	fc^+%	CI 95%	N	)
+++	40.0	25.7-54.3	45				
++	5.1	0-12.5	39	2.5	0-6.0	79	n.s.
+	5.9	0-13.8	34	0.0	0	123	*
(+)	5.3	0-12.3	38	0.0	0	354	***
Neg	13.2	4.0-22.3	53	0.0	0	217	***
Total	14.8	10.0-19.6	209	0.3	0-0.62	773	***

)* 95% confidence interval,)** number of animals per group

The analysed herds were classified according to Fig. 1. Table 7 shows the distribution of herds over classification statuses A to E. Interestingly in summer 2004 (2004-II) a slight shift to higher classes occurred, which resolved in autumn. This effect was observed for 86 herds tested three times in 2004, too. Herds in which faecal culture was positive at least once are listed in parentheses. In total, six herds were culture positive. Data on these herds are listed in Table 8. Three herds (1, 4, BB) were classified as A on each testing occasion; interestingly these herds were classified as having a high prevalence according to Id-positive results (APHIS 91-45-014). One herd (2) was classified as D/E on three occasions, however only 10 of 69 animals were tested on the first three occasions. When the whole herd was tested, one animal turned out to be +++, and faecal culture positive. One herd (5) was classified as A in 2004-I; from three animals (1x (+), 1x +, 1x +++) MAP could be isolated, the +++cow was removed. Both weak positive cows were seronegative on the next occasion (2004-II) and faecal culture was negative, too. Finally one herd (3) was classified as C/B: two animals were Id positive/equivocal both animals were positive in faecal culture, too. Interestingly, the latter three herds were classified as low prevalent or even negative according to Id-positive results. Additionally one herd (3) was classified as B despite negative results in IDEXX-ELISA, since one sample was reactive in Pourquier-ELISA.

Table 7. Herds were classified according to SMAPS (Fig. 1). Distribution of herds over classifications is shown. Seventy-eight and 86 herds were tested 4 and 3 (2004) times, respectively. Distribution of faecal culture positive herds (6 herds) is given in parentheses.

Classification	2003	2004-I	2004-II	2004-III + BB
A	7 (1)	9 (3)	11(2)	8 (3)
B	20	32 (1)	38 (1)	26
C	27	19	31	28
D	56 (1)	40 (1)	14	20
E	8	6	8 (2)	7
Total	118	106	102	89

Table 8: Data on herds with at least one positive faecal culture (fc), number of sera tested, classification of herds with (SMAPS) and without screening (Pq/Id), relative number (%) of IDEXX positive results per herd (Id pos %) and number of animals tested in fc and number of animals positive in fc.

Herd ID	Time	Sera (n)	Herd class SMAPS	Id pos Pq/Ic	fc (animal) (%)	N	fc^{+} (n)
1	2004-I	46	A	A	20	14	5
	2004-II	14	A	A	57		
2	2003	10	D	E	0		
	2004-I	10	D	E	0	16	1
	2004-II	10	E	E	0		
	2004-III	69	A	A	3		
3	2004-I	55	C	B	2		
	2004-II	49	B	B	4	3	2
	2004-III	42	B	B	0		
4	2003	28	A	A	29		
	2004-I	40	A	A	25	37	13
	2004-II	53	A	A	23		
	2004-III	50	A	A	18		
5	2004-I	33	A	A	1	5	3
	2004-II	23	E	E	0		
6	(2004-III)	558	A	A	15	10	9

DISCUSSION

Faecal culture of MAP is laborious, time consuming, slow and expensive. Furthermore, sensitivity of faecal culture was calculated to be 35% to 50% (Collins, 1996; Whitlock et al., 2000). However it is the diagnostic assay of choice for in vivo testing of individual animals. Because of the listed disadvantages of faecal culture, ELISA-serology is practised widely; however these tests differ in sensitivity and specificity. To circumvent these problems and to maximize diagnostic information, testing serum samples with different ELISAs is an alternative. The increased costs for "double-testing" might be decreased by a suitable screening test. Based on these assumptions SMAPS – Sequential MAP Serology – was proposed.

Evaluation of the SMAPS approach, not the validation of single tests, was the aim of the present study. Since each sample is submitted to a screening-test (Svanovir-ELISA) and since only positive samples are retested in two ELISAs of higher specificity, the efficiency of screening was determined. The positive predictive values for serum interpretation on the one hand and stability of subsequent classification of herds on the other were analysed. Faecal culture served as the reference assay and in order to increase sensitivity, faecal samples were collected on three occasions. However faecal cultures were set for only those cows with +++, ++ and + ELISA results in herds classified A, B and C herds. Every (+) animal in D-herds and all ELISA-negative animals in E-herds were tested by faecal culture.

Application of different tests on individual sera is widely used in surveillance of other animal diseases (Brucellosis – ELISA, complement fixation; Enzootic Bovine Leucosis – ELISA, Agar gel immunodiffusion). ELISA is normally used for screening purposes. For MAP the ELISA with the highest sensitivity was used for initial screening, but the relatively high number of positive sera sets a financial limit to the number of follow-up cultures that can be completed. Since between 19.0% and 24.1% of sera were screened positive (Table 2), the screening test is an effective measure to reduce costs.

Since agreement of Pourquier and IDEXX ELISA results is crucial for SMAPS-interpretation, SMAPS was compared to a testing scheme without initial screening. The number of sera that would have been missed by screening was analysed. While in 2003 no +++ results were missed, this value increased in 2004 to 16.7%, 25.0% and 11.8%. However, these differences were still not significant (p>0.05). In contrast the total number of + to +++ samples missed by screening was significant (p<0.05). The same analysis was performed on data from cows for which faecal culture was performed. For this set of data no significant differences were found for + to +++ animals (p>0.05). These data indicate that no significant diagnostic information was lost due to screening. The observation that + and ++ results were missed due to screening might indicate that screening eliminated sera testing false positive in the IDEXX and/or Pourquier ELISA. Another hypothesis is that comparison differences with faecal culture weren't evident because of a lack of sensitivity of faecal culture. Poor reproduction of results close to the cut-off in Svanovir-ELISA might be a problem. This was found for IDEXX-ELISA: 40% of weak positive results (S/P<0.7) were interpreted as negative upon testing of a second sample, while only 10% of sera with initial S/P > 0.7 were negative in a second sample (Hirst et al., 2002). Comparison of frequency distributions for IDEXX and Svanovir ELISA results over S/P-values makes this last explanation very likely (data not shown). Finally it has to be considered that false positive results are probably less frequently reproduced in a second blood sample.

It was surprising that in the infected control herd 41.8% of +++ results were not detected by SMAPS (Table 3). Since this herd is known to be infected, these differences were a major point of concern. However factors in addition to the ones mentioned above (e.g. breeds, regions, management factors especially since herd BB exceeds the size of average Bavarian herds by a factor of ten, and perhaps different strains of bacteria) could be responsible for this phenomenon (Gasteiner et al., 1999). Currently other screening tests are under evaluation in herd BB with promising results.

Positive predictive values were calculated on the basis of faecal culture (Table 5). They were, as expected, highest for (+++) animals and decreased sharply for animals with (++) and (+) results. The observed rate of culture positives in (+) animals might be biased, since (+) reactors in classification A to C herds were not routinely included in faecal culture. However (+++) results are a good indicator for high risk herds, since faecal culture positive animals were almost exclusively found in A-herds and positive predictive values for + and (+) animals are higher in A as compared to Non-A herds. The fact that no difference between A and Non-A-herds was found for ++ animals might indicate an equivocal interpretation of B-herds. Consequently positive faecal cultures in ++ and +reactors indicated the advantage of high sensitivity testing in A herds. Considering the rather low sensitivity of faecal culture, removal of +++ animals has to be discussed (Collins, 1996; Whitlock et al., 2000). Table 5 highlights that animals were scored faecal culture positive if at least one culture was positive during the study. Thus if a culture was positive at the final collection (2004-III) at least some of these cattle might be in a phase of increasing seroreacitivity. (This was the reason the overall number of culture positive animals with negative serology was not determined in the study). For no animal was faecal shedding on three occasions demonstrated; in only three cases was positive culture observed twice. Only in 3/14 cattle (21%) was faecal culture positive twice, however it must be kept in mind that some animals with +++ results or positive faecal culture were removed before the study was finished.

Over the testing period seven to eleven classification A herds were founds. An observed shift of herds in 2004-I and –II to A and B remains to be analysed, however preliminary data suggest that this is partially due to an artefact due to tested herds. Analysis of 86 herds which were tested on three occasions in 2004 clearly demonstrated an increase of B-herds in summer 2004 (2004-II) which decreased in autumn (data not shown). Most of the faecal culture positive herds were grouped at least once in A. For more detailed analysis data for culture positive herds were presented (Table 8). Three different schemes of herd classification were used: SMAPS, classification without screening (Pq/Id) and classification based on Id-positive rates. Almost no differences among these types of classification were observed. Three herds were heavily infected, MAP was readily isolated and herd prevalences exceeded 15% as determined by IDEXX-ELISA (APHIS 91-45-014). Two herds were misclassified as D and E during the study. In one case a +++ animal was removed (herd 5), in the other case a +++ animal was recently bought (herd 2). Since only 10 sera were collected in herd 2, this might have led to misclassification, too. These two herds reflect borderline situations in the further history of Johne's disease control. Finally in one herd no +++ animal was detected, however this herd was still classified as B. In 2004-III no IDEXX ELISA-positive animal was detected, however this herd was classified as B due to a Pourquier-ELISA positive result. Thus testing with two pre-absorbed tests is at least of some benefit.

SMAPS has been demonstrated to be a powerful tool for MAP-surveillance. However because of the small numbers of +++ animals the number of animals to be tested per herd has to be determined carefully. In Bavaria, the standard approach in a voluntary program is for the 30 oldest animals per herd are submitted to serology.

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Value of bulk-milk serology for control of Johne's disease

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ABSTRACT

Several infectious diseases (Brucellosis, Enzootic Bovine Leucosis, BHV1) are controlled by bulk-milk serology (BMS). BMS has proved to be highly accurate and effective and thus it could be a valuable tool for the control of *Mycobacterium avium subspecies paratuberculosis* (MAP) infections. In the present study a registered ELISA (Svanovir, Uppsala) was analysed for BMS. In total 2,677 pairs of blood and individual milk samples were collected from 118 herds. Aliquots of individual milk samples per herd were pooled (n = 104 pools). Sera were tested with three ELISAs (Svanovir, Uppsala; IDEXX, Wörrstadt; Pourquier, Montpellier). Individual milk samples and pooled milk samples were tested in Svanovir-ELISA. Lower sensitivity was found for individual milk samples than serum testing in the same test using recommended cut-offs and lower within-herd prevalences were determined by milk testing. Reducing the cut-off (20%) increased agreement of results as determined by kappa-statistics. Eighty-three to 92% of sera-positive animals (IDEXX and/or Pourquier) were also positive by milk ELISA. With pooled milk, only two herds were negative (OD% 5%), 31 were classified as low prevalence (5%<OD%<15%) and 69 herds high (OD% 15%) prevalence. Optical densities (OD%) were grouped as follows: 10%, 11-20%, 21-30% and >30%. For each group the median, first and third quartile of herd prevalence as determined by serum testing with Svanovir-ELISA was calculated. Although the median of the serological herd prevalence was positively correlated with pooled milk OD%, first and third quartiles of herd seroprevalences overlapped significantly. BMS was not able to distinguish between high and low prevalence herds. Currently BMS is not recommended for diagnosis of MAP on herd level; this does not necessarily exclude BMS for epidemiological research.

Key words: paratuberculosis, serology, milk, ELISA

INTRODUCTION

Bulk milk serology (BMS) is widely used for control and surveillance of diseases (Brucellosis, Bovine Enzootic Leucosis, Bovine Herpesvirus 1) in Germany. These tests are able to detect single positive animals within herds of 50 to 100 cattle, and thus the absence of infection can be demonstrated. On the other hand BMS is used for surveillance of Fasciolosis and Bovine Viral Diarrhoea Virus, however in the case of these test results reflect the infection level/prevalence within herds (Niskanen, 1993; Böttcher et al., 2003; Reichel et al., 2005). BMS could be a valuable tool for monitoring of *Mycobacterium avium ssp. paratuberculosis* infections in cows, too (Nielsen et al., 2000). Recently an ELISA (Svanovir, Uppsala) for BMS using lipoarabinomannan as the antigen was commercialised in Germany (Winterhoff et al., 2002). The aim of the present study was to evaluate its characteristics under field conditions, therefore Svanovir-ELISA results for milk and blood samples and calculated herd prevalences were compared.

MATERIALS AND METHODS

Samples
From 118 Bavarian dairies 2,677 pairs of milk and blood were collected in 2003. Milk samples were conserved with 0.05% NaN₃. Bulk milk of 104 herds was simulated by mixing aliquots of individual milk samples for each herd. Herds were non-randomly selected; participation was determined by cooperation of farmers.

ELISA for serum testing

Sera were tested in three MAP-ELISAs (Svanovir, Uppsala; IDEXX (Scandinavia), Wörrstadt; Pourquier, Montpellier) according to manufacturer's instructions.

SMAPS

The principles of Sequential MAP Serology (SMAPS) are summarized in Fig. 1 (Böttcher and Gangl, 2004; Böttcher et al, 2005). Briefly, sera are screened in a test of higher sensitivity, negative and equivocal sera are defined as "negative". Sera interpreted as test-positive on the first ELISA are then tested in two ELISAs of higher specificity (IDEXX, Wörrstadt; Pourquier, Montpellier). According to the reactivity pattern in these tests, sera are interpreted as "+++", "++", "+" and "(+)" reflecting the probability that a positive result indicates a true case of MAP-infection. The highest serological score defined the herd status, "+++" results in "A", "++" in "B", "+" in "C", "(+)" in "D" and "negative" results in "E".
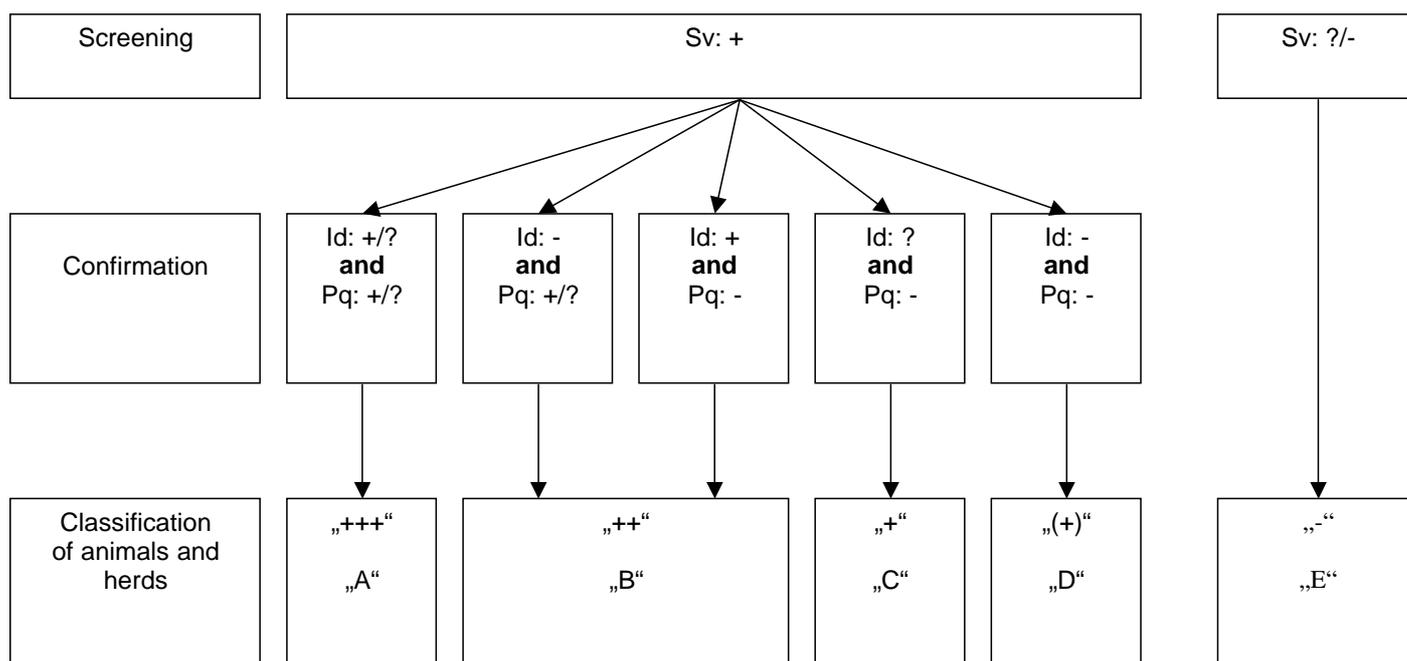
Fig. 1: Sequential MAP Serology (SMAPS, Böttcher and Gangl, 2004)
ELISA: Sv – Svanovir, Id – IDEXX, Pq – Pourquier

ELISA for individual milk and bulk milk testing

Milk samples were tested with Svanovir-ELISA. Testing was performed according to manufacturer's instructions and results interpreted according to cut-offs based on OD percent positive (Sample OD/Positive control OD). Individual milk samples are interpreted as negative if < 26% and as positive if > 56%; equivocal results were those between 26% and 56%. For bulk milk samples, ELISAs were interpreted as negative if below 5%, between 5% and 15% herds are classified as low prevalent and more than 15% indicated a high level of antibody and thus a high infection prevalence herd.

Statistical analysis

Kappa-( )-Indices were calculated according to Sachs (2002). Coefficient of correlation (r) was determined by Excel 2000 (Microsoft).

RESULTS

Sera and individual milk samples were tested with the Svanovir-ELISA. Fig. 2 describes the distribution of reactivity (optical density %; OD%). The third quartile is 50% for sera and 19% for milk. The distribution was compared to the cut-off values, which were 36% and 54% for sera and 26% to 56% for individual milk. Thus the third quartile for serum testing is close to the upper limit of equivocal results, whereas the third quartile

for milk testing is falls below the lower limit of equivocal results. Generally less reactivity for milk samples was observed.

Results on individual animals were compared (Table 1). Data in Table 1 are not evenly distributed: many cattle that were serum ELISA positive in serum were milk ELISA negative. Using cut-offs of 54% and 20% for sera and individual milk, respectively, produced a more even distribution of data (Table 1: data in parentheses). Kappa-indices were calculated on the basis of data from Table 1. Kappa-indices reflect a rather low agreement of results, the highest value (kappa = 0.53) was obtained for serum and milk cut-offs of 54% and 20% respectively (Table 2).
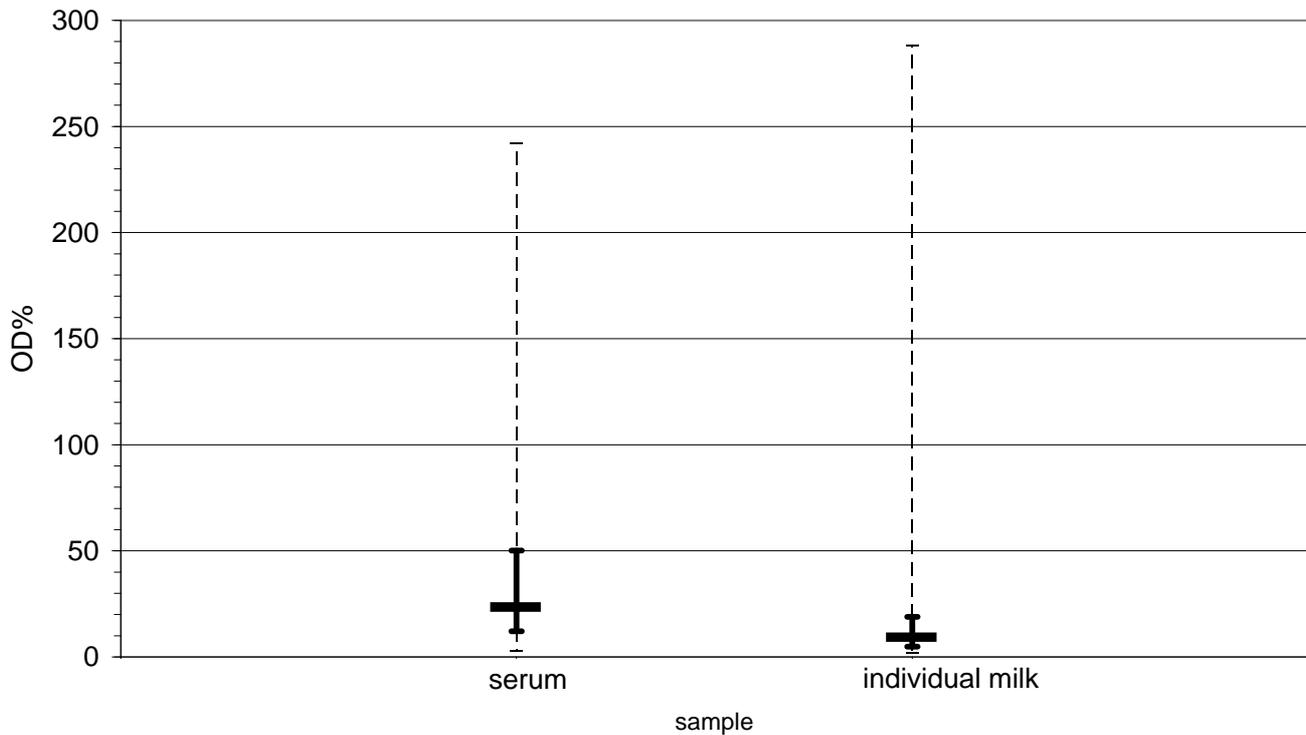
Fig. 2: Distribution of ELISA-reactivity of serum and individual milk samples in Svanovir-ELISA, median, 1st and 3rd quartile and minimum/maximum are shown.

Table 1. Comparison of individual milk and serum results in Svanovir-ELISA. Cut-off for serum testing was 36% to 54%, cut-off for individual milk testing was 26% to 56%. Additionally data for a cut-off of 54% and 20% (data in parentheses) for serum and milk, respectively, are shown

		Individual milk			
		Positive	**Equivocal**	**Negative**	Σ
Serum	Positive	150 (408)	196	295 (233)	641
	Equivocal	17	50	340	407
	Negative	12 (222)	60	1557 (1814)	1629
	Σ	179 (630)	306	2192 (2047)	2677

Table 2. Kappa-(κ)-Indices were calculated for data from Table 1 for interpretation of equivocal results. In the upper left corner κ was calculated for cut-offs 54% and 20% for serum and milk, respectively.

κ = 0.53 (cut-offs: serum 54%, milk 20%)			Individual milk	
			Equivocal results scored as :	
			Positive	**Negative**
Serum	equivocal results	positive	0.39	0.18
	scored as:	negative	0.26	0.29

Within-herd prevalences might be an indicator for MAP infection in a herd. Thus for each herd the prevalence as determined by serum and individual milk testing was calculated. For milk testing two cut-offs

(56% and 20%) were used. Data are summarised in Fig. 3. A positive correlation between within-herd prevalences as determined by serum and milk was observed for both cut-offs (r = 0.63). However using recommended cut-offs for milk testing, within-herd prevalences were underestimated as compared to serological testing in Svanovir-ELISA. Interestingly, herd prevalences below 10% in serum were not detected in milk. When a lower cut-off (20%) was used for milk, within-herd prevalences increased, however prevalences in herds with lower serological prevalences (herd prevalence <10%) were overestimated.

Bulk milk samples were prepared for 104 herds and analysed in the bulk milk test. Only two herds were ELISA negative, while 31 and 69 herds were classified as low and high prevalence, respectively, when recommended cut-offs were used.

classes of serological within-herd prevalence (number of herds per class)
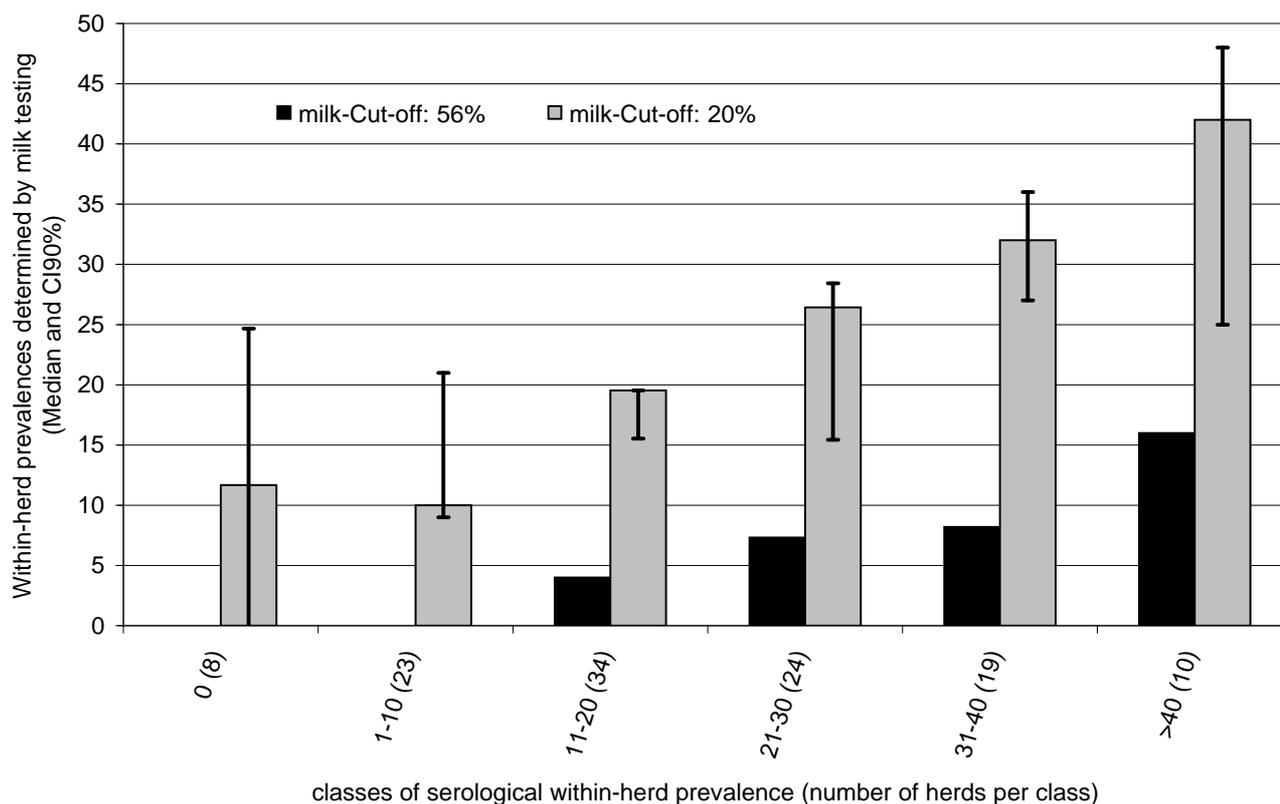
Fig. 3: Within-herd prevalences (HP%) for serum (x) and milk testing (y) are shown. Median results for milk testing are presented, number of herds per class are given in parentheses. Vertical bars are indicating 90% confidence interval (20% cut-off).

Bulk milk reactivity was compared with within-herd prevalences as determined by serum. Four reactivity levels for BMS were defined: 10%, 11-20%, 21-30%, >30%. Although a positive correlation was observed between bulk milk reactivity and serologic herd prevalences (r = 0.42), only BMS-groups 10% and >30% showed useful differences
from serologic within-herd prevalences, since their 90% and 10%-percentiles overlapped only marginally (Fig. 4).
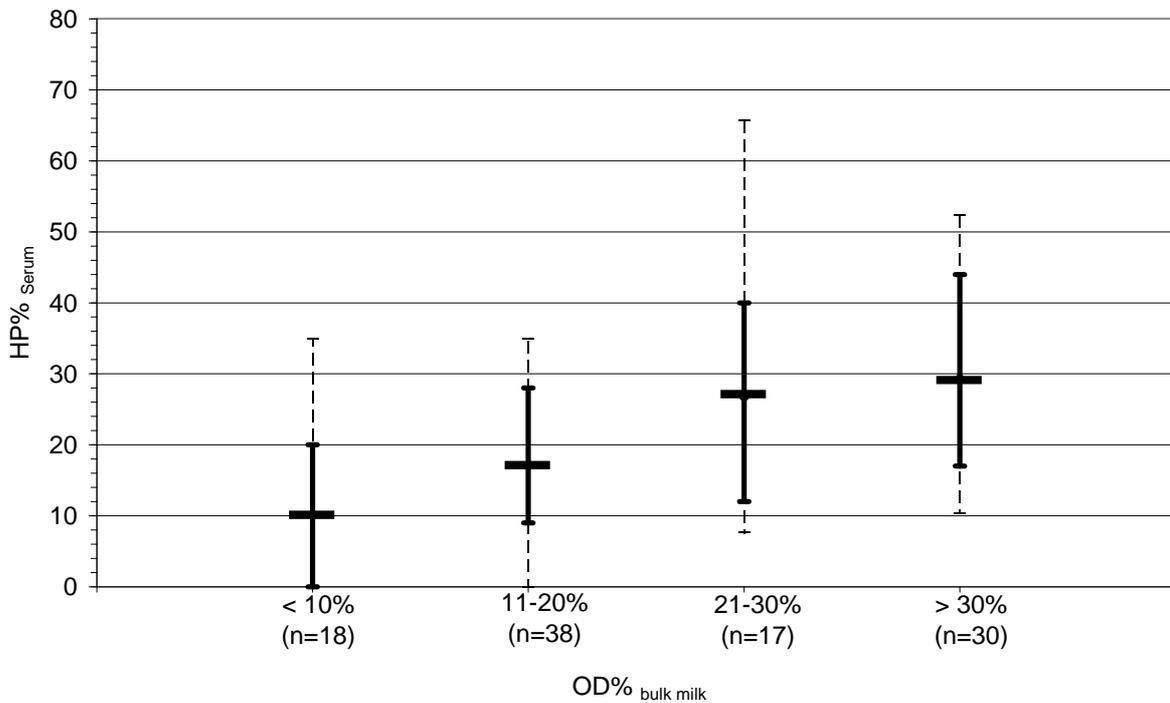
Fig. 4: Comparison of bulk milk reactivity (OD%) with within-herd prevalences as determined by serum testing. Median, 10% and 90% percentiles and minimum/maximum are shown
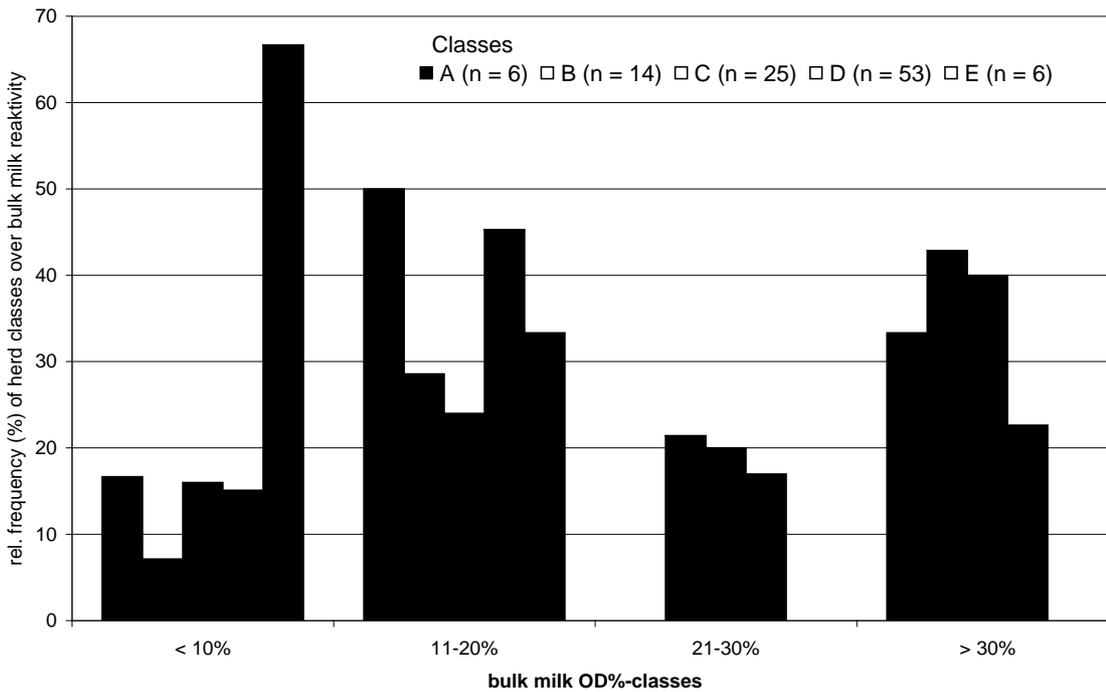
Fig. 5: Herds classified according to SMAPS ranging from highest (A) to lowest (E) likelihood of MAP infection. Relative frequency (%) distribution of classes over bulk milk reactivity is shown.

Table 3: Comparison of SMAPS-results with individual milk results (Cut-off 20%)

SMAPS	Individual milk		
Sera	**Positive (n)**	**Negative (n)**	**% Positive**
+++	12	1	92.3
++	24	5	82.8
+	50	9	84.7
(+)	322	218	59.6
neg	222	1814	10.9

Only Svanovir-ELISA-milk and –serum results have been presented so far. Now, in order to compare milk-ELISA results with other preabsorbed tests (IDEXX, Pourquier) the SMAPS-classification was used. Individual milk samples from animals positive in Pourquier- and/or IDEXX-ELISA (+ to +++) were frequently milk-ELISA positive, too, when a cut-off of 20% was used (Table 3). Using a cut-off of 56%, only 46.2% of +++ and 46.0% of + to +++ animals were detected.

Johne's disease herds have been classified according to SMAPS, and although this is a serological classification, SMAPS was able to detect high risk animals and herds (Böttcher et al., 2005). Herd classification for this study appears in Fig. 5 which shows the frequency distribution of the five SMAPS-classes A to E vs. bulk milk reactivity. Four of six E-herds gave results 10% in BMS and another two herds were between 10 and 20% in BMS. In contrast one of six A-herds was below 10%, an additional 3 herds gave BMS-results between 10 and 20% and only two gave results >30%.

DISCUSSION

The sensitivity of the Svanovir-ELISA is comparatively high when used for serum testing (Jark et al., 1997, Köhler and Burkert, 2003). Therefore Svanovir-ELISA was used for SMAPS as a screening test (Böttcher and Gangl, 2004; Böttcher et al, 2005). Since IgG concentration in milk is 1/20th of that in serum a high sensitivity is a prerequisite for BMS, consequently Svanovir-ELISA might be the test of choice for BMS (Butler, 1983; Winterhoff et al., 2002). The difference of IgG-concentration between milk and serum has to be considered in the ELISA protocol. Background reactivity in milk is often reduced and lower cut-offs and dilutions for milk samples are possible. For Svanovir-ELISA similar cut-offs for milk and serum were recommended, however sample dilution is 1/100 for serum and 1/10 for milk, reflecting only a factor 10. Second (median) and third quartiles for serum testing were approximately twice the value of milk, indicating a still lower sensitivity of milk testing as compared to serum. Based on kappa-statistics serum and milk testing were compared. For recommended cut-offs, kappa was below 0.39 whereas for a cut-off of 20% kappa was 0.53. Similar kappa-indices (0.4 to 0.5) were reported by two other groups (Hendrick et al., 2003; Lombard et al., 2003). The low agreement between serum and milk testing could be explained by the fact that MAP is shed in milk. Since *Mycobacterium phlei*-antigen is used in preabsorbed tests, inhibition of ELISA reactivity by MAP-antigens in milk of infected animals at least seems possible. This effect was demonstrated for BVDV (Obritzhauser et al., 2002).

Using a cut-off of 20% and 54% for milk and serum testing, respectively, roughly 25% of samples are selected by each test (serum and milk), because these cut-offs are close to the third quartiles. But these samples are not necessarily from the same animals. Only two thirds of positives in one sample type are positive in the corresponding sample (Table 1). Since Svanovir-ELISA was used as screening test for serum in our lab, it was of interest to know whether animals with equivocal/positive sera in two other preabsorbed tests were detected in milk, too. SMAPS classification was used for this comparison. Briefly, +++-, ++- and + -reactivities reflecting results in two preabsorbed MAP-ELISAs (+++: Pq ?/+ and/or Id ?/+; ++: Pq ?/+ or Id +; + (Id ?). Using a 20% cut-off 82.8% to 92.3% of +- to +++-animals were detected by milk ELISA, too (Table 3). At this point it has to be stressed that differences between 20% and 26% cut-offs for milk were minor, e.g. 80% to 85% of +- to +++-animals were detected in milk using 26%, however when using 56% fewer than 50% of these animals were detected. Thus a cut-off of 20% turned out to be sensitive enough for screening purposes of milk samples. But this approach has a drawback, since sera have to be collected for further analysis in SMAPS.

Classification of herds by positive serological results was used as an indicator of MAP-infection (APHIS 91-45-014, Böttcher and Gangl, 2004). Predicted within-herd prevalences were compared based on milk and serum ELISAs. Lower prevalences were recorded for milk testing when recommended cut-offs were used. Prevalences assumed to be below 10% based on serum ELISA results weren't detected by individual milk testing (31 herds; Fig. 3). Within-herd prevalences for serum and milk were comparable when a cut-off of 20% for individual milk was used, but in six herds no test-positive milk ELISAs were detected.

Based on these data testing of individual milk samples in Svanovir-ELISA with a cut-off of 20% is suitable for screening purposes and within-herd prevalences are comparable with serologic screening. This finding is in accordance with another study on a LAM-ELISA which found milk testing acceptable (Sweeney et al., 1994). Although agreement within Svanova-ELISA for milk and serum was acceptable, it must be recalled that positive predictive values as determined by faecal culture were rather low for this test (Böttcher et al., 2005).

Bulk milk was prepared in the laboratory in order to exclude further errors due to sampling. Recommended cut-offs for BMS are 5-15% and herds between 5 and 15% are classified as "low prevalence", while herds below 5% are negative and those above 15% are defined as high prevalence. Surprisingly, only 2 of 104 herds were classified as negative, while 31 and 69 were low and high prevalence, respectively. These data are in contrast to another study on 137 herds (78 of the presented herds were included in the present study) in which 3.7% of the tested herds faecal culture positive animals were found (Böttcher et al., 2005).

Consequently, the recommended cut-offs are not useful for BMS. Therefore four groups based on reactivity (OD%) in BMS were defined: 10%, 11-20%, 21-30% and >30%. Each group was compared to within-herd prevalences as determined by serum testing. Although a correlation between BMS reactivity and within-herd prevalence was found, an estimation of within-herd prevalence from BMS reactivity was impossible. Finally these results were compared with SMAPS-classification. Surprisingly, four of six A-herds had BMS-reactivities below 20%, thus A-herds were not sufficiently separated by BMS.

In a separate study herds classified according to SMAPS were tested by faecal culture. In three of six faecal culture positive herds less than 5% of Svanovir-positive animals were detected, thus herds with high seroprevalences are only the tip of the "MAP-iceberg" (Böttcher et al., 2005). Because of its long incubation time diagnosis of MAP has to be done as early as possible to identify even just one infectious animal.

BMS is not recommended for diagnosis of MAP on herd level, since the information provided is limited. In the present study milk samples were collected under controlled conditions, however different systems of bulk milk collection may reduce data validity even further. Additionally, in most farms further tests have to be done to clarify the extent of MAP infection. Serum testing does have several drawbacks: its variable sensitivity (i.e. 15% to 87% reported for one ELISA; Sweeney et al., 1995) and specificity (many seropositive animals may be faecal culture negative; Frederiksen et al., 2004, Böttcher et al., 2005).

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Application of nested PCR (nPCR) to body fluids for the detection of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

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ABSTRACT

A nested PCR (nPCR) probe for IS*900* was developed using peripheral blood and milk samples. Its efficiency in detecting MAP infected cattle was compared with serum ELISA and AGID serology in a cohort of 11 clinical and 46 subclinical lactating Holstein cows from a herd with confirmed paratuberculosis. Placental fluid of 8 pregnant animals from the same herd and of 3 pregnant cows from a beef herd with confirmed paratuberculosis was analyzed for evidence of MAP DNA via nPCR. Semen from a Holstein bull with clinical signs of Johne's disease from the dairy herd was examined for evidence of MAP DNA via nPCR. Infection was confirmed through histopathology for the clinically ill cattle. The detection rate for the nPCR in peripheral blood and milk samples of clinical animals was 100% (11 animals), 100% on ELISA and 55% (6 animals) on AGID in this group. The detection rate of the nPCR was 52% (24 animals) on blood and milk samples of the subclinical animal group as compared to 39% positive or suspicious (18 animals) on ELISA and none on AGID. In 5 of 12 animals (42%) the nPCR identified subclincal animals as infected when these were negative on ELISA and AGID. More animals were detected by nPCR on milk samples than on blood samples. Some cows had both positive blood and milk samples. The clinical bull was nPCR positive on blood and semen samples. Of the 11 pregnant cows 18% (2 animals) tested positive on the allantochorionic placental fluid. Positive nPCR signals obtained in animals with negative ELISA and AGID results make the assay a candidate for detecting a unique subpopulation of animals infected with MAP.

Key words: nPCR, semen, milk, ELISA, AGID, DNA, MAP

INTRODUCTION

Paratuberculosis (Johne's disease) affects ruminants worldwide. Its control is hampered by ineffective methods particularly when applied to the detection of silently infective animals(Whitlock, 1996). Agent detection methods and identification of immunological responses frequently give false negative results. Since there are no methods available for effective management of paratuberculosis such as treatment or vaccination, farmers depend on test and cull programs to control the infection. Thus, a diagnostic test for early detection would be desirable. The objective of our studies were to determine the potential of nested PCR(nPCR) on blood, milk, semen and placental fluid of cattle to detect clinical and subclinical cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The project addressed paratuberculosis not as an infection limited to the intestinal tract but rather as one wherein the organism disseminates hematogenously through blood-borne phagocytes throughout the body.

MATERIALS AND METHODS

A nPCR probe for IS*900* was developed and its efficiency in identifying infected cattle through detecting MAP DNA in peripheral blood and milk samples was compared with the diagnostic ability of ELISA and AGID serology in a cohort of 11 clinical and 46 subclinical lactating Holstein cows from a herd with confirmed paratuberculosis. Subclinical animals were selected by a positive ELISA titer. Fecal culture was not done with animals of this group. Placental fluid of 8 pregnant cows from the same herd and of 3 pregnant cows from a beef herd with confirmed paratuberculosis was analyzed for evidence of MAP DNA

via nPCR. A Holstein bull with clinical signs of Johne's disease from the dairy herd had semen and blood examined for evidence of MAP DNA via nPCR.

Blood collected from the coccygeal vein into vacutainer tubes containing EDTA and 50ml of raw milk collected from all four quarters were gradient centrifuged and centrifuged at 1000 g for 15 min. Blood monocytes retrieved were washed twice in PBS and centrifuged at 500 g for 15 min. Cells were counted, resuspended in 100 uL of 0.2N NaOH, boiled at 110^0C for 20 min to extract DNA and centrifuged at 500 g for 3 min. The sediment of the milk samples was washed 3x in PBS. The samples were counted, resuspended in 100 uL of 0.2N NaOH and boiled as described for the blood. Liquid samples obtained from allantoic fluid via percutaneous abdominocentesis of the standing animal locally anesthetized were centrifuged for 60 min at 1,000g . Pellets were washed and boiled in 0.2 N NaOH as discussed previously. Semen samples collected from a straw (0.5ml) were centrifuged at 3,000 g for 5 min and 1 ml of sediment was boiled in 0.2 NaOH for the extraction of DNA.

An nPCR was performed. Briefly, for the first reaction primers P90,P91 were chosen and primers J1,J2 were chosen for the second reaction. After DNA extraction, 1 uL of the lysate was submitted for PCR. A protocol of 35 cycles was applied for the simple PCR; a protocol of 36 cycles was chosen for the second reaction. A commercial reaction mix (Fail Safe; EPICENTRE, Wisconsin) was used according to the company's specifications. A volume of 10 uL of the PCR product was run on 1.5% agarose gel by electrophoresis in TAE running buffer. Gel inspection was done using ultraviolet light and recorded with a computerized digital camera. DNA extracted from a laboratory strain #295 was used as positive control; sterile water was used a negative control for the PCR assay.

An in-house developed ELISA using a crude, soluble antigen (Allied Monitor, Missouri) tested pre-absorbed sera. Results were calculated from wavelength readings of triplicates and recorded as negative (< 1.5 OD), suspicious (1.5 to 1.9 OD), low positive (2.0 to 2.5 OD) and high positive (> 2.5 OD). The AGID was done with the same crude protoplasmic antigen and final readings were obtained after 48 hours.

All clinically affected animals were euthanized after completion of the study. A complete necropsy was performed and major organs and fetus samples were examined histologically.

Results from the ELISA readings and the PCR tests were statistically compared by McNemar's test (with Yate's correction). A probability value less than 0.05 was considered statistically positive.

RESULTS

The detection rate for nPCR in peripheral blood and in milk samples of clinical animals was 100% (11 animals). All eleven animals were also serum ELISA positive (100%) and 55% (6 animals) in this group were AGID positive. Necropsy confirmed that all animals had histologic evidence of Johne's disease.

Table 1. Clinical and subclinical cattle

Clinical cases N = 11		Subclinical cases N = 46**	
Tests	**Positive**	**Tests**	**Positive**
Blood PCR*	27%	Blood nPCR	4%
Milk PCR*	27%	Milk nPCR	20%
Milk+Blood PCR*	46%	Milk+Blood nPCR	28%
Total	100%		52%
ELISA	100%	ELISA	39%
AGID	55%	AGID	0%

*Combined single + nested   ** Total of 4 groups with different ELISA values

The detection rate of the nPCR on blood and milk samples in the subclinical animal group was 52% (24/46 animals) as compared to 39% positive or suspicious (18/46 animals) on ELISA and none on AGID. (Table 1) (Figs.1-3) Concordance between ELISA and nPCR was 22% for positive results and 30% for negative results. The difference was not statistiscally different. However, it is worth noting that in 5 of 12 animals

(42%) the nPCR identified subclinical animals when these were negative on ELISA and AGID. (Fig. 4) More animals were detected by PCR on milk samples than on blood samples. Some cows had both positive blood and milk samples. (Figs 1,2)
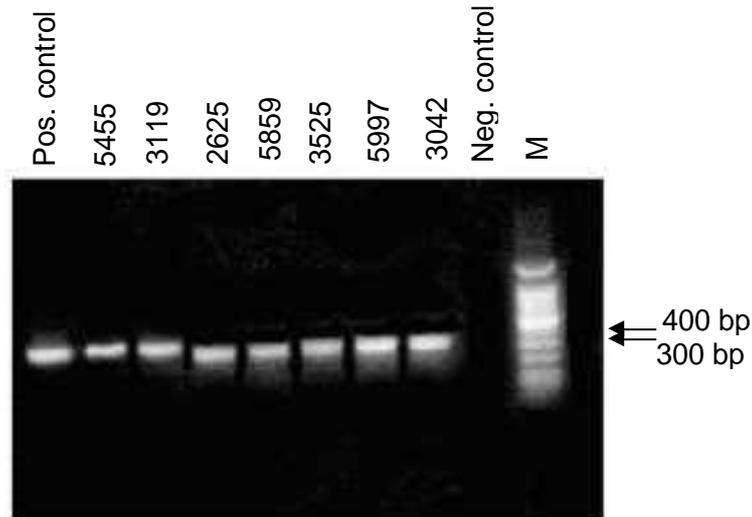
Fig. 1. Selected simplePCR and nPCR amplicons. Cultured blood monocytes of clinical cases with cow numbers on top. M=molecular markers.
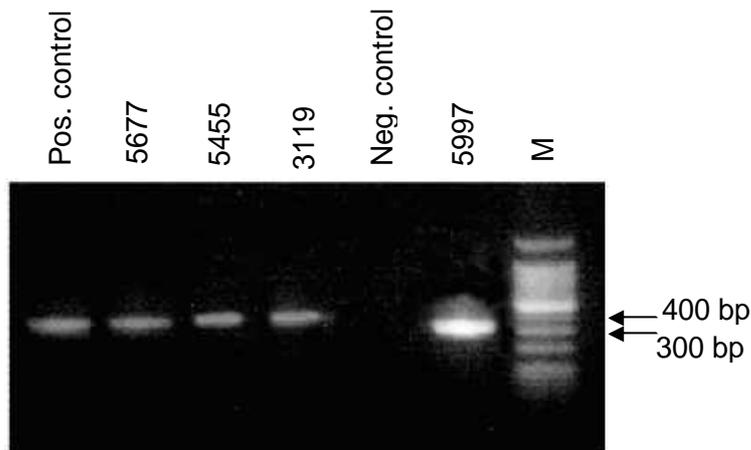
**Fig. 2.** Selected simplePCR amplicons. Milk macrophages of clinical cases with cow numbers on top.
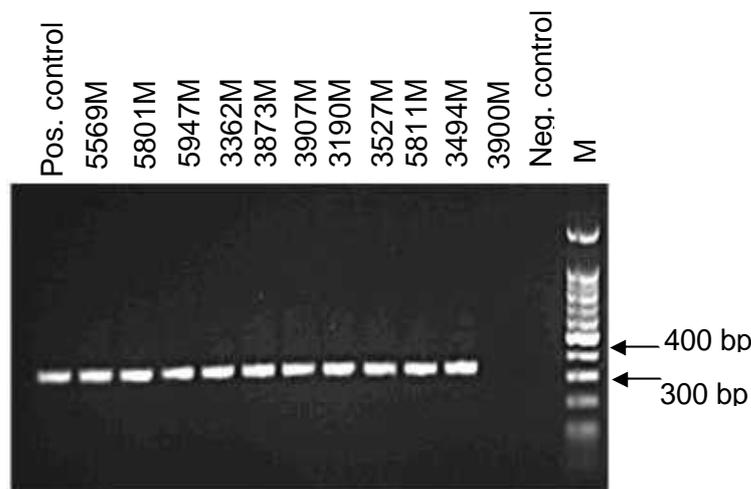
Fig. 3. Selected nPCR amplicons of subgroupof sublinical cows.0000M = milk; M=molecular markers.
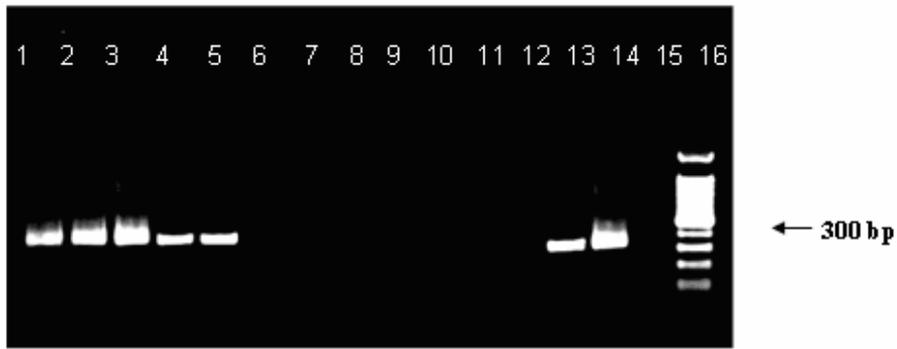
Fig.4. nPCR amplicons of subgroup of subclinical cows with negative ELISA titers.Lane 1=pos.control; lanes2-6=blood amplicons; lanes12,13=milk amplicons; lane 15=neg. control; lane 16=molecular markers.

The clinically ill bull was nPCR positive on blood and semen samples (Table 2, Figs.5, 6). Of the 11 pregnant cows with histopathologic lesions consistent with Johne's disease, 18% (2 animals) tested nPCR positive on the allantochorionic placental fluid. (Fig. 7) Fetal tissue samples were nPCR positive in 4 fetuses (36%).
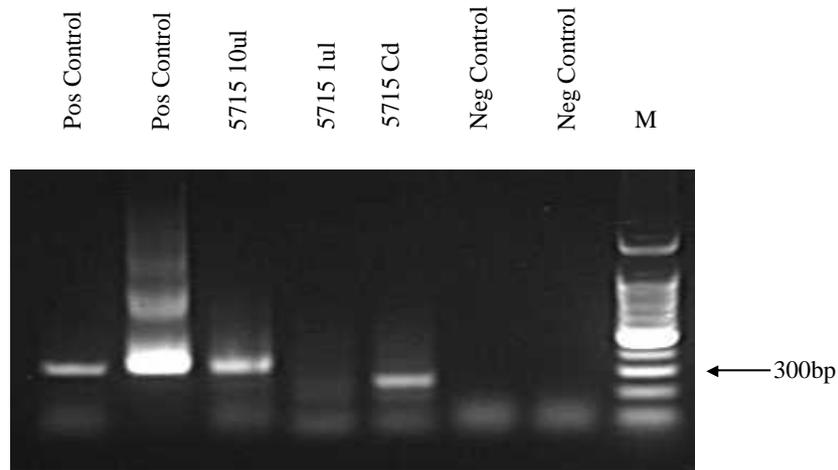
Fig.5. Bull blood; nPCR; ul =monocyte volume;cd =cellular debris.
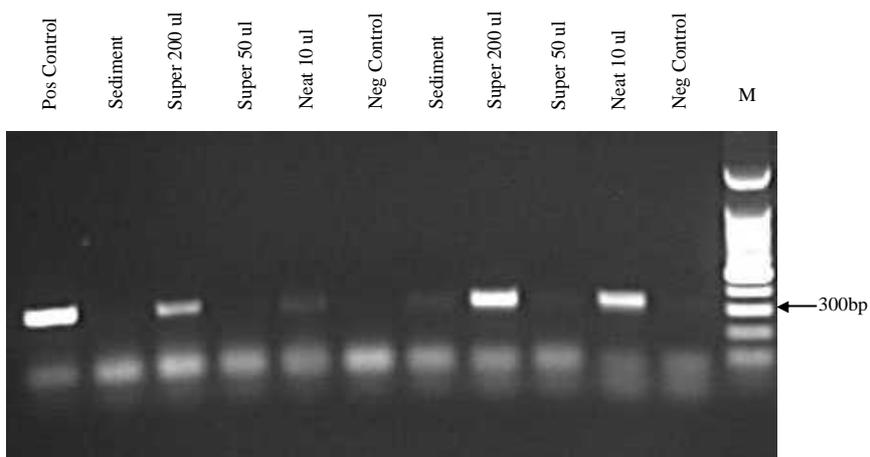
Fig.6.Bull semen; nPCR; ul=volume.Super = supernatant.

Table 2. Sequential data Bull #5715, 4yrs, Holstein

Date	AGID	ELISA	Fecal	P90,91	J1,J2
1/11/02	-	-	ND	ND	ND
11/4/02	+	4.6	ND	ND	ND
11/19/02	-	3.8	-	ND	ND
12/1/02	+	3.5	-	ND	ND
12/17/02	-	4.0	-	neg	pos
1/27/03	-	4.0	ND	ND	ND
1/27/03	Semen			pos	Pos
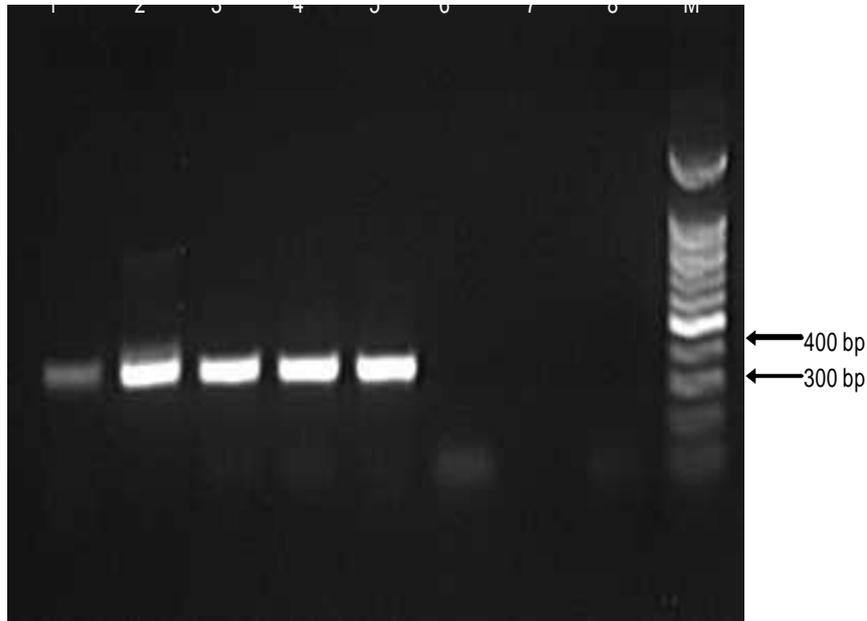
Fig.7. Pregnant cow; nPCR of blood and allantoic fluid.Lane 1=control; lanes2,3,=blood; lanes 4,5=allantois; lane9=neg.control.

DISCUSSION

The concept that MAP disseminates to distant sites from the intestinal tract via blood-borne phagocytes was borne out of observations that the bacilli can be isolated from milk, fetuses, semen and other tissues of infected animals. (Barrington 2003, Giese 2000, Sweeney 1996, Larson 1970) We recently were successful in isolating MAP from peripheral blood monocytes of a clinical cow in support of this concept. (see IAP Newsletter, July 2005)

PCR is a powerful tool to amplify DNA specific to mammalian prokaryotic cells. The insertion sequence IS*900* has been largely employed for the detection of MAP DNA and the primers P90,P91 are used to identify a PCR reaction product at the 413 base pair level (bp) (Vary 1990). PCR assays for IS*900* have been reported to be able to detect as few as 10^4 colony forming units (CFU) per gram of feces (Whipple 1992).

The goal of this study was to develop a simple and cost effective procedure. Thus, a rather crude DNA extraction procedure and the choice of a second set of primers (J1,J2) was made. They served to further intensify the signal already amplified by the first set of primers particularly if these are weak or invisible on agar-gel, thus increasing sensitivity of the test. These primers were chosen to amplify the 5' region of IS*900*.

From the study of a relatively small number of animals the results indicated that the blood or milk nPCR is equally successful at detecting infected animals as is the serum ELISA. (Buergelt 2004a) While the number of clinically affected animals detected was similar with PCR or ELISA methodologies, subclinically affected animals gave positive nPCR signals in subgroups where ELISA readings were negative. Most animals were

detected on milk nPCR and thus milk is the sample that should be selected when economics prevent analysis of both blood and milk.

In-utero transmission of MAP complicates management of the disease through a test and cull program. The application of nPCR as an antemortem test to identify MAP-contaminated placental fluid in late term gestation is technically easy and may be helpful in determining the potential of transmission as a factor in deciding how to manage an animal. (Buergelt 2003, 2044b)

The presence of MAP in semen needs more exploration on a larger scale, but the detection of MAP DNA in one clinical bull supports the few earlier reports of culture isolation of MAP from semen and organs of the male reproductive tract. (Buergelt 2004b, Larsen 1970).

Detection of MAP DNA in body fluids supports the hypothesis of hematological dissemination of the bacilli (Barrington 2003, Juste 2005). Nested PCR testing of peripheral blood, milk, semen and placental fluid samples has potential as an antemortem test to detect clinical and subclinical animals infected with MAP by taking advantage of a hematological phase of circulating infected monocytes in paratuberculosis. Similar to fecal shedding or serologic responses, this hematological phase is intermittent in infected animals. (Barrington 2003, Buergelt 2004a, Juste 2005). Positive signals obtained in subgroups of animals when ELISA and AGID readings were negative make the assay a candidate for detecting a certain subpopulation of infected animals that ELISA fails to detect. The assay should be combined with ELISA testing to identify the largest possible number of infected animals in a herd exposed to MAP, a concept that focuses on different forms of MAP infection described as "complementary sensitivity". Test result agreement between ELISA and nPCR was lower than expected. This discrepancy could be explained by complementary sensitivity: the methods detect different stages or analytes of MAP infection as their respective targets (antibodies vs. bacteria) and these do not occur in parallel (Juste 2005).

ACKNOWLEDGEMENTS

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Predictive value of serologic techniques in the detection of culled cattle in paratuberculosis affected herds

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ABSTRACT

Three Friesian dairy cattle herds with clinical cases of paratuberculosis were included in this study. A total of 485 cattle (> 10 months-old) were tested by ELISA, skin test (ST) and agar gel immunodiffusion test (AGIDT). For a period of one year, the herds were monitored to record which cattle were culled (owners were not aware of the assay results). The relationship between the diagnostic tests results and the possibility of being culled in the following year was statistically evaluated. Forty-three percent of cattle positive to ELISA test, 100% of positive to AGID and 27% of positive reactors to ST were culled in a period of 12 months after performing the assays. Among those cattle showing a negative result to ELISA, 19% were eliminated, as well as 23% and 25% of those negative to AGID and ST respectively. While a significant although not uniform relationship was seen between positivity to ELISA and AGID and the culling of cattle for any reason, no relationship between ST result and culling was seen.

Key words: bovine, diagnosis, ELISA, AGID, skin test, culling.

INTRODUCTION

Infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is widely distributed among ruminant livestock. Its economic importance is derived both from the direct impact on the infected animal health, causing the death of clinically affected animals, and from the indirect losses in animals subclinically affected (Benedictus et al., 1987). It is well known that a great majority of infected animals remain healthy for many years, perhaps their entire life (Chiodini et al., 1984). However, in cows it has been established that MAP infection may decrease milk production (Johnson et al., 2001), increase the number of days open (Johnson-Ifearulundu et al., 2000) or increase susceptibility to mastitis (Merkal et al., 1975).

The early identification of subclinically infected animals would reduce the negative economic impact in the herd, limiting the number of animals likely to develop clinical disease and become fecal shedders. Unfortunately, there is not yet a reliable ante-mortem means of identifying subclinical paratuberculosis because all tests lack good sensitivity and specificity (Chiodini et al., 1984; Kreeger, 1991). Diagnostic tests based on the study of the immune reaction of the host against MAP infection have been widely used. It has been shown that there is a close relationship between the response to immunological tests, the different clinical and pathologic phases of the infection and the amount of excreted mycobacteria (Clarke et al., 1996; Pérez et al., 1997, 1999; González, 2003).

The possible use of these methods for the identification of heavy shedders should be considered. The aim of this work is to evaluate the relationship between the results to humoral and cellular immune-based methods and the culling of cattle, regardless of the cause, in dairy cattle herds.

MATERIALS AND METHODS

Animals
Three Friesian dairy cattle herds (A, B, C) were studied. They had a tuberculosis-free status and a history of clinical cases of paratuberculosis. Serum samples from 485 adult cows (> than 10 months-old) were obtained and the immune response against MAP was investigated. For a period of 12 months after performing the tests records were kept of which cattle were culled for any reason. The owners of the herds were not aware of the results of the immune-based test results at any time during the study.

ELISA
The ELISA was carried out in 189 animals from herd A, 200 from herd B and 85 from herd C. It was performed according to Pérez et al. (1997), using the protoplasmic antigen PPA-3 from MAP (Allied Monitor, Fayette, USA), and protein G (Bio-Rad, Barcelona, Spain) at a dilution 1:1500 as secondary conjugate. Previously, sera were adsorbed in a 1:2 *Mycobacterium phlei* suspension. Samples were processed in duplicate and the same negative and positive control sera were employed. The optical density (OD) results were transformed to an index value by division of the mean OD for each serum by the mean OD for the positive-control serum in each plate. Results were interpreted using the index value of positive $\geq$ 0.8, doubtful (index $\geq$ 0.6 and < 0.8), and negative < 0.6.

Agar gel immunodiffusion test (AGID)
This test was carried out in the same animals as the ELISA test and performed as described by Pérez et al. (1997) using the PPA-3 protoplasmic antigen. The appearance of a clearly definable precipitation line of identity with the reference serum was recorded as a positive result.

Intradermal skin test (ST)
Delayed-type hypersensitivity was tested by intradermal inoculation of 0.1 ml of avian PPD (0.5 mg/ml; CZ Veterinaria, Porriño, Spain) into the skin of the lateral side of the neck. The skin thickness was measured with callipers before and 72 hours after inoculation. Increases in skin thickness greater or equal to 3 mm were considered a positive result. This test was performed in 200 animals from flock A and 195 from herd B and 83 from herd C.

Statistical analysis
A predictive statistical analysis was performed by chi-square test for frequency comparison between the results of diagnostic tests and the culled animals.

RESULTS

ELISA results
From the 474 cows in which the test was performed, 88 (18.6%) were positive. Thirty-six of them (41% of the positive animals) were culled in the following 12 months. Among those negative to ELISA, 19.3% were culled, as well as 34.5% of those showing a result interpreted as "doubtful" (Table 1).

AGID results
Nine cattle (1.9%) out of the 474 in which the test was performed were AGID positive and all of them were culled in the following year (Table 1). From the remaining 465 with negative results, 110 were culled (23.6%).

Intradermal skin test (ST) results
A total of 127 (26.8%) cattle from the 478 were skin-test positive. From them, 34 (27% of positive cows) were culled. Of the 351 test-negative animals 25% were culled. No significant differences were observed between the groups (Table 1).

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Table 1. Percentage of culled animals in a period of 12 months after the testing, according to the result to the diagnostic test and herd. * indicates a significant difference (p<0.001) between the positive and negative culled animals. AGID: agar gel immunodiffusion test. ST: intradermal skin test.

	ELISA			AGID		Skin Test	
	Positive	**Doubtful**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Herd A	43%*	33%	19%	100%*	23%	26%	28%
Herd B	37%*	45%	22%	100%*	28%	35%	30%
Herd C	55%*	18%	13%	100%*	17%	14%	28%
Total	43%*	35%	19%	100%*	23%	27%	25%

DISCUSSION

The most notable result is the clear and significant relationship found between a positive result to ELISA test and the probability of being culled in the following year, since almost half of the ELISA positive cattle were removed from the herds. Humoral immune responses appear late in the paratuberculosis infection, usually associated with the progression of the disease (Stabel, 2000). Positive results to ELISA tests have been associated with animals having with severe lesions associated with clinical signs and heavy bacterial burdens. Other ELISA positives may have multifocal lesions and while still be sublinically affected may soon progress towards more disseminated and diffuse lesions (Clarke et al., 1996; Pérez et al., 1996, 1997). In this study, although culled animals were not followed, the reasons for being eliminated from the herd could be the development of clinical signs of paratuberculosis or other deficits associated with a subclinical infection such as a decrease in the milk production or mastitis (Merkal et al., 1975; Johnson et al., 2001). Further studies would be needed to study the relationship of other factors such as milk production or reproductive status, the response to immunologic diagnostic tests and the culling of cattle in relation to MAP infection.

The high percentage of ELISA positive cows that remain in the herd is remarkable. This could be due to a continued subclinical or perhaps even eliminated MAP infection.

A relationship with culling was more evident in AGID positive cows, since a 100% of them were eliminated from the herd although only 1.9% of the cattle tested were AGID positive. This result was expected since AGID positive animals have been shown to have severe and clinical lesions and high bacterial shedding (Pérez et al.¸1997, 2002; González, 2003) and this probably implies that all of them were culled for showing symptoms of paratuberculosis.

On the other hand, the absence of a relationship between positivity to ST and culling was also notable. This result would be in agreement with the presence of high cellular immune responses thought to be effective in controlling the multiplication of MAP in the early and subclinical phases of the disease. These cellular responses disappear when immune control begins to deteriorate and animals begin showing clinical signs and shedding bacteria and an increase in the antibody response is expected to occur (Chiodini, 1986; Stabel, 2000). Taking this into account, the high percentage of cows showing positive responses to ST (26.8%) indicates that MAP infection is well established in the herds and these animals have been able to mount effective cellular immune responses that control the progression of the disease. Therefore neither production losses nor clinical signs associated with MAP infection were present as a reason for culling.

CONCLUSION

In conclusion, serological positive results, mainly to AGID, can predict the possibility of a cow being culled in the following year. However, ELISA-positive results, although useful, did not consistently predict that sufficient production losses or development of clinical disease would develop within a twelve month period and cause a herd manager to select the cow for culling. Skin testing can be a valuable method for determining the prevalence of the infection, together with the serological techniques in a herd, but it also is not a predictive test for the culling of animals over a year's time.

ACKNOWLEDGEMENTS

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Serodiagnosis of Johne's disease in red deer (*Cervus elaphus*)

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ABSTRACT

An IgG_1 ELISA test has been developed to diagnose infection and disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in deer. Denatured tuberculin (PPDj) and native protoplasmic antigens (PpAg), both obtained from MAP, were used as target antigens in parallel for test development. Results were obtained by ROC analysis (Eng, 2005) of samples from more than 200 deer infected with MAP and 500 deer from farms believed to be free of MAP infection. The cutpoints for the antigens that gave 100% specificity were: PPDj - 60 Eu and PpAg - 40 Eu. At these cutpoints sensitivity values of 81% and 85% were obtained for PPDj and PpAg antigens respectively. When the two antigens are used at a given specificity, but combined serially, native and tuberculin antigens produced a composite sensitivity (91%) higher than either antigen alone at any given specificity. Seropositivity was shown to correlate closely with disease severity. Longitudinal studies carried out over a three year period suggest that this technology can be used to control Johne's disease on deer farms. The performance of the ELISA to date suggests that it may have a role to identify and cull MAP infected juvenile and adult deer.

Key words: diagnosis, IgG_1 ELISA, Johne's disease, deer

INTRODUCTION

Cases of Johne's disease have been diagnosed in farmed deer in New Zealand since 1986 (Mackintosh et al. 2004), more recently in farmed deer in Belgium (Godfroid *et al. 2000*), tule elk (Manning et al. 2004) and white-tailed deer (Davidson et al. 2004) in the USA. Over the past twenty years, Johne's disease has been found at increasing levels in deer herds throughout New Zealand (deLisle et al. 2004). While the disease takes several years for clinical symptoms to present in sheep and cattle, in deer the process from infection to death can progress more rapidly, with animals dying from the disease from eight months of age (Mackintosh et al. 2004). Outbreaks occur in young deer (8-15 months), with losses of up to 15% of animals. Older animals sporadically present with clinical Johne's disease that may be exacerbated by environmental stress or ageing (Mackintosh et al. 2004). Johne's disease can be spread horizontally among adult animals and infection may also spread vertically during pregnancy, since MAP has been isolated from fetal tissues (van Kooten *et al.* 2005). Johne's disease is currently the most important and costly infectious disease affecting farmed deer in New Zealand. The control and eradication of Johne's disease is problematic world-wide, due to the long incubation time before disease presents. The problem has been exacerbated by the lack of MAP-specific diagnostic tests, especially for the diagnosis of subclinical MAP infection. Initial studies in deer (Griffin et al. 2003) suggested that subclinically affected deer produce a stronger seroconversion and higher levels of IgG_1 antibody than has been shown previously in cattle (Collins et al. 2005*)* or sheep (Sergeant et al. 2003). Other studies also indicated that IgG_1 ELISAs have increased sensitivity for diagnosis of tuberculosis (Lightbody et al. 1998) and Johne's disease (Koets et al. 2001) in cattle.

This summary report outlines results using a modified direct IgG_1 ELISA as a diagnostic test for MAP in deer. More comprehensive findings from this study will be published elsewhere (Griffin et al. 2005). Parameters for testing and evaluation of the results were standardised retrospectively following slaughter and necropsy follow up of more than 200 animals, to confirm or exclude the presence of MAP infection.

MATERIALS AND METHODS

Blood samples were obtained from farmed deer properties throughout New Zealand that had MAP infection confirmed by histopathology and microbial culture of samples of animals following necropsy. These results were used retrospectively to develop estimated sensitivity values for the ELISA. Blood samples obtained from more than 500 deer from herds considered free of MAP infection throughout their history were used to establish estimated specificity values for the assay. Denatured tuberculin (PPDj) and native protoplasmic antigens (PpAg), both obtained from MAP were used as target antigens in parallel for test development. The IgG_1 isotype antibody was targeted as earlier work (Chinn et al. 2002) had established that this isotype was more sensitive than IgG-based ELISAs for diagnosis of tuberculosis in deer.

A standard ELISA protocol originally described by Voller (Voller et al. 1979) and modified by Griffin and Buchan (1989) was used in this study, with variations in the type of antigens and antibodies used. Plates used for the ELISA were 96-well micro-titre Maxisorp Immunoplates (NUNC). MAP Protoplasmic Antigen (PpAg) was obtained from Allied Monitor Inc (Fayette, MO, USA) and Purified Protein Derivative of MAP (PPDj) was obtained from CIDC Lelystad (The Netherlands) using the following preparative method. MAP (85B strain) was grown as pellicles on the surface of Watson and Reid media in penicillin flasks at 37ºC for 12-14 weeks. PPDj was prepared from heat-killed cultures in a Koch apparatus for three hours, and subsequently filter-sterilized to obtain a clear fluid. Protein was precipitated by the addition of TCA (4% w/v) and pelleted by centrifugation at 2600 x g. The pellet was washed 4-6 times with 5% NaCl and 0.5% phenol until a pH of 2.6 -2.7 was obtained, at which point it was neutralized by washing with 100mM Na_2HPO_4 (pH 11.0) and re-centrifuged at 2600 x g. This final pellet was suspended in storage buffer at neutral pH (100 mM phosphate buffer containing 19.4% glucose and 0.5% phenol), or lyophilized and stored in the dark if maintained for long periods before use. Plates were coated with antigen preparations (PPDj or PpAg) diluted in carbonate buffer (pH 9.6) to contain 50μg/mL. Fifty microlitres of antigen was used to coat each well. Plates were stored at -20°C, and incubated at 4°C overnight before use. After incubation overnight at 4°C, unbound antigen was washed from the plates by washing them six times in phosphate buffer containing 0.05% Tween 20 (wash buffer). Test serum samples were diluted in wash buffer and added to separate wells for each antigen to be tested. After the addition of serum the plates were incubated for 1 hour at 37°C and washed six times in wash buffer. An unconjugated monoclonal antibody (-IgG1) was added and after incubation, unbound antibody was removed by washing six times. Horseradish peroxidase (HRP) labeled anti-mouse globulin (Biosource International, Camarillo, CA, USA) was added to each well. Substrate solution containing equal volumes of citric acid, Na_2HPO_4 in de-ionized water, H_2O_2 plus 0.4mg/mL of orthophenylenediamine dihydrochloride (OPD) was added to plates which were incubated in the dark at room temperature for 20 minutes. The reaction was stopped by addition of H_2SO_4 and the absorbance read at 490nm using an automated microplate reader (BIORAD – Model 3550). Optical densities (OD) were converted to ELISA units (Eu) by subtracting the OD of the negative serum from the OD of the test serum and multiplying by 100.

RESULTS

Results obtained by ROC analysis (Eng, 2005) of samples from more than 200 deer infected with MAP, confirmed following necropsy, and 500 uninfected control animals are given in Table 1 below. PPDj antigen gave specificity values of 100% at a cut point of 60Eu for PPDj and 40Eu for PpAg. At these cut points and sensitivities of 81% and 85%, were established respectively, for the two antigens. When used in series the composite sensitivity for these two antigens was 87%.

Table 1. Specificity of the IgG1 ELISA assay using different antigens at different test-positive cut points: *60Eu, **40Eu, [#]50Eu

Estimated specificity	Estimated sensitivity		
	PPDj	PpAg	PPDj or PpAg
100%	81%	85%[**]	87%
99.8%	82%	83%[#]	85%

When cutpoints of 50Eu were used for both of the antigens the test had a specificity of 99.8% and sensitivities of 82% and 83% individually with a serial sensitivity of 85%.

The ELISA was carried out repeatedly in a heavily infected deer herd over a two year period (Table 2) where all reactors were culled after the initial test. Whereas clinical disease was found in >20% of animals in 2002, it had completely disappeared by 2004. Animals with seropositivity decreased from 50.3% in 2002 to 5.5% in 2004. Whereas a significant proportion (>40%) of 6-8 month old animals were positive in 2002, no animals in this age category were positive in 2004.

Table 2. Change in seroreactivity over time

	2002	2004
Positive	354 (50.3%)	29 (5.5%)
Negative	351 (49.7)	501 (94.5%)

Seropositivity was shown to correlate closely with disease severity. All deer with severe disease were seropositive (67/67). By contrast, only 77% (77/100) of MAP culture positive animals that had no detectable pathology (subclinicals) were positive in the IgG_1 ELISA.
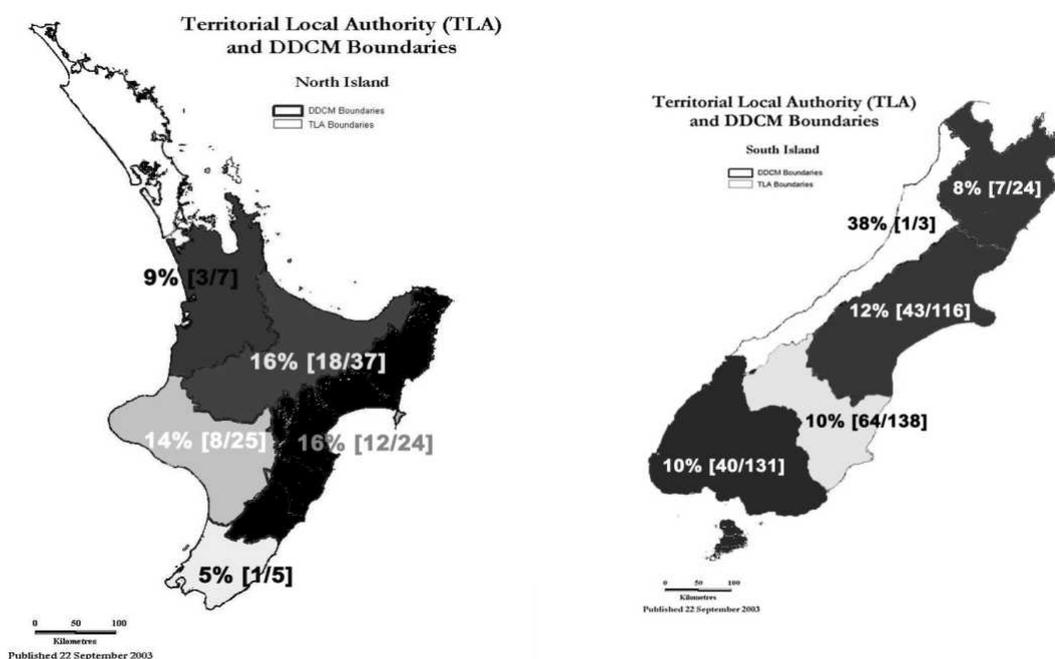
Fig. 1. Percentage of Johne's disease reactivity in deer blood samples submitted for TB diagnosis, and the proportion (in brackets) of farms with animals which had animals with reactivity compatible with MAP infection.

Testing for Johne's disease in blood samples obtained from TB skin test reactors from herds throughout New Zealand (Fig. 1) showed clear evidence of seroreactivity to MAP in a large numbers of deer herds. While these results are presumptive of exposure to MAP, no detailed post mortems were carried to confirm the status of the animals.

DISCUSSION

In recent years the occurrence of Johne's disease in farmed deer has become an issue with an estimated herd prevalence of 5% JD infected herds throughout New Zealand (de Lisle et al. 2003). However, this is likely to be a considerable underestimate as the samples studied by deLisle largely involved tissues from animals with suspect TB lesions found at slaughter that were submitted to confirm or exclude a diagnosis of tuberculosis. Since Johne's disease has become a significant cause of production losses for farmed deer, new technology is needed for the effective diagnosis and control of MAP infection.

Microbiological methods for Johne's disease diagnosis have limitations. Culture of gut tissue homogenates is the most sensitive marker of MAP infection in individual animals. By contrast, whereas cultivation of MAP from faecal samples may give an indication of a property's infection status, it is not sufficiently sensitive to diagnose infection at the individual animal level.

Immunodiagnostic ELISAs have been widely evaluated for Johne's disease diagnosis in a number of target species. These have largely involved pre-absorption of serum samples with *M. phlei* prior to testing with MAP antigens. An absorbed ELISA for sheep produced estimated specificity values of 99% and 95%, with corresponding sensitivity values of 21.9% and 41.5% (Sergeant et al. 2003). Use of an absorbed ELISA on cattle serum showed a sensitivity of 64% in infected cattle over 3 years of age and only 38% for infected animals less than 2 years of age (Huda et al. 2004). Johne's disease in cattle and sheep typically takes several years from subclinical infection before clinical signs appear. By contrast, the earlier onset of disease in deer may result in more obvious patterns of immune reactivity with production of antibody sooner in the course of the disease than is seen in other species.

The approach used in this study was to determine relative levels of antibody reactivity to individual MAP antigens in an unabsorbed ELISA test. The antigens used were both native and denatured antigens from MAP. The specificity of the assay was established using blood samples from a group of animals sourced from uninfected properties, whereas estimated sensitivity was determined in a group of MAP infected deer whose status was confirmed following necropsy by tissue culture.

Receiver operating characteristic (ROC) analysis (Eng, 2005) was used to determine cutpoints which gave 100% test specificity (Table 1). The cutpoints for the antigens that gave 100% specificity were: PPDj - 60 Eu and PpAg - 40 Eu. At these cutpoints sensitivity values of 81% and 85% were obtained for PPDj and PpAg antigens respectively. When the two antigens are used at a given specificity, but combined serially, native and tuberculin antigens produced a composite sensitivity (91%) higher than either antigen alone at any given specificity. Retrospective analysis showed that when a cut point of 50Eu was used for both antigens the overall sensitivity of the assay was 85%.

The performance of the test could be improved further by the use of MAP-specific recombinant proteins (Banantine et al. 2005; Griffin et al. 2005). The results from the IgG_1 ELISA in deer are significantly higher than those obtained with existing tests for MAP infection in sheep (Sergeant et al. 2003) and cattle (Collins et al. 2005). This could be explained by the earlier and more florid presentation of disease in deer (Mackintosh et al. 2004). Previous serodiagnostic studies on tuberculosis in deer show increased levels of antibody to *M. bovis* in tuberculous deer compared with cattle (Griffin and Buchan, 1989: Chinn *et al.* 2001). Overall deer appear to have a greater propensity to produce antibody following exposure to pathogenic mycobacteria than other ruminants.

When the IgG_1 ELISA test was used annually in a heavily infected deer herd and all test positive reactors were slaughtered there was a significant reduction (Table 2) of seropositive animals and elimination of clinical infection. It remains to be established whether repeated use of the ELISA with culling would result in the elimination of subclinical infection over time and whether the process would be cost-effective. Use of the test on TB skin test reactor deer (Fig. 1) showed that a significant number of deer from herds throughout New Zealand had MAP specific reactivity, resulting in false positive reactivity to the intradermal TB skin test. Overall, 33% of the herds tested had some skin-test reactor animals with reactivity that could be more logically explained as a result of exposure to MAP, rather than *M. bovis.* The widespread occurrence of MAP infection throughout New Zealand deer herds will continue to have a major confounding effect on the success of tests for control of TB long term.

ACKNOWLEDGEMENTS

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Interleukin-12 potentiation of the interferon-gamma test rescues day-old blood samples for the diagnosis of paratuberculosis

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ABSTRACT

The interferon-gamma (IFN-γ) test that measures disease-specific cellular mediated immune responses (CMI) is gaining increased acceptance as a diagnostic tool for detecting early immune responses to paratuberculosis. The test is, however, sensitive to the time interval from blood sampling to processing in the laboratory. The general recommendation is a maximum interval of 8 hours from sampling to culture. This is a major obstacle to the routine use of the IFN-γ test in diagnostic or surveillance programs. Potentiating the whole blood IFN-γ test with interleukin-12 (IL-12) rescued antigen-specific IFN-γ production in day-old samples and produced IFN-γ levels that were comparable to those of fresh stimulated samples. Addition of IL-12 to cultures without antigen did not induce non-specific IFN-γ production and potentiation with IL-12 did not induce non-specific IFN-γ responses to PPD in animals that were IFN-γ test negative with fresh samples. Addition of IL-2 was not able to induce comparable increases in IFN-γ responses in day-old samples, and although a combination of IL-2 and IL-12 induced higher levels of antigen-specific IFN-γ than IL-12 alone, this cocktail also induced high levels of IFN-γ in absence of antigen. Thus, the IL-12 potentiated IFN-γ test makes possible the study of antigen-specific CMI responses in stabilized blood samples stored for an extended period of time allowing samples to be submitted to the laboratory by ordinary mail services.

Key words: cattle, paratuberculosis, diagnosis, interferon-gamma, interleukin-12

INTRODUCTION

In vitro measurements of cell mediated immune (CMI) responses are notoriously subject to variation and are less predictable than measures of humoral immune responses. However, quantitative measurements of disease-specific CMI responses, e.g. the interferon-gamma (IFN-γ) test, are gaining increased acceptance as diagnostic tools for many diseases with poor or non-specific production of antibodies in serum. Bovine tuberculosis and paratuberculosis were the first infections for which this test was used in veterinary medicine (Wood et al., 1989; Rothel et al., 1990; Wood et al., 1991; Billman-Jacobe et al., 1992). Even though the IFN-γ test has seemed promising, it has not become widely accepted in diagnostic laboratories. Of the assay's several problems, only the requirement that samples be freshly collected samples will be investigated in this study.

In vitro CMI tests rely on active cellular responses to stimulation by antigen in the assay and resultant proliferation of cytokine, or cytokine-receptor, expression by target T-cells. This process is highly sensitive to the time interval from sampling to stimulation. For the IFN-γ test, samples must be stimulated within 8 hours of sampling for optimal assay performance (Rothel et al., 1992; Jungersen et al., 2002). Although use of day-old samples has been reported to be valid in testing for bovine tuberculosis (Ryan et al., 2000) the feasibility of that approach may be affected by prior PPD skin test sensitization. The need for freshly collected samples is a major obstacle to the routine use of the IFN-γ test in settings other than controlled experimental infections and study herds.

Interleukin-2 (IL-2) is a key activator of T-cells' response to antigenic stimulation. (Lin and Leonard, 2003) The immunomodulatory effects of IL-12 regulation of T_{H1} type immune responses and enhancement of IFN-γ responses to antigenic stimulation is also well known in many animals including bovines (Collins et al., 1998; Collins et al., 1999). The aim of the present study was to investigate if additions to bovine whole

blood cultures of IL-12 and/or IL-2 as co-stimulatory cytokines could potentiate the waning paratuberculosis PPD (Johnin)-specific IFN-γ production in day-old samples. If so, measuring the antigen-specific CMI responses in day-old samples sent to the laboratory by ordinary mail services would be feasible.

MATERIALS AND METHODS

Heparinized bovine whole blood samples were collected from cattle in one Holstein-Friesian (H-F) and one Jersey herd, both of which were known to be infected by MAP. For some studies blood from an H-F stud vaccinated against paratuberculosis (MycoPar®, Fort Dodge Animal Health) was used. Undiluted whole blood samples were incubated for 20-22 hours in 1 ml cultures at 37° C in 5 % CO_2 in the presence of Johnin PPD (10 µg/ml), a negative (PBS) and a positive superantigen control (Staphylococcal enterotoxin B; SEB) (1 µg/ml) as previously described (Jungersen et al., 2002). To day-old cultures were added recombinant cytokines together with the antigens (IL-12 at 10 U/ml and IL-2 at 10 IU/ml). The levels of secreted IFN-γ in culture supernatants were measured by an in-house ELISA with mAbs directed against natural bovine IFN-γ and calculated to pg/ml following log-log transformation of a serial dilution of a sample with a known level of IFN-γ. The amount of bovine IFN-γ in this sample had been previously determined using the Bovigam® ELISA and a recombinant bovine IFN-γ standard. Recombinant bovine IL-12 was produced at IAH (Collins et al., 1998). Recombinant mouse IL-12 and recombinant human IL-2 was purchased from Sigma.

RESULTS

In Fig. 1 the effects of bovine IL-12 and human IL-2 on the antigen specific and the non-specific IFN-γ production in day-old cultures are compared with samples cultured 4 hours after sampling. Without the added cytokines, the PPD-induced IFN-γ levels in day-old samples were 2-5 fold lower than those in fresh cultures. With the addition of IL-12, the IFN-γ levels in 7 of 9 animals were higher than in 4-hour cultures. However, non-specific IFN-γ production in unstimulated cultures occurred. The addition of both IL-12 and IL-2 in combination induced up to an 108-fold increase in PPD-induced IFN-γ production, but also induced very high levels of non-specific IFN-γ production in non-stimulated samples.
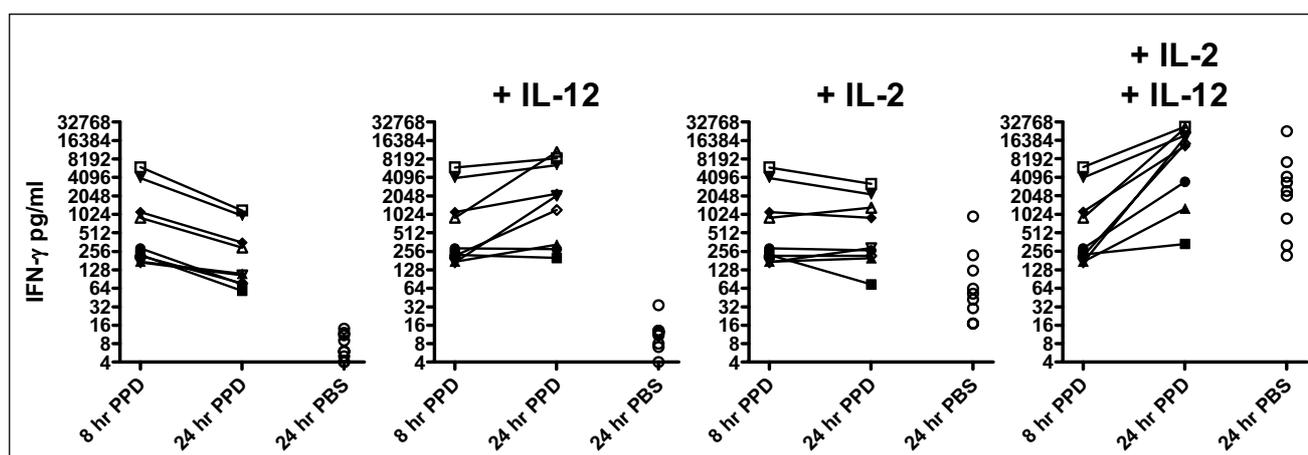
Fig.1. Effect of IL-12 and/or IL-2 addition on paratuberculosis specific IFN-γ response for day-old samples. Whole blood samples were drawn from 9 cows and kept at 20ºC until processed at 4 and 28 hours post sampling. Samples without antigen (PBS) or with johnin PPD were cultured for 22 hours at 37ºC after which supernatants were collected and analysed for IFN-γ.

Different storage temperatures of the blood sample prior to handling influenced the subsequent production of IFN-γ in culture (Fig. 2). Storage of the blood sample at 25ºC resulted in a 5-8 times inhibition of PPD specific IFN-γ production compared to storage at 12-20ºC and a 15 fold inhibition compared to storage at 4ºC, respectively. Storage of the blood sample at 4ºC also resulted, however, in non-specific IFN-γ production in unstimulated samples. No IFN-γ production differences were observed between storage

temperatures from 12-20°C. Subsequent analyses of more samples stored at 4°C (Fig. 3) did not confirm a consistent induction of non-specific IFN-γ at a low temperature and it was decided to continue with refrigerated storage of samples.

Storage temperature of blood sample before culture
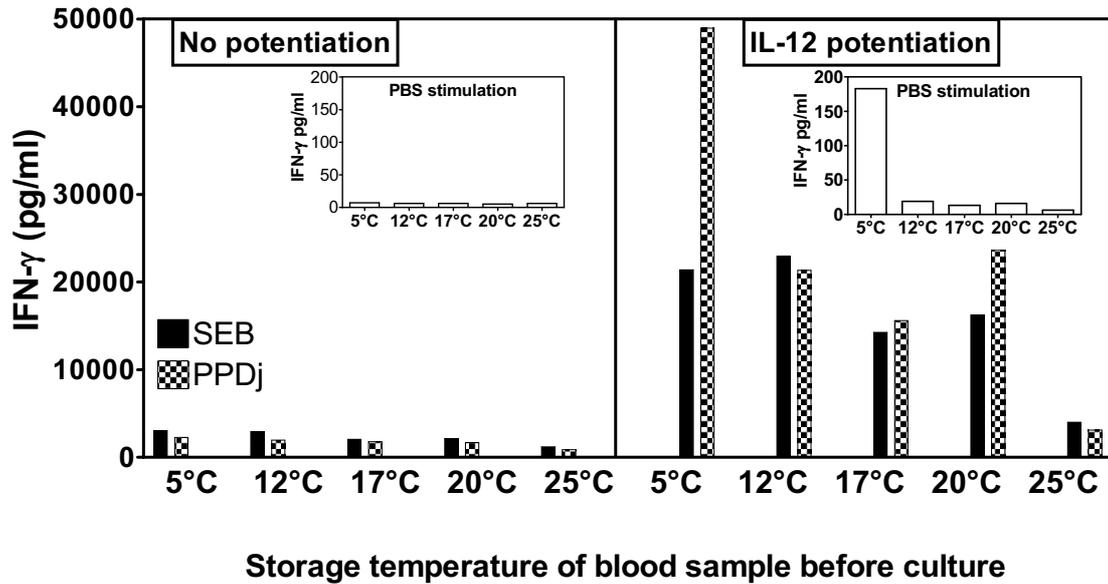
Fig. 2. Effect of storage temperature of blood sample on subsequent IFN-γ production in culture. Blood samples from a bull vaccinated against paratuberculosis were kept overnight at 5, 12, 17, 20 and 25°C before performance of the IFN-γ test with PBS (inserted graphs), johnin PPD and SEB and with or without addition of IL-12.

When recombinant bovine IL-12 and recombinant mouse IL-12 were compared, both cytokines were able to potentiate the paratuberculosis specific IFN-γ response (data not shown), but murine IL-12 consistently induced higher levels of non-specific IFN-γ (Fig. 3) and was omitted from further studies.

IL-12 concentration
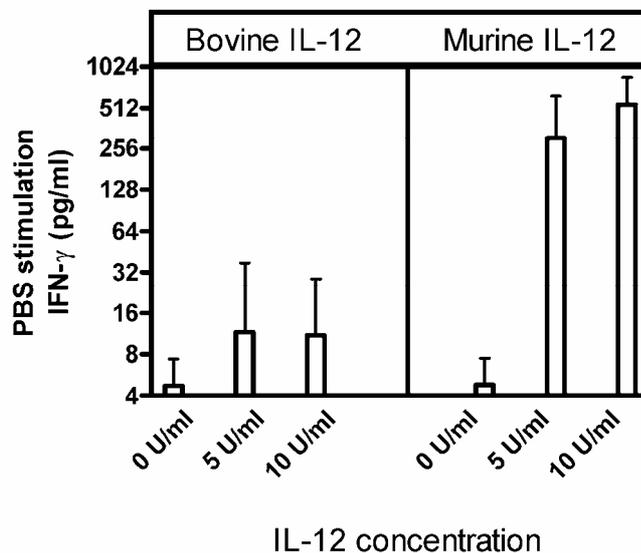
Fig. 3. Induction of non-specific IFN-γ production by bovine IL-12 vs. murine IL-12. Fifteen whole blood samples were refrigerated overnight and cultured 20 hours at 37°C with either PBS, bovine (left panel) or murine (right panel) recombinant IL-12 at 0, 5 or 10 U/ml. The culture supernatants were assayed for IFN-γ production. Results are indicated as the mean of the 15 cows with SD error bars.

Following titration studies of recombinant bovine IL-12 (data not shown) a protocol was designed using samples refrigerated overnight and potentiated with 10 U/ml IL-12 added together with the stimulating antigen. In Fig. 4 the results of this protocol is compared with the standard IFN-γ test (fresh samples, no IL-12) on 31 cows and 36 heifers plus a protocol with non-potentiated stimulation of refrigerated samples

(cows only). Statistical analysis of the paratuberculosis specific IFN-γ responses revealed that the standard IFN-γ test levels were significantly different from both non-potentiated day-old samples of cows (P=0.0025, paired t-test) and from IL-12 potentiated samples of heifers (P<0.0001, paired t-test); this difference was not seen with the IL-12 potentiated day-old samples of cows (P=0.34, paired t-test). Similar results were obtained by the non-parametric Wilcoxon matched pairs test (P=0.0001, P<0.0001 and P=0.13, respectively). However, IFN-γ levels on day-old samples were significantly correlated with levels of fresh samples for cows, irrespective of IL-12 potentiation or not, and heifers (P<0.0001 for all 3 datasets).
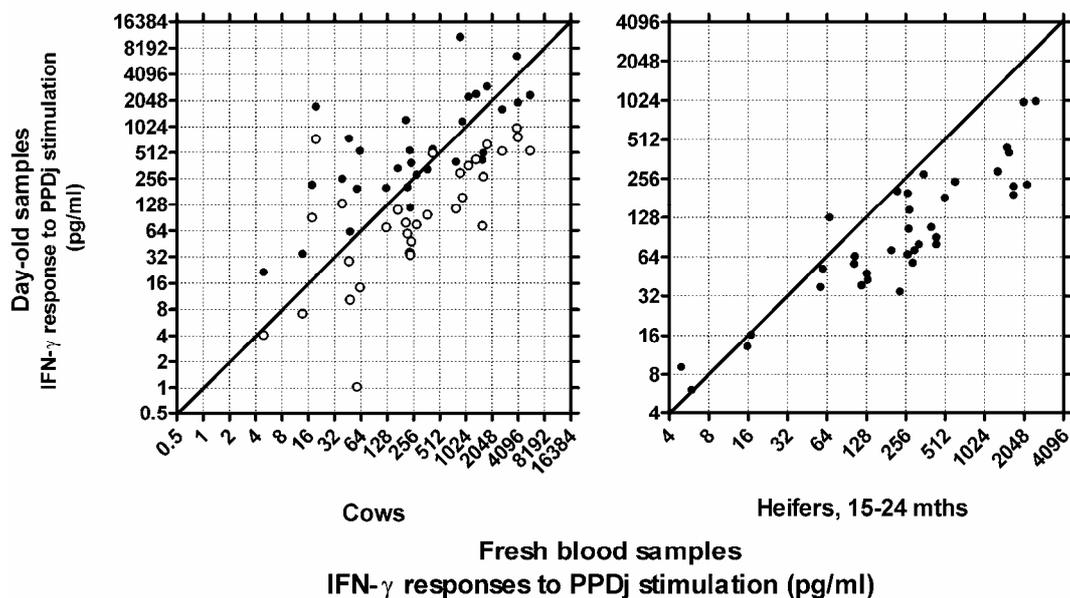
Fig. 4. Correlation between standard IFN-γ test with fresh samples and IFN-γ test performed on samples refrigerated overnight with or without addition of IL-12. 31 cows (left panel) and 36 heifers (right panel) were tested for Johnin PPD IFN-γ response by standard conditions (x-axis) and correlated to IFN-γ response of day-old samples (y-axis) with IL-12 cytokine potentiation ● or without cytokine potentiation ○ (cows only).

DISCUSSION

The addition of IL-12 to whole-blood cultures boosted the production of antigen-specific IFN-γ and permitted the use of day-old blood samples for diagnostic purposes that otherwise would have not been suitable for testing. The IL-12 potentiated antigen-specific IFN-γ production in day-old samples was comparable to that of fresh samples collected from a MAP-infected animal. Non-specific IFN-γ responses were not induced in animals that did not show MAP-specific CMI responses. Addition of IL-12 to samples without antigen did not induce non-specific IFN-γ production. Addition of IL-2 did not to induce comparable increases in IFN-γ responses in day-old samples, and although a combination of IL-2 and IL-12 induced higher levels of antigen-specific IFN-γ than did IL-12 alone, this cocktail also induced high levels of IFN-γ in the absence of antigen.

Other studies have also found a profound reduction of IFN-γ production after overnight storage of samples before stimulation. We have previously found at least a 3-6 fold reduction of Johnin PPD-specific IFN-γ production in 7 animals with paratuberculosis reactivity (Jungersen et al., 2002). In line with this and the present study, Rothel et al. (1992) found a 2 to 7-fold reduction of PPD-specific IFN-γ production in 6 animals following blood storage of 24 hours before stimulation. They also studied the effect of temperature on subsequent PPD-induced IFN-γ production and observed only minimal differences between storage at 4°C, 24°C and 37°C. In the present study, storage temperature at 25°C almost completely abolished IFN-γ production in samples stimulated with Johnin PPD or superantigen. Some non-specific IFN-γ production was observed in samples stored overnight at 4°C, however, this was not a general feature of all samples and storage at low temperatures also yielded the highest level of antigen-specific IFN-γ production. For practical purposes, refrigerated samples are easiest handled and during postal handling of samples in

Denmark and other Northern countries low temperatures may often be attained during overnight transportation anyway. For these reasons refrigerated storage of samples is recommended.

It is surprising that IL-12 addition to blood samples from the heifer group did not augment the level of IFN-γ production of freshly collected and stimulated samples as was observed for adult animals (Fig. 4). We have no explanation for this. The comparison between fresh and day-old samples should be repeated for this age group to rule out a possible experiment artefact before hypotheses regarding differences in level of IL-12 receptor expression or other biological explanations are investigated.

An important issue of IFN-γ production testing is the handling of test-positive animals. For a diagnosis of MAP infection, the test measures the supposedly mycobactericidal CMI response. Among these IFN-γ positive animals, only a limited number will eventually experience progressive disease. It is currently not possible to predict if an IFN-γ positive animal will develop clinical symptoms at a later stage. Another issue is that CMI responses fluctuate somewhat from sample to sample (Huda et al., 2003) and a single IFN-γ test-positive sample from a cow may equally well represent a) a recent CMI response against MAP exposure from other shedding cows in the facility or b) a persistent infection acquired in early calfhood. However, it is the general opinion that the vast majority of chronic, clinical paratuberculosis results from the latter type of infection. For these reasons we believe that the most useful implementation of the IFN-γ test is in herd surveillance, classification and certification which at the earliest can be performed in the heifer group. It is hypothesized that paratuberculosis specific CMI responses in this age group reflects calfhood exposure to the bacteria as shedders are usually not found within this age-group and contact with other age-groups is very limited. This is in agreement with a previous observation that paratuberculosis specific CMI responses among heifers aged 15-24 months may wane with repeated sampling, but rarely increase with time (Huda et al., 2003). Analysis of IFN-γ responses in cattle younger than 15 months are prone to non-specific false-positive reactions (Jungersen et al., 2002), most likely due to innate IFN-γ production by NK-cells (Olsen et al., 2005).

Presently, the IL-12 potentiated IFN-γ test with day-old samples delivered to the laboratory by ordinary postal service is being used to estimate the level of calf-exposure to *M. paratuberculosis* in a number of dairy herds. The assay is used in a management-based eradication programme with yearly screening of 45 heifers in the 15-24 months age group.

CONCLUSION

Recombinant bovine IL-12 potentiates heparin-stabilized blood samples and thus enables the study of antigen-specific CMI responses over an extended period of time. The addition of IL-12 "rescues" day-old blood samples so that they may be submitted to the laboratory for IFN-γ testing by ordinary mail services. This protocol overcomes one of the major obstacles in the practical handling of the IFN-γ test and allows the IFN-γ test to be used as a routine test in the diagnosis and surveillance of paratuberculosis.

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Evaluation of environmental sampling to determine distribution and herd infection status for *Mycobacterium avium* subspecies *paratuberculosis*

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ABSTRACT

A total of 483 environmental samples were collected from 98 dairy operations; 216 (44.7%) were culture positive for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The highest percentage of positive environmental samples were collected from parlor exits (52.3%), holding pen floors (49.1%), common alleyways (48.0%), lagoons (47.4%) and manure spreaders (42.3%). Of the 98 operations tested with environmental sample culture, also obtained were individual fecal culture results from 60, individual serum ELISA from 97 and individual milk ELISA from 34. Of the 50 herds classified as infected by fecal culture, 38 (76.0%) were identified by environmental culture. Two of the 10 operations classified as not infected based on individual animal fecal culture were environmental culture positive. Of the 80 operations classified as infected based on serum ELISA positive test results, 61 (76.3%) were identified as environmental positive. Environmental sample collection and culture is more cost effective than individual animal sampling and identified more than 75% of infected operations in this study. Environmental sampling is another diagnostic tool that veterinarians and dairy producers can use to determine herd MAP infection status.

Key words: *Mycobacterium avium* subspecies *paratuberculosis* (MAP), Johne's disease, environmental sampling.

INTRODUCTION

Johne's disease (JD), or paratuberculosis, is a chronic, progressive disease of ruminants caused by the effects of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) on the gastrointestinal tract. It is transmitted primarily through a fecal-oral route, especially from manure and environmental contamination by infected adult cattle, and young cattle are most susceptible (Sweeney, 1996).

An important component of the U.S. Voluntary Bovine Johne's Disease Control Program is to determine herd infection status for MAP. To help prevent the transmission of MAP, dairy and beef herds providing replacement cattle should know their infection status. Herds acquiring cattle should be fairly confident that the source herd is at low risk for MAP infection. The current requirements for a herd to be considered Level 1 of the test-negative component requires ELISA testing 30 animals followed by fecal culture confirmation of ELISA-positive cattle (USDA, 1991). Since the ELISA is approximately 25-50% sensitive, many infected herds are classified as low risk and enter Level 1 based on this testing scheme.

MAP has been shown to survive in manure slurry long-term at temperatures below freezing (Jorgensen, 1977). A more recent study by Whittington et al. (2004) has shown increased duration of MAP survival in shaded soil and pasture. Lack of shade was shown to decrease survival, presumably due to infrared wavelengths and temperature flux. This evidence suggests that MAP survives for long periods of time in common dairy farm environments. However, the distribution of MAP in the dairy environment is not well documented. A study conducted in Minnesota, USA by Raizman et al. (2004) found that the most common areas to obtain culture positive samples were cow alleyways and manure storage areas. Environmental sampling detected 78% of known infected herds based on previous testing. Further understanding of the relationship between MAP distribution, environmental culture and other standard screening tests would be useful in the development of an environmental culture protocol as a herd-level screening method.

The purpose of this study is to 1) describe the distribution of MAP on U.S. dairy farms and the herd characteristics associated with culture-positive environmental samples and 2) assess the relationship between the MAP culture status in the farm environment and herd infection status as determined by individual cow fecal culture, serum ELISA, and milk ELISA.

MATERIALS AND METHODS

The National Animal Health Monitoring System's Dairy 2002 study surveyed dairy operations in 21 states, representing 82.8% of U.S. dairy operations and 85.5% of U.S. dairy cows (USDA, 2002). One component of the study involved collection and culture of environmental samples for MAP from areas on farms where manure accumulated from a majority of a herd's cows. Operations were selected based on perceived risk factors for MAP infection identified in an earlier questionnaire. Four to five environmental samples and paired serum and fecal samples were collected from March to August 2002. Animals in second lactation and greater were selected for MAP fecal and serum testing, while all lactating animals were tested via milk ELISA. Environmental and fecal samples were cultured using 3 methods in parallel. Serum samples were tested using a commercially available MAP ELISA kit. Milk samples were tested at Antel BioSystems in Lansing, MI, USA. Results of individual animal testing were compared to environmental sampling to determine herd infection status. The correlation between the percent of positive environmental pools and the within-herd prevalence as determined by each individual testing method was evaluated using Spearman's Rank Correlation Test.

RESULTS

A total of 483 environmental samples were collected and 216 (44.7%) were culture-positive for MAP. The highest percentage of positive environmental samples were collected from parlor exits (52.3%), holding pen floors (49.1%), common alleyways (48.0%), lagoons (47.4%) and manure spreaders (42.3%) (Table 1). There was no association between the source of the environmental sample and culture result (p=0.59).

At least one environmental sample was culture positive from 69 (70.4%) of 98 sampled herds. All five environmental samples were positive on 20 (20.4%) operations and only one sample was culture-positive on 15 (15.3%) operations (Fig. 1).

Table 1. Number and percent of environmental samples by source

Environmental Sample Source	Number Positive	Number Negative	Total	Percent Positive	Standard Error
Parlor exits	34	31	65	52.3	6.7
Holding pen floor	26	27	53	49.1	7.4
Common alleyway	61	66	127	48.0	5.9
Lagoon	18	20	38	47.4	9.1
Manure spreader	11	15	26	42.3	11.6
Manure pit	17	24	41	41.5	8.7
Other	49	84	133	36.8	5.5
Total	216	267	483	44.7	3.9

Of the 98 operations tested with environmental sample culture, 60 had individual fecal culture results, 97 had individual serum ELISA results and 34 had individual milk ELISA results. Of the 50 herds classified as infected by fecal culture, 38 (76.0%) were also identified by environmental culture (Table 2). Two of the 10 operations classified as not infected based on individual animal fecal culture were environmental culture-positive. Of the 80 operations classified as infected based on serum ELISA positive test results, 61 (76.3%) were identified as environmental positive. Twenty of the 28 (71%) operations determined to be infected based on milk ELISA testing were identified by environmental culture.
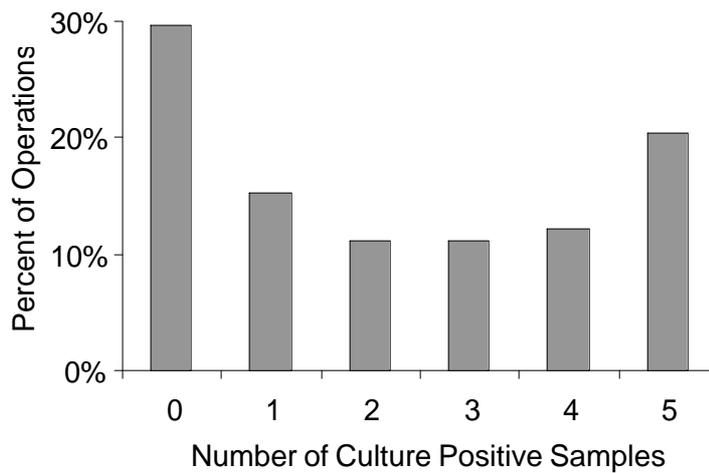
Fig.1. Percentage of operations by the number of culture-positive environmental samples for MAP.

Table 2. Comparison of operation-level environmental and individual sample test results

Environmental sample result	Individual Samples					
	Fecal Culture (n=60)		Serum ELISA (n=97)		Milk ELISA (n=34)	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	38	2	61	7	20	2
Negative	12	8	19	10	8	4
Total	50	10	80	17	28	6
	76%		76%		71%	
	(64-88)		(67-86)		(55-88)	

A significant positive correlation was identified between the percent of positive environmental pools and the within-herd prevalence estimates determined by individual animal testing (p<0.0001). Spearman's rank correlation coefficients were 0.685, 0.531 and 0.648 for fecal culture, serum ELISA and milk ELISA estimates, respectively. The correlation between environmental pools and fecal culture are presented in Table 3.
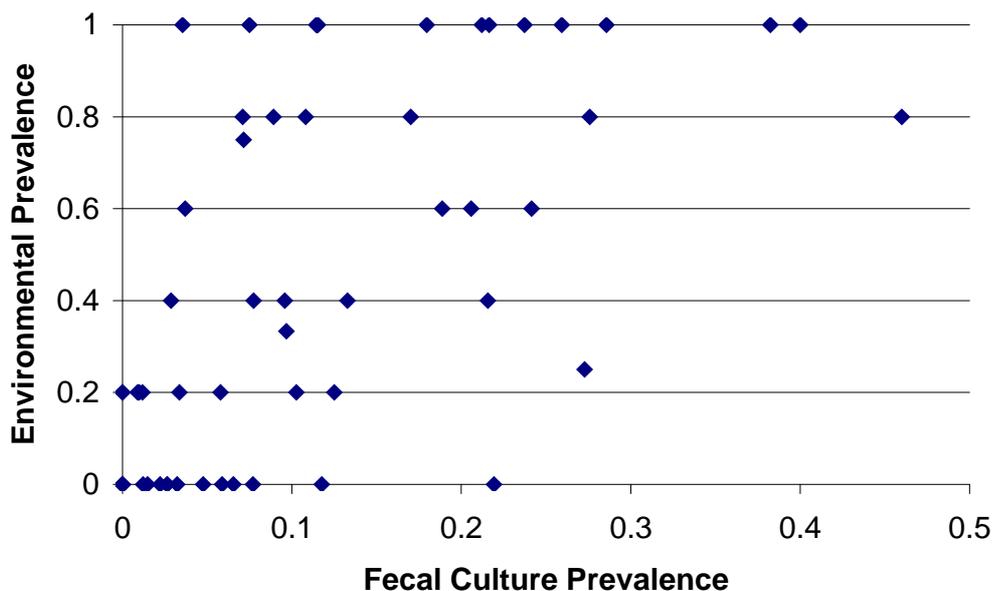
Fig. 2. Operation level percentage of positive environmental pools by fecal culture prevalence.

DISCUSSION

This is the first nationwide study evaluating the distribution of MAP in the environment of U.S. dairy farms and the feasibility of environmental culture to determine herd infection status. The study evaluated herds of different sizes and histories of JD, distributed in different regions of the country.

Testing programs designed to classify herds as uninfected or low-risk currently use serum ELISA and fecal culture testing in series. ELISA sensitivity is poor, however, and many subclinical cows may be test-negative by this method, leading to low-prevalence herds being falsely declared as uninfected. However, individual fecal culture, the most sensitive method available, is expensive and may require 3-4 months to obtain results. Under the voluntary program, animals that test ELISA positive can have fecal culture performed to confirm infection. The sensitivity of fecal culture is also less than 100% and so infected animals may be ELISA positive and fecal culture negative, leading to the herd being incorrectly declared as free of infection. Serum ELISA and individual fecal culture also require handling of individual cattle, which is time-consuming. Therefore, a more sensitive, less intrusive and less expensive method is needed.

The results of this study suggest that individual sampling can be replaced by environmental sampling. Targeted sampling and culture of common alleyways, milking parlor exits, gutters, gutter cleaners, and lagoons or manure pits can reasonably assess herd infection status. The positive correlation between positive environmental pools and within-herd prevalence suggests that a broad estimate of infection within a herd may be determined based on the percentage of positive environmental pools. Environmental sampling provides a convenient and affordable alternative strategy for herd screening, and may eventually replace serum ELISA as an initial herd-level screening process.

CONCLUSION

MAP is commonly found in the environment of infected dairy operations. Environmental sample collection and culture takes less time and is more cost effective than individual animal sampling. More than 75% of infected operations, based on individual animal sampling, were identified using environmental sample culture. Environmental sampling is another diagnostic tool that veterinarians and dairy producers can use to determine herd infection status for MAP.

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Interference of anti-*M. bovis* antibodies in serological tests for paratuberculosis

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ABSTRACT

ELISA assays are broadly used as a diagnostic tool of paratuberculosis. Nevertheless, problems of specificity are due to the great number of shared antigens between MAP and other *Mycobacteria*, especially the closely related *M. avium.* An *M. phlei* preabsortion step in the ELISA eliminates much of the cross-reacting antibody due to environmental bacteria. Despite this, other infections such as bovine tuberculosis, a frequent occurring disease in Brazil and in many other developing countries, may interfere with the specificity of the test. In this study, 97 sera from *M. bovis* infected cows that were skin-test reactive and confirmed as infected by histopathology. Sera were tested in a PPA (paratuberculosis protoplasmatic antigen – Allied Monitor, USA) ELISA that is routinely used for the diagnosis of paratuberculosis. From the 97 sera 25 (26.59%) presented an OD >0.60; the cut-off point for this test was standardized at OD 0.35. From these, twenty sera were randomly chosen for immunoblot analysis. Eighteen sera (90%) strongly reacted with four PPA bands: one 32-36KDa, one 42KDa and two others between 77 and 100KDa. An ELISA-positive control serum sample from a confirmed case of Johne's disease from a region free of tuberculosis also recognized these same four bands. The findings indicate that the proteins are shared between *M. bovis* and MAP. In conclusion, although ELISA tests may be used as valuable tools for the detection of paratuberculosis, interpretation of results in herds known to be infected with *M. bovis* should be done carefully.

INTRODUCTION

The most common immunological tests to identify MAP (*Mycobacterium avium* subsp. *paratuberculosis*) infection are the complement fixation test (CFT), agarose gel immunodiffusion (AGID), and ELISA. ELISA-based methods show the highest sensitivity of serological tests for paratuberculosis (PTB), since these assays are capable of detecting small amounts of antibodies. On the other hand, ELISA is less specific than fecal culture (Collins, 1996) and, therefore, not recommended as the sole diagnostic tool. In Brazil, Marassi *et al.* (2005) reported a sensitivity of 76.7% and a specificity of 70.5% in an improvement of a previously described ELISA recommended as screening test for herds (Ferreira *et al.,* 2002).

MAP shares several antigens with other mycobacteria, including *M. bovis*. Reports show that paratuberculosis can compromise the specificity of bovine tuberculosis (TB) diagnostic tests, and the influence of MAP co-infection on the diagnosis of bovine TB stills needs investigation (Vordemeier *et al.*, 1999). Although the interference of bovine TB on the efficacy of Johne's disease diagnostic tests has not been widely evaluated (Olsen *et al.*, 2001), natural infection with MAP was demonstrated to lead to false-positive reactions in TB skin tests (Buddle *et al.*, 2003).

Since most commercial tests for the serological diagnosis of PTB have been developed in countries where bovine TB is eradicated or at least controlled, minimal effort in wasa taken to evaluate the interference of anti-*M. bovis* antibodies in those tests. However, in various developing countries where both mycobacterial infections occur.the potential for interference of bovine TB in PTB-ELISAs must be understood before recommendations can be made for Johne's disease herd surveillance.

In order to reduce interference and increase ELISA specificity, recent studies focused on the development of tests using new purified immunogenic and species-specific antigens. AhpC, AhpD and 14kDa proteins were used in an ELISA assay in order to discriminate MAP infected from *M. bovis* infected-cattle (Olsen *et*

al., 2001). Recently, another ELISA assay was tested using the ESAT-6:CFP-10 fusion protein with the same purpose and promising results (Waters *et al.,* 2004). Although these antigens could augment specificity levels of immunological assays, the assays were less sensitive and could misdiagnose animals in the early stages of the disease. Regardless of this, new antigens could be helpful to diminish cross reactions between antibodies due to MAP vs. *M. bovis.* (Olsen *et al.*, 2001; El-Zaatari *et al.*, 2002; Waters *et al.,* 2004).

The aim of this study was to evaluate the performance of a PTB-ELISA in an *M. bovis* infected herd without symptoms of paratuberculosis.

MATERIALS AND METHODS

Serum samples
Ninety-seven adult cows with TB from five dairy herds with a previous history of bovine tuberculosis (including clinical cases and recovery of *M. bovis* from slaughtered animals) were studied. At the moment of sample collection, no cattle in the herds had chronic diarrhoea, weight loss or any suspicion or history of paratuberculosis. Infection with *M. bovis* was diagnosed by intradermal tuberculin test. The test consists of the injection of 0.1 mL of bovine PPD (*M. bovis* strain AN5, 1 mg protein/mL), corresponding to 5000 international units (IU) per dose and examination of the site after 72 hours. The interpretation of the results was performed according to the recommendations of the Department of Agriculture in Brazil, i.e., a test-positive animal has more than 4.0 mm of swelling at the site of inoculation. All test-positive animals were slaughtered and cases were confirmed by histopathology and visualization of acid-fast bacilli on Ziehl-Neelsen staining of lesions consistent with tuberculosis, mainly located in lymph nodes and lungs. Serum from a confirmed case of bovine paratuberculosis with a strong ELISA result located in a TB-free region was used as a control (kindly provided by the Johne's Testing Center, U. Wisconsin, USA).

Study design
The study was conducted in two steps. First the 97 sera were tested by PPA-ELISA as follows. From those, twenty test-positive sera were randomly chosen for the western blot analysis.

PPA-ELISA protocol
ELISA was conducted as previously described (Marassi *et al.*, 2005). Briefly, Paratuberculosis Protoplasmic Antigen (Allied Monitor, USA) was used as a capture antigen. It was diluted in a carbonate buffer (9.6 pH) and adsorbed in a 96-well plate overnight at 8°C. Each well was coated with 100μL of antigen solution (0.07mg/mL) and blocked with 2% casein in TBST (Tris 10mM, 0.9% NaCl, 0.2% Tween 20). *M. phlei* extract (5mg/mL*)* was mixed with an equal volume of serum for 60 minutes with constant agitation at 37°C. After that, the *M. phlei* – serum suspension was incubated overnight at 8°C., the pre-adsorbed sera were added to the wells (100μL/well) and incubated for one hour at 37°C. Monoclonal bovine IgG linked to alkaline phosphatase was used as a conjugate. Immune complexes were detected by addition of p-NPP substrate (1mg/mL) with incubation for 30 minutes at room temperature in the dark. Three wash steps with TBST were included after incubation with the first and second antibody. Sera were tested at the dilution 1:100 and all assays were run in duplicate. Any assay with a between-well coefficient of variation of >15% was repeated. The sample's optical density (OD) was measured by a wavelength of 405nm. Final results were expressed as the ratio S/P, obtained by dividing the mean OD value (S) of a given serum divided by the mean OD (P) of the positive control. Thus, sera were classified as positive when their S/P was equal or higher than 0.35.

Western-blot
Twenty micrograms of PPA were applied to a 12% SDS-PAGE gel and protein electro-transferred onto nitrocellulose membranes using a mini-Protean II system (Bio-Rad laboratories, USA), according to the manufacture's protocol. Membranes were blocked in a solution of 5% of casein (Sigma-USA) in TBST (Tris 10mM, 0.9% NaCl, 0.2% Tween 20) overnight at 8°C. *M. phlei* was mixed with an equal volume of serum for 60 minutes with constant agitation at 37°C. After that, the *M. phlei* – serum suspension was incubated overnight at 8°C. Serum samples were used at a dilution of 1/100 and incubated for one hour. A 1/5000 IgG bovine conjugated with alkaline-phosphatase (Sigma-Aldrich laboratories, USA) was added and the

membrane was incubated for one hour at room temperature. Three wash steps of five minutes with TBST were included after incubation with the first and second antibody. The antibody-antigen complex was detected using a solution of 4mL of BCIP/NBT (Sigma-Aldrich laboratories, USA) for 10 minutes. The reaction was stopped with distilled water.

RESULTS

From the 97 tested sera, 39 (40.2%) were test-positive with PPA-ELISA. From those, fourteen presented weak reactions bordering the 0.35 cut-off of the test and twenty-five (25.6%) presented SP > 0.60. Those animals were considered as strong positives and their samples were tested twice. As stated, none of these cattle presented symptoms characteristic of paratuberculosis nor had any herd a previous history of Johne's disease (Fig. 1).
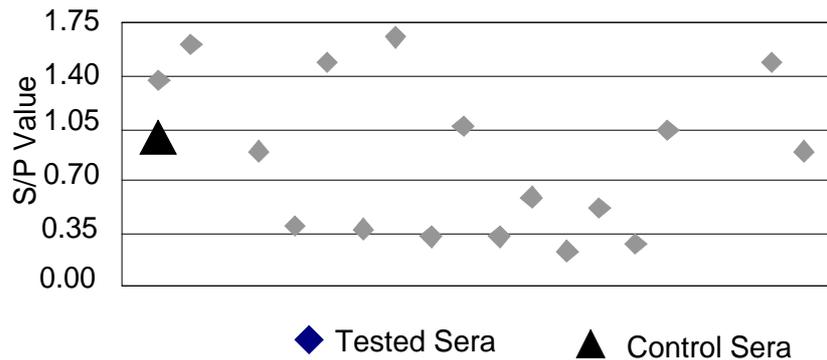
Fig. 1. Performance of 20 selected sera at ELISA. Cut-off value: 0.35.

From those strongly ELISA-positive sera, twenty were randomly chosen for western blot analysis in order to identify the immunodominant bands that reacted with these sera. This analysis demonstrated that 18 sera (90%) reacted strongly with four PPA bands: one 32-36kDa, one 42kDa and two others between 77-90kDa. The positive control serum also recognized these same four bands, indicating the bands are shared by *M. bovis* and MAP (Fig. 2).
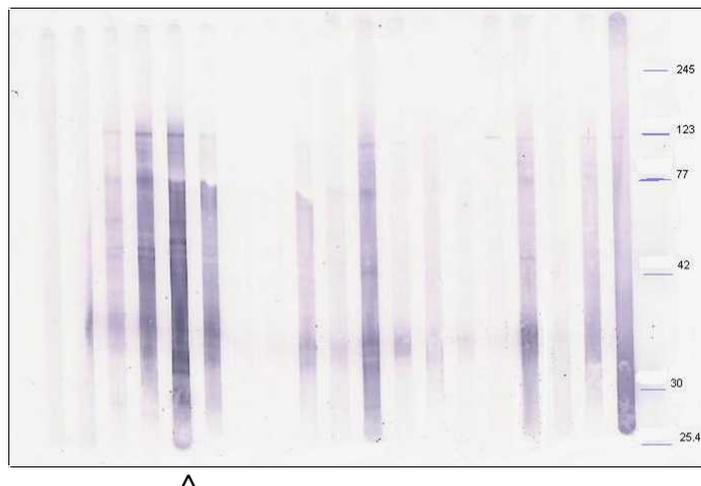
Fig. 2. Western blot analysis of 20 sera from *M. bovis* infected cattle. Arrow indicates the control sera.

DISCUSSION

Tuberculosis is an endemic bovine infection in many developing countries which is controlled through periodic PPD testing and culling of the reactive animals. The usefulness of serologic surveillance of mycobacterial infections is influenced by several variables, such as the nature of the capture antigen used,

the evolution of the disease with the switch from cellular to humoral immunological response, the genetic of the exposed herd, and the epidemiologic conditions of each country (Köhler *et al.,*2001).

Cross-reacting antibody responses due to infection by different mycobacterial species can interfere with diagnosis (Waters *et al.,* 2004). Immunological assays for paratuberculosis diagnosis were developed to identify the infection in a herd earlier than with the culture test. Research efforts have been made to develop serological assays employing purified and species-specific antigens in order to identify subclinical paratuberculosis free of interference due to other mycobacterial infections (El-Zaatari *et al.,* 1997).

Commercial PTB-ELISAs have a supposedly high specificity as a consequence of the preadsorbtion step with *M. phlei* which is believed to remove most of the cross-reactive antibodies formed during infection with related environmental mycobacteria. Nevertheless, few studies have evaluated the anti-*M. bovis* antibodiy interference in those tests (El-Zaatari *et al.*, 1997, Olsen *et al.*, 2001).

Olsen *et al.* (2001) reported that the specificity of commercial assays for paratuberculosis may be very low and noted that commercial ELISAs may be unable to distinguish between PTB and TB. Those results are in agreement with the present study. We studied only naturally *M. bovis* infected animals and found that sera from these animals strongly reacted to several PPA antigen proteins. These findings lead to the supposition that some proteins of PPA are shared between MAP and *M. bovis,* which could confound the diagnosis of both mycobacterial diseases.

In the present study, twenty-five (26.6%) of the animals with tuberculosis were strongly reactive to PPA. All of these animals were confirmed as cases of tuberculosis based on typical lesions and the presence of acid-fast organisms; these findings also indicate that they were in an advanced phase of the disease. As in many other mycobacterial infections, it is expected that animals in advanced stages of tuberculosis produce antibodies against the infectious agent, *M. bovis.* Many researchers have demonstrated the presence of several proteins shared between mycobacterial species (Mutharia *et al.,* 1997; Olsen *et al.,* 2001; Waters *et al.,* 2004). Therefore, antibody cross-reaction to shared antigens with these sera was not an unexpected. The most likely reason for this finding is that commercial antigen-based diagnostic tests for PTB use as antigens a crude mixture of whole-cell proteins that may not be completely specific for MAP (Buddle *et al.*, 2003; Huntley *et al.*, 2005).

Sera of animals in different stages of PTB have been reported to react to a 34kDa protein (Gilot *et al.,* 1994)**,** while a 32kDa molecule was identified in MAP as well as in *M. bovis* that produced cross-reactive responses. (Amadori *et al.***,** 2002). We believe those proteins may be related to the 32-36kDa band observed on this study's Western Blot analysis.

In the present study, sera also reacted with a 42kDa component of PPA. This band was also identified as an intracellular molecule of MAP which cross-reacted with *M. bovis* and the *M. avium* complex (Harris & Barletta, 2001). Further studies are being conducted to obtain a better definition of the nature of the two bands between 77-90kDa.

CONCLUSION

Serologic tests are valuable tools for control and diagnosis of paratuberculosis and tuberculosis infection. Therefore, further studies using specific antigens to reliably differentiate between anti-*M. bovis* and anti-MAP antibodies are imperative for the control of the two diseases in developing countries where both infections may occur simultaneously.

ACKNOWLEDGEMENTS

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Isolation, PCR identification and real-time quantification of *M. paratuberculosis*

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ABSTRACT

Sensitivity and specificity are of principal concern when testing for MAP (MAP). Inhibitors or a surplus of DNA in a sample can be deleterious using PCR methods because sensitivity is reduced or perhaps false negative results may arise. If these difficulties were removed would detection of MAP be more frequent? If the total assay time is very short, and if the isolation and detection process could be automated, wouldn't that be welcomed? Our solution to these issues is using immunomagnetic separation (IMS) whereby ferromagnetic beads bind the bacteria within 30 minutes. During washing the bacteria are permeabilized and the nucleic acids reside on the beads. Further washing removes non-specifically bound inhibitors and finally PCR-grade DNA is eluted from the beads. A revolutionary asymmetric real-time PCR using sequence specific primers for the amplification of MAP sequences and an internal control with monochromal multiplexing at SYBR Green wavelength allows the quantification of MAP sequences and the detection of the internal control with economical real-time cyclers. If a real-time cycler is not available or higher throughput is needed, the amplicons of product and internal control can be detected with a hybridization detection kit as a real-time read-out with hybridization sensitive to as low as 0,3 fg of MAP-DNA. Switching from conventional PCR to real-time PCR is thus made easy as the same amplification protocol can be used on all cyclers.

Key words: *Mycobacterium avium* subsp. *paratuberculosis,* immunomagnetic separation, real-time PCR, monochromal multiplexing, milk, faeces

INTRODUCTION

Assay sensitivity and specificity are principal concerns in testing for MAP (MAP). In serving the life science community with high quality products for ultra sensitive applications, we decided to develop a PCR-based test for milk and faeces. Before starting we made a catalog of demands both from the academic and economic point of view. Sample preparation should incorporate almost all bacteria from 1-25 mL of whole milk and 1-5 g of faeces, while excluding nonspecific DNA and inhibitors of PCR. The assay should be simple to perform using affordable laboratory reagents, be capable of automation and permit effective recovery of named bacteria sequences. The PCR should be applicable in real-time mode and include hybridization detection. The PCR should be very sensitive, highly specific, fast, quantitative, easy to set up and robust. Moreover it should incorporate an internal control with monochromal multiplexing in order to use inexpensive real-time cyclers, a decontamination system and one setting for all cyclers. We describe the development of a new system for detection and quantification of MAP including sample preparation and PCR fulfilling these demands.

MATERIALS AND METHODS

Sample Preparation
Bacterial DNA
To avoid laborious disruption of MAP which has the tendency to grow in clumps, and to perform cell counting for comparison, we prepared the DNA of pure strains and used known amounts of bacterial DNA to optimize the PCR. For validation, the DNA from ten different isolates of bovine and human MAP strains (CIP 103964, 103967, 103968, 103971, 103972, 103973, 103974, 103975, 103976, 103977) and 18 non-

MAP bacteria (DSM 20662, DSM 20569, DSM 20617, DSM 20481, DSM 20069, ATCC 19433, NCTC 1803, ATCC 12228, NCTC 662 NCTC 10662, DSM 20630, NCTC 7464, NCTC 9001, NCTC 11994, ATCCC 14028, DSM 43999, DSM 44244, DSM 46621), abundant in bovine faeces and milk, were isolated using Nucleospin (Macherey-Nagel, Düren, Germany) according to the manufacturers protocol and quantified by fluorimetry.

Raw milk samples
Immunomagnetic bead separation was performed with the ParaStatus™ Isolation Kit (Aureon Biosystems, Vienna). Briefly, 1 mL of raw cow's milk and 10 μL of *M. avium* beads were pipetted into a 1,5 mL microcentrifuge tube and rotated at 10 rpm for 30 min at room temperature. The bacterial/bead complex was washed three times with Wash&Lysis Buffer, once with Wash Buffer II. The DNA was eluted with 40 μL Elution Buffer at 95°C, the beads removed and the residual neutralized with neutralization buffer. Alternatively 8 mL milk were centrifuged to pellet the mycobacteria, the pellet was resuspended in 200 μL PBS and DNA purified with High Pure PCR Template Preparation Kit (Roche, Basel) and analyzed with ParaStatus™ PCR Kit using 4 μL of eluate without BSA in a 20 μL reaction.

Pasteurized milk samples
Centrifugation of 25 mL of the sample resulted in a bacterial pellet bacteria, the supernatant was discarded and the pellet resuspended in 1 mL TPBS. This was transfered to a 1,5 mL Eppendorf tube. The remaining steps proceeded as described in raw milk sample preparation for immunomagnetic bead separation.

Bovine faecal samples
One g of faeces was resuspended in 10 mL TPBS. The diluted faecal mixture was vigorously shaken to produce a homogenous suspension. This suspension was applied to a stomacher filter bag, incubated for 5 minutes and 1 mL of the filtrate used in the ParaStatus™ Isolation Kit as described for milk.

PCR assay
Target DNA was amplified with ParaStatus™ PCR Kit which uses two sequence specific primers for MAP and two primers with target sequences for the internal control. Both the desired product and the product of internal control are quantified with SYBR Green which causes an increase in the fluorescent signal. The signal of the internal control is measured after the 45th cycle. After completion of the PCR a melt curve is performed on the amplicons producing melt peaks for the product and the internal control. Amplification of this IS*900*-based PCR was conducted in 20 μL and under the following conditions: 1 initial cycle of denaturation and activation at 95°C for 5 min., 65 cycles of denaturation at 95°C for 10 sec., annealing, extension and quantification at 67°C for 34 sec. on Light Cycler (Roche, Basel), iCycler (BioRad, Hercules) or Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City).

Highest sensitivity was achieved using 1 μL BSA (1mg/mL), 6 μL Amplification Mix, 10 μL of iQ™ SYBR Green Supermix (BioRad, Hercules) or SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City) or Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad) and 3 μL DNA preparation from milk or 1 μL DNA preparation from faeces with 2 μL water.

Detection
Using the ParaStatus™ PCR Kit the quantification of signal in real-time mode was performed during the annealing and extension cycle at 67°C at SYBR Green wavelength. Amplification performed on Biometra T-Gradient Thermoblock (Biometra, Göttingen) or on any of above mentioned real-time cyclers can be further analyzed with ParaStatus™ Detection Kit as the ParaStatus™ PCR Kit does contain fluorescein labeled primers allowing a post-PCR hybridization detection. After PCR the amplicon is diluted with 100 μL Wash&Hybridization Buffer and each 50 μL are applied to a microplate well coated with a probe for IS*900* PCR-product and to a well coated with a probe for the internal control product. After 15 min. the wells are washed, incubated each for 15 min. with 50 μL Anti-Fluorescein-HRP conjugate, washed and incubated for 15 min. with TMB substrate. After stopping the reaction with 50 μL 1N H_2SO_4, the absorbance is read at 405 nm with blank at 620 nm.

RESULTS

PCR assay
PCR sensitivity
We calculated from the published sequence (NC_002944, 4829781 bp. MAP str. k10, complete genome) that one MAP bacteria contains 5 fg DNA and 17 repetitive IS*900* elements. As can be seen from Fig. 1, ParaStatus[TM] PCR Kit was able to detect a single copy of the IS*900* element. Please be aware that this assay is run with monochromal multiplexing at the same SYBR Green wavelength for quantification and internal control and the internal control reaches threshold around the 45th cycle. Confirmation was done by running melt curves as shown in Fig. 2.
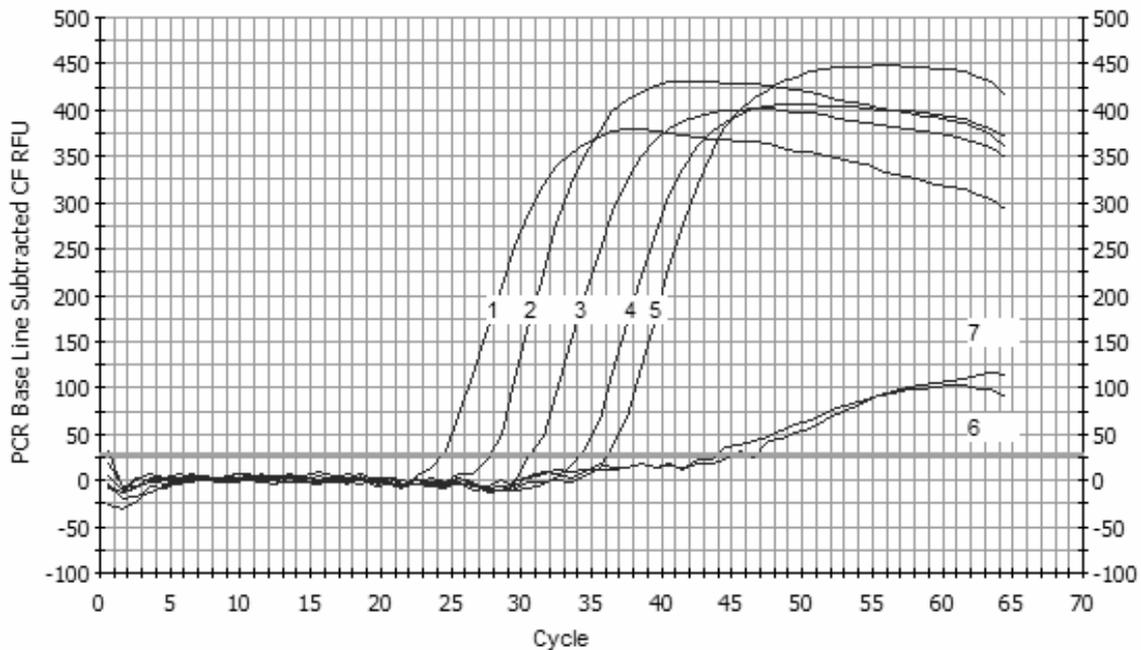
Fig. 1. PCR amplification of MAP DNA dilutions from CIP 103964 on iCycler.
Line 1, 2, 3, 4, 5, 6 correspond to DNA at an end concentration of 3 pg, 300 fg, 30 fg, 3 fg, 0.3 fg and 0.03 fg respectively. Line 7 is water as a negative control. The threshold cycles are at 24.3, 27.7, 30.5, 34.1 and 36.1 for positive samples as well as 43.7 and 46.6 for negative results, which is the amplification of the internal control. These data demonstrate that the PCR efficiency for positive samples is extremely high with this setting.
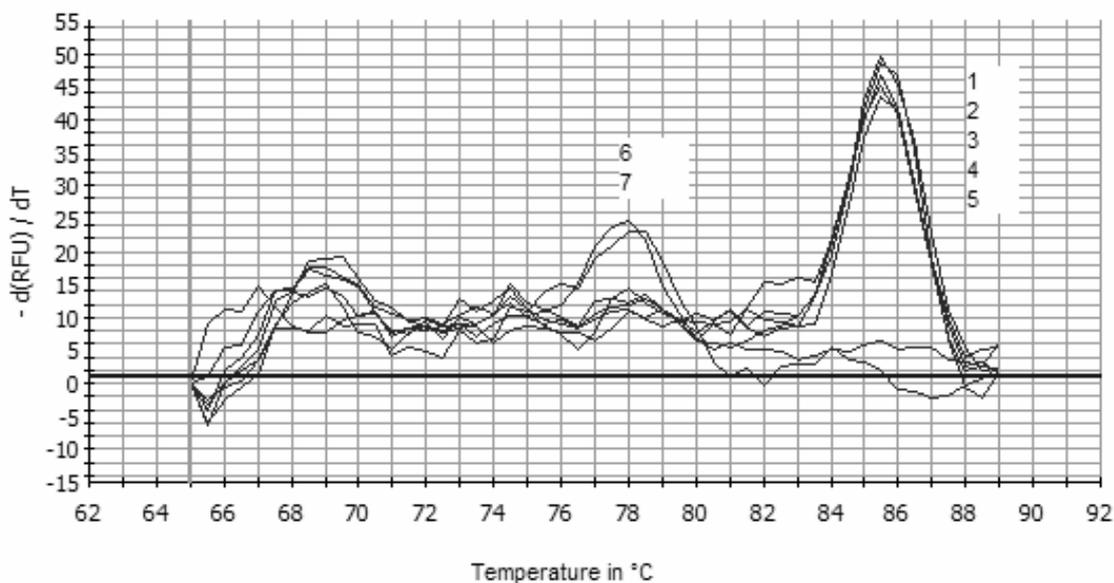
Fig. 2. Corresponding melt curves of amplification in Fig. 1 of products and internal controls on iCycler.

The melting temperature of 78°C for the internal control and 85.5°C for MAP product on the BioRad iCycler helps to confirm the product and ensures PCR integrity. The melting temperature for the internal control on Roche Light Cycler is 76.5°C, on Applied Biosystems 7500 Real-time PCR System 75.5°C. The melting temperature for the MAP product is 84.5°C on Roche Light Cycler and 83°C on Applied Biosystems 7500 Real-time PCR System (data not shown).

With these same settings the other MAP isolates were analyzed. All strains were clearly positive by real-time PCR and hybridization occurred at similar low detection levels as shown in Table 1. An optical density of > 0.05 is considered to give a positive result. As the PCR uses 65 cycles many reactions lead to primer depletion thus giving high signals in the hybridization test. If a positive sample's signal is very high, the amplification of the internal control is very weak and thus no internal control is detected.

Table 1. Optical density of hybridization test of IS*900* PCR of MAP strains amplicon MAP is the specific probe, IC is the internal control probe.

Bacteria	MAP	IC	Result
Negative Control	0.029	1.107	-
MAP # CIP 103964	>3.000	0.030	+
MAP # CIP 103967	2.903	0.026	+
MAP # CIP 103968	>3.000	0.023	+
MAP # CIP 103971	2.952	0.026	+
MAP # CIP 103972	2.935	0.034	+
MAP # CIP 103973	2.906	0.028	+
MAP # CIP 103974	2.822	0.032	+
MAP # CIP 103975	2.808	0.018	+
MAP # CIP 103976	2.783	0.027	+
MAP # CIP 103977	2.737	0.021	+

PCR specificity
Five ng of bacterial DNA isolated from non-MAP bacteria was analyzed with the PCR and showed no signal in real-time PCR except for the internal control. No greater amount of DNA was applied to the specificity test as from theoretical reasons it is not possible to isolate more than 5ng with the ParaStatus[TM] Isolation Kit. The hybridization test confirmed the absence of the MAP PCR amplification product and the integrity of the PCR by detecting internal control sequences as can be seen by the results from Table 2.

Table 2. Optical density of hybridization test of IS900 PCR of non-MAP strains amplicon MAP is specific probe, IC is probe for internal control

Non-MAP bacteria	MAP	IC	Result
DSM 20662	0.020	1.129	-
DSM 20569	0.018	1.197	-
DSM 20617	0.020	1.203	-
DSM 20481	0.015	1.141	-
DSM 20069	0.025	0.490	-
ATCC 19433	0.024	1.100	-
NCTC 1803	0.029	1.298	-
ATCC 12228	0.030	0.905	-
NCTC 662	0.029	1.022	-
NCTC 10662	0.022	0.795	-
DSM 20630	0.036	0.710	-
NCTC 7464	0.024	1.028	-
NCTC 9001	0.030	1.087	-
NCTC 11994	0.031	0.837	-
ATCC 14028	0.027	1.051	-
DSM 43999	0.029	1.079	-
DSM 44244	0.025	0.981	-
DSM 46621	0.020	0.732	-
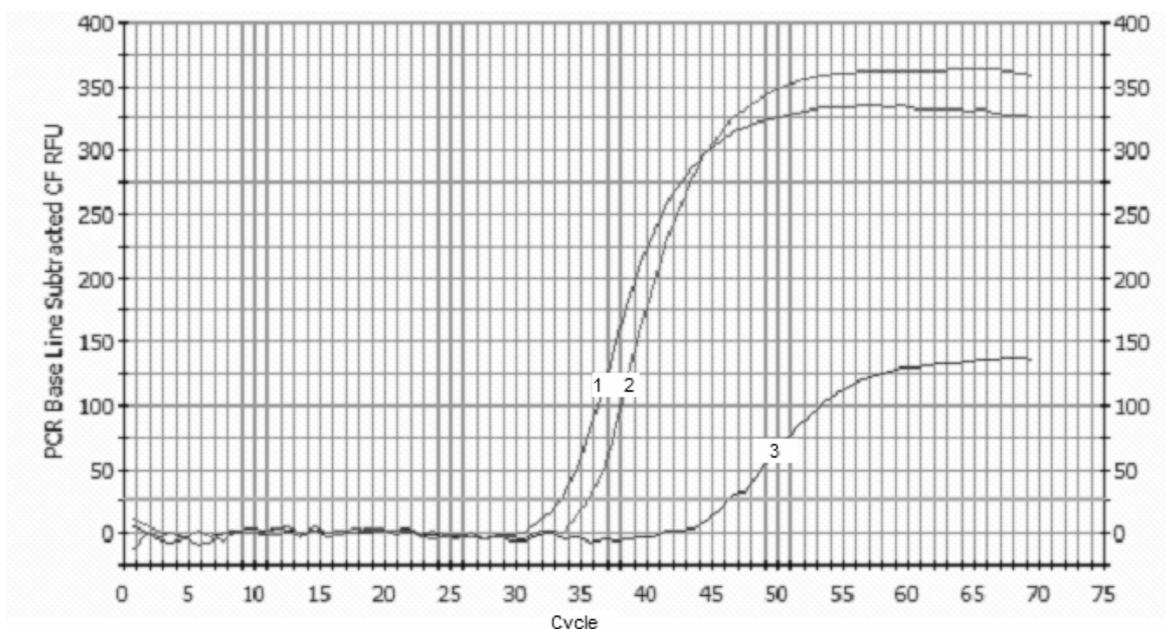
Fig. 3. PCR amplification of MAP-DNA from spiked raw milk on iCycler.
Line 1: 1 mL milk with ParaStatus™ Isolation Kit; Line 2: High Pure PCR Template Preparation Kit from 8 mL milk, Line 3: Water as negative control showing the internal control. The steep ascent of the signal confirms the high purity of the DNA.

Sample Preparation
Sensitivity
Twenty-six individual samples of raw milk were analyzed using ParaStatus™ Isolation Kit and ParaStatus™ PCR Kit. From these samples one was found to be positive. Twenty five mL of six samples of commercially available pasteurized milk were analyzed as described and two were found positive. Cow's faeces free of MAP as well as faeces spiked with MAP and faeces from a bull with Johne's disease were received from LaboVet (Vienna). All spiked samples and all samples from the bull were found to be PCR-positive. All other samples were negative for MAP with a positive internal control.

DISCUSSION

Milk and faeces are clinical specimens for paratuberculosis diagnosis. Both specimens are difficult to prepare for PCR due to excessive nonspecific DNA and PCR inhibitors. Therefore in many cases the DNA eluted from conventional preparation methods is of poor quality and the eluted material must be diluted in order to amplify the PCR. To overcome these problems immunomagnetic bead separation (IMS) methods with superparamagnetic beads have been developed (Khare et al. 2004, 0). We have developed a new class of magnetic particles covalently coupled with purified antibodies raised against epitopes on *M. avium* cell walls. These beads bind to the bacteria during the incubation step and are attracted by the magnet with a much higher force than superparamagnetic beads. Moreover, in many IMS methods the beads are washed with non-stringent buffers like PBS which do not remove inhibitors from the beads (Grant et al. 1998, 0). We have developed buffers which effectively remove inhibitors and are compatible with IMS thus leading to very high recovery of bacteria from the sample since during elution a form of "bead beating" is done.

The MAP PCR is an asymmetric real-time PCR system using specific primers for the amplification of MAP sequences. The amplicon size positively affects both the sensitivity and reliability of this PCR process. Short PCR products can be amplified more efficiently from difficult samples such as formalin fixed paraffin wax embedded samples (Ryan et al. 2002, 0). Monochromal multiplexing at SYBR Green wavelength allows the quantification of MAP sequences and the detection of the internal control to ensure integrity of the PCR process. In case a real-time PCR thermal cycler is not available or higher throughput is required, this PCR kit may be combined with the Aureon ParaStatus™ Detection Kit as the amplification protocol is equally suitable for real-time PCR or conventional PCR.

Many PCR methods have been developed for the detection and quantification of MAP sequences. In virtually all reported PCR amplifications unspecific products arise which lower the amplification yield and require hybridization confirmation either during or post PCR (Möbius et al. 2005, 0). We designed primers according to isothermal rules which result in very high annealing temperatures, thus excluding unspecific priming and leading to a two step PCR (Himmler et al.2000, 0). Besides these advantages, the design results in very short amplification products giving high yield and sensitivity in this sequence specific PCR which can be analyzed with SYBR Green. The design of a very short amplification product permits a post PCR melt curve to confirm the amplification products. The specificity of all primers and hybridization probes was validated by Blast search (BLASTN Version 2.2.10).

The internal control is efficiently reduced by using a very low primer and target concentration in order to keep the yield of the MAP product high to produce high sensitivity. This layout further makes monochromal multiplexing (Siraj et al. 2002, 0) at SYBR Green wavelength possible even in the quantitative mode as the internal controls reache threshold far after the amplification product of only one MAP-IS*900* copy.

If a real-time cycler available, the amplicons can be detected with specific hybridization for the MAP IS*900* sequence and internal control in a microplate format within one hour. As the PCR primers are partially fluoresceinated and supplied in asymmetric concentrations, hybridization is done without denaturation to minimize the number of steps in the simple protocol. The probes used on the microplate are short and therefore very specific even at room temperature hybridization.

The amplification mix is supplied in one vial and includes all primers and the internal control, thus making the setup of the reaction very easy and reducing possible handling errors during setting up the PCR. The protocol was adapted to be compatible with all real-time and non real-time cyclers. If needed, the amplification can be performed with dUTP and UNG to prevent contamination.

CONCLUSION

With the Aureon ParaStatus ^{TM} Sample Preparation Kit and the Aureon ParaStatus ^{TM} PCR Kit we were able to detect MAP in raw milk, pasteurized milk and bull's faeces making screening for MAP feasible and economic. Milk is very healthy food and should be free of MAP whether or not it is the causative agent for Crohn's disease to exclude any possible threat for the consumer.

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Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle and uninfected herd-mates

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ABSTRACT

The objectives of this study were: 1) determine the frequency with which uninfected and infected cows "switch" from positive to negative ELISA results when tested on multiple sequentially-obtained samples, 2) determine whether fecal culture or ELISA becomes positive first in infected cattle and 3) compare the change in ELISA OD values over time for infected and uninfected cattle to determine if a rise in OD value (but below the "cutoff") could provide an early indication of infection. Approximately 3,000 serum samples from 545 cows were analyzed using a commercially available ELISA. Cows had fecal and serum samples collected every 6 to 12 months. Of the infected cows, 38% had at least one ELISA positive serum sample. Of these, 50% were fecal culture-positive at least one test prior to becoming ELISA-positive, 38% were positive for the first time on fecal culture and serum ELISA concurrently, and 12% were ELISA-positive at least one test prior to being detected by fecal culture. Of the infected cows that remained in the herd after a positive ELISA result, 75% remained ELISA-positive on all subsequent ELISA tests. For cows that were seronegative, the difference between ELISA OD values on serial test dates was compared for infected and uninfected cows. There was no difference between groups, and detecting a rise in ELISA OD value below the cutoff was not useful in discriminating between infected and uninfected seronegative cows.

Key words: serology, mycobacterium, diagnosis, paratuberculosis

INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic enteric infection of cattle and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Most infected cattle do not exhibit the typical clinical signs of Johne's disease which include diarrhea, weight loss, and edema due to protein-losing enteropathy. Identification and removal of infected animals, along with management efforts to reduce transmission, are important aspects of many control programs. Testing of serum for antibodies to MAP using ELISA on a herd basis is recommended for identifying infected and uninfected premises, and as a screening test to identify possibly infected individual animals for additional testing.

When the sensitivity of ELISA tests has been investigated, the percent infected animals seropositive is strongly influenced by the stage of infection. Whereas 85% to 90% of animals with clinical signs of Johne's disease were seropositive by ELISA, 15% or fewer of subclinically infected cattle shedding few MAP organisms in feces were seropositive (Sweeney 1995).

This has led to the conclusion that early in the infection, MAP fecal shedding (albeit light), precedes detectable antibodies production, but as infection progresses to more advanced stages, and fecal shedding is heavier, antibody production begins. One objective of this study was to compare the time of onset of fecal MAP shedding relative to detectable antibody production in cows with multiple samples obtained sequentially over time.

When the sero-status of individual animals changes on repeated ELISA testing, producers and veterinarians may become frustrated and lose confidence in the laboratory or the diagnostic test results. In one study, 39.5% of cattle with a positive serum ELISA test result were test-negative on a subsequent test, but the infection status of these animals was not confirmed using organism detection methods. (Hirst 2002)

Reasons for a change in sero-status could include inter-laboratory, plate-to-plate and well-to-well variability in the assay, especially important when individual results have an O.D. value close to the critical cutoff value for results interpreted simply as positive / negative. (Collins 1993, Sweeney, 1995) In the aforementioned study (Hirst 2002), cows with ELISA results close to the cutoff were more likely to have a change in sero-status on subsequent tests. Another possible cause of change in ELISA status is variable concentration of antibody in the animals' serum, due to changes in production or loss of serum antibodies by the animal. The second objective of the study reported here was to determine the repeatability of ELISA results when cows were sampled on multiple sequential dates.

Although ELISA results are usually reported as "positive" or "negative", in one report, negative ELISA O.D. results that were just below the recommended cutoff point were 15 times more likely to come from infected cattle than uninfected cattle, based on likelihood ratios. (Collins 1993) In the current study, we chose to investigate the possibility that multiple sequentially obtained ELISA results from individual animals might provide more useful information than a result obtained from one sample at a single point in time. The objective was to compare changes in ELISA O.D. values over sequential samples for infected cows and uninfected herd-mates to determine if a rise in O.D. could allow earlier detection of infection.

MATERIALS AND METHODS

Serum samples were selected from archived specimens in our laboratory. Serum samples were collected once or twice annually from adult cattle in naturally infected dairy herds over a 10-year period. Shedding status was determined by culture for MAP on individual fecal samples obtained each time that serum samples were collected. Each serum sample was tested for antibodies to MAP by ELISA (HerdChek® *Mycobacterium paratuberculosis* antibody ELISA Kit, IDEXX Laboratories, Westbrook ME) according to manufacturer's recommendations. In this system, the patient's ELISA O.D. result is compared to that of a positive control, giving the S/P ratio. This ratio is compared to the cutoff of 0.25 to determine if the sample is "positive" or negative". Fecal culture results were then compared with ELISA results of infected animals to determine which test yielded a positive result earlier in the animal's life. Also, sequential ELISA results were examined to determine the frequency of "switching" from a positive result interpretation to negative. The change in S/P ratio among sequential tests (delta-S/P) was compared for seronegative infected cows and seronegative uninfected cows to determine if a rise in S/P ratio could predict infection status.

RESULTS

The study included a total of 2,953 serum samples from 282 infected cows and 263 uninfected cows. For the infected cows, 102 were never seropositive, 50 were fecal culture positive prior to becoming ELISA positive, 38 became positive on both tests simultaneously, and 12 were positive first on ELISA. Of the seropositive infected cows that were not culled and had subsequent ELISA testing performed, 24% subsequently had a negative ELISA result, but they were again ELISA positive on subsequent tests. Amongst uninfected cows with a positive ELISA result, 95% were ELISA-negative when retested.

Examination of delta-S/P revealed no difference between infected and uninfected cows. A rise in S/P ratio (but still below the cutoff of 0.25) could not accurately discriminate between infected and uninfected cows.

DISCUSSION

The results of this study confirm that most often, fecal shedding of MAP begins before antibodies are detected in serum by ELISA. Very few cows were ELISA-positive prior to becoming fecal shedders of MAP. This fits the paradigm that early during the infection, MAP is "controlled" by a cell mediated immune response, and that later in infection, as humoral immunity develops, the infection progresses and fecal shedding begins.

Overall, the number of seropositive cows that subsequently became seronegative in this study (42%) was similar to that of the previous study (39%).(Hirst 2002) The "switching" occurred much less frequently for infected cows than for uninfected cows. In the uninfected cows that "switched", there was usually only a single seropositive result, often just above the cutoff S/P value, and when those samples were tested using a different ELISA system, half of them were negative. On the other hand, the infected cows "switched" to negative much less frequently. When they did switch, they were usually negative only on one test, then reverted back to positive again. These negative serum samples, when retested on another ELISA system, remained negative. Thus the switch to seronegative status in those infected cows may have reflected actual antibody production fluctuations or loss (e.g., via colostrum) in the animals.

Prior to the start of the study, our hope was to show that infected cows would show a rise in S/P ratio from one sample to the next prior to becoming seropositive. This would permit earlier detection of infected cattle in herds where periodic testing is employed, by comparing current results with earlier results for individual animals. Unfortunately, the results of this study did not show any benefit to this practice. A rise in S/P ratio did not permit discrimination between infected and uninfected cows, beyond that achieved by examining a single ELISA result and comparing it to the cutoff of 0.25. It is possible that this "prodromal" rise in S/P ratio was missed in many cases, and more frequent testing might identify the rise in S/P that occurs as the S/P approaches the cutoff point. However, in most commercial systems, more frequent testing would not be practical.

The results of this study confirm that in most cases, fecal shedding of MAP by cattle precedes the development of antibodies detectable by ELISA. However, the fact that a small percentage of cases did become ELISA positive prior to fecal shedding illustrates that different cattle are detected by different test methods. The change in S/P ratio over time did not provide further discrimination between infected and uninfected cows beyond that already achieved by the use of a single sample result.

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Detection of *Mycobacterium avium* subsp. *paratuberculosis* in formalin- fixed, paraffin embedded tissues of goats by IS*900* PCR

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ABSTRACT

The objective of the present study was to optimise a DNA extraction procedure for formalin-fixed, paraffin embedded tissue sections for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by IS*900* specific polymerase chain reaction (PCR) in cases of naturally occurring paratuberculosis in goats. A total of 60 paraffin blocks (33 from small intestine and 27 from mesenteric lymph nodes) with various grades of histological lesions were used in the study. Three DNA extraction procedures were evaluated and the results analysed by PCR. The PCR carried out directly on the supernatant of tissue pellets disrupted in PBS by beating with zirconium/silica beads or on following DNA purified from the supernatant using a DNeasy kit (Qiagen) did not yield consistent results. The combination of bead beating rehydrated tissue pellets in the lysis buffer provided in the DNeasy kit, overnight proteinase K digestion and subsequent DNA extraction and ethanol precipitation gave consistent results and were adopted for all paraffin sections thereafter. The overall sensitivity of the PCR was 76% of all the samples that included various grades of lesions showing none or few to abundant acid-fast bacilli (AFB). The sensitivity of the assay was over 86% (38/44) in all paraffin sections having clearly and easily demonstrable bacilli. 50% of the tissue sections with rarely detectable AFB were also positive in the PCR implying a high quality of the DNA obtained by the procedure. There was no significant difference between the sensitivity of the PCR analyses carried out on intestinal vs. mesenteric lymph node tissues. The results of this study suggest that IS*900* PCR on formalin-fixed, paraffin embedded histological sections is a practical and important tool for confirming diagnosis of paratuberculosis in goats, where fresh tissues for bacterial culture or PCR are not available due to cold storage and shipment problems. The procedure also can be adopted on the archived tissues.

Key words: *Mycobacterium avium* subsp. *paratuberculosis,* goat, diagnosis, Johne's disease, histopathology, IS*900,* polymerase chain reaction.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Johne's disease), a chronic granulomatous infection of domestic and wild ruminants (Kreeger, 1991; Stevenson and Sharp, 1997; Tripathi et al., 2002). The disease is prevalent in most countries including India (Singh et al., 1996; Tripathi *et al.*, 1999; Rajukumar *et al.*, 2001) and causes significant economic losses to the cattle, sheep and goat industries. The organism has been implicated as a causal or exacerbating agent in human Crohn's disease (Chamberlin et al., 2001; Naser et al., 2002) and can infect other monogastric species (McClure et al., 1987; Grieg *et al.*, 1997; Beard et al., 2001). MAP shares more than 90% DNA homology with *M. avium* subsp. *avium* (Imaeda et al., 1988; Saxegaard and Baess,, 1988). The bacterium differs from other subspecies of the *M. avium* group by its dependence on mycobactin for growth in culture (De Voss *et al.*, 1999) and by the presence of multiple copies of the insertion sequences IS*900* and IS *Mav* 2 (Green et al., 1989; Strommenger et al., 2001).

A presumptive diagnosis of Johne's disease from necropsy materials is made on the basis of histopathology of intestinal and mesenteric lymph node (MLN) tissues showing granulomatous lesions and acid-fast bacilli (AFB). The definitive diagnosis, however, is achieved by isolation of mycobactin-dependent mycobacteria and subsequent confirmation by demonstration of specific genes of MAP (Green et al., 1989; Strommenger et al., 2001). Bacterial culture, though considered the 'gold standard', is very slow, time

consuming and labour intensive with poor sensitivity. Also bacterial culture from paucibacillary cases may be unrewarding. Radiometric culture method has reduced the incubation period without substantially improving the sensitivity and has specialized laboratory requirements (Whittington et al., 1999a).

Since routine specimens for histopathologic analysis are prepared in standard fixatives, fresh tissues are generally not available for isolation or molecular confirmation. Two methods are available for confirmation of infection with fixed tissue: immunohistochemistry (Plante et al., 1996) and in situ PCR (Sana et al., 2000). PCR is a useful tool that can be applied on a variety of biological specimens (Whittington et al., 1999b). Recently, the PCR assay has been used for direct detection of nucleic acid in DNA extracted from formalin-fixed paraffin sections in most mycobacterial infections of humans and animals including paratuberculosis (Plante et al., 1996; Miller et al., 1997, 2002; Whittington et al., 1999b, Fiallo et al., 1992; Coetsier et al., 2000; Mahaisavariya et al., 2005). Detection of the IS900 sequence has been mostly carried out from cases of Johne's disease in cattle and sheep and less frequently on goat samples. Various DNA extraction protocols from formalin-fixed, paraffin sections of paratuberculosis have been attempted in different laboratories with varied results (Plante et al., 1996; Miller et al., 1997, 2002; Whittington et al., 1999b).

The aims of this study were to optimize a procedure of DNA extraction from formalin-fixed, paraffin embedded tissue sections and to evaluate the efficacy of IS900 PCR for retrospective diagnosis and confirmation of MAP infection in paraffin blocks prepared from naturally occurring cases of paratuberculosis in goats.

MATERIALS AND METHODS

Paraffin blocks and preparation of sections
A total of 70 blocks of small intestine and mesenteric lymph nodes from naturally infected goats with different disease status and healthy uninfected goats were selected. Two four-micron sections were cut from each block and stained with haematoxylin and eosin (H&E) and by the Ziehl- Neelsen (ZN) techniques. In addition, 2x20 micron sections were cut from each block and transferred to a sterile eppendorf for DNA extraction. A separate disposable knife was used for each block and before sectioning, the microtome and the working table were thoroughly disinfected with 70% alcohol to prevent cross-contamination of samples.

H&E and ZN-stained sections were examined for the presence of characteristic histological lesions and the presence of AFB. The efficiency of the PCR test was assessed on 60 paraffin blocks (33 of small intestine and 27 mesenteric lymph node) from 34 animals with paratuberculosis and 10 additional blocks (5 each of small intestine and mesenteric lymph nodes) from healthy animals. Tissues were fixed in 10% buffered formal saline and allowed to fix for between 2 and 7 days (Luna, 1968). These paraffin blocks were 1-8 years old and maintained in the Goat Disease Laboratory of Division of Pathology at IVRI, Izatnagar.

Histopathology
In all cases, histologic lesions were arbitrarily categorized in 3 grades on the basis of severity and distribution of lesions and presence of acid-fast bacilli. Grade 1 lesions were characterised by focal macrophage granuloma and diffuse infiltration of lymphoid cells at times mixed with macrophage infiltration in the mucosa. In mesenteric lymph nodes (MLN), small focal granulomas were well delineated in the cortical areas and mainly composed of macrophages. AFB were observed occasionally or very rarely (score 0 to +) (Fig. 1). Grade 2 lesions consisted of multifocal epithelioid granulomas in the intestinal mucosa that also extended to the submucosal region in a number of cases. AFB were numerous mostly in singles and also in small and large clumps and were easily detectable in the lesions (score ++ to +++) (Fig. 2). The lesions in MLN were multiple, sometimes coalescing with each other with an AFB score + to +++. Grade 3 lesions were most severe and characterised by the presence of large epithelioid granulomas loaded with clusters of AFB, frequently filling entire villous and crypt areas in the mucosal regions (score +++) (Fig. 3). In MLN also, granulomatous lesions were diffuse with abundant AFB.
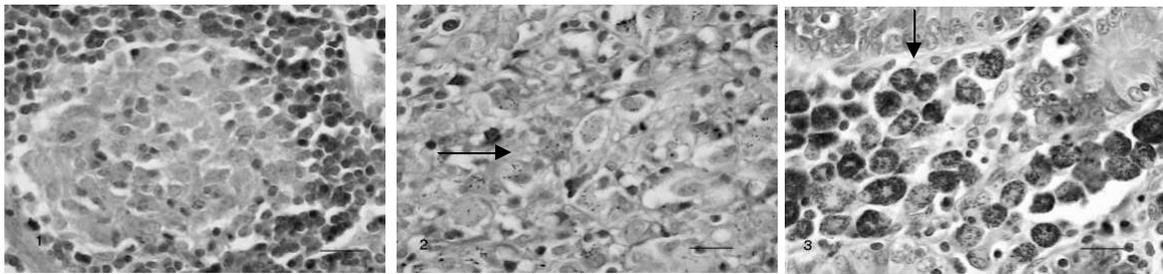
Fig 1. Mesenteric lymph node: focal granulomatous lesion without demonstrable acid-fast bacilli (score 0). Zeihl Neelsen's method, counterstain methylene blue. Bar= 40µ;
Fig. 2. Small intestine: granulomatous lesions showing numerous acid-fast bacilli in singles (arrow) (score ++). Ziehl Neelsen's method, counterstain methylene blue. Bar= 40µ;
Fig. 3. Small intestine: diffuse granulomatous lesions loaded with cluster of acid-fast bacilli (arrow) (score +++). Ziehl Neelsen's method, counterstain methylene blue. Bar= 40µ;

DNA extraction from paraffin sections
Removal of paraffin from tissue sections was achieved by the addition of 1 ml xylene. Sections were vortex mixed thoroughly and then kept for 10 min at room temperature (RT), followed by centrifugation at 13,000 x g for 10 min. This process was repeated once before extracting the pellet twice with absolute ethanol. After xylene and ethanol extraction, three procedures for DNA extraction were evaluated using five known positive samples to determine which procedure gave the best template for IS_900_ PCR.

Procedure 1. Pellets were dissolved in 100 µl of phosphate buffered saline (PBS) and transferred to a screw-capped microcentrifuge tube containing washed 0.1mm zirconium beads (Stratatech Scientific Ltd, UK) and 'ribolysed' (Hybaid Ribolyser, Hybaid, UK) at 5.5 meters per second for 2 cycles of 40 seconds. The PCR was carried out directly using 2 and 5 µl of the supernatant.

Procedure 2. The supernatant was transferred to a fresh eppendorf tube and the DNA was then extracted using the DNeasy Tissue kit (Qiagen, UK), following the manufacturer's instructions. 5 µl of the DNA was assayed by IS_900_ PCR as described previously.

Procedure 3. Tissue pellets were suspended in 180 µl of the ATL buffer of the DNeasy Kit and ribolysed with the condition described in the first procedure (frothing did not pose much problem in collecting ~ 180 µl of the supernatant if lids of the eppendorfs were tight). Supernatant was transferred to a fresh eppendorf and further processing was carried out as per instructions in the kit except for overnight digestion with proteinase K (PK). Eluted DNA in a final volume of 200 µl was further concentrated with 5M sodium acetate (1/10 volume) and 2.5 volumes of absolute ethanol in the presence of a DNA carrier (Mussel glycogen) and kept at -20^0C overnight. DNA was pelleted, washed with 70% ethanol, dried and finally resuspended in 25 µl of sterile distilled water. 5 µl of this DNA was used for PCR analysis.

DNA for the positive control in the PCR was taken from high quality plug DNA prepared from caprine isolates for pulsed-field gel electrophoresis analysis according to the procedure of Hughes _et al._ (2001).

PCR analysis
DNA extracted from all paraffin sections was analysed for the presence of IS_900_ sequences. PCR was carried out as previously described (Sanderson et al., 1992) with primers 90 (5'-GTT CGG GGC CGT CGC TTA GG) and 91 (5'- CCC ACG TGA CCT CGC CTC CA) amplifying a segment of 400 bp. The amplification was carried out in 50 µl reaction mixture containing 1X PCR buffer, 2.5mM MgCl2, 2.5 mM of dATP, dGTP, dCTP and dTTP, 1 U of Taq polymerase (Promega, UK), 5 µl of primer 90 (5uM) and primer 91 (5uM), and 5 µl of extracted DNA. The PCR conditions were 95^0C 5 min, 40 cycles of 95^0C-1 min, 55^0C 1 min, 72^0C 1 min and final extension at 72^0C 5 min. Positive and negative controls were included with PCR reactions. For PCR control, DNA template was replaced with sterile distilled water and amplified similarly. The size of the product was confirmed by conventional gel electrophoresis on a 1% agarose gel in 1 x TBE using 100 bp molecular size marker (Promega).

RESULTS

Three DNA extraction procedures were applied to paraffin sections of 5 confirmed cases of Johne's disease and known to contain rarely (2) to abundantly (3) detectable AFB. IS900-PCR analysis of DNA extracted using the first procedure detected three out of five positives. The results were not consistent with either of the two amounts of DNA template used. The positive results with 2 μl of template were not obtained with 5 μl template and vice-versa. None of the samples extracted using the second procedure was positive by IS900 PCR. The third procedure gave the best results with four out of five samples testing positive by IS900 PCR and was adopted subsequently for DNA extraction from all paraffin sections.

The results of histopathology with regard to severity of lesions and bacterial burden, and PCR are presented in Tables 1and 2 and Fig. 4. The sensitivity of PCR in grade 1 lesions with rare or no AFB of all intestinal and MLN sections was found to be 60%, for grade 2 lesions 81.8% and for grade 3 lesions it was 88.8%. The overall sensitivity of IS*900* PCR was found to be 76.7%, but when cases with easily detectable AFB were considered, the sensitivity rose to 86.4% (Table 2). There was no significant difference in the sensitivity of PCR between intestinal and MLN tissue sections. Six cases with multifocal and diffuse lesions containing abundant AFB were negative in the PCR analysis. When results of PCR on both tissues of 26 animals were considered separately, intestinal and MLN tissue PCR detected 76.9% and 80.7% animals, respectively. But, when either of the two tissue PCR results was considered, over 96% animals could be diagnosed as PCR-positive. None of the DNA samples from 10 blocks of healthy goats yielded positive PCR results.

Table 1. IS*900* PCR results according to grade and bacterial burden in formalin-fixed, paraffin sections from intestine and mesenteric lymph nodes of goats.

Case No	Small intestine			Mesenteric lymph node		
	Histological grading	AFB	PCR Results	Histological grading	AFB	PCR results
1	I	0	-ve	I	0	ND
2	I	+	+ve	II	+	+ve
3	I	+	+ve	I	0	+ve
4	I	0	+ve	I	0	-ve
5	I	0	+ve	II	+	+ve
6	I	0	-ve	I	0	-ve
7	I	0	+ve	I	0	-ve
8	I	0	+ve	II	+	+ve
9	I	0	-ve	I	+	+ve
10	I	+	-ve	I	+	+ve
11	I	0	+ve	II	+	-ve
12	II	+	+ve	ND	ND	ND
13	II	+++	+ve	I	0	-ve
14	II	+++	+ve	I	+	+ve
15	II	+++	-ve	II	++	+ve
16	II	++	+ve	II	++	+ve
17	II	+++	-ve	ND	ND	ND
18	II	++	-ve	ND	ND	ND
19	II	+++	+ve	I	++	+ve
20	II	+++	+ve	II	+++	+ve
21	II	+++	+ve	III	+++	+ve
22	II	+++	+ve	II	+	+ve
23	III	+++	+ve	II	++	+ve
24	III	+++	+ve	II	+++	+ve
25	III	+++	+ve	III	+++	+ve
26	III	+++	-ve	III	++	-ve
27	III	+++	+ve	III	+++	+ve
28	III	+++	+ve	III	+++	+ve
29	III	+++	-ve	III	+++	+ve
30	III	+++	+ve	ND	ND	ND
31	III	+++	+ve	II	+++	+ve
32	III	+++	+ve	ND	ND	ND
33	III	+++	+ve	ND	ND	ND
34	ND	ND	ND	III	+++	+ve

Table 2: PCR results on paraffin sections on intestine and mesenteric lymph node (MLN) tissue sections of animals according to bacterial burden

	Section with AFB	Section without or rare AFB	Section of all MLN	Section of all SI	All SI+MLN section
Number	44	16	27	33	60
PCR +ve	38	8	21	25	46
% Sensitivity	86.4%	50%	77.8%	75.8%	76.7%
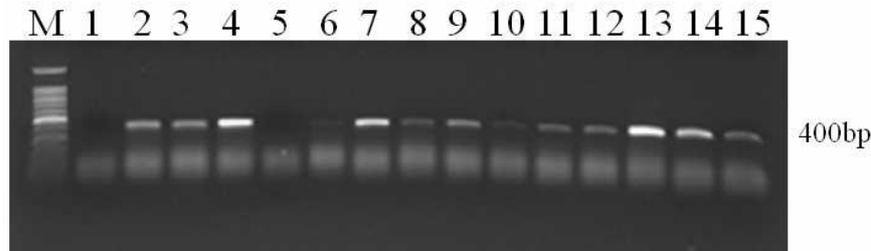
Fig. 4. IS*900* PCR on formalin-fixed, paraffin embedded tissue sections of small intestine and mesenteric lymph node from paratuberculous goats. Lane M-100bp ladder, Lane 1-negative control, Lanes 2-14 –test samples, Lane 15-positive control.

DISCUSSION

Out of three procedures tried, the first procedure yielded PCR positivity in 60% cases but the results were inconsistent, possibly due to the presence of inhibitors or concentration of target and host DNA present in the samples. Several methods such as boiling and freezing (Miller et al., 1997), PK digestion followed by DNA extraction with a kit (Cooper et al., 1997), freezing and thawing in buffer containing calcium chloride followed by PK digestion without further DNA purification (Plante et al., 1996) have been used for extraction of DNA from paraffin tissues. Whittington et al. (1999b) compared efficacy of these methods and reported that the method of Miller et al. (1997) to be the most simple and sensitive. We tried the simplest method of ribolysing xylene and ethanol extracted tissue pellets in PBS followed by PCR and obtained inconsistent results. There was no amplification of DNA template from any of 5 samples extracted by second procedure, most probably due to incomplete lysis of the bacteria by PK.

The third procedure involving ribolysation of tissue pellets directly in ATL buffer of Qiagen kit, followed by PK digestion overnight and concentration of DNA might have resulted in better quality and quantity of DNA for PCR assays. The advantage of this procedure is that it primarily uses commercial kit reagents except for final concentration, and hence uniformity in the results is expected among different laboratories.

The overall sensitivity of PCR was 76% of all samples examined from confirmed positive cases of Johne's disease with various grades of lesions showing rare or a few to abundant AFB. The sensitivity of the assay was over 86% in all the paraffin sections with multibacillary lesions and was comparable to sensitivities of 71-88% (Whittington et al., 1999b), 80% (Miller et al., 1997) and 90% (Plante et al., 1996) reported previously. It was evident from the study that sensitivity of PCR increased from grade 1 to grade 3 lesions suggesting that a positive correlation existed between the extent of lesions and total AFB burden, and PCR results. Whittington et al. (1999b) compared the efficacy of amplifying long (413 bp) and short (229 bp) fragments of the IS*900* gene in detecting MAP from the paraffin sections and reported higher sensitivity with short fragments (88%) in comparison to long fragments (71%). The fragment (400 bp) used in this study is comparable to the long fragment of Whittington et al., (1999b) but the sensitivity observed was comparable to their results amplifying a short fragment. This suggests that the quality of DNA obtained in this study may have been better.

The sensitivity of PCR involving sections showing no or rare AFB (paucibacillary lesions) was found to be 50%, far better than those of Whittington et al. (1999b) reporting only 12.5% sensitivity in 8 sheep exhibiting paratuberculous lesions without visible AFB. The increased sensitivity in our study is most likely due to two

20-micron sections used compared with two 5-micron sections used by Whittington et al. (1999b). It may be possible that detectable bacteria could have been present in a total of 40-micron section but not in 4 micron sections examined microscopically in ZN staining. Inconsistent PCR results have been reported between consecutive sets of sections prepared from the same block due to uneven distribution of AFB within lesions especially when the overall burden of bacteria was low (Whittington et al., 1999b). Coetsier et al. (200) observed 50% PCR positivity in paucibacillary tissues known to be infected with MAP, *M.bovis*, *M.avium* and *M.tuberculosis* using three 20-micron sections. Fiallo et al. (1992) used six 10-micron sections for PCR detection of important mycobacterial infections of humans and animals. The other reason for PCR positivity in tissue sections with rare or no AFB could be due to the presence of cell wall deficient forms or spheroplastic forms of MAP as IS*900* sequences have been demonstrated in biopsy materials and paraffin sections of intestines in Crohn's disease patients (Hulten et al., 2001; Sechi et al., 2004)

Though the PCR assay is capable of detecting a single copy genome of MAP, the sensitivity of IS*900* PCR on clinical samples has been reported to be equal or less than the culture method (Stevenson and Sharp, 1997). At least 6 cases showing abundant AFB (in singles and clumps) were negative by PCR. The sensitivity of PCR on formalin-fixed, paraffin embedded tissue sections was reported to be dependent upon the tissue type, copy of the genes and length of fragment to be amplified, concentration of target and host DNA, PCR inhibitors in extracted DNA, duration of fixation, type of fixation and age of paraffin blocks (Greer et al., 1991a, b; Marchetti et al., 1998; Karlsen et al., 1994). The most likely factors influencing the sensitivity of PCR in our study could be the prolonged duration of fixation in 10% formalin degrading MAP DNA or inhibitory effects of formalin and paraffin on Taq polymerase activity (Plante et al., 1996). The duration of formalin fixation of tissues used in the present study was not evaluated and the possibility of fixation after 3-4 weeks in some cases was not ruled out. Significant inhibitory effects of 10% formalin on PCR amplification of *M. leprae* in biopsy tissues have been observed when fixation time exceeded 24 hrs (Fiallo et al., 1992). They report the fixation of tissues for PCR confirmation of parartuberculosis infection in 50-70% ethanol could be an alternative when shipment of fresh tissues on ice is expensive.

There were at least 8 cases (mostly grade 1), where only one of the tissue sections yielded positive results suggesting the need for incorporating both intestinal and MLN tissues in the PCR protocol, especially from mild cases with low bacterial load. The specificity of PCR on paraffin embedded sections appears to be very high as none of the sections from healthy intestinal and MLN tissues were positive. A stringent protocol was adopted during preparation of sections from paraffin blocks and separate laboratory spaces for DNA extraction, PCR mix preparation and finally DNA template addition before amplification.

The results of this study suggest that the sensitivity of the IS*900* PCR on formalin-fixed, paraffin embedded histological sections from paratuberculosis cases in goats depends on the burden of AFB and the quality of DNA extracted. It could be used for retrospective diagnosis and epidemiology of paratuberculosis especially in a hot climate where keeping and shipping fresh tissues under cool conditions is difficult and tissues are routinely preserved in 10% formalin for histopathological diagnosis.

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Tissue PCR and its comparison with other tests for the diagnosis of natural paratuberculosis in goats

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ABSTRACT

An IS*900* polymerase chain reaction (PCR) for detecting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in tissues was developed and compared with the other tests in 41 goats naturally infected with paratuberculosis. On histological examination, paucibacillary (12) and multibacillary (29) granulomatous lesions were observed. The protocols for DNA isolation and PCR were standardized on caprine tissues. Positive PCR amplifications of 626 and 314 bp fragments of IS*900* gene were detected in 37 (90.2%) of 41 animals. The specificity of the PCR products were confirmed by restriction digestion with *Pvu*II enzyme and sequence analysis of cloned PCR products that showed more than 97% homology with the published sequence of IS*900* gene. A positive johnin test with varying skin thickness was observed in 25 (73.5%) of 34 animals. The agar gel immunodiffusion (AGID) test and absorbed-ELISA detected 29 (85.2%) and 32 (94.1%) of 34 animals, respectively. MAP was isolated in mycobactin J containing slopes in 35 (85.3%) of 41 goats. The results of the present study revealed that ELISA was most sensitive amongst the ante-mortem assays and tissue PCR was most sensitive amongst the post-mortem tests.

 Key words: goat, paratuberculosis, polymerase chain reaction, agar-gel immunodiffusion, enzyme-linked immunosorbant assay, histopathology

INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic bacterial disease of domestic and wild ruminants caused by a slow growing acid-fast bacterium called *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The clinical disease is in cattle is characterized by an intermittent and incurable diarrhoea, progressive weight loss and emaciation. Pathological manifestations include granulomatous enterocolitis, lymphadenitis and lymphangitis (Clarke, 1997). The disease is known to occur worldwide and causes considerable economic losses to the livestock industry. Efficient diagnostic methods for the detection of early and subclinical cases of paratuberculosis are not available, and control and eradication of the disease remains a daunting task since its first report more than a century ago.

The cell-mediated immune response elicited in the early stage of the infection is detected by the johnin skin test that is based on the delayed type hypersensitivity (DTH) reaction and the release of IFN- in blood (Jungersen et al., 2002; Kalis et al., 2003). Although bacterial culture is considered a 'gold standard' for the diagnosis of paratuberculosis, it is time consuming, expensive and less sensitive especially in subclinical cases in which numbers of viable bacteria are low or present in non-cultivable spheroplastic form (Chiodini et al., 1984; Manning and Collins, 2001). Serologic tests such as the enzyme-linked immunosorbant assay (ELISA), agar gel immunodiffusion (AGID) and complement fixation tests (CFT) offer rapid detection of antibodies to MAP in clinical cases, but they have varying degree of sensitivity and specificity, particularly in subclinical infection (Milner et al., 1990; Collins, 1996). The polymerase chain reaction (PCR) assay is a rapid and specific method for the detection of MAP in clinical materials (Vary et al., 1989). Identification of insertion sequences (IS*900* and IS*Mav2*) specific to MAP has facilitated the rapid diagnosis of MAP infection (Green et al., 1989; Strommenger et al., 2000).

Limited studies are available on the application of the PCR assays on the infected tissues from paratuberculosis-affected cattle (Bauerfiend et al., 1996), buffaloes (Sivakumar et al., 2005), sheep

(Gwozdz et al., 1997) and goats (Dei et al., 1995; Munjal et al., 2004). Also, the comparative efficacy of PCR with other clinical and postmortem tests is largely uninvestigated in the diagnosis of caprine paratuberculosis.

The objective of the present study was to develop a tissue PCR and compare it with other commonly used clinical and postmortem tests in the diagnosis of naturally occurring paratuberculosis in goats.

MATERIALS AND METHODS

Animals

A total of 41 adult goats of both sexes originating from a known MAP infected farm were used in this study. On the basis of regular clinical and faecal examination, goats diagnosed with paratuberculosis were separated from the herd and maintained in an animal shed. Of these, 34 animals were available for single intradermal johnin and serological testing and 7 others were brought for necropsy. The goat breeds were Black Bengal, Jamunapari breeds and their crosses. They showed clinical signs of soft pasty faeces with occasional diarrhoea and maintained a good appetite until the terminal stages of the disease. Most of them were in poor condition and some were emaciated at the time of necropsy.

Johnin test

All the available animals (n=34) were tested by a single intradermal injection of 0.1 ml of johnin PPD (Biological Products Division of the Institute) in the neck region. As per the instruction of the manufacturer, an increase of >2 mm in thickness of skin at the injected site after 72 hrs was recorded as positive for paratuberculosis infection.

Blood and tissue samples

Blood was collected from goats (n=34) at the jugular vein prior to intravenous pentothal euthanasia (Thiopental, Ranbaxy Inc, New Delhi, India). Sera was stored frozen at $-20^{O}C$. Gross lesions were recorded and representative tissue samples from small intestines (duodenum, jejunum, ileum and ileo-caecal valve), associated mesenteric lymph nodes (MLN), ileal and jejunal Peyer's patches, caecum, and colon were collected in 10% buffered neutral formalin for histopathology. Two adjacent portions of the distal ileum next to ileocecal valve and associated MLNs were collected for bacterial culture and PCR and were stored at $-20^{O}C$ until used.

Histopathology

The tissues preserved in 10% formalin were processed by conventional histological technique. Sections of 4μ thicknesses were prepared and stained with haematoxylin and eosin (H&E) and Ziehl-Neelsen (ZN) methods.

Tissue PCR

DNA was extracted from tissue samples of the 41 paratuberculous goats and 10 healthy goats (negative for paratuberculosis by histology and culture) by the method described by Sivakumar et al., (2005). Two sets of primers, BN1: 5' GTT ATT AAC GAC GCC CAG C-3' and BN2: 5' ACG ATG CTG TGT TGG GCG TTA G-3' primers flanking a region of 626 bp (Sivakumar et al., 2005), and BA5: 5' - CTG GCT ACC AAA CTC CCG A-3' and BA6: 5' – GAA CTC AGC GCC CAG GAT –3' primers flanking a region of 314bp (Bauerfiend et al., 1996) of IS900 sequence of MAP were used in the study. The PCR amplification and cycling conditions were similar to those described earlier (Sivakumar et al., 2005; Bauerfeind et al., 1996). The sensitivity or detection limit of PCR was determined by testing the DNA isolated from the intestinal and lymph node tissues spiked with MAP G-12A strain (caprine) and 10-fold serial dilution of purified DNA isolated from culture of the same strain. Positive and negative control and a PCR control (replacing DNA template with sterile distilled water) were always included in the amplification protocol. The size-confirmed specific PCR products were cloned in pDrive vector (Qiagen, Hilden, Germany) and transformed into DH$_5$ strain of *E.coli* as described previously (Sambrook and Russel, 2001). The recombinant clones were selected and sequenced in an automated nucleotide sequencer. The sequences were analysed with the published sequence of IS*900* (Accession No: X16293).

Bacterial culture
Faecal (n=34) and tissue (n=41) samples were processed for bacterial culture as described previously (Tripathi et al., 1999; Rajukumar et al., 2001). Positive colonies were identified on mycobactin J slopes as small, round white colonies and they were confirmed by IS*900* PCR.

Agar gel immunodiffusion test (AGID)
AGID was performed as described previously (Sherman and Gezon, 1980). The appearance of a clear precipitation line was recorded as positive and a hazy line as weakly positive. Absence of any line was recorded as negative.

Absorbed ELISA
Protoplasmic antigen (capture antigen) from MAP 316F strain and adsorbing antigen from *M.phlei* were prepared as described previously (Rajukumar et al., 2001). The ELISA procedure and the determination of positive results were adapted from Rajukumar et al. (2001).

Table 1. Diagnostic test results.

Case	Johnin	AGID	ELISA	HP	ZN	TC	FC	PCR
1	+	+	+	+	MB	+	+	+
2	+	−	+	+	PB	+	+	+
3	+	+	+	+	MB	+	+	+
4	+	−	+	+	PB	−	−	−
5	+	+	+	+	MB	+	−	+
6	+	+	+	+	MB	+	−	+
7	−	+	+	+	MB	+	−	+
8	−	+	+	+	MB	+	+	+
9	−	+	+	+	MB	+	+	+
10	+	+	+	+	MB	+	+	+
11	+	+	+	+	MB	+	+	+
12	+	+	+	+	MB	+	+	+
13	+	+	+	+	MB	+	+	+
14	+	+	+	+	MB	+	+	+
15	−	+	+	+	MB	+	+	+
16	+	+	+	+	MB	+	+	+
17	+	+	+	+	MB	+	+	+
18	+	+	+	+	MB	+	+	+
19	−	+	+	+	MB	+	+	+
20	+	+	+	+	MB	+	+	+
21	+	+	+	+	PB	+	+	+
22	+	+	+	+	MB	+	+	+
23	+	−	−	+	PB	−	−	−
24	−	−	−	+	PB	−	−	−
25	+	+	+	+	MB	+	+	+
26	−	+	+	+	MB	+	+	+
27	+	+	+	+	PB	+	+	+
28	+	+	+	+	PB	+	+	+
29	+	+	+	+	MB	+	−	+
30	−	+	+	+	MB	+	+	+
31	−	+	+	+	MB	+	+	+
32	+	+	+	+	MB	+	+	+
33	+	−	+	+	PB	−	−	−
34	+	+	+	+	PB	+	−	+
35	ND	ND	ND	+	MB	+	ND	+
36	ND	ND	ND	+	MB	+	ND	+
37	ND	ND	ND	+	PB	+	ND	+
38	ND	ND	ND	+	PB	−	ND	+
39	ND	ND	ND	+	PB	−	ND	+
40	ND	ND	ND	+	MB	+	ND	+
41	ND	ND	ND	+	MB	+	ND	+

HP= Histopathology, ZN= demonstration of acid-fast bacilli, TC= tissue culture, FC= Faecal culture, MB= Multibacillary, PB=Paucibacillary,

Statistical analysis
The sensitivities of various tests in MB and PB cases were analyzed statistically using Chi square test (Snedecor and Cochran, 1989).

RESULTS

The summary of results of histology, johnin test, AGID, ELISA, culture and PCR are presented in Table 1. The results of histologic findings were used as the basis for calculating the sensitivity of all other tests employed in this study (Table 2).

Table 2. Comparative efficacies of different tests

Tests employed	Overall sensitivity (%)	MB Sensitivity (%)	PB Sensitivity(%)
Tissue PCR	90.2 (37/41)	100 (29/29)*	66.6 (8/12)
Tissue culture	85.3 (35/41)	100 (29/29)*	50.0 (6/12)
Faecal culture	73.5 (25/34)	84 (21/25)*	44.4 (4/9)
Johnin test	73.5 (25/34)	68 (17/25)	88.8 (8/9)
Absorbed-ELISA	94.1 (32/34)	100 (25/25)*	77.7 (7/9)
AGID	85.2 (29/34)	96 (24/25)*	55.5 (5/9)

The values in parenthesis indicate the number of animals positive upon number of animals tested; * significantly higher in comparison to paucibacillary cases at 5% level of significance.

All 41 goats were found to have lesions consistent with paratuberculosis. Lesions were divided into paucibacillary (PB) and multibacillary (MB). The ileoceacal valve from all these had granulomatous lesions with AFB. The PB lesions were observed in 12 goats devoid of any clinical signs except for slight reduction in the body weight per the history collected from the farm manager. Grossly, small intestines showed thickening and occasionally mucosal corrugations and enlargement of the MLNs. Histological lesions consisted of epithelioid granulomas and diffuse infiltration with lymphocytes in the intestine and in MLN (Fig.1) with few acid-fast bacilli (AFB). The remaining 29 goats had obvious clinical signs that consisted of loose and pasty faeces, progressive loss in the body weight, and emaciated body condition. The necropsy changes consisted of thin subcutaneous tissues, gelatinized mesenteric fat, and cording and knotting of lymphatics. Small intestines had a distended lumen in most cases and mucosae were thickened and corrugated. MLNs were enlarged several times and had agelatinous capsules. The cortex revealed grayish white areas and was not distinctly demarcated from the medulla. On histological examination, the intestines and MLNs had typical granulomatous inflammatory changes consisting of multifocal epithelioid granulomas (Fig.2) or large sheet of epithelioid cells loaded with large number of AFB (Fig.3).
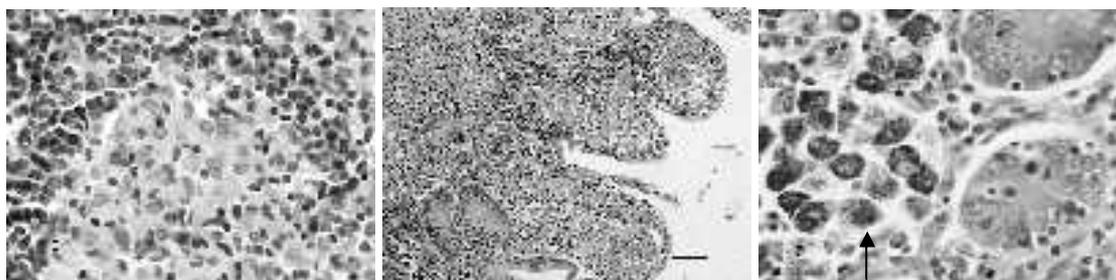
Fig.1. Mesenteric lymph node: granulomatous lesions in the paracortex. H&E. Bar= 40µ. **Fig.2.** Small intestine: diffuse granulomatous lesions showing numerous epithelioid macrophages. H&E. Bar= 40µ. **Fig. 3.** Small intestine: diffuse granulomatous lesions loaded with cluster of acid-fast bacilli (arrow). ZN method. Bar= 40µ.

The analytical sensitivity of tissue PCR had previously been found to be 230 bacteria/g spiked tissue and 1.8×10^{-13} g DNA isolated from pure culture of MAP. Tissue samples from 37 of 41 (90.2%) goats were positive in the PCR using both sets of primers (Fig.4 and 5). All 10 healthy goats were negative in PCR. The PCR products with BN1 and BN2 primers, following digestion with *Pvu*I enzyme yielded two DNA bands, one at 414 bp and other 212 bp (Fig.6). Cloning and sequencing of the PCR products (626bp) revealed more than 98% homology with the published sequences of IS*900* (Green et al., 1989).
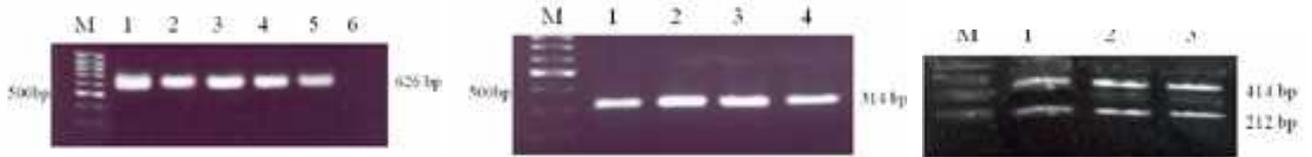
Fig.4. Agarose gel electrophoresis of PCR products amplified using BN1 and BN2 primers. Lane M: 100bp DNA molecular marker, Lane 1: Positive control, Lanes 2-5: tissue samples, Lane 6: Negative control.
Fig.5. Agarose gel electrophoresis of PCR products amplified using BA5 and BA6 primers. Lane M: 100bp DNA molecular marker, Lane 1: Positive control, Lanes 2-4: tissue samples.
Fig.6. PCR products of BN1 and BN2 primers digested with restriction enzyme PvuII. Lane M: 100bp DNA molecular marker, Lane 1-3: PCR products digested with PvuII enzyme

MAP was isolated on mycobactin J slopes from 35 of 41 (85.3%) tissues and 25 of 34 (73.5%) faecal samples. About 50% of PB cases were negative for bacterial isolation. Of 25 (73.5%) goats positive in the johnin test, there was an apparent difference in severity of the DTH reaction between PB and MB cases. Most PB goats showed a stronger DTH reaction as evidenced by increased skin thickness of more than 4 mm. While all PB cases showed positive DTH reaction, 5 MB cases were negative in the DTH reactions. The AGID test was positive in 29 of 34 (85.2%) goats. Of the 5 AGID negative goats, 4 were PB and only one was MB. The ELISA was positive in 32 of 34 (94.1%) goats tested, which also included 7 PB cases.

DISCUSSION

Efficacy of tissue PCR for the diagnosis of paratuberculosis in goats has been rarely studied (Dei et al., 1995; Munjal et al., 2004). In the present study, a DNA extraction procedure and PCR cycling conditions were optimised for IS900 PCR specific detection of MAP genetic material in caprine tissues that had histologic lesions of paratuberculosis. The PCR assay was compared with bacterial culture and commonly used immunological tests such as johnin test, AGID and ELISA.

All 41 goats had characteristic gross and histological lesions of paratuberculosis as described previously (Thomas, 1983; Corpa et al., 2001). The PB and MB lesions observed in the study were akin to "mixed lesions" and "severe lesions", respectively, reported by Corpa et al. (2001).

In the present study, approximately 90% of goats were positive in the PCR assay. Few studies have demonstrated MAP DNA in tissues from spontaneously occurring paratuberculosis in goats. Munjal et al. (2004) detected IS900 sequences in MLN tissues of 10% of 277 goats and considered the test as a 'gold standard' for assessing the efficacy of a lipoarabinomannon (LAM) ELISA. The results of the present study corroborated well with those reported previously on naturally (Gwozdz et al., 1997) and experimentally infected sheep (Perez et al., 1995). The sensitivity of IS900 PCR has already been reported to be higher on infected tissues than on other clinical samples (Perez et al., 1995; Stevenson and Sharp, 1997). In the present study, in addition to all MB cases, 8 (66%) PB cases were also detected positive in the PCR suggesting that while tissue PCR was almost 100% sensitive in clinical and MB cases (Gwozdz et al., 1997), it can also detect PB cases as reported previously in buffalo (Sivakumar et al., 2005). The negative PCR results in 4 PB cases could possibly be due to low number of bacteria present in tissues i.e. below the detection limit (230 bacilli/g) of the procedure used in the study.

The presence of multiple copies of IS900 gene sequence and its specificity were the criteria for its extensive use in the diagnosis of paratuberculosis infection in animals (Green et al, 1989; Vary et al., 1990). However, the report of IS900-like sequences in other mycobacterial species (Englund et al., 2004)) necessitates further confirmation of the amplified products. In this study, the specificity of PCR products was confirmed by restriction enzyme and sequence analyses, and PCR using another set of primers (BA5 and BA6) as reported previously (Sivakumar et al., 2005). None of the tissues from healthy goats were positive in PCR suggesting high specificity of the test. However, inclusion of a confirmation step for amplified products (such as RE digestion, Southern blotting or sequencing of PCR products) in the PCR protocol for the diagnosis of paratuberculosis would only increase the cost of the test, and hence may not

be adopted routinely. A single step PCR targeting multiple genes specific for MAP could be a better alternative (Rajeev et al., 2005).

Most PB cases were positive in the johnin test suggesting strong CMI responses as described previously (Chiodini et al., 1984; Collins, 1996; Clarke, 1997). The DTH positivity in 17 MB cases showing mild to severe reactions and also positive in serological tests suggested that CMI and humoral immune responses were elicited concurrently in goats for quite some time before CMI waned and became undetectable by the johnin test. This was confirmed in the present study as 32% (8/25) of MB cases were negative in the johnin test indicating a reduction in the CMI response as the infection progressed to an advanced stage. Various criteria have been used for positive interpretation of the results of the johnin test. This includes ≥ 2 mm, $\geq$ 5mm and double fold increase in the skin thickness. In the present study ≥ 2 mm increase in the thickness was considered positive (OIE, 2004), which could explain the higher sensitivity of the johnin test in comparison to an earlier report (Paliwal et al., 1984).

In the present study, bacterial cultures from tissues identified 85% and from faeces 73.5% of the infected animals in accordance with the findings of earlier studies that identified 76-86% of the goats with clinical disease (Gezon et al., 1988; Thomas, 1983). The sensitivity of the bacterial culture was almost 100% in MB cases, but it was about 50% in PB cases. This suggested that the number of bacilli in the clinical samples influenced the results of bacterial culture (Collins, 1996; Manning and Collins, 2001).

The overall sensitivity of AGID was found to be 85.2%, better than what was (51-72.5%) reported earlier (Sherman and Gezon, 1980; Thomas, 1983; Gezon et al., 1988; Dimareli-Malli et al., 1992; Garcia Marin et al., 1992). This was because the study involved mostly clinical cases. The higher sensitivity of the test in MB cases as also observed by Garcia-Marin et al. (1992) supports the importance of the test in the clinical confirmation of paratuberculosis in goats (Sherman and Gezon, 1980; Thomas, 1983). The specificity of the AGID test is almost 100%, and it is as reliable as the bacterial culture in making the confirmatory diagnosis (Collins, 1996: Sherman and Gezon, 1980). All culture positive animals, except for one, were positive in the AGID test in this study suggesting that MB cases are rarely misdiagnosed by the AGID test. On the other hand, negative results in AGID should not be used for declaring the animals free from MAP infection since the test has lower sensitivity (Sherman and Gezon, 1980; Thomas, 1983).

The ELISA in goats has been less extensively evaluated than with cattle and sheep. Sensitivities from 54 to 90% have been reported for absorbed-ELISA in goats depending on levels of infection in herds (Garcia-Marin et al., 1992; Burnside and Rowley, 1994; Rajukumar et al, 2001; Dimareli-Malli et al, 2003). In the present study, a 94% sensitivity level was obtained for an absorbed-ELISA on histologically confirmed cases, emphasizing its value in the diagnosis of paratuberculosis in goats. High reliability can be placed on ELISA results in animals from endemically infected farms with MAP (Collins, 1996).

CONCLUSION

The tissue PCR, bacterial culture and serological tests showed higher sensitivities in multibacillary goats in comparison to paucibacillary goats. Among all the tests employed in the present study, the absorbed-ELISA as an ante-mortem test and the tissue PCR as post-mortem test show high sensitivity and may be adopted for the diagnosis of clinical paratuberculosis in goats.

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Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets

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ABSTRACT

We here describe the identification and characterization of three novel secreted *M. avium* subsp. *paratuberculosis* (MAP) antigens of 9 kDa, 15 kDa and 34 kDa (MAP2609, MAP2942c and MAP2942c resp.) by screening a genomic expression library with serum from a naturally infected clinical cow. The 9, 15 and 34 kDa antigens display strong homology to previously described *M. tuberculosis* antigens, TB 8.4, MPT53 and Erp, respectively. Furthermore these antigens were shown to be recognized by antibodies in subclinical infected cattle.

INTRODUCTION

M. avium subsp. *paratuberculosis* is the causative agent of paratuberculosis, or Johne's disease, a chronic enteritis in ruminants currently responsible for substantial worldwide economic losses for farmers and the dairy industry. The bacterium is believed to infect ruminants very early on in life, but is persistent for several years before the onset of clinical symptoms. Management and eradication programs to control the infection are severely hampered by the lack of a sensitive (and specific) test capable of detecting early host exposure to MAP. Current immuno-diagnostics are based on crude antigen mixtures, and a more detailed dissection of the antigenic make-up of MAP will be necessary to develop improved tests. A whole range of new antigens has in recent years been identified and characterised. These include the cell wall derived glycolipid lipoarabinomannan (Jark, Ringena *et al.* 1997), and protein antigens with molecular weights of 12 kDa (Cobb and Frothingham 1999), 14 kDa (Olsen, Tryland *et al.* 2001), 18 kDa (bacterioferritin; (Elsaghier, Prantera *et al.* 1992)) 19 kDa (AhpD;(Olsen, Reitan *et al.* 2000)), 23 kDa (superoxide dismutase; (Mullerad, Hovav *et al.* 2002)), 24 kD (p24BCD; (Elsaghier, Prantera *et al.* 1992)), 30 kD (P30; (Burrells, Inglis *et al.* 1995)), 34 kD (Gilot, De Kesel *et al.* 1993), 35 kD (El-Zaatari, Naser *et al.* 1997), 42 kDa (White, Whipple *et al.* 1994), 44.3 kDa (Mutharia, Moreno *et al.* 1997), 45 kD (AhpC; (Olsen, Reitan *et al.* 2000)), 65 kD (Hsp65; (el-Zaatari, Naser *et al.* 1995)), 70 kD (Hsp70; (Stevenson, Inglis *et al.* 1991)). Some of these antigens are now being evaluated for the development of new, more sensitive diagnostics for paratuberculosis.

Instead of the frequently used antibodies from immunised mice, we have here used antibodies derived from a naturally infected cow to identify antigens for development of a more sensitive diagnostic test. By screening a genomic expression library we identified three secreted antigens with a molecular weight of approximately 9, 15 and 34 kDa. Characterisation of these antigens revealed that these are different from the antigens identified by previous approaches. Recombinant purified forms of the three antigens were shown to be broadly recognized by antibodies in infected subclinical ruminants, indicating that these antigens may represent important targets for the immune response generated during a natural infection in ruminants.

MATERIALS AND METHODS

Genomic expression library
A genomic expression library was constructed using the λTripleEx™ expression vector according to the Clontech manual (PT3003-1) and Stratagene Gigapack® III Gold Packaging manual. Genomic DNA was purified from 4 weeks old cultures of Watson Reid broth containing 2 mg/L mycobactin P using the CTAB/NaCl method, essentially as described by Ausubel (Ausubel 1992). Genomic DNA isolated from MAP strain 316F was partially digested with *Tsp*509I and fragments with an average size of 1.5 kbp, obtained by sucrose gradient centrifugation, were ligated into *Eco*RI-digested, dephosphorylated λTriplEx arms. The packaging reaction was carried out using Gigapack III Gold Packaging Extract and host strain *E.coli* XL1Blue (Clontech (S0924)). The genomic library was shown to contain 1.2 x 10e6 individual phages. Amplification in *E.coli* XL1Blue resulted in a phage titer of 3.5 x 10e9 PFU, which contained 80 % recombinants with an average insert size of 1.5 kb. Statistical analysis (Clarke and Carbon 1976) showed this library to be representative (>99.9%) of the MAP genome.

Serum samples
The genomic expression library was screened using a serum sample (3869) taken from a cow naturally infected with MAP. Infection of the cow was confirmed by culture positive faeces samples in two previous years. The cow and the herd of origin had no history of bovine tuberculosis, avoiding potential cross-reactivity. Serum from cow 3869 was adsorbed according to El-Zaatari (El-Zaatari, Naser *et al.* 1997) using *E. coli* strain XL1-blue. The efficiency of adsorption was evaluated by immunoblotting against a MAP sonicate and *E.coli* lysate*.*

Immunoscreening and characterization of positive clones
Screening of the λTripleEx™ expression library using adsorbed serum sample 3869 at a 1:50 dilution, and conversion of positive λTripleEx™ recombinants to pTripleEx™ clones was carried out according to the Clontech manual (PT3003-1).

Overexpression and purification of recombinant antigens
Mature forms of the 9, 15 and 34 kDa antigens were expressed as poly-histidine tagged fusion proteins in *E.coli* XL1Blue using the vector pQE80 (Qiagen). Standard PCR amplification was carried out based on genomic DNA from MAP strain B854 to amplify the DNA fragments encoding the mature form of the 9, 15 and 34 kDa antigens. Oligo nucleotide forward-primer 5'-CGC GGA TCC GAC CCC ATG GAC GCG ATC-3' at carrying a *Bam*HI restriction site and reversed primer 5'-CGC AAG CTT GAA GTT GTT ACA GGT GAC CG-3' carrying a *Hin*dIII restriction site were used to amplify a DNA fragment encoding mature 9 kDa antigen, oligo nucleotide forward-primer 5'-GCC GGA TCC GAC CGC CTG CAA TTC ACC GC-3' carrying restriction site *Bam*HI and reversed primer 5'-GGT AAG CTT CAG CGC AGC GCC GCC ACC C-3' carrying restriction site *Hin*dIII were used to amplify a DNA region encoding mature 15 kDa antigen, oligo nucleotide forward-primer 5'-GCC GCC ACC ATG ACC GAC GAC CGC CTG CAA TT-3' carrying restriction site *Bam*HI and reversed primer 5'-GCG CAG CGC CGC CAC CCG GG-3' carrying restriction site *Hin*dIII were used to amplify a DNA fragment encoding mature 34 kDa. The amplified DNA fragments were cloned into the vector pCR4-TOPO (Invitrogen) according to the manufacturer's instruction manual, and the sequences of inserted DNA fragments encoding mature 9, 15 and 34 antigenic polypeptide were subsequently confirmed by DNA sequencing as being identical to those determined for the original recombinants that were derived from MAP 316F genomic DNA. All three DNA fragments were subcloned using the corresponding restriction sites of expression vector pQE80 and transformed to *E.coli* XL1Blue according to standard procedures. Expression in *E.coli* XL1blue was induced by IPTG as described by the manufacture (Qiagen, "The QIAexpressionist"; fourth edition- January 2000). By a similar strategy, as described above we selected 65 kDa (Hsp65) and 70 kDa (Hsp70) antigens from our MAP expression library using monoclonal antibodies (P.Willemsen and J.Thole, data not shown). For comparison with the immunological recognition of the 9, 15 and 34 kD antigens, these two well-known MAP antigens were likewise expressed and purified as recombinant histidine-tagged proteins. In addition, we expressed and purified MPB83, a secreted *M.bovis*-specific protein as the control antigen in our immunological analysis.

RESULTS

Approximately 10^6 individual phage plaques from the amplified λTriplEx™ expression library were screened using serum 3869. Eighty six positive phage recombinants were identified, purified, converted to plasmid pTriplEx™ recombinants and subjected to analysis by DNA sequencing. Sequence analysis of pTriplEx™ clones was performed by cycle sequencing (BigDye™ Terminator Cycle Sequencing v2.0, PE Biosystems, Foster City, CA) on an ABI Prism 3700 DNA analyzer (Greenomics, Wageningen, The Netherlands) using the 5' sequencing primer 5'-TCCGAGATCTGGACGAGC-3' as indicated in the Clontech manual (PT3003-1). Sequence data were compared with genomic sequence data of strain MAP K-10 (http://www.cbc.umn.edu/ResearchProjects/AGAC/Mptb/Mptbhome.html) and strain *M. avium subsp. avium* 104 (http://www.tigr.com): The sequence analysis revealed that the majority of these recombinants (55), represented 3 distinctive open reading frames, each containing part of a signal sequence, and a mature protein with a molecular weights of approximately 9, 15 and 34 kDa, respectively. To determine the full length of these genes, the region 5 ' to sequences carried by the pTriplEx™ recombinants were characterised from DNA fragments amplified by reversed oligo-nucleotides using the genomic DNA from MAP316F.

9 kDa antigen
Three of the 55 recombinants represented *MAP*2609, an antigen of approximately 9 kDa. The gene sequence predicts a signal sequence followed by a mature protein of 82 amino acids, suggesting that this is a secreted antigen. The mature protein displays 100% homology to ORFs predicted by the genome of *M. avium* ss.*avium* 104 and 73% similarity to ORF Rv1174c, and ORF Mb1207c of *M. tuberculosis* H37Rv and *M. bovis,* respectively. In *M. tuberculosis* and *M. bovis* these proteins have been characterized as secreted antigens denominated TB8.4 or SA-5K, respectively (Coler, Skeiky *et al.* 1998; Freer, Florio *et al.* 1998). For SA-5K it was shown that the corresponding gene is expressed during the course of infection (Bottai, Batoni *et al.* 2003).

15 kDa antigen
Seventeen recombinants represented an antigen of approximately 15 kDa. This antigen displayed strong homology with the secreted MPT53 antigen of *M. tuberculosis*. The determined gene sequence predicts a mature ORF of 135 amino acids (*MAP*2942). The 15 kDa antigen displays strong homology (82-99%) with predicted ORFs on the genomes of other mycobacteria including *M. avium ss. avium* and *M. bovis*. The mature protein is preceded by a predicted signal sequence of 32 amino acids suggesting that the 15 kDa antigen, like its identified counterpart in *M. tuberculosis*, is a secreted antigen.

34 kDa antigen
Thirty five of the 55 recombinants represented a 34 kDa antigen that displayed strong homology to the previously identified Erp-family of secreted antigens (de Mendonca-Lima, Picardeau *et al.* 2001). The sequence of a 1175 bp region encoding this 34 kDa antigen (*MAP*0210c) is displayed in Fig. 3. The predicted gene encodes a mature protein of 314 amino acids that is preceded by a putative signal sequence of 20 amino acids. An identical ORF is predicted on the genome of MAP strain K10. The 34 kDa antigen contains a highly conserved N-terminal region, a central region containing 14 PGLTS-motifs, and a C-terminal region rich in proline and alanine residues arranged as GAAGALP-motifs (Fig. 1). The overall gene homologies between different mycobacterial Erp family members as well as the number of repeats can vary extensively. The *M. avium avium* member is 98% homologous to MAP and contains 15 copies of the PGLTS-motif in the central region. The *M. tuberculosis* Erp-member has an overall homology of only 35%, and contains 11 PGLTS motifs in the central region. The relevance of these differences remains to be investigated, but may affect their level of immunological cross-reactivity.

Antibody recognition in infected cattle
To further analyze the immunological significance of the three secreted antigens, the purified mature forms of the 9, 15 and 34 kDa antigen as poly-histidine tagged fusion proteins from *E.coli* were subjected to immunoblotting using pooled sera from infected cows (see Fig. 2). SDS-PAGE and immunoblotting was carried out using the Mini-Protean II electrophoresis and blotting apparatus of Bio-Rad according to the manufacturer's protocol. Proteins separated by SDS-PAGE were stained using Coomassie Brilliant Blue (0.1 %; from Merck). For immunoblotting, reactive bovine antibodies were detected using prot G conjugated with horseradish peroxidase (1:1000, Zymed laboratories Inc., San Francisco, USA)). Reactivity was

visualized using 3, 3', 5, 5'-tetra methyl benzidine as a substrate As shown in panel B, a pooled serum from cattle infected with MAP clearly reacted to the 15 and 34 kD antigens whereas a weak recognition of the 9 kDa and Hsp65 antigen was found. No reactivity to the Hsp70 and the *M. bovis* specific antigen MPB83 antigen was found.
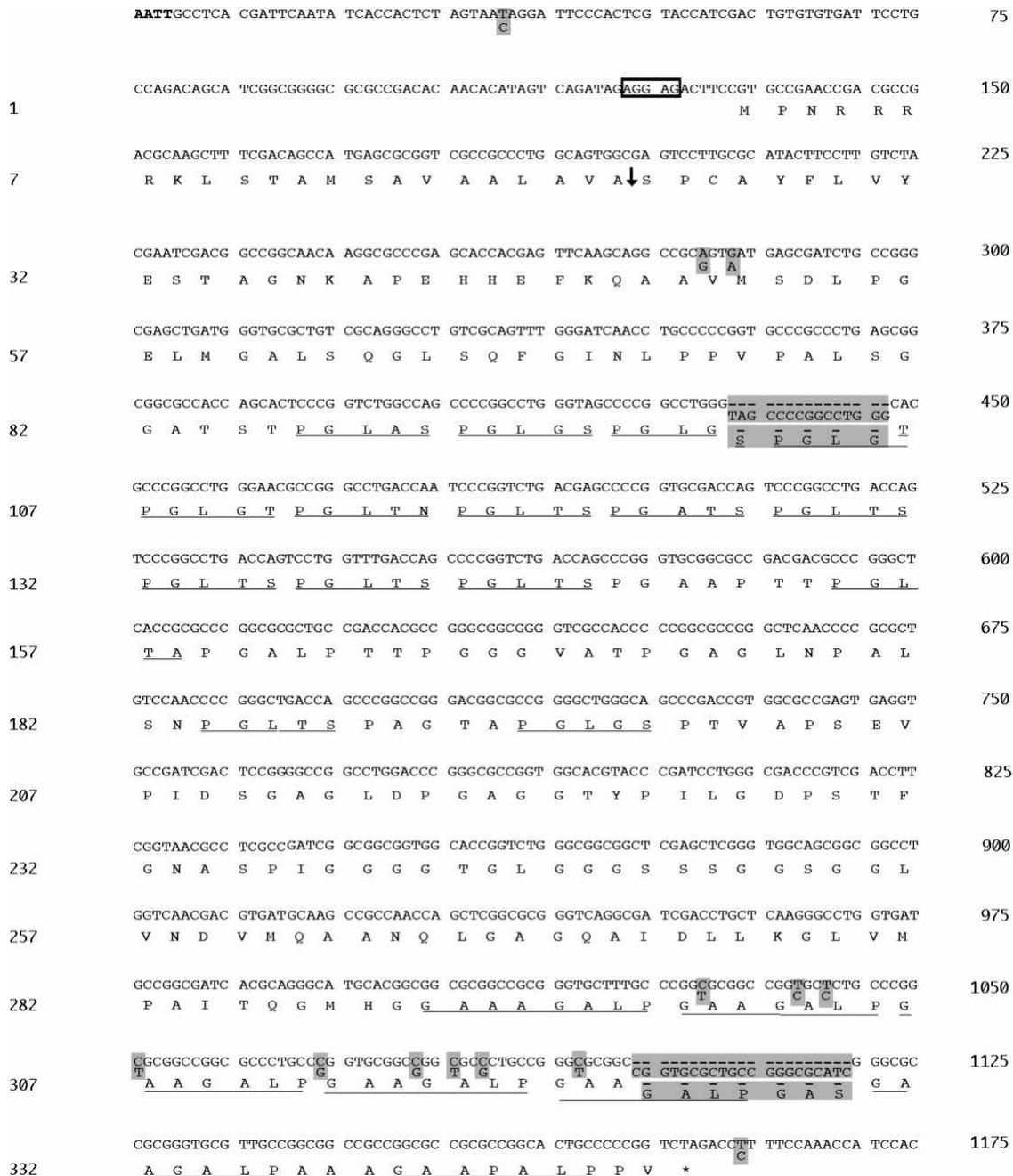
Fig. 1. Nucleotide sequence of the gene encoding the MAP 34 kDa antigen and resultant amino acid sequence. A potential signal peptidase cleavage site is indicated by an arrow. A potential ribosome binding site is indicated by a box. The AATT sequence at positions 1-4 (indicated in bold) represents the 3'-end of the MAP DNA fragment carried by the 35 pTriplEx recombinants selected by serum 3869. The 14 PGLTS and 6 GAAGALP repeating motifs that are present in this protein are underlined. The 34 kDa antigen of the closely related *M. avium ss. avium* strain 104 is almost identical but differs in that it carries 15 PGLTS and 7 GAAGALP motifs. Different nucleotide sequences and subsequent different amino acid sequences of *M. avium ss. avium* are presented on a line below the corresponding sequences of MAP as shaded boxes.

We used two categories of sera from MAP infected cows as assessed by repeated positive fecal cultures. Firstly, 7 sera from naturally infected but subclinical cows obtained during routine surveillance diagnostics. The second category consists of 4 sera from cows that were in the clinical stage of infection. An ELISA-

format was used in which we coated each well with 100 µl of a 5µg/ml solution of single antigens. Using an arbitrary cut-off value of OD>0.3 at a serum dilution of >1/80, 11 out of 11 (100%) of serum samples from subclinical and clinical stage cows reacted with the novel 9 and 15 kDa novel antigens, and 10/11 (91%) with the novel 34 kDa antigen (Table 1). In these two groups 11/11 (100%) recognised Hsp65, whereas 7/11 (64%) recognised Hsp70. None of these antigens showed reactivity with control serum from an *M. bovis* infected animal.
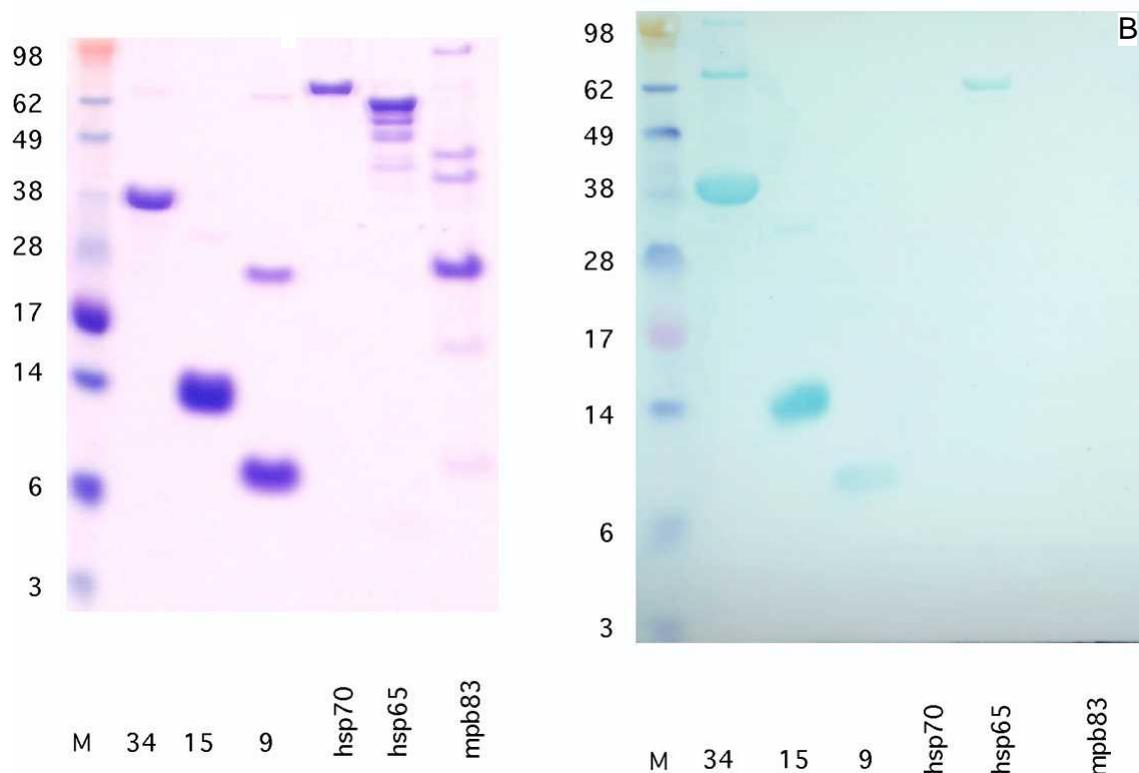
Fig. 2. Reactivity of antibodies in a pooled serum samples from MAP infected cattle (panel B) to purified 9 kDa, 15 kDa, 34 kDa, Hsp70 and Hsp65 antigens of MAP and MPB83 of *M. bovis.* Coomassie Brilliant Blue SDS-PAGE of purified antigens are shown in panel A. Positions of molecular size markers (M) are indicated to the left.

Table 1. Reactivity of cattle sera to purified 9 kDa, 15 kDa and 34 kDa antigens of MAP in an ELISA. For comparison, Hsp65, Hsp70, a total antigen extract (strain B854) and MAP sensitine johnine (CIDC-Lelystad, the Netherlands) was included in the test. Substrate absorbance was measured in a Bio-Tek Model EL312 Automated Microplate Reader (BIO-TEK Instruments, Inc.) at 450 nm (E450). Values are expressed as maximum sera dilutions giving an absorbance higher than 0,3. Dilution values ≥80 are in bold and are considered as positive.

	Serum	34 kDa	15 kDa	9 kDa	Hsp65	Hsp70	B854 total antigen	Johnin
Subclinical	1362	320	640	>1280	320	80	>1280	>1280
	1364	160	<1280	>1280	320	80	>1280	>1280
	1366	80	320	160	160	80	>1280	>1280
	1369	320	640	>1280	>1280	320	>1280	>1280
	1371	320	640	>1280	160	40	>1280	>1280
	1372	160	320	>1280	640	40	>1280	>1280
	1376	320	320	640	160	40	>1280	>1280
Clinical	3869	>1280	>1280	>1280	>1280	320	>1280	>1280
	3053	320	320	>1280	320	80	>1280	>1280
	3068	640	>1280	>1280	320	40	>1280	>1280
	3096	<10	>1280	>1280	320	80	>1280	>1280
M. bovis serum	Oryx4	10	40	40	20	<10	>1280	320
Score (≥80)	Subclinical	7/7	7/7	7/7	7/7	4/7	7/7	7/7
	Clinical	3/4	4/4	4/4	4/4	3/4	4/4	4/4
	Bovis serum	0/1	0/1	0/1	0/1	0/1	1/1	1/1

CONCLUSION

In an attempt to identify the antigens of MAP during natural infection, we used a serum sample from a naturally infected cow to screen a genomic expression library. Fifty-five of the 86 positive clones that were identified could be dissected into three groups each representing a secreted antigen of 9, 15 and 34 kDa, respectively. Whereas the remainder of the positive clones represents other antigens that are still under study, the findings of this study may indicate that secreted antigens represent a frequent target of the natural immune response to infection to MAP.

None of the three antigens identified thus far was found to represent any of the earlier antigens that were identified previously. The 9 kDa antigen has corresponding antigens in *M. tuberculosis* and *M. bovis*. Studies have shown that these antigens exhibit a poor specificity in serodiagnosis of tuberculosis (Batoni, Bottai *et al.* 2001), whereas as a vaccine the antigen induced protection on challenge with virulent *M. tuberculosis* (Coler, Campos-Neto *et al.* 2001). Its potential use as a diagnostic marker for paratuberculosis or for development of vaccines is under study.

The two other secreted antigens described in this study, a 15 and a 34 kDa antigen have corresponding antigens in other mycobacteria, including *M. bovis* and *M. tuberculosis*. The 15 kDa antigen is homologous to MPB53 and MPT53, secreted antigens expressed by *M. bovis* and *M. tuberculosis*, respectively, that were shown to be moderate antibody targets in the immune response during bovine and human tuberculosis (Wiker, Michell *et al.* 1999),(Johnson, Brusasca *et al.* 2001), but almost undetectable as secreted antigens of *M. avium* and other nontuberculosis mycobacteria. Therefore, our findings that the 15 kDa antigen is recognized by a serum of a natural infected cow are significant. Similar to MPT53 (Wiker, Michell *et al.* 1999) we found a region in the 15 kDa antigen with strong homology to an active thioredoxin site, displaying a C-X-X-C motif. The significance of this finding is currently unknown. MPT53 did not serve as an alternative substrate for thioredoxin reductase (Wiker, Michell *et al.* 1999).

The 34 kDa antigen was shown to be strongly homologous to the Erp family of secreted proteins, initially identified in *M. tuberculosis* (Berthet, Rauzier *et al.* 1995). These proteins contain a conserved N-terminal region, with a central region composed of a number of repeats based on a PGLTS motif, and a conserved C-terminal region rich prolines and alanine residues (de Mendonca-Lima, Picardeau *et al.* 2001). The *M. bovis* homologue has been shown to be a very dominant target of the antibody response in cattle during tuberculosis infection (Bigi, Alito *et al.* 1995). The data shown here indicate that the antigen may be similarly dominant in the antibody response during infection with MAP in cattle. Despite their relatedness in composition, the overall homology between the 34 kDa antigen and distantly related members such as the *M. tuberculosis* and *M. bovis* homologues is only 35%, mainly due to the homology between their conserved N-terminal regions. Application of the specific central and C-terminal regions may perhaps be applied for developing tools that discriminate between infection with paratuberculosis and tuberculosis. Similarly, the C-terminal region of another 34 kDa antigen identified by a specific monoclonal antibody has been applied for the specific serodiagnosis of paratuberculosis (De Kesel, Gilot *et al.* 1993). Despite the limited set of sera tested the novel antigens showed a high concordance with respect to the ELISA results. All sera from MAP infected cattle were positive except for serum 3096 (clinical) in combination with the 34 kDa antigen, underlining the necessity of using multiple recombinant antigens when establishing an ELISA for diagnostic purposes. A high titer anti-*M.bovis* (Oryx4) serum cross-reacted with total MAP (B854) antigen and Johnin, but not with the purified recombinant antigens. This probably reflects the different expression profiles between *M. bovis* and MAP cells when encountering the host immune system, rather then differences between the MAP antigens and their highly homologous *M. bovis* counterparts. It is clear, however, that the antigenic properties of these three novel MAP antigens justify further assessment of their diagnostic applicability.

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Evaluation of three PCR assays for identification of Mycobacterium avium subsp. paratuberculosis from liquid culture

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Abstract

Surveillance for Johne's disease frequently relies upon culture of samples (tissue, fecal and environmental) for isolation of *M. paratuberculosis* (*M. ptb.*). This mycobacterial species is one of several genetically similar species belonging to the *M. avium* complex. PCR assays have been developed to discriminate among them. Accurate identification of members of this complex remains a difficult task however, especially if more than one mycobacterial species is isolated from a sample. Our aim was to evaluate the performance of three PCR assays that use different target sequences (2 university-developed: "A" and "B", and one commercially available: "C") to identify *M. ptb.* when an acid-fast organism is isolated from culture. The three PCR assays were evaluated with isolates obtained from radiometric culture of 261 fecal or tissue samples chronologically submitted for Johne's disease surveillance. Since both genetic target and growth pattern evidence was found of mycobacterial co-isolates from 74 cultures, these were analyzed separately. For the 187 cultures considered to contain only one mycobacterial isolate, PCRs A and B agreed 97% of the time with no statistically significant difference between their results. A comparison of PCR C with assays A and B yielded statistical significant differences with 67% and 68% agreement, respectively. When comparing each of the PCR assays (A, B, and C) for the 74 cultures believed to contain mycobacterial co-isolates, agreement was poor. (A:B - 31%, A:C - 78%, B:C - 53%.) Based on this study, isolation of more than one mycobacterial species is not rare, and their presence can interfere with the ability of the PCR assay to accurately identify *M. ptb.* The interpretation of results from molecular methods for diagnosis of *M. ptb.* from clinical samples should be done carefully. The use of multiple genetic targets is recommended as an effective practice to identify the presence of more than one mycobacterial species.

Immune parameters and vaccine efficacy in Mycobacterium avium subspecies paratuberculosis experimentally infected sheep

D Begg, J Frank Griffin

Abstract

Johne's disease in ruminants is caused by the pathogenic bacterium *Mycobacterium avium* subsp. *paratuberculosis* (Map). Currently available Map commercial vaccines protect against clinical disease but not infection. Using an experimental model of ovine Johne's disease this study examined the proprietary Johne's vaccine Neoparasecä and an Aqueous formulation of Map 316F (AquaVax). Detailed immunological examinations including lymphocyte transformation assays, FACS analysis, IFN-γ and antibody detection from blood and gut associated lymphoid tissues was carried out on animals after vaccination and challenge with virulent Map to identify markers of protective immunity. Neoparasecävaccination provided significant protection against disease while AquaVax did not. Diseased animals had different immune responses from different gut lymphatic tissues which correlated with the histopathology observed. Immune animals had stronger cell-mediated responses and altered proportions of CD4$^+$, CD8$^+$, CD25$^+$ and B cells in blood, spleen and the gut lymphatics, than diseased animals.

Comparison of two methods for the detection of paratuberculosis in dairy cows in Israel: milk and serum ELISA

M Chaffer, S Friedman, O Koren, M Freed, Z Beider, E Ezra, D Elad

Abstract

In large paratuberculosis control program, requiring the screening of more than 15.000 cows per year, such as in Israel, bleeding cows for the serum ELISA test, becomes costly and demanding. In the last years, milk ELISA has been developed to check the disease. Milk recording is performed monthly in Israel. More than 100.000 cows, about 88% of the cows in the country, are checked monthly through the milk to which a preservative is added. Examining the same samples for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) could significantly reduce the resources needed for the control program. In this study, a commercial milk ELISA for detection of antibodies against MAP was evaluated using milk and preserved milk. In the first stage blood and composite milk was collected from 735 milking cows from four farms. In the second stage blood was collected from 987 milking cows from five farms. Milk was collected on the Milk Recording test day, by the Central Milk Laboratory representative and preserved with bronopol. Serum and milk were tested, according to the manufacturer's instructions, by commercial kits: IDEXX and Pourquier, respectively. Fecal samples from positive cows were cultured and part of these results is pending. The level of agreement between results for serum and milk samples was determined by the kappa statistic. The agreement between the 2 tests assessed using Kappa value was excellent (0.82) when comparing composite milk with serum. Comparison between serum and preserved milk showed that 43 cows were positive in both tests, 6 in serum ELISA and 15 with milk ELISA, the rest of 923 cows were negative in both. The agreement between the tests was good (0.80) Composite milk or preserved milk from the National Milk Recording seems to be a good alternative for serodiagnosis of paratuberculosis in cows.

Evaluation of five antibody detection tests for bovine paratuberculosis

Michael T. Collins, Scott Wells, K R Petrini, J E Collins, R D Schultz, Robert H. Whitlock

Abstract

Five diagnostic tests based on ELISA technology for bovine paratuberculosis were evaluated using individual serum or milk samples from 359 dairy cattle in seven paratuberculosis-free herds and 2,094 dairy cattle in seven *M. paratuberculosis*-infected dairy herds. Three independent laboratories using three different culture procedures completed fecal cultures for *M. paratuberculosis* on these cattle and found 417 cows to be shedding *M. paratuberculosis* in their feces. An animal that was fecal culture-positive for *M. paratuberculosis* by any of the three laboratories was considered a confirmed case of infection. The specificity of three ELISAs (two on serum and one on milk) was 99.8%. The specificity of the remaining two ELISAs, both done on serum, was 94.9% and 84.7%. Four of the five ELISAs evaluated produced similar sensitivity in detecting fecal culture-positive cattle (27.8% - 28.9%). Serum ELISA "D" had the lowest specificity (84.7%) and the highest sensitivity (44.5%). If the case definition for *M. paratuberculosis* infection was based on the culture results of a single laboratory instead of the combined results of three laboratories, ELISA sensitivity estimates were 45.7% to 50.0%. With the exception of ELISA D, assay agreement was high (kappa 0.66 to 0.85) for categorical assay interpretations (positive or negative) but linear regression of quantitative results showed low correlation coefficients ($r^2 = 0.40$ to 0.68) due to the fact that ELISA results for some cows were high in one assay but low in another assay. Likelihood ratio analysis showed a direct relationship between the magnitude of ELISA result and the odds of a cow shedding *M. paratuberculosis* in its feces. If used judiciously and interpreted quantitatively, these ELISAs are useful tools in support of paratuberculosis control programs in dairy herds.

Evaluation of real time PCR of pooled fecal samples for detection of Mycobacterium paratuberculosis in cattle

C Couquet, S Chatonnet, A Fremont, L Rebeyroles, Y Versmisse, B Blanchard

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of Johne's disease. The ability to detect MAP rapidly and accurately is important for cattle breeding. The present study compares the results under real time PCR technique on individual feces and on pools of 2 feces on the one hand to the results under coproculture considered as the reference method on the other hand. One hundred bovine fecal samples were selected by the laboratory (LDAR87) for MAP detection. Individual fecal samples were analyzed under real time PCR and were used to create pooled fecal samples. The 100 samples were randomly pooled by two and analyzed by PCR. 31 fecal samples, which were positive under coproculture, were mixed with negative coproculture feces. For each experiment, 1g of feces was used. DNA was extracted from fecal suspension using the ADIAPURE$^{\lozenge}$ kit (Adiagene). PCR was performed using the ADIAVET PARATB PCR$^{\circledR}$ kit (Adiagène). While 31% of the samples were positive under coproculture, 34% of the samples turned out to be positive under PCR on individual samples and 36% of the samples under PCR on pools of 2 samples. The PCR test results concurred with the coproculture results. Under PCR tests on pooled samples more feces where positive than under PCR on individual samples. The differing results concerned weakly contaminated feces. These differing results could be due to the heterogeneity of the feces. These data indicate that the use of real time PCR on pooled fecal samples is as effective as coproculture to detect MAP without decrease of sensitivity.

Impact of Corynebacterium pseudotuberculosis infection on serologic surveillance for Johne's disease in goats

H F Cushing, C Wolf, S Hietala, Elizabeth J.B. Manning

Abstract

In goats and sheep, false-positive results due to antibody produced by *Corynebacterium pseudotuberculosis* (C. PsTB) infection are reported to occur on serologic assays for *M. a. paratuberculosis* (*M. ptb.*). This issue of compromised specificity was evaluated by testing 557 adult goats from 8 Midwestern goat herds. Assays for *M. ptb.* infection include radiometric fecal culture and two commercially available Johne's disease ELISAs: one USDA-licensed for cattle only (ELISA-1) and one USDA-licensed for cattle, sheep and goats (ELISA-2). A case of *M. ptb.* infection was defined as a fecal culture-positive goat from a herd with known clinical cases of Johne's disease. A hemolysin inhibition assay (HI) was used to detect C. PsTB antibody. A case of C. PsTB infection was defined as a goat with an HI titer ≥ 1:8 from a herd with confirmed cases of C. PsTB infection. Three infection status groups were evaluated. Group A (herds believed free of *M. ptb.* and C. PsTB infection; 181 goats) tested negative on all *M. ptb.* fecal cultures and HI tests. Five goats were test-positive on both ELISAs and two goats were test-positive on ELISA-1 only. In Group B (*M. ptb.* infected; no C. PsTB infection; 41 goats) all animals were HI negative. Six goats were fecal culture positive and strongly positive on both ELISAs. ELISA-1 identified two additional goats as *M. ptb.* test-positive. For Group C (C. PsTB infected; no *M. ptb.* infection; 335 goats) all fecal cultures were negative and 91% were HI test-positive with a range of HI titers from 1:8 to ≥1:256. In this population two goats were test-positive on both *M. ptb.* ELISAs. ELISA-1 however produced 85 additional test-positive results. These data confirm that false-positive *M. ptb.* ELISA results may occur for goat herds infected by C. PsTB and demonstrate that false-positives arise more often for ELISA-1 than ELISA-2.

Identification of Mycobacterium avium subsp. paratuberculosis in tissue and faecal material of sheep by PCR. Comparison with serological data

L De Grossi, F Di Donato, G Micarelli, A Cersini, E Sezzi

Abstract

Not only does paratuberculosis cause dramatic economical loss in sheep farming but furthermore attracts attention because of the possible relationship with Crohn's disease in human subjects. There is scant information on the ovine disease and the validity of diagnostic methods is better known in bovine paratuberculosis. In addition, some strains of MAP are very difficult to culture, so the golden test can't be used easily in sheep. This study was aimed at screening paratuberculosis in the sheep population of Viterbo province and at obtaining further information on diagnostic methods. The investigation was at an slaughteron 300 adult sheep from 100 breeding centres. Diagnostics were carried out by PCR using IS*900* as primer. Several specimens (faeces, mesenterial limph nodes, mammary limph node, ileocecal valve) from each animal were tested. Out of these 300 sheep, 83 blood samples were also tested with ELISA. Forty-five animals were negative in both tests, 27 animals were positive in tissue specimens and among 10 were positive in faeces with PCR. With ELISA, 21 animals were positive and 10 were doubious. Comparing PCR and ELISA, 18 sheep resulted being positive in both tests, while 3 animals were only positive in ELISA and 9 were positive in PCR only in tissues. The authors suggest that differing positive results may reflect different stages of the disease. The ELISA S/P value has been also under study and debated.

Direct DNA extraction coupled with culture increases the sensitivity of M. a. paratuberculosis detection

L de Juan, J Álvarez, J Bezos, A Aranaz, B Romero, A Mateos, L Domínguez

Abstract

The gold standard for paratuberculosis diagnosis in ruminants is culture. This technique is 100% specific but occasionally the sensitivity is low due to: 1) certain *M. a. paratuberculosis* strains that are difficult to grow (sheep type); 2) biological sample; 3) clinical stage of the animal; and 4) intermittent shedding of *M. a. paratuberculosis* in faeces. The objective of this study was to determine the usefulness of a complementary diagnostic technique to improve the sensitivity of the diagnosis. The protocol is based on a direct DNA extraction from tissues (ileocecal valve and lymph node, and mesenteric lymph node), and specific PCRs aimed at the IS*900* and f57 sequences. Positive and negative controls were included in all the tests to evaluate the performance of the method. A total of two hundred and thirty-six tissue samples from two different farms, where paratuberculosis was confirmed by cultured, were tested: 105 samples from a caprine flock, and 131 tissue samples from a cattle herd. The selection of this group of animals was made on basis of the type of *M. a. paratuberculosis* strains (Collins et al. 2002), since they were infected with *M. a. paratuberculosis* isolates belonging to the cattle and sheep type, respectively. The application of this new technique based on direct DNA extraction allowed the detection of animals both culture positive and negative. This fact is really important mainly in farms with animals infected with *M. a. paratuberculosis* strains belonging to the sheep type, since isolation of the agent is difficult and in several occasions false negative results are obtained. An extra advantage to this protocol is that results are obtained in two days when compared with the long incubation period for culture. Moreover, the DNA obtained could be subjected to a classification by PCR for epidemiological purposes, allowing a prompt implementation of control measures. *Attendance to this Congress was sponsored by EU-funded project SSPE-CT-2004-501903.*

Detection of Mycobacterium avium subsp. paratuberculosis from goats by Sequence Capture PCR

Berit Djønne, Ingrid Olsen, A Storset, M Valheim, S Nilsen, Maria Hallock, G Holstad

Abstract

Isolation of *Mycobacterium avium* subsp. *paratuberculosis* by culture is time consuming, and four months incubation time is necessary. PCR methods, on the other hand, tend to have low sensitivity in clinical samples, due to excessive non-specific DNA, low number of bacteria and high content of inhibitors. Therefore, a sequence capture method, followed by a dot blot assay to identify *M. a. paratuberculosis*, has been developed (Halldórsdóttir et al., 2002). The aim of the present study was to evaluate this method on tissue samples from goats infected by *M. a. paratuberculosis*. Seven goat kids experimentally infected by *M. a. paratuberculosis* and one uninfected kid were used for the study. At week 106-117 post-infection the animals were euthanized, and necropsies and histopathological examination were performed. Lesions compatible with *M. a. paratuberculosis* infection were found in six infected goats and acid-fast bacilli were present in 5 of them. Samples from duodenum, jejunum, ileum, caecum, colon, and the jejunal, ileocolic, colic and mediastinal lymph nodes were examined by cultivation and sequence capture PCR. The **PCR** method could detect 100 cfu/gram tissue. *M. a. paratuberculosis* was detected by culture from five infected animals. From one goat bacteria were isolated from the intestinal lymph nodes only. In the other four animals, bacteria were cultured from different segments of the intestine and lymph nodes. Sequence capture PCR detected *M. a. paratuberculosis* from all infected goats, including the two goats where no bacteria could be isolated. From 3 goats, PCR positive samples were detected in the intestinal lymph nodes only. *M. a. paratuberculosis* was not detected by any method from the uninfected goat. The present study shows that sequence capture PCR might be an alternative to culture to detect *M. a. paratuberculosis* from goats. Reference Halldórsdóttir S, Englund S, Nilsen SF, Olsaker I. Vet Microbiol. 2002. 22, 327-40. *Attendance to this congress was sponsored by the EU-funded project SSPE-CT-2004-501903.*

MGIT, liquid culture system for detection of MAP in bovine fecal samples

Terry L Fyock, Raymond W. Sweeney, Robert H. Whitlock

Abstract

Liquid culture has been used to identify *Mycobacteria* in liquid culture for several decades, only recently have non-radiometric liquid culture methods been available for *M. paratuberculosis*. Our laboratory has evaluated a number of variables in both sample processing and culture media to enhance MAP growth and reduce time to detection (TTD). Objectives: 1)To develop an enhanced sample processing method and improved media formulation to reduce TTD. 2) To determine the relationship between MAP cfu in the sample to TTD. Materials & methods: Sample processing components evaluated included: fecal sample size; inoculum size; HPC percentage; antibiotic concentrations and types: (ANV, PANTA and Nisin). Media component variables included: egg yolk (EY) volume; EY sources; species of EY, OACC, and OADC, among others. Results: Increased EY volume up to 2,000 ul per tube reduces TTD by the equivalent of nearly two logs of MAP compared to 60 ul EY. Both ANV and PANTA reduce TTD compared to no antibiotics, but vancomycin is more inhibitory than naladixic acid or amphotericin B. Nisin has promise since it has minimal inhibitory effect on MAP growth. Different sources of EY have little effect on MAP growth in liquid culture. Reduced HPC percentage (0.3%) is well tolerated with little increase in contamination, and on occasion does enhance MAP recovery, but not consistently. Increased inoculum size reduces TTD up to a limit of 1,000 ul but does result in a higher contamination rate. Increased MAP concentrations reduce TTD proportionately. Repeated experiments with MAP alone, MAP spiked fecal samples and field samples positive on HEYM showed a high degree of correlation between TTD and MAP cfu in the original sample. Conclusion: Liquid culture system with MGIT has proven to be a robust diagnostic culture system that has the potential to replace traditional culture using solid media (HEYM).

Validation of a commercial ELISA kit for assessment of the humoral immune response in Mycobacterium paratuberculosis infected sheep and goats

S Gumber, Graeme J. Eamens, Richard Whittington

Abstract

Mycobacterium paratuberculosis causes Johne's disease, an economically significant problem in ruminants in most countries. Serological tests for *M. ptb.* are useful in estimating the prevalence of infection when it is established in herds/flocks and for the confirmation of diagnosis in animals showing clinical signs. The present study was designed to evaluate a commercial ELISA kit (Institut Pourquier) under Australian conditions and to compare it with the existing agar gel immunodiffusion (AGID) test for the diagnosis of ovine and caprine paratuberculosis. Serum samples from 2200 uninfected sheep and 946 uninfected goats varying in breed, age and geographic locations were tested to estimate diagnostic specificity for each species. The diagnostic sensitivity of assays for sheep was estimated by testing 2550 sheep serum samples from various infected properties, while for goats, 125 serum samples from infected animals were tested. Results for plates were accepted or all samples on the plate were retested according to decision-limit criteria for positive and negative control sera, while individual samples with a coefficient of variation (CV) for paired replicates of more than 15% were also retested. The CV of positive and negative control sera was 9.7 and 11.4%, respectively. Diagnostic specificity and sensitivity were calculated at different cut point ratios and results were analysed by age, geographic location, and histological lesion type and culture results

Real-time PCR-systems for confirming direct detection of Mycobacterium avium subsp. paratuberculosis

D Herthnek, Goran Bölske

Abstract

Although the IS*900* gene is considered specific for *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a PCR directed against it still has to be confirmed with other methods, as cross-reactions with related IS elements in other mycobacteria have been demonstrated. Real-time PCR has the advantage over nested PCR in minimising the risk of contamination, but is still very sensitive. However, confirmation of positive samples by sequencing of amplicons cannot easily be achieved when using real-time PCR. We therefore developed two new real-time PCR systems targeting other parts of IS*900*. The new primers and probes were designed to be as specific as possible and to complement our primary PCR system. Special attention was paid to the similarity between IS*900* and the equivalent gene in strain 2333. Another system of primers and probe was designed for the gene f57, also regarded specific for MAP. However, as the MAP genome contains multiple copies of IS*900* but merely one copy of f57, this system ought to be less sensitive. The newly designed systems were tested on several mycobacterial strains to elucidate specificity. They are also being applied to clinical samples that are positive for MAP in our primary PCR system. Evaluation of the sensitivity is in progress. When applied on 77 non-MAP mycobacterial strains, one each of the alternative IS*900* systems gave weak positive reactions to an isolate of *M. avium* subsp. *avium* from a horse and to an *M. kubicae*-like isolate from a cat, respectively. Strain 2333, that is positive with our primary system, showed negative with all the three new systems. The level of unspecific reactions in all of the four PCR systems is probably very low and the new systems should therefore be reliable for confirmation of the primary system. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

Detection of Mycobacterium avium subsp. paratuberculosis in bovine semen by real-time PCR

D Herthnek, S Englund, Peter Willemsen, Goran Bölske

Abstract

Although semen is not a common way for spread of paratuberculosis, isolation of MAP from semen has been reported. However, as the risk of spread by artificial insemination is not fully investigated, there is a demand for a semen test intended for use in paratuberculosis free herds. The rapidity, sensitivity and specificity of real-time PCR would greatly benefit the detection of MAP. We have applied the following method on artificially infected bovine semen. Samples of diluted MAP free semen were spiked with MAP, counted in bürker-chamber since CFU-count usually underestimates the number of detectable genomes. The MAP suspension used was also washed from free DNA to avoid overestimating the sensitivity of the test. Incubation with lysisbuffer and proteinase K followed by mechanical disruption by beadbeating released the DNA, which was recovered with phenol/chloroform extraction. Real-time PCR with a fluorescent probe targeting the insertion element IS*900* could detect as few as 10 MAP per sample of 100 µl semen. PCR-inhibition was monitored by the addition of an internal control to the samples and it was shown that the applied method was sufficient for removing inhibiting components.
Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903

Effects of vaccination on Johne's disease AGID and ELISA tests in experimentally challenged baby goats

Murray E. Hines II, M Pence, C Baldwin, L Whittington, J Musgrove, Sharif S Aly

Abstract

The effects of vaccination on Johne's disease AGID and ELISA test results were evaluated in a group of 80 goat kids enrolled in a vaccine challenge study. Four vaccine combinations, including cell wall-competent (CWC) alum adjuvant, CWC-QS21 adjuvant, cell wall deficient (CWD) alum adjuvant and CWD-QS21 vaccines were evaluated. Kids were vaccinated at one and four weeks of age with each vaccine or a sham control vaccine consisting of alum adjuvant. Kids were challenged orally with 6.0×10^9 organisms in four divided doses (1.5×10^9 organisms per dose) using a confirmed goat isolate of *Mycobacterium avium subsp. paratuberculosis* (MAP). Both AGID (Cornell method) and ELISA (Biocor ®) tests were performed pre-vaccination, post-vaccination/pre-challenge and monthly thereafter. Infection status was determined by a combination of necropsy findings and fecal/tissue culture of necropsy tissues. Half the kids within each group were necropsied at 6 months post-challenge and remaining kids were necropsied at 9 months post-challenge. Results indicate overall sensitivity and specificity in sham-vaccinated challenged and unchallenged control kids for all post-challenge samplings were 60% and 100% for AGID, and 70% and 100% for ELISA. At 6 months post-challenge sensitivity and specificity were 22.2% and 100% for AGID, and 55.6% and 100% for ELISA in sham vaccinated control groups. At 9 months post-challenge sensitivity and specificity were 40% and 100% for AGID and 80% and 100% for ELISA in sham vaccinated control groups. None of the vaccines resulted in positive AGID or ELISA results post-vaccination/pre-challenge. Time to first positive AGID test was reduced by 2 months in all vaccinated groups, but time to first positive ELISA was not affected. Overall post-challenge AGID sensitivity was reduced >50% by the CWC-QS21 vaccine, but specificity was generally unaffected by any vaccine. Overall post-challenge ELISA sensitivity was reduced by all vaccines with CWC-QS21 eliciting a 3 fold reduction and CWC-alum a 2 fold reduction in sensitivity. The CWD-QS21 vaccine reduced overall post-challenge ELISA specificity by 3 fold and had a high false positive rate (66.7%), but only mildly affected sensitivity.

Comparison of the diagnostic value of the Complement Fixation Test (CFT), Polymerase Chain Reaction (PCR) and the culture methods for detecting Mycobacterium avium subsp. paratuberculosis in sheep and goats

J Ikonomopoulos, B Kantzoura, E Fragiadaki, I Pavlik, M Bartos, J C Loukas, M Gazouli

Abstract

Objective: *Mycobacterium avium* subsp. *paratuberculsosis* (MAP) is the cause of Johne's disease that affects mainly ruminants. However the growing concern about the spread of MAP is largely due to its implication in Crohn's disease of man. In the present study, we compare three methods, PCR, serology and culture, in detection of MAP from sheep and goats. Material and Methods: For this purpose, we compared the ability of detection of MAP in seemingly healthy sheep and goats from South-West Greece area. From a total of 30 flocks, 632 sheep and goats had fecal, serum, and whole-blood samples examined, respectively, by culture, the Complement Fixation Test (CFT), and the Polymerase Chain Reaction (PCR) targeted to IS*900*, respectively. Results: The PCR produced positive results in 5.4% of the cases and the CFT produced positive 4.4% and 14.4% dubious results. Fecal cultures were negative in all but a single case. From the 408 samples that were examined both by PCR and CFT, the concord between the two tests for the CFT-positive results was 7.1%. The percentage of agreement between PCR and CFT-negative results was 95%. CFT and PCR produced similar results, since positive reactors were estimated as 4.4% and 5.4%, respectively. Although the expected failure of PCR to detect all the positive reactors with a single blood sample is probable due to the absence, or the decreased concentration, of MAP-circulating antigen in the blood, after production of adequate amounts of specific antibody. However, false positive CFT-results could also be a source of the latter or attributed to the antigenic incompatibility of the MAP strains that were detected by PCR and the antigen used in our CFT assay, which was designed with reference to cattle strains of RFLP type B-C1. Our failure to record more culture-positive results could be attributed to MAP-strains of low viability, which is consistent with the inability of our single isolate to be subcultured. Conclusions: Given the aforementioned problems with the methods tested here, we cannot recommend to use of a single method for safe detection of MAP in sheep and goats. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

Evaluation of fecal culture pooling methods for detection of Mycobacterium avium subspecies paratuberculosis in a beef herd

S M Jensen, Jason E. Lombard, Franklyn Garry

Abstract

Given the increased cost of whole herd fecal culture for the detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection, studies evaluating fecal pooling in dairy cattle have been conducted. This beef cattle study evaluated individual fecal samples, strategically pooled samples, and collection order pooled samples in detecting infected animals. Individual fecal samples were collected from 174 beef cattle and subsequently divided into three aliquots for individual animal testing, strategic pooling and ordered pooling. Each sample pool included 4-5 individual samples and all testing was performed concurrently. Individuals were selected for a strategic pool based on their ranked age whereas order pooled samples were based on order of collection. Nineteen of the 174 individual samples, 6 of the 35 strategic pools, and 2 of the 35 ordered pools were culture positive. Four of the six strategic pools and one of the two ordered pools that were culture positive contained at least one of the 19 individual samples found to be culture positive. Both individuals classified as heavy shedders were detected by strategic pooling, while only one heavy shedder was detected by ordered pooling. Of the positive pools, two strategic pools and one ordered pool contained no samples found to be positive upon individual culture. One sample within each pooling method was found to contain two culture positive individuals. The results of this preliminary beef study suggest that bacteriologic culture of strategically pooled samples may provide a more reliable method for detection of MAP infected animals as compared to ordered pooling. However, pooling of samples from beef herds where the majority of infected animals are moderate to low shedders may not be a sensitive enough method because it significantly reduces the ability to detect MAP infection compared to individual fecal culture.

Validation of fecal culture method and a serum ELISA for the diagnosis of paratuberculosis in sheep and goats

Poychronis Kostoulas, Leonidas Leontides, C Billinis, C Enøe, M Florou, M Sofia

Abstract

Objective: Latent-class models were used to determine the sensitivity (Se), specificity (Sp) and predictive values (PV) of a faecal culture (FC) method and a serum ELISA for paratuberculosis in dairy sheep and goats. Materials and methods: One hundred sera and faecal samples were collected from each of four dairy sheep and goat flocks with a history of clinical paratuberculosis. Faecal samples were cultured in Herrold's Egg Yolk medium supplemented with mycobactin J and antibiotics. Sera were tested using a commercial serum ELISA. The ELISA results were interpreted at the recommended cut-off and values reduced by 25 and 50%. Assuming independence, Bayesian estimates and posterior 95% credible intervals (PCI's) of the Se's and Sp's were obtained. Estimates of P's and the PV's and 95% PCI's were also estimated for each sub-sample of the data. Results: In sheep the Se and Sp of the FC were 19% (5%-41%) and 97% (94%-99%), respectively, while in goats they were 11% (4%-21%) and 98% (95%-99%), respectively. The Se and Sp of the serum ELISA in sheep and goats were 39% (17%-67%), 97% (94%-99%), 64% (46%-83%) and 94% (89%-97%) respectively, at the recommended cut-off. In either species, lowering of the cut-off improved Se and positive PV's of serology with minimal loss in Sp's and negative PV's. Conclusions: The Se of serology differs between sheep and goats. Species-specific cut-off selection in small ruminants is recommended. The performance of the ELISA could be improved by lowering of the cut-off. The sensitivity of the particular FC was quite low; in combination with its laborious and time-consuming process it cannot be suggested as a routine diagnostic procedure.

Sensitivity and specificity of a commercial Johne's disease ELISA in dairy herds of southern Chile

J Kruze, Gerdien van Schaik, M Pradenas, F Haro, Armin Mella

Abstract

Blood and feces were collected from 1,333 lactating cows in 27 dairy herds of southern Chile between September 2003 and August 2004. Blood samples were collected in 10 ml vacutainer tubes and the sera frozen at -20°C until tested using a commercial ELISA kit (IDEXX Laboratories, Inc.). Duplicate samples were assayed following manufacturer recommendations and the S/P ratio for each sample calculated using an automated ELISA reader (IDEXX). Rectal fecal samples were collected using individual polyethylene sleeves, transported to the lab, and cultured within 24h on Herrold's Egg Yolk Medium (HEYM) and mycobactin J (3 tubes) and HEYM without mycobactin J (1 tube). Prior to culture, 2g of each fecal sample was suspended in 35 ml HPC solution and incubated at 37°C overnight. It was then centrifuged and the pellet re-suspended in an antibiotic solution containing nalidixic acid, vancomycin, and amphotericin B, again incubated at 37°C overnight. A 0.15 ml aliquot of each suspension was used to inoculate all HEYM tubes which were incubated at 37°C for 16 weeks. Colonies resembling *M. paratuberculosis* and showing mycobactin-dependence were tested by IS*900* PCR. *M. paratuberculosis* was isolated from 10 (37.0%) herds and 54 (4.1%) cows. By ELISA, herd-level prevalence was 74.1% (20 herds with ≥1 ELISA-positive cow) and cow-level prevalence was 6.5% (86). ELISA sensitivity and specificity, relative to a single fecal culture, were 38.9% and 94.9%, respectively. The kappa value for ELISA and culture agreement was 0.263. These results show ELISA accuracy similar to that of other reports and that Johnne's disease is widely distributed in dairy herds in southern Chile. A control program to reduce its prevalence is urgently needed. *Grant research supported by FONDO SAG, Ministry of Agriculture, Chile*

Validation of commercially available ELISA tests for the serodiagnosis of paratuberculosis in Germany

H Köhler, B Burkert, I Pavlik, I Moser, P Möbius, G Martin

Abstract

In preparation of a national paratuberculosis guideline, which was implemented recently, a validation study was performed including four commercial ELISA tests. The tests are based on different Map antigens, a protoplasmic antigen (test A), a whole cell antigen (test B), a cell wall antigen (LAM, test C) and a protoplasmic antigen of a *Mycobacterium avium* subspecies (test D). Two tests include an absorption step of the samples with *M. phlei* (test A and B) and two tests are also approved for milk samples (test A and C). Blood serum was collected from 286 dairy cattle from four farms considered free of paratuberculosis (negative panel) and from 110 cows shown to be infected with Map by at least one positive result of the cultivation of faeces and/or up to nine organ samples (positive panel). The positive panel included clinically and sub-clinically diseased animals. Blood, milk, faecal and organ samples of the positive panel were collected at the same day. Milk samples were tested from 115 of the negative and 44 of the positive animals. The relation of antibody detection to shedding of Map, to the extent of gross pathological lesions and to clinical disease was calculated for tests A, B and C. The following characteristics were determined for the four tests: A strong positive relation of antibody detection to the actual shedding state was determined for test A and B. Antibody detection was more often positive in animals with medium to large gross pathological lesions, and in animals with clinical disease. In the national paratuberculosis guideline, the use of serological tests with a specificity of more than 99 % is recommended. **Sensitivity (%) Blood serum /milk Specificity (%) Blood serum /milk** Test A 53.6 / 54.5 99.9 / 98.3 Test B 55.5 / - 94.8 / - Test C 70.0 / 54.5 76.6 / 88.7 Test D 40.0 / - 94.8 / -

Improvement of an in-house ELISA for bovine paratuberculosis serology in Brazil

C D Marassi, L S Fonseca, R Ferreira, W Lilenbaum, W M Oelemann

Abstract

Paratuberculosis (Johne's disease) is chronic enteritis in cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). In Brazil, few reports describe the isolation of the organism. The gold standard test for Map is the isolation from tissues or feces. Moreover, bacterial growth is slow and test results are available only after four to six months of incubation. Furthermore, shedding of bacilli at levels detectable by fecal culture is irregular and does not occur during the early stages of infection, which compromises the sensitivity of this methodology. The most common immunological tests to identify Map infection are complement fixation test (CFT), agar gel immunodiffusion (AGID) and ELISA. In Brazil, commercial ELISA kits are imported and too expensive to be used as part of diagnostic laboratorial routine. Apart from that, their use has not yet been approved in the country. The aim of the present study was to improve an original assay PPA-ELISA protocol established by our group, and to determine sensitivity and specificity of the modified test. In a first step, we introduced modifications that minimized plate-to-plate and between-well variations, thus making the test more stable and reliable. In the second part of this study, a panel of 106 sera samples was tested by this modified PPA-ELISA protocol in order to estimate its sensitivity and specificity. The new assay presented overall sensitivity of 76.9% and specificity of 70%. Our study demonstrated that this assay could be recommended as a valuable diagnostic tool for paratuberculosis in Brazil and other developing countries.

Development of a luciferase reporter phage assay for identification of Mycobacterium avium subsp. paratuberculosis

S E McCusker, Lucy M. Mutharia

Abstract

Existing methods of *Mycobacterium avium* subsp. *paratuberculosis* (Map) detection require at least seven weeks to obtain visible growth on solid media. Molecular identification methods are available but are unable to identify the bacterium as live or dead. Mycobacteria specific luciferase reporter phage were utilized to develop an assay for identification of Map. Assay conditions were optimized and inhibitors were identified. Of the phage phAE39, phAE40 and phAE85 tested, phAE85 consistently produced the brightest luminescence in Map and was chosen for use in the assay. Phage phAE85 was capable of infecting all of the 18 Map strains tested. Results indicated that the brightest luminescence was produced with phAE85 at an MOI = 100. Substrate buffer composition was examined and the optimal buffer was chosen. Under optimized conditions the minimum detection limit of the assay is 100 cells. Serum components and detergents were found to inhibit phage binding. The use of IMS beads in the LRP assay will also be discussed.

Agreement of three ELISAs for Johne's Disease

Shawn McKenna, H W Barkema, Greg Keefe, Donald C. Sockett

Abstract

During a ten-month period in 1999, 994 serum samples were randomly collected from dairy cows at slaughter in eastern Canada. The sources of these cattle were from all four Atlantic Canadian provinces along with some cows from the state of Maine. The serum was used in part, to assess the agreement of three commercially available ELISAs for *Mycobacterium avium* subsp. *paratuberculosis*. Two ELISAs were indirect absorbed ELISAs licensed for use in North America, the third was an indirect non-absorbed ELISA currently licensed for use in Europe. Typical comparisons of ELISAs in the past have compared test performance such as sensitivity and specificity and not agreement. Overall, there was poor agreement between the three ELISAs. The highest and lowest kappa values were 0.33 and 0.18, which is fair and poor agreement, respectively. Tissue culture of ileum and associated lymph nodes was also performed using the Trek ESP® Culture System II. When only tissue culture–positive cattle were compared, the ELISAs had better agreement (kappa = 0.37 to 0.51). The proportions of positive tests, however, were significantly different among the three ELISAs. The poor agreement among the three ELISAs outweighs the fact that these tests have low sensitivity. The implications are greatest when the tests are used at the cow level to make individual animal decisions, which is not the recommended method on the product labels. At the cow level, if the result obtained from one ELISA is positive, using a different ELISA in a pre-clinical animal has a high likelihood of giving a different result due to low predictive values of positive test results. Additionally, the classification of a herd with a low *M. ptb.* prevalence will differ among the three ELISAs.

Test performance evaluation of three ELISAs for Mycobacterium avium subsp. paratuberculosis using tissue and fecal culture as comparison standards

Shawn McKenna, Greg Keefe, H W Barkema, Donald C. Sockett

Abstract

Three serum ELISAs for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (*M. ptb.*) were evaluated against culture of tissue and feces samples from 994 dairy cows collected at slaughter between January and October 2001. Culture of ileum and associated lymph nodes was performed using the Trek ESP® Culture System II. Overall, cultures for *M. ptb.* were positive in 160 (16.1%) of the 994 cows and 36 (3.6%) were fecal culture-positive for *M. ptb.* Two of the ELISAs evaluated were absorbed indirect assays and the third was a non-absorbed indirect assay. Estimated sensitivities of the absorbed ELISAs when compared to tissue culture were 8.8% (95% CI: 4.4 – 13.1%) and 6.9 % (3.0 – 10.8%), while the unabsorbed ELISA has a sensitivity of 16.9% (11.0 – 22.7%). Specificities were 97.6% (96.6 – 98.6%), 96.0% (94.7 – 97.4%) and 90.8% (88.8 – 92.7%) respectively. When compared to fecal culture, the sensitivities of the absorbed ELISAs were 16.6% (4.6 – 28.8%) and 13.9% (2.6 – 25.2%), respectively, and the sensitivity of the unabsorbed ELISA was 27.8% (13.1 – 42.4%). Specificities were 97.1% (96.0 – 98.1%), 95.9% (94.6 – 97.2%) and 90.1% (88.2 – 92.0%), respectively. Measurement of test accuracy was performed using receiver operator characteristic curves, and the area under the curves (AUC) for the absorbed ELISAs when tissue culture was the standard were 0.553 and 0.547, while the unabsorbed ELISA had an AUC of 0.540. When fecal culture was the comparison standard, the AUC of the absorbed ELISAs was 0.575 and 0.574, while the unabsorbed ELISA was 0.529. Overall, the sensitivities of the ELISAs when compared to tissue culture were low. The apparent advantage of the unabsorbed ELISA with respect to sensitivity is at the cost of lowered specificity and test accuracy.

Comparison of a gel diffusion test, three absorbed ELISAs, a faeces-PCR kit and faecal culture for diagnosis of caprine paratuberculosis in France

P Mercier, J Martin, C Bertin, C Courtes, B Durand, M L Boschiroli, K Laroucau

Abstract

It has become evident that paratuberculosis in France is not rare and, in particular, very few available data exist concerning its prevalence among dairy goat flocks. In this animal species, vaccination is no longer applied and therefore, control of paratuberculosis is based on preventing transmission of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from goats to kids by management measures, supported by culling of animal excreting bacteria by the faecal route (Map shedders). For undertaking any prevalence study, a simple and reliable diagnostic tool should be applied. Although faecal culture is the most accurate test for identifying Map shedders, this technique is long (up to 16 weeks or more for results to be available) and expensive., For this reason, a study was designed to evaluate more rapid and/or more sensitive alternative diagnostic methods. We compared three absorbed commercial ELISA tests, an AGID test, a commercial faeces PCR kit and faecal culture. Sera and faeces were collected in 12 flocks (480 animals, 40 per flock) of a major caprine breeding area. These flocks were originally considered paratuberculosis free or paratuberculosis infected based on their disease history (clinical signs or serological results). Four different age groups (10 animals per group) were studied: 0-1 year; 1-2 years; 2-3 years; 3 years and more. The infectious status of these flocks, after analysis of the results obtained with these new methodologies, showed us that only two flocks could really be considered Map free. Sensitivity and specificity of each test were estimated, taking faecal culture as reference. Their concordance was calculated by different statistical methods. The intra-flock prevalence and theprevalence in each age group for each flock were also evaluated. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

Application of different methods for the diagnosis of experimental paratuberculosis in goats

Sarvam Kumar Munjal, Bhupendra Nath Tripathi, O P Paliwal, J Boehmer, Matthias Homuth

Abstract

The diagnosis of paratuberculosis is a major problem. As part of investigating diagnostic strategies for paratuberculosis infection, serial results from various diagnostic methods in progressive experimental infection in goats has been evaluated. 23 goat kids were divided into three groups: the infected, contact and control, comprising 10, 5 and 8 goats, respectively. Animals of the infected group were orally inoculated on 7 occasions after every 2 days with 5 ml of inoculum containing 2 x 10^9 *Mycobacterium avium* subspecies *paratuberculosis* per ml. Lymphoycte proliferation test using johnin PPD detected paratuberculosis infection from 60 days post infection onwards. Johnin PPD was found to be a better antigen for the proliferative assays as compared with sonicated antigen. Delayed type hypersensitivity also detected an early infection but found to be non-reliable and non-specific. Faecal smear examination after staining with Ziehl Neelsen's (ZN) technique detected more goats as positive than bacterial culture and polymerase chain reaction (PCR) assay. The PCR assay was found to be a better method than bacterial culture and had shown better sensitivity in the tissue samples as compared with feces. Tissue impression smear examination after staining with ZN technique has same detection rate as the bacterial culture and fecal PCR. Lipoarabinomanan (LAM) based ELISA was better than indirect ELISA and agar gel immunodiffusion (AGID) test, and started detecting goats from 150 days post infection onwards. Indirect ELISA was found to be more sensitive than AGID test. Histological examination was confirmatory and detected 5 goats as positive. The result indicates that no single test provides an accurate diagnosis in the early stages of infection. As the disease progresses, ELISA could be a sensitive method for the screening of animals.

Isolation of atypical mycobacteria from the environment in cattle herds with high and low seroprevalence to Mycobacterium avium subsp. paratuberculosis

B Norby, G T Fosgate, Allen J. Roussel, Elizabeth J.B. Manning, Michael T. Collins

Abstract

Introduction: Testing of beef herds in Texas for paratuberculosis has identified herds with high proportions of seropositive results. In some of these herds, several atypical mycobacteria were isolated from fecal samples. The objective of this study was to investigate the association between atypical mycobacteria in the environment and 1) herds with high or low seroprevalence to *Mycobacterium avium* subsp. *paratuberculosis* (*M. ptb.*) as well as 2) chemical characteristics of the environment. Methods: Six beef cattle herds with a high seroprevalence for *M. ptb.* and three closely located herds without a previously high seroprevalence were identified in 2003. In 2004, between six and 13 environmental samples were sampled from these nine farms. Environmental samples were collected from water and soil. Soil samples were cultured using the BACTEC® system for mycobacteria. Acid fast organisms were tested with IS*900* PCR, and mycolic acid analyses using HPLC was used to characterize IS*900* negative mycobacteria. The Wilcoxon test was used compare the proportion of environmental mycobacteria positive samples for the two herd types and the t-test was used to compare chemical characteristics and isolation of atypical environmental mycobacteria. Results: Atypical mycobacteria isolated from soil and water included non-identifiable mycobacteria (n=3) *M. scrofulaceum* (n=2), *M. avium* complex (n=2), *M. avium-intracellulare-scrofulaceum* complex (n=2), *M. terrae* complex (n=1), *M. gordonae* (n=1), *M. abscessus* (n=1), *M. asiaticum* (n=1). Overall, 17.5% and 0.03% of samples were positive for atypical mycobacteria for herds with high and low *M. ptb.* seroprevalence (P=0.24). Isolation of atypical mycobacteria from soil was positively associated with high iron content (P<0.001), low pH (P<0.001), low Calcium (P=0.008), and high Manganese (P=0.004). In water, isolation of atypical mycobacteria was positively associated with high Manganese (P<0.001), high Potassium (P=0.002), and low pH (P=0.034). Conclusions: Environmental atypical mycobacteria may be ingested by cattle and possibly cause cross reactions on antibody tests for *M. ptb.*

Using real-time PCR to solve mycobacterial problems

Jim O O'Mahony, C Hill

Abstract

Due to its slow growing nature, detecting *Mycobacterium paratuberculosis* (MAP) in biological samples can be problematic. Moreover, determining viability is fraught with complications and technical difficulties. Our aim was to employ real-time PCR as a means of resolving the fundamental challenges associated with determining viability and estimating mycobacterial numbers. Initially we developed a robust and efficient DNA extraction protocol for mycobacteria from milk and clinical samples. It was found that the combination of a guanidinium based lysis buffer, physical grinding and a silica based purification column worked optimally for mycobacterial DNA. Subsequently, a real-time PCR assay was designed and optimised for the IS*900* region of MAP which had a sensitivity of 40cfu/ml (in less than 3 hours). Quantitative analysis was then carried out on artificially contaminated milk samples using this assay and the correlation between actual and predicted numbers of mycobacteria was assessed. Subsequently, a novel approach was used to assess mycobacterial viability using an assay based on real-time PCR and mycobacteriophages. After designing and optimising a real-time PCR for mycobacteriophage D29, the phage and mycobacterial host *M. smegmatis* were mixed in different ratios. In all cases the initial phage DNA levels were calculated by real-time PCR and samples were removed over time until all mycobacterial cells were lysed. DNA was extracted from samples removed at each time point and the total amount of phage DNA was determined using quantitative real-time PCR. As D29 can only infect living cells, a notable increase in phage DNA was used as an indirect measure of the viability of mycobacteria. We have shown that real time PCR is rapid, delivers reproducible quantitative information and offers a powerful alternative to conventional means of detecting mycobacteria in milk and clinical samples.

Contribution of atypical mycobacteria to false-positive reactions to serum ELISA test for paratuberculosis

Jason Osterstock, Allen J. Roussel, G T Fosgate, B Norby, Elizabeth J.B. Manning, Michael T. Collins

Abstract

Introduction: Serum ELISAs are a key element of herd-based screening programs for paratuberculosis in cattle. The tests have a reported specificity of 96.8 to 99%; however, some herds have an apparent false-positive rate that exceeds what would be predicted based on these specificity estimates. The objective of this study was to evaluate the effect of inoculation of cattle with atypical mycobacterial isolates originating from feces of cattle in herds with demonstrated high false-positive rates using commercially available serum ELISAs. Methods: Nineteen beef calves were selected from a single herd for use in this study. Calves were confirmed to be mycobacterial fecal culture negative using radiometric culture methods and seronegative for paratuberculosis with ELISA 1. All calves in the study had an S/P ratio of 0.00 at the start of the study. Calves were randomly assigned to one of seven treatment groups. Calves in six of the treatment groups were administered an injection of killed mycobacterial isolates in an oil adjuvant and the final group received an adjuvant only control (n=2). Isolates included *Mycobacterium avium* complex (n=3), *M. intracellulare* (n=3), *M. scrofulaceum* (n=3), *M. celatum* (n=3), *M. terrea* (n=3), and *M. avium* subsp. *paratuberculosis* (*M. ptb.*) (n=2). All calves received the injection subcutaneously. Results: Serum ELISA 1 performed four weeks after administration of the isolates demonstrated increases of S/P ratios in all calves receiving *Mycobacterium avium* complex, scrofulaceum, intracellulare, and paratuberculosis. Using the recommended cut-off for positive test results (S/P > 0.25), the following proportions were classified as positive: *M. avium* complex (2/3), *M. scrofulaceum* (0/3), *M. intracellulare* (3/3), *M. ptb.* (2/2). Calves in the control group remained seronegative (S/P = 0.00). Samples evaluated using ELISA 2 remained negative in all calves. Conclusions: Based on these results, it appears that atypical mycobacteria can induce humoral immune responses in cattle that contribute to false-positive serologic reactions.

Application of different methods for the detection of M. avium subsp. paratuberculosis (Map) in Argentina

Fernando A. Paolicchi, M J Zumárraga, A Gioffré, I Etchechoury, C Morsella, K Cirone, A Cataldi, M I Romano

Abstract

Introduction: Paratuberculosis has a high prevalence in Argentina. Some regions of the country show seroprevalence between 2.5 to 51.5 %. The purpose of the present study was to evaluate the efficacy of different methods to detect paratuberculosis in dairy cattle herds in Argentina. Materials and methods: Fifty-two cows were selected from a dairy herd in Argentina. Sera from the animals were analyzed by indirect ELISA, using Map protoplasmatic antigen. Nine recombinant antigens were used in IFN-γ assay, 3 of Map (Lpp24, P34 and Bacterioferritin) and 6 of *M. bovis* (ESAT-6, CFP-10, 2624, 3747, Tpx, Trbb). Responses to recombinant antigens and to aviar PPD were analyzed by an *in vitro* assay for IFN-γ (Bovigam). Faeces, milk and in-line milk filters culture, following decontamination with hexadecylpyridinium (0.75%w/v) for 24 h, were done in tubes containing Herrold's culture medium supplemented with mycobactin (2mg/L) and pyruvate (4g/L). Samples were also tested by IS*900*-PCR. Results: The results showed that 45 out of 52 (86.5%) animals were positive to ELISA. Fourteen animals were culture positive in faeces (14 of 52, 27%). All the animals that excreted the bacteria through faeces were ELISA-positive. Only one of these animals was culture positive in milk. However we were able to isolate one colony from the in-line milk filter at farm level. Nine animals showed significant levels of IFN-γ with aviar PPD (9 of 52, 17%). All IFN-γ positive samples were ELISA-positives and faecal culture positives. Unfortunately, in the present study the use of recombinant antigens in IFN-γ assay did not enhance the specificity of the standard test using aviar PPD. We obtained negative PCR results in all samples. Conclusion: In a control programme, an ELISA test would be a good first step for identifying the affected herd, following of culture of faeces.

Comparative study between the Johne's absorbed ELISA and complemement fixation test for diagnosis of bovine paratuberculosis

Gela Petriceanu, R A Radulescu, A Răgălie

Abstract

Several serological tests (ELISA, CFT, AGID) are available for the detection of antibodies against *M. a. paratuberculosis*. The purpose of this study was to estimate the sensitivity and specificity of two methods for serological diagnosis of paratuberculosis. Materials: The immune responses were investigated on serum samples from 1955 cattle from a contaminated farm and 1923 cattle from a paratuberculosis free area. Methods: CFT: at a serum dilution of 1:5 or higher was considered positive; Johne`s absorbed ELISA for screening and verification: assays were performed with the commercial kits according to the manufacturer`s instructions. Results: Of the 1955 cattle from the contaminated area, 96 (4.9%) samples were positive in ELISA-abs. but in CFT only 22 (1.1%) were positive and 40 (2.0%) samples were doubtful. Concordance of the positive results of the two tests was 2.1% in infected cattle. Of the 1923 cattle from free area in the first year, 35 were positive and 25 doubtful in CFT and in ELISA-abs. only 20 positive samples were detected. At the second control we found in ELISA a smaller number positive samples than in CFT and doubtful samples from first control were negative in the second control. Positive serological results obtained in both lots were confirmed or infirmed by bacteriological and histological analysed. Conclusions: The comparative investigations of the ability to detect antibodies to *M. a. paratuberculosis* demonstrated that ELISA was better than CFT, with sensitivity of 90.5 % compared to 83.8% for CFT and a specificity of 99.4% compared to 96.6 % for CFT. ELISA is after numerous authors, the most sensitive method comparative to CFT and AGID. The reading and computerised interpretation of the results allow the utilisation of the method on large scale in the programme of surveillance and eradication of bovine paratuberculosis.

Validity and costs of pooled fecal culture to detect Mycobacterium avium subsp. paratuberculosis in dairy cows in southern Chile

M Pradenas, J Kruze, Gerdien van Schaik

Abstract

Mycobacterium avium subsp. *paratuberculosis* (Map) is a fairly prevalent disease in the southern part of Chile. For farmers that want to control the disease it is expensive to detect the infected cows with diagnostic tests. One way to decrease the costs is to use pooled fecal culture instead of individual tests. The objective of the study was to compare the validity and costs of pooling with individual fecal culture and an individual ELISA. Individual fecal and blood samples were taken from 50 cows >2 years old in 12 herds located in the south of Chile. In total, data on 598 dairy cows were obtained. Blood samples were tested for antibodies with an ELISA. The fecal samples were cultured for 16 weeks on solid media (HEYM). Both individual samples and pools of 5 and 10 animals were formed and cultured. Colonies suspected to be Map were confirmed with a PCR (IS*900*). The sensitivity of the pools to detect shedding cows was compared with the sensitivity of individual culture and ELISA results. Costs of pooling were compared with costs for individual fecal culture and for individual ELISAs. In total, 7% (42) individual samples were culture positive for Map. The pools with 5 and 10 animals detected 43.2% and 46.4% of the culture positive pools, respectively. The sensitivity differed for the level of shedding (higher for pools with high shedders than for pools with low shedders) and with the prevalence within a herd (higher sensitivity in herds with a higher prevalence). Pooling had a similar sensitivity as an individual ELISA; the moderate to high shedders were detected. However, pooling considerably decreased the costs, which should be weighed against a lower sensitivity compared with individual culture. The optimal testing strategy depends on the prevalence in a herd, the goal of the testing and the willingness to pay of the owner

Comparative evaluation of the results from the surveillance and confirmation methods applied in ruminants' paratuberculosis

R A Radulescu, Gela Petriceanu, A Ragalie, S Popescu, S Ion, A Niculae

Abstract

Evaluations of the methods involved in surveillance and diagnosis of ruminants' paratuberculosis in Romania are presented. The methods used were: complement fixation test (CFT), indirect enzyme-linked immunosorbent assay (iELISA-abs), enzyme immunoassay for the detection of bovine IFN-γ (EIAs-γ-IFN), agar gel immunodiffusion (AGID), delayed-type hypersensitivity (DTH), bacterioscopicy (B), and histological tests (HT). Samples were obtained from 2400 cattle of which 787 were infected and 595 sheep, all from infected flocks. The number of positive and doubtful results obtained in CFT was greater in sheep samples (38.1%) than the cattle samples (5%). ELISA showed fewer positive results: cattle 7.9% and sheep 12.4%. The percentage of cross-reactions (false-positve) was greater in sheep than in cattle. The AGID used in this study was performing approximately equal to CFT. The difference in results of the assays assessing cell-mediated immune response (DTH and EIAs-γ-IFN) was a non-significant, though EIA-γ-IFN was more sensitive than DTH with 5.5% positive. The data processing from the confirmatory tests (bacterioscopy and histology) showed that from 31 examined cattle, acid-fast bacilli were found in faeces and in the tissues of 38.7% of the subjects and only in smears from the intestinal affected mucosa and cut-surfaces of mesenteric lymph nodes in 29% of the subjects. In sheep samples the percentage of the faecal shedders was greater than cattle though in investigated sheep the specific lesions were more discrete than those observed in cattle. **Method Cattle Sheep No. of samples P+D reactions No. of samples P+D reactions** CFT 2400 119 595 227 AGID 125 114 55 21 iELISA-abs 2400 190 595 74 DTH 54 12 20 14 EIAs-γ-IFN 54 15 0 0 AP, B, HT 31 26 55 38 P-positive, D-doubtful, N-negative, AP-anatomopathological findings

Detecting CMI responses to Mycobacterium avium ssp. paratuberculosis infection in calves born to naturally infected dams

S Robbe-Austerman, S Young, A O'Connor, M V Palmer, Judy R. Stabel

Abstract

Ideally the dairy and beef industries should identify and remove MAP infected animals before producers incur the expense of developing heifers. The cell mediated immune (CMI) response to MAP occurs early in infection. Therefore, tests that detect a CMI response (skin reaction against PPD and INF-γ ELISA) may be useful for detecting animals in the early stages of infection with MAP. Our objectives were to evaluate the age a CMI response could be detected in calves and the accuracy and reliability of the skin test and INF-γ ELISA in MAP infected and non-infected calves. Materials and methods: Seventeen calves born from naturally infected Johne's cows were removed at birth, fed unpasteurized milk from their dams, housed separately until weaning and then grouped in two pens according to age. At 4-6 months, 6-8 months and 12-13 months each calf was tested for the presence of CMI response using the skin test and the INF-γ ELISA®. Calves were necropsied at 12-13 months of age and tissues were cultured. Results and discussion: Ten of 17 calves were tissue culture positive for MAP and 7 negative. Of the ten MAP culture positive calves the skin test identified 5 at 4-6 months of age, 4 at 6-8 months and 7 at 12-13 months. Of the ten MAP culture positive calves the INF-γ ELISA identified 2 calves at 4-6 months of age, 4 at 6-8 months and 10 at 12-13 months. Of the seven MAP culture negative calves, all tested negative using the skin test except three animals at 12-13 months. The INF-γ ELISA identified one calf as having CMI response to MAP at 4-6 months of age, 6 as positive at 6-8 months and again at 12-13 months. In tissue culture positive calves the skin test and INF-γ ELISA appears best able to identify a CMI response at 12-13 months. Even though the INF-γ ELISA identified 6 of the 7 non-infected calves as positive, it may be the standard positive cutoff of 0.100 + control plasma is not adequate in young calves.

Comparing the probability of MAP tissue culture positivity in CMI negative and CMI positive sheep

S Robbe-Austerman, Judy R. Stabel, M V Palmer, A O'Connor

Abstract

Our objective was to evaluate the sensitivity and specificity of a previously defined antigen and calculate the relative risk of CMI positive sheep in MAP tissue culture positive sheep. A johnin PPD (NVSL lot 0201) was evaluated in 2 non-infected sheep flocks (288 sheep) and found to have a skin test specificity (Sp) of 93.1 (95% CI 89.5-95.7) and a IFN-γ ELISA Sp of 96.5 (95% CI 93.7-98.3). This PPD was then used for skin testing and the IFN-γ ELISA every 6 months for three years in 4 MAP infected flocks. During the study period, sheep that tested positive twice on skin test and all culled sheep, greater than 6 months of age, were submitted for tissue culture for MAP. 95 sheep were evaluated, 31 were tissue culture positive, 64 were tissue culture negative. Using tissue culture as the reference test, the final skin test prior to culling had Se of 84% (95% CI 66-96) and Sp of 56 (95% CI 43-69). The IFN-γ ELISA had Se of 87 (95% CI 70-96), and Sp of 70% (95% CI 58-82). The prevalence of tissue culture positive animals was substantially higher among skin test positive sheep (48%) compared to the prevalence of tissue culture positive animals among skin test negative sheep(12%) (Relative risk: 4, 95% CI: 2,9). The prevalence of tissue culture positive animals was substantially higher among INF- γ positive sheep (59%) compared to the prevalence of tissue culture positive animals among skin test negative sheep (8%) (Relative risk: 7, 95% CI: 3,19).

Association of atypical mycobacteria with positive ELISA results for paratuberculosis

Allen J. Roussel, G Fosgate, Elizabeth J.B. Manning, Michael T. Collins

Abstract

Introduction: We observed beef herds with an unusually high proportion of cattle that were seropositive to a commercial serum ELISA test for paratuberculosis, but without corresponding clinical disease in the herd's history. Our hypothesis was that mycobacteria other than *M. avium* subsp. *paratuberculosis* (*M. ptb.*) in the cattle's environment caused these false-positive serum ELISA results. Methods: Cattle from 6 herds with previous evidence of unusually high seroprevalence and 3 control herds geographically near an affected herd, but with no seropositive animals on a previous test were selected. All mature cattle in each herd were tested for antibody and fecal shedding. Serum was analyzed using 2 commercial ELISA kits and feces were cultured using the BACTEC7 system. ELISA 1 was the kit used during the previous testing period. Acid-fast organisms were tested by IS*900* PCR to confirm *M. ptb.* Mycobacteria negative for IS*900* were characterized by mycolic acid analysis using HPLC. Results: Results of serology and culture are in Table 1. The proportion of cattle on affected ranches from which non-*M. ptb.* mycobacteria were isolated was significantly greater compared to the proportion on unaffected ranches using a Mann-Whitney test (p< 0 05) The non-*M. ptb.* isolates were submitted for identification to a maximum of 10 per herd. The mycobacteria isolated included *M. scrofulaceum* (n=28), *M. avium* complex (n=17), *M. asiaticum* (n=7), *M. avium* (n=6), *M. celatum* (n=6), *M. intracellulare* (n=3), *M. kansaii* (n=3), *M. mucogenicum* (n=2), *M. nonchromogenicum* (n=1), and *M. terrae* complex (n=1). Conclusions: There is an association between a high seroprevalence to the serum ELISA for paratuberculosis and the prevalence of non-*M. ptb.* mycobacteria in the feces of cattle. This may explain the occurrence of unusually high seroprevalence to the serum ELISA in some herds without a corresponding prevalence of infection with *M. ptb.*

Table 1. Median (range) of cattle positive to serologic tests and fecal culture

	% pos ELISA 1	% pos ELISA 2	Proportion of cattle with non-Map	Proportion of cattle with *M. ptb.*
Affected	0.33 (0.10-0.75)	0.10 (0.02-0.40)	0.22 (0.10-0.72)	0.02 (0.02-0.07)
Control	0.04 (0.04-0.06)	0.00 (0.00-0.02)	0.04 (0.00-0.06	0.00

Application of a rapid DNA extraction method with real-time fluorescence PCR for the detection of Mycobacterium avium subsp. paratuberculosis in raw milk

Juliana Ruzante, W L Smith, C V Jaravata, Ian Gardner, J S Cullor

Abstract

The screening of milk for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) DNA using PCR can be problematic due to the presence of agents in raw milk that can inhibit or hinder the PCR reaction. The purpose of this study was to evaluate a DNA extraction method commonly used in forensic science, for its potential application in screening for MAP DNA in bovine raw milk. Bulk tank milk from a herd repeatedly tested negative for Johne's disease was spiked with different log concentrations of MAP (from approximately 10^4 to 1 CFU/ml). Forty five milliliters of the sample was centrifuged and the pellet was placed onto FTA card media (Whatman®) for DNA extraction. Total DNA was eluted from the card after appropriate washing steps, and real time PCR using fluorescence resonance energy transfer (FRET) probes (Roche Lightcycler®) targeting the IS*900* sequence was performed. The assay was able to reliably detect samples containing approximately 2 logs CFU/ml MAP and was able to detect samples containing only one log CFU/ml in a majority of spiked samples. The DNA extraction and PCR protocol was able to be completed in 4 hours and also lends itself well to high sample throughput and the archiving of samples. The novel protocol can potentially contribute to the improvement of MAP control programs and to the assessment of the quality and safety standards of milk and dairy products since it provides a rapid screening method for both hospital and bulk tank milk, reducing the numbers of samples to be cultured.

Comparison of ELISAs on milk and serum for the detection of paratuberculosis in dairy goats

Miguel Salgado, J Kruze, Michael T. Collins

Abstract

Early detection of *Mycobacterium paratuberculosis*-infected animals is important for infection control and reduction of economic losses. Serologic tests using ELISA technology are popular because they are fast, inexpensive, and easily automated. Use of milk instead of serum for antibody detection could potentially make paratuberculosis surveillance even easier, more affordable and thus more accessible for the dairy goat industry. The aim of this study was to determine whether ELISA results obtained on milk can predict those for serum using a commercial ELISA kit on samples from dairy goats. Eight chilean goat herds were selected for participation in this study. Blood and milk samples were collected from all female goats > 2 years-old from October 2004 to February 2005; 394 total goats. Serum and milk samples were tested in duplicate wells using a commercial *M. paratuberculois* antibody test kit (IDEXX laboratories, Inc). Association between categorical milk and serum ELISA results (using S/P ≥0.25 as positive cut-off) was evaluated using McNemar's Chi-square test and agreement using the kappa statistic. Correlation between serum and milk ELISA S/P values was evaluated by linear regression. All statistical analyses were done using GraphPad InStat Program version 3.00. Six of the 8 herds evaluated, showed at least one positive result for either serum or milk ELISA. Out of 394 animals sampled, 66 (17%) were ELISA-positive using serum and 37 (9.4%) were ELISA-positive using milk samples. Only 2 herds had 100% of animals with negative results for both sample types. Categorical results on all 394 paired milk and serum samples tested were in good agreement (kappa = 0.68). Linear regression of S/P values on results for paired serum and milk samples showed moderate correlation (r^2 = 0.40). These results suggest that the ELISA used to test milk samples may be a potentially useful and convenient method to estimate herd prevalence of paratuberculosis in dairy goat herds.

Development and evaluationof a Real Time-PCR-method for the detection of Mycobacterium avium ssp. paratuberculosis

H Schönenbrücher, A Abdulmawjood, M Bülte

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) has been identified as the causative agent of paratuberculosis (Johne´s disease) in ruminants. The involvement of this microorganism in Crohn's disease (CD) in humans has been uncertain. Food (e.g. milk and milk products) has been discussed as a potential vector. A basic requirement for the generation of prevalence data of MAP is the development of reliable detection methods. Until now the investigations of food as a possible vector for the pathogen have been focussed on milk and milk products. The authors present a development and validation of a new Real Time PCR-Assay for the specific detection of MAP. In the present studies different MAP-specific markers have been included. The method will be used as a methodical platform for the analysis of bovine fecal-, bovine tissue samples, human biopsies and food (including meat and meat products). To circumvent the inhibitory effects of the matrices, different sample preparation techniques are evaluated. *The work is part of the research project "Human-Food-Environment-Mycobacterium avium ssp. paratuberculosis and Morbus Crohn? (funded by Ministry of Science and Arts, Land Hessen, Germany, http://hrzntweb-v1.hrz.uni-giessen.de/forschungsbericht)".*

Genomic polymorphisms for diagnostics of Mycobacterium avium subspecies paratuberculosis

M Semret, D C Alexander, P de Haas, P Overduin, D van Soolingen, Debbie Cousins, M A Behr

Abstract

M. avium subsp. *paratuberculosis* is an emerging pathogen of mammals, and is actively being investigated as a possible zoonotic agent. The lack of reliable diagnostic assays has hampered rational assessment of the prevalence of this organism in humans and animals. We have used a comparative genomics approach to reveal genomic differences between *Mycobacterium avium* subsp. *paratuberculosis* and its close relative *M. avium* subsp. *avium*, a highly prevalent environmental organism. From computational and DNA microarray-based study of two prototype strains, *M. avium avium* strain 104 and *M. avium paratuberculosis* strain K10, we have uncovered 2 types of large sequence polymorphisms (LSPs); those present in the former but missing in the latter (LSP^{A}s), and those only present in the latter (LSP^{P}s). We examined the distribution of 3 LSP^{A}s and 17 LSP^{P}s across a panel of 383 *M. avium* complex isolates in order to determine their potential utility for development of accurate diagnostic tests. Our results show that absence of 2 of the LSP^{A}s (LSP^A 8 or LSP^A 14) was 100% specific for the identification of *M. avium paratuberculosis*. In contrast, only 7 of the 17 LSP^{P}s show a comparable degree of specificity. Of these 7, LSP^{P}2 and LSP^{P}15 were also highly sensitive for the identification of *M. avium paratuberculosis*, while the remaining 5 LSP^{P}s were only variably present in *M. avium paratuberculosis*. These data indicate LSPs best suited for diagnosis of *M. avium paratuberculosis* and highlight the limitations of in silico-driven comparisons of prototype sequences for development of diagnostic assays.

Comparative genomics of M. avium subsp. paratuberculosis strains isolated from different host species

M Semret, S Sreevatsan, D M Collins, M A Behr

Abstract

M. avium subsp. *paratuberculosis* (MAP) is an emerging pathogen of livestock and other mammals, in which it causes inflammatory bowel disease. Transmission from farmed animals, usually from cows, to wildlife or other "unusual" hosts has been well documented through the use of genotyping methods. However some strains seem to cluster with specific hosts. For instance sheep (S) strains, and some strains infecting bison (B), appear to be distinct from classical cattle (C) strains both phenotypically (more difficult to culture) and by molecular typing methods (IS*1311*-based). The basis for these genotypic and phenotypic differences is poorly understood. From whole genome DNA microarray-based comparative genomic studies, we have determined that there is extensive genomic diversity among the subspecies of the *M. avium* complex, with large sequence polymorphisms (LSPs) differentiating between MAP and other members of the complex. Since analysis of a restricted set of strains has revealed that MAP strains form a relatively homogeneous group, we hypothesised that LSPs may be a source of genetic diversity among phenotypically and genotypically distinct strains. To that end, we performed DNA comparisons of 3 S strains, 3 C strains, and one bison isolate, using *M. avium* subsp. *avium* (MAA, strain 104) as the referent. In addition to previously described LSPs, we found that a 16kb sequence, present in MAA 104 and in S strains, appears to be missing from the C strains and the bison isolate tested. Conversely, a 7kb sequence present in MAA 104 and C strains appears to be missing from the S strains tested. These results suggest that some LSPs may discriminate between different MAP strains. Confirmation and testing across a larger number of isolates is underway.

Comparative evaluation of culture, ELISA and PCR in screening of milk samples for the detection of Mycobacterium avium subsp. paratuberculosis infection in lactating goats

Shri N. Singh, S Kumar, A V Singh, I Sevilla, Ramon A. Juste, V K Gupta

Abstract

Prevalence of MAP infection in goat's milk of organized and farmer's herds was studied using culture and PCR and plate-ELISA. Milk samples (53) were from (3 goat units) of organized herds (CIRG), whereas 255 milk samples from CIRG and 67 from nearby villages were tested by milk ELISA. Fat and sediment layers were cultured and DNA was isolated and whey tested for ELISA. Antigen was prepared from *Mycobacterium avium* subsp. *paratuberculosis* (bison type) strain S 5 of goat origin. DNA isolated from decontaminated milk samples, was amplified using IS *900* primers. PCR and products were of 229 bp as in case of standard MAP S 5 (Bison type) strain. Incidence of MAP milk samples from organized goatherds was 69.8% (43.3% in fat and 45.2% in sediment) by milk culture and 54.7% by milk ELISA whereas, IS*900* PCR on fat and sediment layers (106) of milk samples, detected 48.6% positive goats. Incidence of MAP infection in the milk samples of apparently healthy Sirohi, Marwari, Jakhrana and Barbari goat breeds was 66.6%, 75.0%, 33.3%, 80.0% and 66.6%, 50.0%, 33.3% and 20.0% in milk culture and milk ELISA, respectively. Incidence in apparently weak Jamunapari goats was 69.2% by culture of milk samples (fat = 23.0% and sediment = 69.2%) and 76.9% by milk ELISA. Culture of fat and sediment layers exclusively detected 35.1% and 37.8% positive goats, respectively. Positive goats were pauci and multi bacillary/. Maximum colonies appeared around 75 days post incubation. However colonies continue to appear up to 120 days of post incubation. Milk samples (241) of lactating goats from 5 organized herds milk ELISA test was 70.1 % whereas in farmer's herds 58.2%. Incidence was 30.0, 58.8 and 38.8 percent in Makhdoom, Jhandipur and Farah villages, respectively. Culture was superior than milk ELISA and PCR for the detection of MAP infection in goatherds.

Differential expression of genes encoding CD30L and P-selectin in cattle with Johne's disease: Progress toward a diagnostic gene expression signature

K Skovgaard, C B Pudrith, S N Grell, P M Heegaard, Gregers Jungersen, Paul M. Coussens

Abstract

Commonly paraTB in cattle is diagnosed by serum enzyme-linked immunosorbent assay (ELISA), by detection of the pathogen by cultivation of individual faecal samples, or by *in vitro* measurement of cell mediated immune responses using the IFN-γ test. There is an ongoing need for developing new diagnostic approaches as all currently available diagnostic tests for paraTB may fail to detect sub-clinical infection. We used cDNA microarrays to simultaneously measure expression of over 1,300 host genes to help identify a subset of gene expression changes that might provide a unique gene expression signature for paraTB infection. In the present study, non-stimulated total leukocytes isolated from ten sub-clinical paraTB infected cows were examined for genes being expressed at significantly different levels than in similar cells from control cows with the same herd background. We included cattle (Holstein) from two locations (Denmark and USA) for the microarray experiment. Our results indicate that expression profiles of at least 52 genes are different in total leukocytes from *M. paratuberculosis* infected cattle compared to control cattle. Gene expression differences were verified by quantitative real-time reverse transcriptase polymerase chain reactions (qRT-PCR) on the same group of cattle (Holstein) used for the microarray experiment. In order to assess the generality of the observed gene expression, a second and different group of cattle (Jersey) was also examined using qRT-PCR. Out of the seven genes selected for qRT-PCR, CD30 ligand (CD30L) and P-selectin were differentially expressed in freshly isolated total leukocytes from paraTB infected and control animals of both breeds of cattle. Although further work is clearly needed to develop a more complete gene expression signature specific for paraTB, our results demonstrate that indeed a subset of genes in total leukocytes are consistently expressed at different levels, depending upon *M. paratuberculosis* infection status.

Testing for Mycobacterium avium subspecies paratuberculosis infection in asymptomatic milk herd

J Szteyn, A Wiszniewska

Abstract

For the first time paratuberculosis of cattle appeared in 1970. Since then, the competent authorities have not recorded any cases of that disease. Currently, as in the other European Union countries, paratuberculosis is a disease that is subject to compulsory reporting. During routine examination of cattle in the area of Z.u?awy for tuberculosis an increased number of cattle with positive reaction to avian tuberculin in skin test was recorded. That formed the base for conducting examinations for paratuberculosis. During the first stage, three herds of dairy cattle where positive reactions to avian tuberculin had been recorded were selected for further examination. Blood for serological tests using ELISA test by IDEXX was collected from animals older than two years. The first tests were carried out in 2001 and presence of antibodies specific for MAP was found in all three herds. The serological tests were repeated at 6-month interval. Presence of antibodies specific for MAP was confirmed in all three herds. The number of animals with positive results of ELISA test was: in the first herd 1 (0.65%), in the second 1 (0.80%) and in the third 2 animals (22.2%.). Unfortunately between the first and the second testing both animals tested positive in herd three were sold. After 6 months the following results of ELISA tests were obtained: in herd one 2 doubtful reactions (S/P between 0.15 and 0.30), in herd two 2 positive and 6 doubtful while in herd three there were no positive or doubtful reactions. The third round of tests gave 2 animals tested positive and 2 doubtful in heard one, one positive and 3 doubtful in herd two and 1 positive in herd three. The examinations confirmed presence of paratuberculosis in herds of dairy cattle. The lack of appropriate diagnose of the disease was the reason for spread of paratuberculosis in the cattle herds. Elaboration of a program for control of paratuberculosis in Poland seems to be required. *Supported by WAMADAIREC project OLK1-CT-2002-30401*

Detection of Mycobacterium avium subsp. paratuberculosis IS900 in lesions of bovine paratuberculosis by a supersensitive in situ hybridization technique

Shogo Tanaka, T Kai, N Yamada, K Kawasaki, M Sato

Abstract

In process of intercellar survival of *Mycobacterium avium* subsp. *parutuberculosis* (MAP) during asymptomatic phase of the infection, it becomes harder to demonstrate MAP in tissue sections by established histological and immunohistochemical techniques by reason of transformation into cell wall deficient forms of the mycobacteria. In order to address this issue, a supersensitive in situ hybridization (ISH) technique was employed. Formalin-fixed and paraffin-embedded mirror sections of mesenteric lymph nodes were obtained from 5 cows clinically infected with MAP. A FITC-labeled double strand PCR probe targeted for MAP IS*900* was synthesized using the DNA extracted from ATCC19698 strain by PCR technique with a pair of reported primers. Slides were deparaffinized and treated with 10 µg/ml of proteinase K at 37ºC for 20 min, following incubation with hybridization solution containing 1 µg/ml of the purified probe for 16 hr at 50 °C. After wash with 5x, 2x and 0.2x SSC, immunological detection and visualization of hybridized probe were performed by a tyramide signal amplification method using a commercial kit (GenPoint™ Fluorescein, DakoCytomation, Tokyo). Specific signals for MAP IS*900* were detected in macrophages, epithelioid cells, multinucleate giant cells and Langhans-type giant cells located in the cortex of mesenteric lymph nodes. Positive IS*900* signals were also found in small number of macrophages in the medullary sinus and macrophages infiltrating in the perivascular connective tissue. Interestingly, positive signals were detected in epithelioid cell tubercles in which no acid-fast bacilli were found by Ziehl-Neelsen method and immunohistochemistry for the mycobacteria.To confirmspecificity of positive IS*900* signals, competitive in situ hybridization using the FITC-labeled probe and non-labeled probe was performed. This tyramide signal amplification ISH procedure provides a way to detect cell wall deficient forms of MAP and to investigate the pathogenicity of this type of the mycobacteria.

A light cycler based real-time PCR assay for the detection of Mycobacterium avium subsp. paratuberculosis in food samples

T Tasara, R Stephan

Abstract

Significant public health concerns have been raised that *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the etiological for Johnes' disease in world dairy and beef herds, may also be involved in some cases of human Crohn's disease. Therefore animal food products are potential sources for human infection with MAP. PCR methods are potential tools for rapid monitoring animal food products for MAP contamination but a number of challenges remain. These include sample material associated PCR inhibition as well as MAP DNA template isolation difficulties in food matrices. Moreover, the IS*900* element targeted by most of the current MAP PCR protocols may not be highly specific for MAP organisms. In order to address some of these challenges, we developed a real-time PCR protocol that amplifies the f57 sequence as an indicator for MAP presence. This PCR method incorporates an internal amplification control template to monitor for potential PCR inhibition or failures that would otherwise lead to false negatives in routine PCR screening of food samples. The developed assay's specificity was confirmed through analysis of 10 MAP isolates and 63 isolates of other bacterial species. This real-time PCR assay has a linear quantitative amplification range from 2×10^1 to 2×10^6 copies of MAP f57 target on purified DNA. In parallel an optimal strategy for MAP DNA isolation from artificially contaminated milk samples was also developed. The combined protocols when applied to MAP spiked raw milk samples, a minimum detection limit of 10 MAP cells per ml was achieved starting from 10 ml samples. This entire procedure can be accomplished within a single working day making this an ideal assay for rapid large scale MAP monitoring. The assay's performance in naturally contaminated meat and milk samples is currently under evaluation on samples collected from the slaughter houses and raw milk bulk tanks throughout Switzerland

Comparison of BACTEC and MGIT systems for detection of M. paratuberculosis

G Thomas, Elizabeth J.B. Manning, Michael T. Collins

Abstract

The BACTEC 460 system (modified 12B medium and 460 instrument) was the first liquid culture system adapted for detection of *M. paratuberculosis* (*M. ptb.*). It has proven to be fast and reliable for both detection and quantification of the organism from clinical samples. This system is no longer in commercial production and has been replaced with a non-radiometric system; a culture medium designed specifically for *M. paratuberculosis* growth called the Mycobacterial Growth Indicator Tube (MGIT) paraTB and an instrument, the MGIT 960. The purpose of the present study was a head-to-head comparison of the BACTEC 460 and MGIT 960 systems on 1,128 consecutively submitted clinical samples (feces and tissues). The samples originated from domestic and wild animals from diverse locations (21 U.S. states). Samples were split into two portions and processed using the previously described HPC-filter concentration protocol. All cultures that gave a positive signal in their respective instrument were subcultured on a blood agar plate to detect contaminating microbes and stained (Ziehl-Neelsen) for detection of acid-fast bacteria (AFB). AFB-positive cultures were subcultured to HEY with and without mycobactin and also tested by multiplex PCR to identify *M. paratuberculosis*. In total, 921 (81.6%) of samples were signal-negative by both systems. Contamination rates (no AFB detected) were 6.8% for MGIT cultures and 5.9% for BACTEC cultures. AFB were detected by one or both systems from 102 (9.0%) samples. Of these 102 AFB-positive samples, from 23 (22.6%) both systems detected *M. ptb.*, from 16 (15.7%) only MGIT detected *M. ptb.*, from 5 (4.9%) only BACTEC detected *M. ptb.*, and for 58 (58.9%) the AFB detected were mycobacterial other than *M. ptb.* The MGIT system was more sensitive ($p<0.05$) and faster (23 versus 33 days to detection; $p<0.009$) than the BACTEC system. MGIT technology is an improvement over BACTEC system for *M. ptb.* detection.

Validation of the Pourquier ELISA to detect antibodies against Mycobacterium avium subspecies paratuberculosis in individual milk samples and bulk milk samples of Dutch dairy cows and herds

C van Maanen, Gerdien van Schaik, A van der Meulen, M Waal, P Franken

Abstract

The objective of the study was to determine the diagnostic performance of the Pourquier ELISA for detection of antibodies against Map in individual milk samples and in bulk milk samples as compared with serum samples/seroprevalence. Milk and serum samples were tested and results were interpreted according to the instructions of the manufacturer. For individual milk samples the relative sensitivity was estimated by testing a panel of paired serum-milk samples from seropositive cattle (n=80), and the specificity was estimated by testing a panel of 500 individual milk samples from Map-free certified herds. Feasibility of bulk milk testing was investigated by titrating 65 positive individual milk samples in negative milk. Also 400 bulkmilk samples from herds with a known within-herd seroprevalence were tested. Specificity of bulkmilk samples was investigated by testing 100 bulkmilk samples from Map-free certified herds. Although S/P values of individual milk samples were clearly lower than those of the corresponding serum samples, S/P values correlated well (r = 0.87). The relative sensitivity for individual milk samples as compared with serum samples was 80 % at the cut-offs recommended by the manufacturer. Titrations of positive milk samples showed a clear relationship between S/P value and titre. Although there was a fair correlation between S/P values of bulk milk samples and within-herd seroprevalence (r = 0.70), the agreement between bulk milk results and herdstatus (2 or more seropositive cows) was poor, corresponding with a sensitivity of 30 % and a specificity of 99.4 % at the cut-off recommended by the manufacturer. In conclusion, the diagnostic performance of the Pourquier ELISA for individual milk samples creates opportunities for a cheaper and more feasible testing scheme, while the diagnostic performance for bulk milk samples is still under evaluation, but seems to be less promising.

Field validation of the Pourquier ELISA to detect fecal shedding of Mycobacterium avium subspecies paratuberculosis in Dutch dairy cows

Gerdien van Schaik, C van Maanen, P Franken

Abstract

The objective of the study was to determine the validity of the Pourquier ELISA to detect Map shedding Dutch dairy cows corrected for possible factors that can influence the performance of the ELISA. The dataset consisted of the test results of 1027 cows from 16 infected Dutch dairy herds that participate in a Map control program and the test results of 1057 cows in 22 Map-free farms that participated in a Map certification program. On these farms, blood- and fecal samples were collected from all dairy cattle over 3 years old. The serum was tested with the Pourquier ELISA and the fecal samples were cultured on solid media for 16 weeks. Logistic regression models (culture positive (1) or negative (0)) were used to determine the optimal cutoff values and the validity of the ELISA with the ELISA S/P value, age, herd size, test date, herd ID, and prevalence as covariates. The cumulative probability of being a fecal shedder of 20%, 50% and 80% were used to derive the cutoff values. Age, herdsize, test date and herd were not significant and only the ELISA S/P value and the within-herd prevalence were positively associated with fecal culture result. The Pourquier ELISA was a highly specific (99.3%) and fairly sensitive ELISA (51.3%) relative to fecal culture. The ELISA detected 75% of the moderate to high shedders and the specificity did not significantly increase when based on certified free-herds only relative to all fecal culture negative cows. Optimal cutoff values differed between herds that differed in prevalence and the infection status of an individual cow should be based on a herd-specific interpretation of the ELISA. In conclusion, the Pourquier ELISA is a good test for a control program because the low proportion of false positive results and the large proportion of the moderate to high shedders that are detected.

Production impact of sub-clinical manifestations of bovine paratuberculosis in dairy cattle

M A Villarino, E R Jordan

Abstract

Bovine paratuberculosis (Johne's Disease) is a chronic debilitating disease of cattle. The disease is widely distributed and dairy veterinary practitioners and dairy producers are becoming aware of the benefits of establishing Johne's Disease control measures. Although there is no doubt that the clinical disease can cause significant economic losses, the sub-clinical consequences of the disease are not well documented. The objective of this investigation was to evaluate the production impact of sub-clinical manifestations of Johne's Disease in dairy cattle based on sero-conversion using an ELISA test. To accomplish this objective, a retrospective cohort study in a commercial dairy located in northwest Texas was conducted. Production data were collected starting April, 2001 using a paired comparison scheme. The cohort consisted of cows of similar age (+/- 30 days), lactation, and origin. Production parameters (lifetime milk production, milk per lactation) and individual performance parameters (days in milk, days in dairy, cull date and reasons for culling) were collected from 120 ELISA positive and 120 ELISA negative for Johne's Disease cows, for as long as the animals were maintained on the premise. Statistical comparison (t-test) and linear regression analysis on the obtained data were conducted. Our results to date indicate a significant reduction in milk production (4,090 kg lifetime milk production per animal) from ELISA positive cows when compared to ELISA negative cows. Also, more ELISA positive cows became lame (5x), developed respiratory disease (1.25x), digestive disease (1.83x), and mastitis (2x) than ELISA negative cows. Currently, 28.33 % of the ELISA positive and 60.34 % of the ELISA negative animals remained in the herd. The reduction of milk production in the ELISA positive cow started in the second lactation and remained for the rest of her productive life, regardless of when the cow was detected as ELISA positive.

Large scale detection of Mycobacterium paratuberculosis (M.ptb) in faecal samples using immunomagnetic capture and TaqMan PCR

Peter Willemsen, R Ruuls, M Damman, Douwe Bakker

Abstract

Objective: Development of a rationalized approach for a large scale detection of M.ptb from faecal samples. Materials and methods: A primer pair (mpf/mpr) and probe was developed for the detection of IS*900*, which complied to TaqMan PCR demands avoiding false priming to closely related mycobacterial IS sequences. Furthermore, a mimic DNA template was developed which enabled detection of PCR inhibition while not interfering with IS*900* detection. M.ptb was isolated from faecal samples using immunomagnetic separation (IMS) with monoclonal antibodies directed against surfaces antigens of M.ptb. Determination of sensitivity was carried out using spiked faecal samples and samples with known cultural status. M.ptb cells for the purpose of spiking were obtained from liquid cultures by filtration through a 5 µm filter and subsequent harvesting in PBS containing 0.05% Tween-20 and 10 % glycerol. The amount of cells/ml was determined microscopically in a counting chamber (Weber Scientific International Ltd., England). Results: Using IMS, in combination with RT-PCR, an average of 10-100 cells per gram of faeces could be detected. Since cultural data show that one cfu corresponds to clumps of 10- 50 cells this sensitivity could also be expressed as ~1 cfu per gram of faeces. Comparison using a limited set of faecal samples with a known cultural status showed a good agreement (κ=0.76). Although subsequent implementation of the IMS protocol for a robotized screening on a Biomek FX(Beckman) requires a reduction of sample size this approach still facilitates a large scale and high-through put screening of faecal samples detecting 10 cfu's/gram faeces.

Mycobacterium avium subsp. *paratuberculosis* – its plans for survival

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For over a century research workers have been extending our knowledge of paratuberculosis, the disease caused by *Mycobacterium avium subsp. paratuberculosis* (MAP). In this paper I aim to review how this organism has developed a "lifestyle" that ensures its continuing survival and therefore presents a challenge to scientists, veterinarians and the livestock industries.

So what are these plans for survival? As I see it MAP has the ability to infect various host species with variable affects and has developed the means of perpetuating itself within these host species by *intraspecies transmission*. Such is its desire to survive that MAP has also developed the ability to be spread from one species to another (*interspecies transmission).* To achieve either or both these transmission routes the organism has relied on its ability to survive in the *environment* – the third element which I cover in this paper.

INTRASPECIES TRANSMISSION

Although paratuberculosis is primarily a disease of domestic ruminants, with cattle being the most economically significant host worldwide, infection with or without overt disease has been recorded in ruminant and non ruminant wildlife species (Ayele et al, 2001, Beard et al, 2001) and in some monogastric animals.

As an enteric pathogen it is not surprising that the major route of intra-species transmission is considered to be the faecal–oral route. MAP has included in its plans for survival its ability to infect an animal and be excreted in normal faeces before clinical signs become apparent and laboratory tests are able to confirm the infection. However as the disease progresses in domestic ruminants, affected animals shed increasing numbers of the organisms. Bovine faeces becomes increasingly liquid thus disseminating the organism more widely over their bodies and in the environment. The fact that in cattle the disease is precipitated by parturition facilitates widespread dissemination of infection via the liquid faeces to newborns. That the disease may go into remission until the next parturition allows MAP the opportunity to infect two calf crops before the dam succumbs.

With coprophagic animals e.g. the rabbit, the faeco-oral route of transmission does not have to rely on the chance encounter of the organism with its host. In the rabbit, coprophagia is a routine practice between the lactating doe and its offspring, thus ensuring infection of the next generation at what is generally considered the most vulnerable age.

Table 1. Intraspecies transmission

	Faeco-oral	Intra uterine	Milk	Semen
Cattle	+	+	+	+
Sheep	+	+	+	?
Goats	+	+	+	?
Deer	+	+	+	?
Rabbits	+	?	+	+

Faecal-oral transmission was for some time considered the sole route of intraspecies transmission. However MAP was already a few steps ahead and had developed more direct and more certain routes of infecting the next generation namely across the placenta and in colostrum and milk. It has been shown that in cattle the risk of transmission through transplacental and milk routes increases with the advancing stages of the disease. Risk is low in the early stages rising to 40 per cent of foetuses being infected in clinical cases with similar levels of infection being recorded for milk and colostrum (Giese and Aherns, 2000). More recent studies have shown that transplacental infection occurs in sheep (Lambeth et al, 2004), goats (de

Juan et al, 2005), deer (van Kooten et al, 2005) and possibly rabbits. (Judge et al, 2005a) MAP has been recovered from milk samples from sheep, goats, rabbits and humans (Naser et al, 2000). Could it be that human foetuses are also infected in utero since the human placenta has the most intimate relationship with the uterine vessels and MAP has been recovered from the blood of patients with Crohn's disease (Naser et al, 2004)?

What is the outcome of in utero infection? Does it progress to disease or does the foetus tolerate the infection? What is the outcome of a low level infection over a long period as occurs with milk? There is minimal information available to help us answer these questions.

INTERSPECIES TRANSMISSION

On a worldwide economic basis cattle are the most important species to be affected by MAP with sheep in second place and other farmed ruminants being well behind. The ability of MAP to be transmitted from one species to another requires exposure (through shared grazing or milk) and a strain of MAP that can survive in multiple hosts. Therefore grazing management of farmed livestock is a major factor in transmission among domestic species. Thus in many areas across the world dairy cattle are kept exclusively on units but the situation for beef cattle varies across the globe. In some areas the situation will be very similar to that for dairy cattle but in other areas beef cattle and sheep frequently cograze – a situation encouraged for the control of internal parasites. Similarly small ruminants may be managed in exclusive situations while in others, particularly in extensive systems of management, sheep and goats may share the same pastures.

As for potential spread between domestic livestock and wildlife the opportunity of interspecies transmission will vary depending on the opportunity for the species to share grazing. In many parts of the world various species of wild deer and feral goats will graze the same pastures as domestic cattle, sheep and goats. In Scotland rabbits are a major wildlife species on many beef cattle and sheep enterprises.

One major factor which facilitates interspecies transmission in animals is they do not avoid the faeces of other grazing species, in sharp contrast to the absolute aversion by domestic ruminants to faeces of their own species. This was demonstrated by Daniels et al (2001) and later Judge et al (2005b) and their colleagues in Scotland that calves will ingest significant numbers of rabbit faecal pellets, one of which can carry an infective dose. Small ruminants have more selective grazing habits. The list of wildlife shown to have been infected with MAP continues to grow. Many will be dead-end hosts: although they are infected either they don't excrete MAP or excrete the organism at very low levels and/or their faeces are likely to be repulsive for grazing ruminants e.g. foxes and stoats who eat infected rabbits.

Interspecies transmission can also occur via milk. It is not unknown for lambs and kids to be fed cows' milk or for goats' milk to be fed to lambs. Humans of course drink the milk from cattle, goats and sheep and eat cheese made from such milk. Many studies have been conducted on the effect of pasteurisation of MAP infected milk and it would appear that the pasteurisation standards set for *Mycobacterium bovis* are insufficient for its near relative MAP.
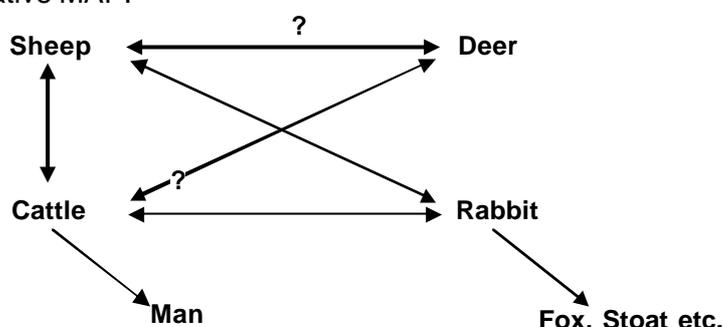

Fig. 1. Interspecies transmission in the wild

At this and recent colloquia we have heard of improving methods of typing different isolates of MAP. It would appear that some strains of MAP show species specificity but there are others which can be recovered from a variety of domestic species and wildlife. (Motiwala et al, 2004) In addition to providing the

essential evidence to support interspecies transmission with animals these improved typing methods are invaluable in establishing the potential zoonotic role for MAP.

A summary of the possible interspecies spread of MAP in the wild is shown in Fig. 1.
Interspecies transmission may occur, as with the case of rabbits and cattle by direct ingestion of faecal material. In many cases interspecies transmission will depend on the dispersion of MAP from faecal material onto the pasture thus creating a contaminated environment.

THE ENVIRONMENT

The environment, to include grazing area and water supplies, is the pool into which MAP is shed by infected animals. What happens to MAP in this pool is very much dependent on the ability of MAP to survive outside the body of its hosts under varying conditions of temperature, humidity, pH and irradiation of the cell wall and soil type. The hardiness of the organism in the environment is a reflection of the structure of the cell wall of the organism – another plan of MAP.

Early studies in the northern hemisphere and more recent studies in Australia (Whittington et al, 2004) have shown that MAP is capable of prolonged survival on pasture, this being up to 55 weeks in a dry fully shaded environment with much shorter times in unshaded locations where infra red and ultra violet is considered the detrimental component of sunlight. Over the period of the field experiments it was noted that not only was there a reduction in the number of culture positive samples but there was an increase in the incubation period required in culture to reach peak growth index, findings consistent with a gradual decline in the viability of the organism. However, MAP is not beaten; it appears to ultimately go through a dormancy period induced by unfavourable environmental conditions, which when they become favourable again, allow the bacterial cells to regain their ability to divide and thus become detectable. Whether these cells retain their ability to infect susceptible hosts has not been determined.

The effect of rainfall on the distribution of MAP may be dependant on the form of faeces passed by hosts (e.g. large cow pats, trail of liquid faeces, individual pellets etc.) The type and structure of the soil and the slope of grazing areas could result in MAP being leached from the faecal mass into the soil or could run off the surface. In either case there is a potential for contamination of rivers, lakes and reservoirs, the source of drinking water for animals and humans alike. Earlier in the conference we heard of studies in Wales by Pickup and colleagues and in Northern Ireland by Rowe and colleagues of the recovery of MAP from water. That MAP survives in reservoir sediments for up to 50 years again demonstrates its tenacity. However field studies in Australia (Whittington et al – 2003) have shown the infectivity of soil/pasture and water/sediment samples to decline significantly over a five month period.

It was generally considered that an acid pH favoured the survival of MAP and thus the application of lime (basic pH) would be detrimental. However the recovery of organisms after 72 hours at pH 12.5 in calcium hydroxide treated slurry shows that MAP is tougher than both *M. bovis* and *Brucella abortus*. (Flynn et al, 2005).

Within animal buildings there is widespread faecal contamination of floors, walls, feed and water troughs. In MAP-infected herds the level of bacterial contamination reflects the prevalence of MAP in the animal population. Direct deposition of faeces in feeding/drinking receptacles may occur and the organism may be carried on footwear and vehicle wheels to other parts of the livestock unit.

The ingenuity of MAP is further shown by the recovery of the organism from nematode larvae (Whittington et al, 2001), larvae of *Eristalis tenax* and adult *Diptera* (Kopecna et al, 2005) thereby suggesting a possible vector role for invertebrates in the transmission of MAP.

In this brief review on the transmission of MAP I have endeavoured to give examples of how this organism has established itself in a large number of animal species through intra and interspecies spread and its survival tenacity in the environment. What is clear is that factors that affect the ability of MAP to be introduced and maintained in animal populations are many and varied. The challenge is therefore for us to

develop practices to prevent the infection of susceptible animals with MAP. Adopting improved farm management practices would appear to be a crucial first step.

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Epidemiology of Johne's disease: Recent developments and future trends

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ABSTRACT

Because of the prolonged incubation period and associated difficulties in studying Johne's disease, the epidemiology of this condition is still not well understood, despite the efforts of many researchers over the years. However, with the advent of new technologies and methods, progress in understanding the epidemiology of this complex disease is still being made.

Recent studies have focused on factors affecting transmission of infection, particularly levels of excretion, intrauterine transmission and transmission via semen and milk in sheep, and the role of recently infected young cattle in transmission. Risk factors for herd and animal level infection are also of considerable interest, although no clear pattern of identifiable risk factors have emerged, other than herd size and numbers of introductions. Further studies on transmission and risk factors will hopefully progressively clarify some of these issues over the next few years.

The currently available screening tests for Johne's disease are limited by the chronic nature of the disease, and their relatively poor sensitivity, particularly in the early stages of infection. Recent developments have led to refinement of methodologies and sampling strategies to improve the efficiency and cost-effectiveness of the current tests, particularly for herd and flock screening. However, new technologies and techniques are required before any substantial improvement in test performance at the individual animal level can be expected. These advances will hopefully arise over the next few years from ongoing studies using genomic and proteomic techniques to identify suitable testing targets indicative of early infection.

Simulation modelling has also been used extensively in recent years to help understand the epidemiology of Johne's disease and for the evaluation of management and certification options. As better data on epidemiological parameters becomes available the models are becoming more sophisticated and should become increasingly more reliable.

INTRODUCTION

Johne's disease, or paratuberculosis, is a syndrome characterised by chronic, granulomatous enteritis affecting mainly ruminants, and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease is characterised by infection of young animals early in life and a long incubation period, so that clinical disease usually only occurs in adults. Johne's disease is now recognised as an increasingly important source of economic loss to producers and has become the focus of national and on-farm control programs in the last 10-20 years (Kennedy and Benedictus, 2001).

Because of the prolonged incubation period and associated difficulties in studying Johne's disease, the epidemiology of this condition is still not well understood, despite the efforts of many researchers over the years. However, with the advent of new technologies and methods, progress in understanding of the epidemiology of this complex disease is still being made, and this paper summarises some of the advances made over the last few years, including some of the findings presented at this colloquium.

This paper focuses primarily on transmission of infection, risk factors for infection, developments in testing technologies and strategies and modelling of Johne's disease.

TRANSMISSION

Spread of paratuberculosis is primarily via the faecal-oral route, with clinically affected animals excreting large numbers of organisms and causing significant environmental contamination. Young animals are exposed to faecal contamination of the udder, fodder and the environment, providing ample opportunity for exposure. Although the faecal-oral route appears to be the main method of transmission, other methods can also be important in some situations.

Important factors affecting the successful transmission of MAP include the level of excretion by infected animals, the route of excretion, the source of exposure and the susceptibility of exposed animals.

Excretion levels
Recent research has attempted to quantify patterns of excretion of MAP from infected animals. The level and frequency of MAP excretion depend on the stage of disease and the type of lesions. For example, sheep with multibacillary lesions were found to be excreting MAP organisms continuously, whereas companion animals from the same flock, but which did not have detectable lesions excreted MAP organisms only intermittently, possibly due to undetectable infection or due to passive passaging of organisms through the gut (Whittington et al., 2000b). In the same research, the mean excretion rate for five sheep (4 with multibacillary lesions and 1 with no detectable lesions) was in excess of 10^8 organisms per gram of faeces (>8 x 10^{10} per sheep per day), considerably higher than previous estimates of $10^5 - 10^6$ per gram of faeces in cattle (Jorgensen, 1982).

Route of excretion
As discussed above, the primary route of excretion is via faeces. However, MAP excretion via milk and semen and intra-uterine infection of the foetus have been demonstrated previously in cattle (Stehman, 1996; Sweeney, 1996) and also more recently in sheep (Eppleston and Whittington, 2001; Lambeth et al., 2004). MAP excretion via these routes is usually more frequent in animals with more advanced disease or that are showing clinical signs (Sweeney, 1996; Lambeth et al., 2004).

Source of infection
Until recently, the main source of infection has generally been assumed to be older animals in the clinical or pre-clinical phase of disease. Clinical cases of Johne's disease often excrete very large numbers of organisms (see above), and therefore represent the main source of exposure. Similarly, adult animals are more likely to be in more advanced stages of disease, and are therefore also a higher risk for intrauterine and trans-mammary transmission.

However, recent research has shown that calves and younger age cattle may excrete MAP organisms soon after infection (Bolton et al., 2005; van Roermund and de Jong, 2005; Weber et al., 2005a). Modelling of MAP transmission has also supported the hypothesis that infected calves provide an important contribution to the spread of infection in infected cattle herds (van Roermund et al., 2002; Mitchell et al., 2005). Although this source of exposure may be of lesser significance in heavily infected herds, it suggests that young animals from infected herds or flocks are an important factor in disease transmission and may pose a greater risk of spreading infection than was previously considered likely.

Studies of environmental samples from infected dairies have also identified high-risk areas for environmental contamination with MAP, and that levels of MAP contamination were correlated with percentage of culture-positive faecal-pools in the dairy herd (Raizman et al., 2004). Areas with high proportions of samples positive included cow alleyways and manure storage areas, while lower (but still important) proportions of positive samples were observed for the calving area and sick cow pens. Proportions of positive samples were lowest in water run-off and postweaned calf areas.

Susceptibility of exposed animals
In cattle, young calves have generally been regarded as highly susceptible to MAP infection, with susceptibility declining with age (Sweeney, 1996). There has been a general assumption that a similar situation exists for sheep, although this has been based on extrapolation from cattle rather than on any sheep-based research. However, recent research is starting to shed more light on the situation in sheep. In one study, there was no difference in infection rates of lambs, weaners or adults following similar levels of

exposure to MAP under natural conditions of heavy contamination (Reddacliff et al., 2004). This finding suggests that any age-resistance effect in sheep might not be as clearly defined as in cattle, and that under conditions of heavy challenge adult sheep can be just as susceptible to infection as young sheep. In contrast, observations on one recently infected property in Australia found that infection was strongly clustered in sheep exposed as lambs and that infection was not detectable in sheep exposed as adults, suggesting that some age-related resistance does occur but may be dependent on level of exposure. Additional research is still required to further clarify this situation, but age-related resistance to infection in sheep appears to be less-pronounced than is assumed to be the case in cattle.

RISK FACTORS FOR MAP INFECTION

The identification of risk factors for Johne's disease presents significant challenges to researchers, particularly because of the chronic nature of the disease, and the lack of reliable diagnostic tests for detection of infection. In addition, introduction of infected animals is generally recognized as the main risk factor for introduction of herd infection, so that most studies have concentrated on factors associated with prevalence or severity of disease in infected herds or flocks, rather than the presence or absence of infection.

Cattle
A number of recent studies have been undertaken to investigate possible risk factors for Johne's disease in cattle. Because of the limitations of test performance, these studies generally focused on risk factors for seropositivity for Johne's disease (at either cow or herd level), rather than for confirmed infection, and were mainly in dairy cattle. Risk factors that were identified included:

- herd size (Hirst et al., 2004; Muskens et al., 203);
- purchasing of replacements and rate of importation of replacements (Chi et al., 202; Hirst et al., 2004); and
- previous occurrence of clinical signs of Johne's disease (Hirst et al., 2004).

Increased risk of infection with increasing herd size could be related to the larger numbers of introductions into the herd (Hirst et al., 2004) or to increased cattle density resulting in greater environmental challenges (Muskens et al., 203). Purchasing of replacement animals is a logical risk factor for herd-infection with Johne's disease and the greater the number of animals introduced the greater the likelihood that an infected animal will be introduced. In contrast, the occurrence of clinical signs of Johne's disease is probably a reflection of the presence of disease and perhaps of a higher prevalence of disease, rather than a rsk factor for infection.

In addition, in one study in beef cattle herds in Texas, USA (Roussel et al., 2005), risk factors for seropositive animals included:
- species of cattle (seroprevalence was higher in *Bos indicus* than *Bos taurus*)
- geographic location in the State of Texas; and
- water source (seroprevalence was higher for cattle watered on a running stream or river.

However, only 7% of the seropositive cattle were positive on follow-up faecal culture, so that many of the serological reactions are likely to be false-positive results. Therefore, the risk factors identified are not necessarily associated with the occurrence of Johne's disease, and could be associated with some other factor leading to false positive serological reactions, highlighting the problems of basing Johne's disease studies on the results of serology.

One study, undertaken in Scotland, investigated risk factors for the suspected occurrence of Johne's disease on individual farms based on official records and owner knowledge of farm history to identify case and non-case farms, rather than using serology (Daniels et al., 2002). Risk factors identified for case farms included:
- herd size (total number of sheep and cattle);
- frequency of application of manure to pasture;

- number of rabbits present;
- wildlife access to stored feed; and
- type of water supply for cattle (piped or piped and open vs open only).

The effect of some of these factors was unexpected, and may have been due to confounding by farmers on infected farms taking active control measures. For example, application of manure to pasture "often" was associated with a lower risk of Johne's disease than application sometimes or never. Similarly, use of an open water source was associated with a reduced risk compared to use of piped water, despite the use of open water sources being potentially subject to contamination and hence posing a higher theoretical risk.

Finally, one Australian study investigated the occurrence of clinical cases in infected dairy herds (Ridge et al., 2005). This study found no association between the continued occurrence of clinical cases or serological reactors and three recommended control measures for Johne's disease in dairy cattle. The control measures considered were: early removal of calf from dam; separating unweaned calves from adult cattle and effluent from adult cattle; and not grazing of weaned calves on pastures grazed by adult cattle or that have been treated with effluent from adult cattle. The lack of significant effect of these measures might have been due to the relatively small sample size in the study. The study did identify that feeding of antibiotic contaminated waste milk and providing water to calves were associated with an increased occurrence of cases while allowing cows to calve in paddocks was associated with decreased occurrence of cases. The reasons for these associations are unclear, but it is interesting to note that all of these factors are associated with the early neonatal period, supporting previous hypotheses that this is the most critical period for infection of calves.

Although an association between soil type (particularly acid soils) and occurrence of Johne's disease has been suggested (Johnson-Ifearulundu and Kaneene, 1997) and application of lime to pasture was found to be protective in one previous study (Johnson-Ifearulundu and Kaneene, 1998), the results of recent studies have been mixed. Two of the recent studies discussed above which specifically considered (acid) soil type as a potential risk factor found no evidence of association (Daniels et al., 2002; Muskens et al., 203). However, one other study of the spatial distribution of infected herds in Indiana found that there was significant clustering of infected herds according to soil type (Ward and Perez, 2004), with infected herds more likely to occur on low-silt, sandy-loam or loam soils (soil types that are likely to be more acid). These findings were similar to the findings of a previous study on association between soil type and Johne's disease in sheep in Spain (Reviriego et al., 2000).

Sheep
One study in Australia has investigated risk factors for Johne's disease infection in sheep flocks (Lugton, 2004). This study used a postal questionnaire to owners of known-infected sheep flocks to evaluate risk factors for the level of disease observed on the farms. In this study, a large number of potential risk factors were considered, as well as several different measures of the occurrence of Johne's disease on the study farms. Because of the design limitations, further investigation is required to confirm the potential risk factors identified. In addition, there were no identified factors that were consistently associated with the occurrence of Johne's disease across multiple analyses.

Risk factors with a statistically significant association with occurrence of Johne's disease in the various analyses are summarized in Table 1.

Additional analyses were undertaken with the seasonality of occurrence of cases and the occurrence of scouring as a clinical sign for OJD. The interpretation of these analysess was unclear and not considered further here.

Summary
To summarise, there are still no clearly identified risk factors for MAP infection and clinical disease at either the animal or herd/flock levels, other than herd size and introduction of potentially infected animals. Additional evidence supporting the role of soil type and acid soils is inconsistent, but this area perhaps warrants further research. Additional research focused on farm-level risk factors for infection and disease,

such as that currently being undertaken in sheep in Australia, may provide more insight into this complex area.

LIMITATIONS OF CURRENT TESTING FOR JOHNE'S DISEASE

One of the greatest difficulties in understanding the epidemiology of Johne's disease is the limitations of the available screening tests. These limitations are mainly associated with the chronic nature of the disease, and the fact that current tests have very poor sensitivity in the early stages of infection. Currently available tests are mainly based on serology (eg ELISA or AGID), faecal culture in solid or liquid media and PCR testing on faeces, milk or tissue samples for the detection of the unique DNA sequence IS900.

Recent research on screening tests for Johne's disease has focused mainly on:

- optimising sampling strategies to achieve acceptable sensitivity at reduced cost;
- adapting existing tests to new samples or approaches; or
- refining and improving existing tests and technologies.

Table 1. Summary of risk factors for occurrence of Johne's disease in known infected flocks in Australia (Lugton, 2004).

Outcome measure	Factor	Comment
Farmer observed cases of Johne's disease	Estimated time the flock had been infected	> 10 yrs compared to 2-10 yrs and < 2 yrs.
	Altitude	> 800m. The reason for this difference is unclear.
	Predominant breed of sheep	Fine wool merinos vs other breeds. This might be an inherent difference in susceptibility or could be due to management factors that were not captured elsewhere.
	Treatment of illthrifty sheep	Removal from flock vs drenching or no treatment. This probably reflects a response to more severe disease than a causal factor.
Estimated incidence of deaths due to Johne's disease	Lamb marking percentage	This is most likely a result of Johne's disease rather than a cause.
	Age at culling	Association between incidence of Johne's disease and an older age at culling is probably a result of the need to retain sheep for longer in higher-incidence flocks.
	Proportion of quality pasture	Flocks with a higher proportion of quality pasture had a higher reported incidence, but the reason for this association is unclear.
	Weeks of hand feeding	Flocks that hand fed for longer had a higher incidence, possibly associated with greater exposure to faecal contamination of fodder on the ground.
The youngest age at which sheep die from Johne's disease	Incidence of disease	This was the one factor that accounted for most of the variation in age of youngest cases.
	Soil texture	Cases occurred at a younger age in flocks on sandy soils. This could be associated with soil acidity, although soil pH and fertilizer application that affect soil pH were not important in any of the models.
	Proportion of quality pasture	Flocks with a higher proportion of quality pasture had cases at a younger age. Again the reason for this association is unclear.
	Breeding replacement sheep	Introduction of replacements reduced the age of youngest cases, possible by reducing the overall level of contamination on the farm.
	Treatment of illthrifty sheep	Removal from flock vs drenching or no treatment. This probably reflects a response to more severe disease than a causal factor.
	Age at weaning	Weaning at > 5 months of age. This may be a result of a longer period of exposure to high levels of contamination as highly susceptible lambs.

Pooling of faecal samples for culture is being investigated or applied in a number of countries and for both sheep and cattle (Whittington et al., 2000a; Tavornpanich et al., 2004; Fyock et al., 2005a; Weber et al., 2004; van Schaik et al., 2003b; Antognoli et al., 2005). Pooled culture is primarily used as a tool for identification of infected herds or flocks or conversely for certification of low-risk herds and flocks. It has also been evaluated for identification of individual infected animals for culling, but has limited value for this purpose depending on prevalence of infection (van Schaik et al., 2003b). Environmental sampling has also been used as an alternative to faecal sampling for the identification of high-risk areas on dairy farms and for evaluating the effectiveness of control programmes (Fyock et al., 2005a; Raizman et al., 2004; Lombard et al., 2005).

Johne's disease ELISA's have also been adapted, particularly for use with milk instead of blood samples. These have been used or proposed for both individual cow testing and for bulk milk testing. Although the milk ELISA shows promise as an alternative individual animal test to serology (Nielsen et al., 2002; Hendrick et al., 2005; Collins et al., 2005), it is likely to be of limited value as a bulk milk test for surveillance purposes (Nielsen et al., 2000; Weber et al., 2005b). Despite this, simulation studies suggest that bulk milk screening might be a useful tool to support milk quality assurance programmes (Weber et al., 2005b). Similarly, culture of bulk milk samples has also been evaluated, but was found to be less sensitive than bulk-milk PCR (Stabel et al., 2002).

Some existing tests have also been adapted or improved by uptake of newer technologies. For example, liquid culture media have the advantage of supporting more rapid growth of MAP and hence shorter culture periods for confirmation of infection (Fyock et al., 2005b). Early liquid culture systems relied on radioactivity in the media for detection of growth. Newer technologies have now allowed the use non-radioactive media, providing a more useful and efficient culture process with the potential to replace existing solid and liquid media systems (van Schaik et al., 2003a; Fyock et al., 2005b).

Despite these advances that have been made in broadening the application and efficiency of testing systems, there have been no fundamental changes to the approaches used, so that the available tests are still limited in their ability to detect early infection in individual animals.

ROLE OF MODELING

Considering the difficulties and cost involved in conducting large studies on Johne's disease, modeling of the disease has become increasingly popular in recent years. Most of these models are based on core aspects of our understanding of the epidemiology of Johne's disease (for example age-related resistance to infection, the effect of calf-management practices and the long pre-clinical period) to predict the course and dynamics of infection in infected herds or flocks. Most models also incorporate stochastic elements to reflect uncertainty about some of the epidemiological parameters. The models have been used for a number of different purposes, including:

- to further investigate the epidemiology and dynamics of the disease in infected herds (Pouillot et al., 2004; Mitchell et al., 2005);
- to evaluate alternative strategies for management and control of Johne's disease in infected herds (Groenendaal et al., 2002; van Schaik et al., 2003b; Dorshorst and Collins, 2005); and
- to evaluate national or industry-wide strategies for herd-certification and disease management (Weber et al., 2004; Weber et al., 2005b; Tavornpanich and Gardner, 2005).

Although such models have proved very useful in supporting recommendations for on-farm control or for development of national control and certification programmes, they are still limited by the underlying assumptions and the lack of detailed knowledge of some aspects of Johne's disease epidemiology. As our understanding of the epidemiology of this complex disease improves so will the reliability and utility of the various models being used.

FUTURE PERSPECTIVES

What can we expect (or hope for) from the future for our understanding of Johne's disease epidemiology?

1. Further research and understanding of transmission mechanisms and risk factors for infection, including age-susceptibility and infectious dose, the role of environmental factors such as soil type and the identification of other risk factors that can be manipulated to help control the disease.

2. More basic research into the epidemiology and pathogenesis of Johne's disease utilizing new technologies such as genomics and proteomics.

3. Development of new tests and testing technologies based on the outcomes of the fundamental research and other new technologies being developed in the bio-medical field. New tests are likely to focus particularly on the early detection of infection.

4. Sophisticated models based on sound epidemiological data to predict disease transmission and progression and the impact of a complex mix of control measures on occurrence of both infection and clinical disease.

Perhaps the day is coming when the farmer can use a pen-side test to identify at-risk animals before their first joining and log onto the internet and answer some simple questions about his/her farm to receive customized advice (based on a sophisticated model running remotely) on how best to manage the disease in the current farm environment, for both individual infected animals and the herd as a whole.

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Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in calves: implications for infection control and management

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ABSTRACT

It is widely accepted that most infections caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) occur in utero or in the neonatal animal. The challenge, however, has been to detect the infection in the young animal to facilitate more prudent animal management decisions. The objectives of this study are to determine whether: 1) fecal shedding of MAP can be detected in naturally infected calves, 2) there is a relationship between MAP test positive cows (ELISA or fecal) and fecal shedding in their offspring, 3) there is an association between fecal shedding in the calf and their ELISA test status, and 4) pooled fecal sample testing is sensitive enough to detect MAP shedding in calves. This is a two year longitudinal, prospective study using dairy calves of four age groups from seven herds in Michigan with known MAP prevalence. Fecal, and blood samples, are obtained from ten calves in each of the four age groups at 3 month intervals. The TREK® liquid culture system is used for fecal culture and the Biocor® ELISA for blood. Preliminary results (study 40% complete) showed fecal shedding was detectable in calves with a higher proportion of shedding calves being from ELISA or culture-positive dams. Few calves were ELISA positive (2.2%). There was an association between ELISA positive calves and dam test status but little association with concomitant calf fecal status. Also, pool size may be critical in detection of fecal shedding in calves, with five calves per pool being more sensitive than ten calves per pool.

Key words: calves, dams, shedding, longitudinal study, liquid culture

INTRODUCTION

Johne's disease, *Mycobacterium avium subsp. paratuberculosis* (MAP), is a chronic infectious inflammatory enteric disease of both domestic and non-domestic ruminants. This disease was first described in 1895 in Germany and its etiology was characterized as an acid fast bacillus (Twort,1910). Although paratuberculosis is a disease characterized over a century ago, over the past three decades it has emerged as a major economic factor in the US dairy industry. The cost to the individual US dairy producer is between $22 and $27 per cow, across all herds, with a possible $250 million total annual price tag.(Ott et al.,1999). Although widely variable prevalence figures have been published (Adaska et.al.,2003; Hirst et.al.,2004), a recent study placed the percent of infected herds in Michigan at 52.4% (Ifearulundu-Johnson et al.,1999) and a national random sample reported that 2.5% of the individual animals tested across the US were fecal culture positive (USDA-NAHMS,1997).

Calves are often infected before the age of six months (Sweeney,1996) via feces or colostrum and transplacental infection also occurs (Kopecky et al.,1967; Seitz et al.,1989; Sweeney et al.,1992). However, MAP is a slow-growing bacteria and development of clinical signs may take 2-5 years (Harris and Barletta,2001) which presents a challenge to a producer's management of the infection. Attempts to detect naturally infected calves have been unsatisfactory (McDonald et al.,1999; Ayele et al.,2004) due to infrequent shedding at low levels. Fecal culture has been an ineffective method for detecting low level shedders (Kim et al.,2002) and its overall sensitivity may not surpass 33% (Whitlock et al.,2000). Due to reports of increased sensitivity of the recent TREK®ESPII liquid culture system (Stitch etal.,2004) this study assesses the ability of this liquid culture system to identify fecal shedding in naturally infected dairy calves.

MATERIALS AND METHODS

Study design
This is a longitudinal, prospective, two year study involving calves from seven commercial dairy herds in the southern portion of the State of Michigan, USA.

Criteria for herds inclusion
Seven herds were selected for this study due to known test prevalence levels (1 – 42% based on fecal liquid culture-TREK®ESPII), their involvement in the ongoing USDA Johne's demonstration project, and the availability of invaluable individual cow test data. With the exception of one 80 cow Jersey herd, the herds are Holstein and herd sizes ranged from 110 to 511 cows. These herds represented both grazing and confinement management styles.

Selection of calves
Ten heifer calves from each of four age groups: 0-3 months, 4-6 months, 7-14 months, and 15-24 months were selected for fecal culture and ELISA testing during each herd visit.   An attempt was made to test all heifer calves from fecal culture-positive dams. Forty calves from each herd were tested at approximately three month intervals, repeated eight times, for a total of approximately 1,600 samples when the study ends.

Sampling
Fecal samples were collected per rectum from each calf using individual latex gloves and sterile water for lubrication. At least 6 grams of fecal material were collected; 2 grams each for the five and ten calf pooled sample as well as 2 grams for the individual calf culture. Blood was collected (5 cc) from the jugular or caudal tail vein for ELISA Paracheck® (Biocor) testing.

Fecal Culture Processing
Individual fecal samples were labelled and refrigerated at 5°C and submitted to lab within 1-2 days. Samples from each age group of 10 calves were divided into 2 pools of 5 as well as one pool containing all 10 calves. Using separate tongue depressors individual samples were transferred to the pooling vial and manually mixed for one minute. The pooled samples were submitted at the same time.

Laboratory
All samples were tested at the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University, a USDA certified lab for both liquid (TREK®) and solid (HEYM) media fecal culture for Johne's disease. The Cornell method (Stabel, 1997) was used in preparation of fecals for culture. The samples were placed in the TREK® ESP Culture System Ⅱ incubator that measures a decrease in O2 pressure. This is a semi-quantitative test and positive samples were described as high shedders if it took 7-21 days to turn positive, moderate shedders if 22-28 days to positive, and low shedders if positive at 29- 42 days. If not positive by day 42, calves were classified as not shedding. Positive and negative controls were used with each batch of forty samples and the positive control was the same across the study i.e. a fecal sample obtained from a serially confirmed low shedding cow. All positives from liquid culture were confirmed with both acid fast staining and real-time IS*900* PCR (Kim et. al.,2004). The blood was centrifuged and the serum tested for MAP antibodies by the Paracheck® (Biocor) ELISA using the manufacturers recommended procedure.

RESULTS

To date 40% of the study is complete and the following are the preliminary results:

Objective 1- Detection of fecal shedding
Of the 583 samples cultured (Table 1), 12 samples (11 calves) were positive (2.1%) with 9 low shedders, 2 moderate shedders, and 1 heavy shedder (Table 1). Of the four culture-positive calves tested the 2nd time, 1 was positive again and 3 were negative but the pools containing these 2 of these 3 were positive. All but

two of the fecal shedding calves were from the first three age groups (14 months) and 3 of these were < 6 months of age.

Table 1. Dam vs. calf test status.

	Pos. calves (fecal)	Neg. calves (fecal)	Total
Pos. Dams (fecal or ELISA)	8	91	99
Neg. Dams (fecal and ELISA)	3	481	484
Total	11	572	583

Table 2: Ages of calves with Positive Fecal Samples and Shedding Level

	0 – 3 Mos.	4 – 6 Mos.	7 – 14 Mos.	15 – 24 Mos.	Total Fecal Shedders
# Positive Fecal Samples	1 (low)	2 (low)	5 (low) 1 (moderate) 1 (heavy)	1 (low) 1 (moderate)	12

Objective 2 – Relationship between fecal shedding in calf and dam test status (test-positive dam = positive fecal or ELISA test)
Ninety-nine calves from test-positive dams were tested; 91 calves were culture negative and eight (8.1%) were culture-positive. For calves from 484 test-negative dams, three calves were culture-positive (0.6 %) and the rest culture negative. The calculated relative risk of the calf being culture-positive if from a test-positive dam is 13.3 (8.1%/0.6%) based on these preliminary results.

Objective 3 – Relationship between positive fecal shedding and ELISA results:
Of the 583 blood samples tested, 13 were ELISA positive (2.2%) but only one animal had both a positive fecal and positive ELISA test indicating nearly no relationship between detectable fecal shedding in calves and their ELISA status. However, 8/13 of the sero-positive animals were from test positive dams (fecal or ELISA).

Objective 4 – Sensitivity of pooling samples
Of the 8 initial positive individual fecal samples that were in 8 separate pools (10 samples per pool), one pool was positive. We lowered pool size to 5 at that juncture (while also continuing to run pools of 10). With the subsequent 4 positive samples we demonstrated 2 positive pools of 5 while the pools of 10 were negative. We also had three positive pools (2 pools of five and one pool of 10 containing calves in one of the pools of 5) that contained no test positive individuals. However, they each contained an animal that had tested fecal positive on a prior test.

DISCUSSION

Five of 7 herds had test-positive calf samples and the youngest calves testing positive (6 months of age) came from the two highest prevalence herds (14% and 42% respectively). The fact that some of the fecal positive calves tested negative subsequently leads one to believe that either they are low/sporadic shedders or there may be pass-through of the organism. Follow-up sampling may help answer this question. Finding that 11/12 positive fecal tests and the 6 positive pools came from the 339 samples gathered from the four highest prevalence herds leads one to speculate that if fecal culture or pooled fecal culture is used as a tool, it would be most effective in herds with a high prevalence of Johne's disease. There seems to be a relationship between dam status and fecal shedding as 8/11 (73%) calves shown to be shedding by a positive individual fecal culture came from test positive dams that comprised only 17% (93/583) of the total dams tested. The fecal and ELISA positive calves originated almost exclusively from the four herds with the highest test prevalence. The proportion of test positive dams in this group of four herds was found to be 57/339 (19%).

Although nearly the same proportion of fecal samples were positive (12/583) as were ELISA positive (13/583), only one calf was positive for both simultaneously suggesting that a combination of testing types may be a better way of assessing the infection level in young animals. Quantitative assessment of ELISA

tests may also be a more valid and informative diagnostic method than simple positive/negative ELISA interpretations (Collins et. al.,2005).

Finally, with only 1/12 of the fecal positive samples turning their pool of ten positive, one may infer that pools of ten are too large for the low shedding levels in calves (Wells et al., 2002; Wells et. al., 2003; van Schaik et al., 2003). We now use 2 pools of five and a pool of ten for each group of ten calves and results seem to have improved. Pooled fecal samples containing five calves in conjunction with environmental testing (Raizman et al., 2004) may be a feasible, inexpensive early predictor of the success of management changes in herds with a high prevalence of MAP. An explanation for positive pools comprised of individually test-negative individuals may be that the distribution of MAP is uneven in a fecal sample, especially with light shedding animals such as calves. (Kalis et. al., 1999; Visser, 1999).

CONCLUSION

Preliminary results indicate that it is possible to detect fecal shedding in calves. It is hoped that, by serially testing these animals as calves and later as cows, more questions may be answered to help us manage, control, and ultimately eliminate Johne's disease from our dairy cattle industry.

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Abattoir surveillance of paratuberculosis in farmed deer in New Zealand

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ABSTRACT

Continuing surveillance for paratuberculosis provides crucial information for monitoring the success of a disease control programme. However, surveillance can be difficult and expensive. In this paper we describe the efficient use of abattoir surveillance for documenting the spread of paratuberculosis in farmed deer. Deer farming is a major industry in New Zealand with approximately 2 million deer raised on pasture on 5000 farms. Bovine tuberculosis has been a major concern for the deer industry and a series of measures, including abattoir surveillance are being used to successfully control this disease. However, abattoir surveillance revealed that the macroscopic and microscopic features of infections with *Mycobacterium avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* (MAP) can be very similar to those of caused by *Mycobacterium bovis.* Tissue samples from gut associated lymph nodes of suspect cases of bovine tuberculosis identified at slaughter have for the last 20 years been cultured for MAP as well as *M. bovis.* The findings from these examinations have provided an developing picture of paratuberculosis in farmed deer in New Zealand. Since the 1980s, over 1550 cases of MAP have been identified from samples collected from abattoirs as part of the bovine tuberculosis control programme. The large increase in the number of cases and infected herds over the last five years documents the spread and worsening problem of paratuberculosis in deer. Repeat isolations from herds indicate that some herds have been persistently infected for many years. A recently developed PCR test revealed that the "bovine" strain of MAP was present in 94 of the 98 infected herds examined, and the "ovine" strain was present in the remainder. This highlights that both sheep and cattle are potential new sources of infection for deer.

Key words: farmed, deer, PCR, strain typing, surveillance

INTRODUCTION

There is a paucity of good information on the prevalence and incidence of paratuberculosis in cattle and sheep. This reflects the deficiencies of current diagnostic tests and the costs of obtaining statistically robust data. Without reliable surveillance data it is very difficult to develop accurate estimates of the national cost of paratuberculosis or determine whether economic losses are increasing or diminishing.

The deer industry in New Zealand has a unique opportunity to gather surveillance data on paratuberculosis. A feature of paratuberculosis in farmed deer is that some infected animals develop macroscopic lesions that are indistinguishable from those caused by *Mycobacterium bovis* (de Lisle et al 2003). Necrosis is visible macroscopically, a feature that is extremely rare in other ruminant species with paratuberculosis. Abattoir surveillance for bovine tuberculosis is routinely carried out on all slaughtered farmed deer. Formalin fixed and fresh samples are taken from lesions resembling bovine tuberculosis from all lines of deer slaughtered for histopathology and mycobacterial culture respectively. Since 1990, all 'suspect' lesions from the mesenteric lymph nodes of deer submitted for culture for bovine tuberculosis (*M. bovis)* have also been cultured for paratuberculosis. Initially, this was carried out by the inclusion of an additional culture slope (Herrold's egg yolk medium containing mycobactin), and more recently by the inclusion of an additional BACTEC vial containing egg yolk and mycobactin.

A PCR test designed to detect IS*900* has been used to identify MAP in some selected cases from the abattoirs. More recently, a PCR test was developed to distinguish the "bovine" and "ovine" subtypes of MAP (Collins et al. 2002)*.* This test has been used to examine isolates of MAP from 98 different deer herds.

The results of testing between 1985 and 2000 have been published previously (de Lisle et al. 2003). The objective of this paper is to show the continuing spread of paratuberculosis in farmed deer in New Zealand by reporting information from 2001 to 2004.

MATERIALS AND METHODS

The samples, culture procedures and PCR used to detect MAP directly in tissues were those described previously (de Lisle et al. 2003). The majority of samples were submitted from cases identified at slaughter in abattoirs as being macroscopically typical of or suspected of bovine tuberculosis. A small number of samples came from clinically affected animals slaughtered on farm.

Since 1990, all requests for mycobacterial culture of mesenteric lymph nodes of farmed deer containing lesions with the macroscopic appearance of tuberculosis have, in addition, been routinely cultured for MAP as well as other mycobacteria. From 1986 to 1999 a culture procedure used a decontamination step with 0.35% cetylpyridinium chloride and Herrold's egg yolk medium supplemented with mycobactin. Subsequently, the culture procedure changed to the use of a liquid culture system using BACTEC 12B vials (Becton Dickinson, USA) supplemented with egg yolk, mycobactin and antibiotics (PANTA, Becton Dickinson, USA) (Whittington et al. 1999).

Bacteria obtained through culture were identified as MAP based on one of the following criteria:
    (a) Slow growing, acid-fast staining, small bacillus that was mycobactin dependent.
    (b) Slow growing, acid-fast staining, small bacillus containing IS*900*.

The PCR test for the detection of MAP directly in tissues used a single set of primers previously described by Collins et al. (1993). The PCR used for identifying "bovine" and "ovine" types of MAP was that recently described by Collins et al. (2002). The test was applied directly to BACTEC 12B vials from which MAP had been isolated. The isolates examined using the subtyping PCR were selected on the basis of one isolate per herd and included infected herds throughout New Zealand.

RESULTS

The number of cases of microbiologically confirmed cases of Johne's disease is summarised in Fig. 1 and the cumulative number of infected herds in Fig. 2. The change in the number of cases of paratuberculosis and the number of infected herds observed since 1999 cannot be attributed to the use of a different culture procedure, to variations in the number of deer slaughtered or to different sampling regimes. The 598 infected deer herds represent approximately 12% of all herds in New Zealand. Many of the herds show evidence of persistent infection with cases identified up to 13 years. There were 53 herds from which positive samples were received on 5 or more occasions. These herds were known to have been infected for an average of 4.7 years.
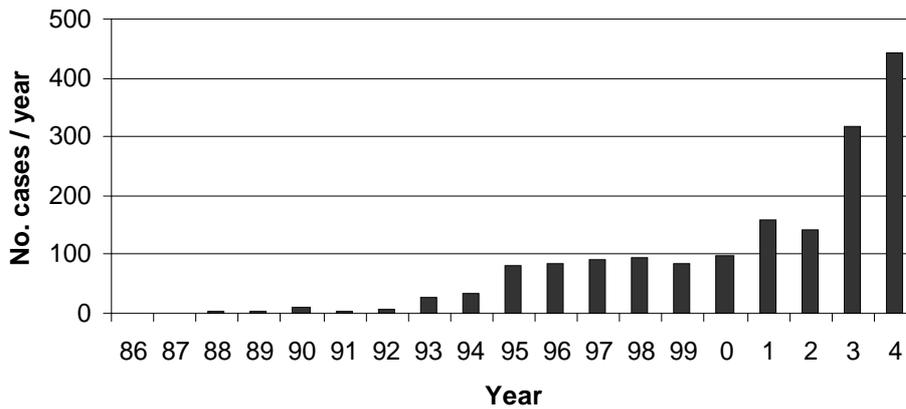
Fig. 1. Annual number of cases of paratuberculosis. Diagnosis based on the isolation of MAP and/or a positive test by an IS*900* PCR applied directly to tissues.
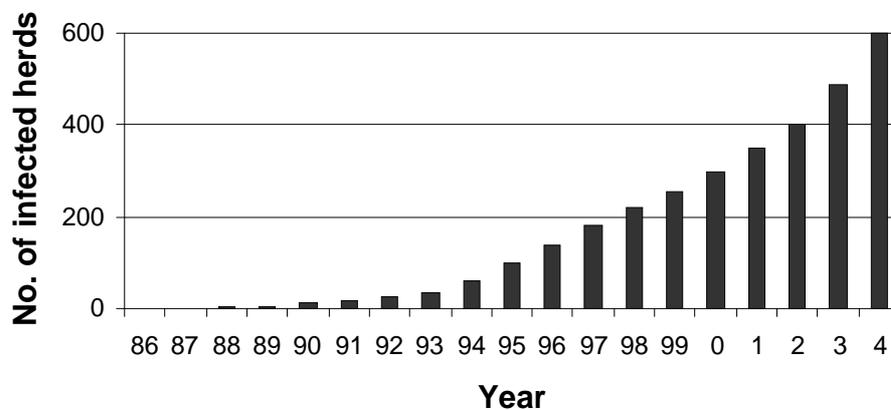
 Fig. 2. Cumulative number of infected herds. Diagnosis based on the isolation of MAP and/or a positive test by an IS*900* PCR applied directly to tissues.

The macroscopic appearance of paratuberculosis in deer is often difficult to distinguish from that of other mycobacterial infections, especially bovine tuberculosis. In 2004, MAP was isolated from 390 farmed deer with macroscopic lesions identified at slaughter as being either "Typical" or "Equivocal" of bovine tuberculosis. Of these, 34 (8.7%) were classified as being "Typical" of bovine tuberculosis. During the same period, *M. bovis* was isolated from 98 deer with lesions identified at slaughter and categorised using the same criteria as that for the paratuberculosis cases. Of these *M. bovis* cases, 43 (43.9%) were categorised as "Equivocal" and the remainder as "Typical" of bovine tuberculosis. The distribution of the macroscopic lesions of the 2004 cases from which MAP was isolated is summarised in Table 1.
Of the 98 different strains examined by the subtyping PCR, 94 of them were the "bovine" subtype and the remaining 4 the "ovine" subtype.

Table 1. Distribution of macroscopic lesions of the 2004 cases from which MAP was isolated. The "mesenteric" site includes the ileocaecal valve lymph node as well as the ileojejunal nodes.

Site	No. (% total)
Mesenteric lymph nodes only	307 (78.7%)
Retropharyngeal nodes only	55 (14.1%)
Mesenteric & retropharyngeal nodes	19 (4.9%)
Mediastinal lymph node	6 (1.5%)
Submandibular lymph node	3 (0.8%)
Head nodes (combined figure)	77 (19.7%)

DISCUSSION

The data presented in this paper is based on the detection of deer with macroscopic lesions which resemble those of bovine tuberculosis. Only a relatively small percentage of deer infected with MAP develop this type of lesion, with the remainder having the more "classical" lesion with little or no necrosis. Consequently, some infected herds, especially those that have recently been infected and have not had any clinical disease, will most likely not be detected by the abattoir surveillance procedure operating in New Zealand. Even taking this qualification into account, the abattoir data reveals valuable information. In the last four years, the number of infected herds has almost doubled and now approximately 12% of the deer herds in New Zealand are known to have been infected. Many herds are known to have been infected for several years, indicating that once paratuberculosis has been detected in a herd the infection persists. The number of infected herds has reached such a level that some control options such as quarantining infected herds are no longer practicable. Furthermore, the inadvertent purchase of infected deer is becoming increasingly more likely.

An unexpected finding from the abattoir surveillance has been the isolation of MAP in lesions outside the lymph nodes of the small intestine, especially the retropharyngeal lymph node. Culture-positive lesions were observed in some deer only in the retropharygeal lymph nodes, while in other deer they were observed in this node as well as in mesenteric nodes. Whether or not this indicates that deer may be infected through the cranial routes in addition to the small intestine is unknown.

PCR analysis of isolates of MAP from 98 different farms revealed that 94 of them were the "bovine" subtype. The initial strain typing study carried out at the start of the epidemic of Johne's disease in farmed deer in New Zealand, when the typing was based on IS900 restriction fragment length polymorphisms, revealed 17 isolates were the "bovine" subtype and three the "ovine" subtype (de Lisle et al. 1993). The fact that the "ovine" subtype continues to be isolated from only a small percentage of cases of clinical paratuberculosis in deer indicates either that this subtype is less infective for deer than the "bovine" subtype, or that deer are exposed to the "ovine" subtype much less often than to the "bovine" subtype. The increase in the number of infected herds and the number of infected deer being detected at slaughter is of major concern to the deer industry in New Zealand. The figures highlight the urgent need for cost-effective measures to control paratuberculosis in deer.

Unfortunately, some of the options used by dairy farmers, such as rearing calves in isolation of their mothers, are not practicable in deer. Vaccination using oil-adjuvanted products are protective in deer but cannot be used in New Zealand because they interfere with diagnostic tests for bovine tuberculosis.

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Epidemiology of paratuberculosis in two red deer (*Cervus elaphus*) populations of Trentino (Northern Italy)

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ABSTRACT

The objective of this study was to estimate the prevalence and describe the epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in two distinct areas of Trentino. Both areas are characterized by high densities of red deer and domestic livestock (cattle and small ruminants) that share pasture. During the five-year period 1998-2002 the intestines of 246 red deer killed or found dead were examined in the western sector of the province. Between 1999 and 2002 the study was extended to the eastern part of the province, where the viscera of 156 red deer were collected. Isolates of MAP were isolated in 194 of the 402 carcasses examined by bacterial culture (HYEM). The results obtained show a significant difference in the prevalence of MAP in the two populations examined (66.2% in the western sector and 18.6% in the eastern sector). The typing of the strains identified as MAP by using IS*900*-based PCR technique was carried out by means of PCR-REA on the IS1311 sequence. The results of this analysis confirmed that the strains isolated in the deer were "cattle type". The infection has been reported in other sympatric species of wild ungulates, present in both study areas, such as roe deer, chamois and ibex, as well as non-ruminant species, such as fox and hare. In order to verify the existence of a species-specific cycle of MAP, phylogenetic analysis by AFLP was carried out to clarify the role of each wild species in the epidemiology of paratuberculosis in the area.

Key words: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), epidemiology, red deer, PCR, AFLP.

INTRODUCTION

Paratuberculosis is a chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that affects both domestic and wild ruminants. Recent studies have revealed that MAP can also infect non-ruminant species (Beard *et al.*, 1999; Greig *et al.*,1999). Cervids are particularly susceptible to MAP infection, and manifest clinical signs at a young age (Pacetti *et al.*, 1994; De Lisle & Collins, 1995; Stehman, 1996).

In Italy the first reported case of paratuberculosis in wild ruminants was identified in two red deer originating from Stelvio National Park in the early 1990s (Pacetti *et al.*, 1994). The disease also infects other sympatric species of wild ungulates found in the Alps such as ibex (*Capra ibex*), chamois (*Rupicapra rupicapra*), roe deer (*Capreolus capreolus*), and mufflon (*Ovis musimon*). The estimated prevalence of infection in these species varies by geographic area and the diagnostic technique used (Tolari *et al.*, 1987; De Meneghi *et al.*, 2000; Gennero *et al.*, 1993; Ferroglio *et al.*, 2000; Robino *et al.*, 2000, Nebbia *et al.*, 2003). However, the role played by wild ungulates and non-ruminants in the epidemiology of paratuberculosis is still poorly understood.

The objectives of this study were to estimate the prevalence and describe the epidemiology of MAP in wild red deer in two areas of Trentino (Northern Italy), and understand the implications for other species of wild

animals, both ruminant and non-ruminant. The results of this study may prove useful to both wildlife and livestock managers.

MATERIALS AND METHODS

Survey area

The study was carried out in two areas of Trentino (Fig. 1) characterized by high densities of red deer and domestic livestock (cattle and small ruminants) that share pasture. One study area is in the western sector of the province including the Trentino sector of the Stelvio National Park and the surrounding areas (Peio, Rabbi and Vermiglio Reserves). The other study area is in the eastern sector of the province, including the Paneveggio-Pale di S.Martino Natural Park and its surrounding areas within the Travignolo watershed (Moena, Predazzo and Primiero Reserves) (Fig. 1 and Table 1). During the summer in the western sector of the province there were about 1,630 cattle and 390 small ruminants, whilst in the eastern sector there were 3,100 cattle and 4,730 small ruminants.
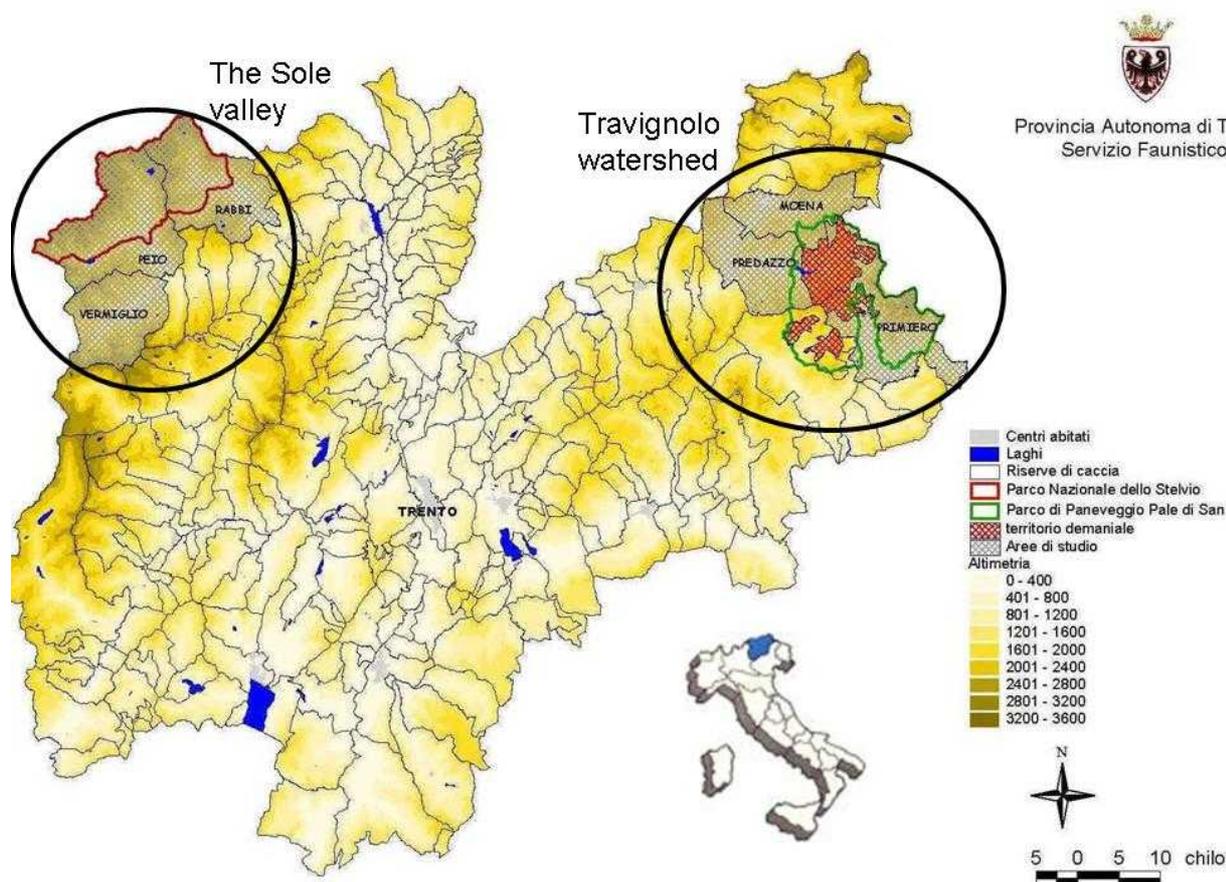
Fig. 1. Map of Trentino Region showing the two areas sampled in the survey

Table 1. Characteristics of the study areas and of the red deer populations surveyed

	Western sector of Trentino	Eastern sector of Trentino
Altitude (min - max) (m)	1500-3770	1200-2300
Total area (hectare)	9850	7085
Red deer density (head/100 ha)		
1998	11.5	3.7
1999	13.2	6.6
2000	14.1	6.6
2001	9.3	6.9
2002	12.6	7.3

Collection of samples
During the five-year period 1998-2002 in the western sector of the province, the internal organs of 246 red deer, killed during hunting seasons, or found dead, were examined and samples collected for culture. The study was extended to the eastern part of the province, in 1999, where, up to 2002, the viscera of 156 red deer were collected (Table 2). The age, sex, weight and biometric measures were recorded for each animal. The small and large intestine was examined at necropsy for visible lesions of paratuberculosis. Samples for bacteriologic culture were taken from the ileum, ileo-cecal-colic valve and mesenteric lymph nodes. The anatomo-pathological lesions found during autopsy were classified as type A, B, C on the basis of severity, location and distribution (Perez *et al.,* 1996). During necropsy both the kidneys and the peri-renal fat were removed in order to evaluate the body condition by Kidney Fat Index (KFI). Further analyses were carried out to exclude the possibility that lesions were caused by other bacteria or viruses. To evaluate the possible involvement of other wildlife species thought to be susceptible to paratuberculosis infection, analyses were carried out on 228 roe deer (*Capreolus capreolus*), 88 chamois (*Rupicapra rupicapra*) and 6 ibex (*Capra ibex*) obtained from the study areas. Non-ruminant species, such as foxes (*Vulpes vulpes*) and hares (*Lepus europaeus*) were included in these analyses from 2000 onwards.

Table 2. Red deer survey data

Red Deer	Trentino: Western sector	Trentino: Eastern sector	Total
Hunted	151	149	300
Found dead	95	7	102
Total carcasses examined	246	156	402

Bacteriological culture
For every carcass, culture was completed to detect MAP in a pool of tissues consisting of the proximal part of the ileum, the ileo-cecum-colic valve and the mesenteric lymph nodes. Tissue homogenates were prepared by following the methods outlined below. Five to ten grams of tissue were decontaminated by adding Butterfield buffer and leaving overnight at room temperature. Each sample was then subjected to trypsin digestion (0,4 % in PBS) (15 ml), homogenised in a stomacher blender, and left to stand for 15 minutes at 37°C. The supernatants were centrifuged at 1800-2000 rpm for 10 minutes and each pellet was resuspended in 7 ml of sterile distilled water. 5 ml of each supernatant sample was finally resuspended in 10 ml of sterile distilled water and 1.65 ml of decontaminant Benzalkonium chloride (BAC 0,3%), and incubated overnight at room temperature. 200 µl of inoculum was added to each of three slants of Herrold's egg yolk medium. The first slant contained mycobactin J (2 mg/l) and amphotericin B (50 mg/l) (HEYM); the second slant contained HEYM with sodium pyruvate (4 g/l); and the third slant contained HEYM with cloramphenicol (30 mg/l) (Belletti e Zavanella,1987). All the tubes were incubated at 37°C and examined once every 4 weeks for up to 16 weeks. The strains isolated were classified according to the definition criteria proposed by Stevenson (1996). The identification of the mcyobacteria as MAP was carried out by considering the morphological characteristics of the colonies, which appeared non-pigmented and wrinkled with a protruding centre, and biological characteristics, such as slow growth and mycobactin dependence, confirmed by both Ziehl-Neelsen stain and PCR.

Molecular typing of MAP isolates
The strains isolated by culture were screened by PCR for the presence of the MAP species-specific IS*900* insertion sequence. The following primer sequence was used: forward (p90) GAAGGGTGTTCGGGGCCGTCGCTTAGG and reverse (p91) GGCGTTGAGGTCGATCGCCCACGTGA. These PCR primers amplify a 413-bp target sequence. In order to identify the bovine or ovine type of strain isolated, the PCR-restriction endonuclease analysis (REA) based on IS*1311* sequence was used. Strains identified as MAP by PCR-IS*900* that were isolated from cattle, small ruminants and red deer were typed by PCR using primers: forward (M56) GCGTGAGGCTCTGTGGTGAA, reverse (DD2) GTCGGGTTGGGCGAAGAT. The PCR-amplified product, resulting in a 909-bp probe, was digested by the restriction endonuclease *Hinf* I. The PCR products were subjected to agarose gel electrophoresis. Two negative controls and one positive control (DNA from *Mycobacterium phlei* ATCC 11758, *Mycobacterium flavescens* ATCC 14474, and *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698), were included in each amplification run.

Kidney Fat Index calculation
The Kidney Fat Index (KFI) proposed by Riney (1955) is a quick and easy method for assessing body fat and is commonly used as an indicator of body condition in ungulates. To determine the KFI, both kidneys were extracted from each carcass along with all the peri-renal fat. The kidneys were then dissected from the fat and the two weighed independently. The KFI was calculated using the following equation: KFI= (kidney fat weight/kidney weight) * 100.

Kidney weight is included in the index to allow a comparison between animals of different sizes, assuming that the kidney weight is proportional to body weight. (Mitchell _et al._, 1976; Finger _et al._, 1981).

Statistical analysis
Chi-square tests based on contingency tables were carried out to test for an association between prevalence and (I) sex, (II) age class, (III) type of animal (hunted or found dead) and (IV) KFI/weight, with analyses stratified by geographical areas. Continuous variables were dichotomised on either side of the median value. The median of KFI/weight was calculated for each age class by sex. The level of risk was estimated using an odds ratio according to the Mantel Haenszel method. Confidence intervals on annual prevalence by geographical areas were estimated assuming a hypergeometric distribution in the data.

RESULTS

MAP was isolated in 194 (48.2%) of the 402 deer examined. The prevalence of infection varied in the western sector, from a maximum of 80.0% (95%CI: 68.20 - 88.63) in 1999 to a minimum of 55.0% (95% CI: 45.26 - 64.37) in 2001. In the eastern sector, the maximum prevalence was 36.4% (95%CI: 16.92 - 60.77) in 1999, and the minimum was 5.9% (95%CI: 2.21 – 13.24) in 2001. Prevalences calculated using the data from the hunted animals only did not differ significantly.

In the western sector of the province the average prevalence of infection was 66.2%, significantly higher than the one in the Travignolo watershed, which was 18.6% (x^2 = 75.88; _P_ = 0.000). There was no significant difference (_P_ >0.05) in the prevalence of the infection in relation to the median of weight, median of KFI, and cause of death (hunted or found dead), stratified by geographical areas.

In both study areas, the sex of the animal was not associated with infection prevalence (western Trentino x^2 =0.537 ; _P_ = 0.309; eastern Trentino x^2 =0.372; _P_ = 0.318). For different age groups, the distribution of the infection was similar to what was found by other authors (Pacetti _et al._, 1994; Manning _et al._, 1998; Godfroid _et al_,. 2002). The greatest percentage of positive results from culture examinations in both sectors were found in the youngest animals. There was a significant difference between the values of prevalence found in calves and yearlings in the western area (x^2 = 10.25; _P_ = 0.017) (Fig. 2). Such values tend to decrease in adults examined. The age-related prevalence pattern can be explained if we consider mortality caused by the infection. The disease decreases the life expectancy at birth of each individual, according to the prevalence of infection observed over the entire population: a lower disease prevalence in adults occurs when infected animals in the population die at a young age. Furthermore, it would seem that adults may act as a vector of the infection. In the eastern area, the youngest age group had the highest prevalence of infection however, it was not significantly different from other age classes.

The odds ratio for the western area was 9.06 (95%CI: 5.59 – 14.68).
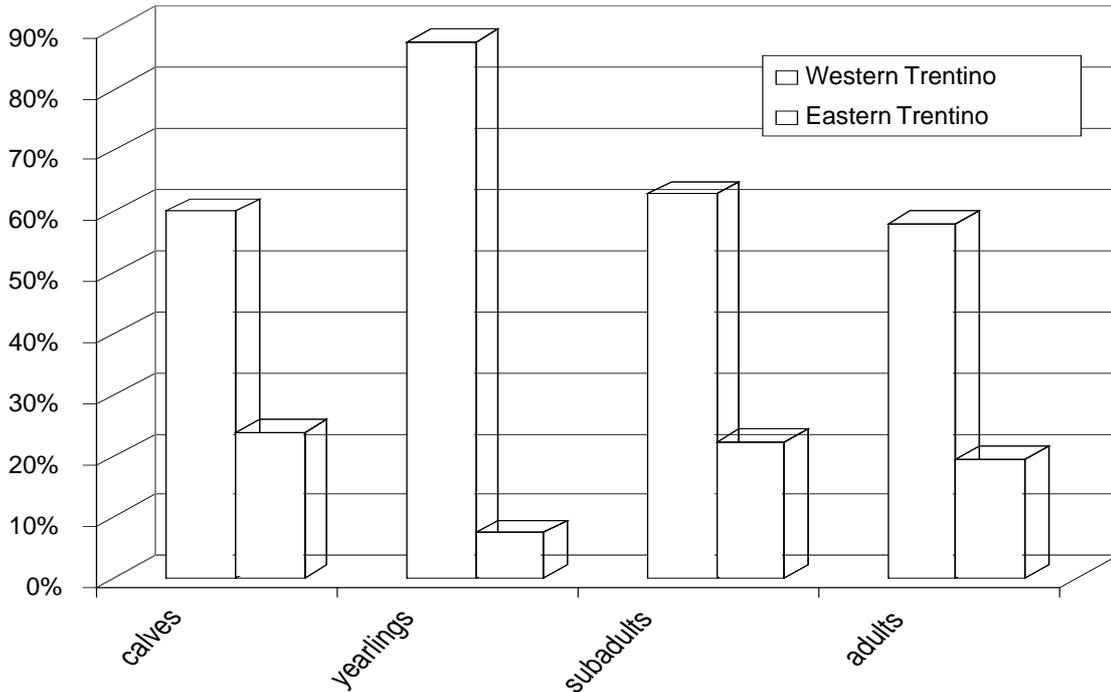
Fig. 2. Comparison between the prevalence of paratuberculosis infection in the western and eastern sectors in relation to age

The infection was also found in other species sympatric with wild ungulates from both study areas such as roe deer, chamoix and ibex (Table 3) as was seen in previous research in Italy and abroad (Tolari *et al.*, 1987; *et al.*, 2000; Ferroglio *et al.*, 2000; Pavlík *et al.*, 2000; Robino *et al.*, 2000; Machackova *et al.*, 2002; Nebbia *et al.*, 2003). Natural infection of paratuberculosis was not limited to ruminants: similar to observations in Scotland (Beard *et al.,* 1999; Greig *et al.,* 2002) MAP was also isolated from fox (*Vulpes vulpes*) and hare (*Lepus europaeus*) (Table 3). Furthermore, according to the authors mentioned above, in foxes, the anatomo-pathological lesions concerned only the lymphatic tissues, whilst no lesions related to paratuberculosis were observed in hares.

Table 3. Prevalence of MAP in different wild species examined from 1999 to 2002

Species	Western area		Eastern area	
	No.	Percent positive	No.	Percent positive
Roe deer	106	24.4%	122	18.8%
Chamois	27	33.3%	61	22.9%
Ibex	0	0%	6	16.7%
Fox	39	7.7%	26	3.8%
Hare	11	18.2%	0	0%

In order to verify whether a domestic-wild animal infection cycle exists, or whether MAP behaves as a species-specific pathogen, a phylogenetic analysis of isolated MAP by means of AFLP is underway. Strain typing, carried out by means of PCR-REA on the IS1311 sequence, confirmed that isolates were of the "bovine type".

The primary lesions found in deer that were culture positive to the culture were located in the intestine. The lesions affected, to varying degrees, the distal tract of the jejunum, the ileum, the ileo-cecum-colic valve, the cecum and the local lymphatic system. A broad range of lesions were observed, varying by location, severity and distribution. In some cases the distal small intestine and the lymphatic system appeared normal or lightly congested. In other cases typical bovine lesions were apparent such as thickening and corrugation of the mucosal layer of the small intestine, edema, and enlargement of mesenteric lymph nodes, Peyer patches and ileo-cecum-colic valve. The considerable variety observed made it possible to classify the lesions according to location, severity and distribution, useful to the study of the disease in the

different categories, distinguished by age, sex and type of recovery (killed or found dead). On the basis of these data and taking as a reference the classification of the immunohistochemical lesions suggested by Perez *et al.* (1996), an attempt was made to classify the observed lesions into three main classes and further subclasses (Fig. 3).

Class A: Effects limited to lymphatic system with thickening Peyer patches, enlargement of mesenteric lymph nodes and/or thickening of lymphatics in mesentery and serosa.

Class B: Catarrhal or catarrhal-haemorrhagic enteritis confined to the ileum.

Class C:
    C1: catarrhal or catarrhal-haemorrhagic enteritis associated with severe lesions to the ileum-cecum-colic
    C2: chronic enteritis with thickening of the wall and wrinkling of the mucosa
    C3: lesions from both class C1 and C2 associated with severe enlargement of mesenteric lymph nodes
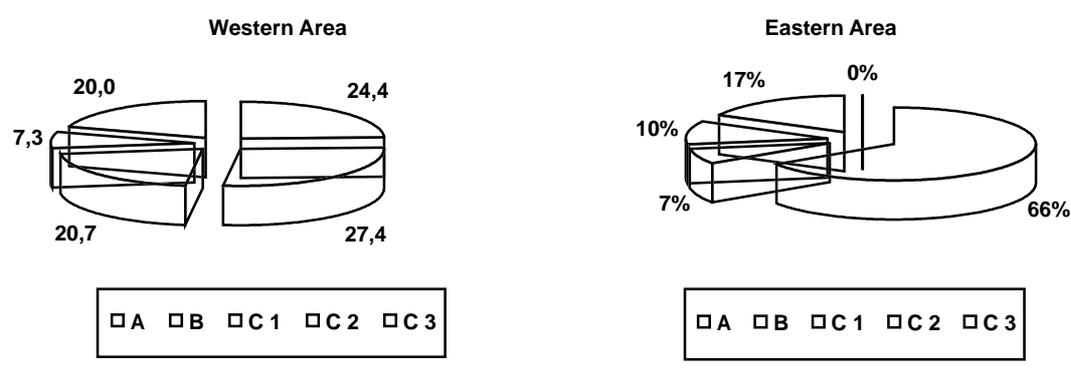
Fig. 3. Prevalence of lesions observed in positive animals in the western and eastern areas

No significant differences were found in the distribution of lesions between hunted and found dead subjects, an indication that paratuberculosis was not affecting population dynamics. A similar result was obtained when evaluating the distribution of lesions by age groups in each study population.

CONCLUSION

The results showed a significant difference in the prevalence of MAP between the two deer populations examined. The statistical analysis highlighted that area of origin is a significant risk factor: for study animals from the Travignolo watershed area, characterized by a much lower density of deer than in the western area, the risk of infection was lower.

The isolation of MAP in many wild species, ruminant and non-ruminant, as documented in the current study has implications for animal health and for the control of the disease., Besides transmitting the infection to conspecifics, infected animals may act as a reservoir of infection for other species, particularly domestic ruminants, with which they share the pastures. Conversely, infected domestic livestock may also be the source of infection for wildlife.

Strain typing, carried out by means of PCR-REA on the IS1311 sequence, confirmed that the isolates were the bovine strain. This may suggest a risk of transmission of the infection between domestic and wild animals during the summer season, when mountain pastures are shared by all species. (Moreira *et al.*, 1999; Pavlik *et al.*, 2000; Robbi *et al.*, 2000; Machackova *et al.*, 2002; Nebbia *et al.*, 2003).

In the two populations of deer analysed, it appears that paratuberculosis at even a quite high prevalence has not had a significant effect on population dynamics. Significant differences were not found between

hunted animals and those found dead in both the areas (western area: 2 = 2.41; P = 0.121 ; eastern area: 2 = 0.36; P = 0.546); furthermore, in animals found dead, only mild lesions were seen at necropsy.

It is necessary to continue molecular biology research to phylogenetically characterize MAP in order to evaluate the relationship among the different strains isolated. A better understanding of the role of the wildlife, non-ruminants included, in the maintenance and transmission of paratuberculosis will allow a better understanding of the risk factors to wildlife and disease control strategies in domestic ruminants. Considering the possibility of interspecific transmission, through contaminated pastures, the current detection and cull policy will be inefficient for the control of the disease in areas where livestock interact with wildlife.

ACKNOWLEDGEMENTS

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A thirty-three year history of the epidemiology, diagnosis and eradication of bovine paratuberculosis in a sub-tropical environment

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ABSTRACT

There are few descriptions of the biology of bovine paratuberculosis in a sub-tropical environment. We therefore analysed data collected over a thirty three year period in a sub-tropical region of New South Wales, Australia. The objective was to describe attributes of the epidemiology and control of the infection. Records of all cattle herds in the study area were reviewed and those with a history of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) between 1971 and 2004 were identified. Records contained comprehensive descriptions of each outbreak and had been collected and maintained by the senior author for over half of the study period. Descriptive analysis of the 78 herds with a history of MAP infection revealed 54% had achieved eradication with the proportion of successes higher in beef herds (70%) compared to dairy herds (32%). Herds infected earlier in the study period took longer to be diagnosed and longer to eradicate the infection compared to herds that were infected more recently. De-stocking was a key component of successful eradication programs but had a limited impact in dairy herds with evidence of chronic infection and within-herd transmission. There was no evidence of spread between herds other than that due to the introduction of infected animals or sharing of contaminated facilities. Improved diagnostic tools, flexible eradication programmes and education of herd owners are possible reasons why MAP infection is being more successfully managed in recent years.

INTRODUCTION

One impediment to the control of paratuberculosis in cattle is that most of the information on epidemiology and ecology is derived from studies of animals housed or grazed in temperate climates. In Australia, a large proportion of cattle are grazed in sub-tropical and tropical regions. Although bovine paratuberculosis is extremely rare or absent in most of these locations, the infection can possibly enter with the importation of cattle from moist and temperate regions where paratuberculosis is more common. Thus, information describing the course of the infection herds kept in warmer climates is needed and would benefit the national approach for controlling paratuberculosis in Australia.

Obtaining the information required for herds in non-temperate climates is problematic because the infection is almost entirely absent from these northern areas of Australia or occurs only at very low levels (Anonymous, 2004). However, there is one region in northern New South Wales that experiences a sub-tropical climate that has been subject to regulatory control of paratuberculosis for a protracted period. Historical information from regulatory and advisory activities in this region outlines important aspects of the epidemiology of the disease as it occurs outside of temperate grazing systems. The bovine paratuberculosis data for this region were therefore obtained and analysed to define the following: the prevalence of infected herds in 2004, the number of new cases over time, the process by which herds were classified as infected, the route by which herds become infected, whether transmission occurs between neighbouring herds, and the impact of timing of herd diagnosis on the success of eradication.

MATERIALS AND METHODS

Data for this study comes from the records held by the Casino Rural Lands Protection Board (Casino RLPB) located in north eastern New South Wales, Australia. Casino RLPB covers about 500,000 hectares of grazing country and woodland varying from undulating to flat and experiences rainfall of moderate to high intensity, primarily in the summer (median annual rainfall of 1154mm). It is one of New South Wales' 48 RLPBs that manage animal health and livestock identification on behalf of the Government of New South Wales and local land holders. The current livestock population of Casino RLPB is approximately 180,000 adult beef cows in 2,450 herds and 19,500 dairy cattle in 84 commercial herds. Beef cattle are almost exclusively managed on pasture with some supplementation with grain and conserved fodder during dry-periods. Dairy cattle are predominantly of the Friesian-Holstein breed. They graze primarily on sub-tropical pasture as part of a family-run enterprise and receive varying amounts of supplements during milking according to individual management preferences. Dairy cattle calve all-year-round while calving of beef breeds occurs most commonly in the spring.

Records on the paratuberculosis infection status of all cattle herds in the Casino RLPB have been maintained since 1960 and have been compiled since 1988. During this period data on the paratuberculosis status of herds and on activities related to diagnosis and eradication were obtained in several ways. Firstly, owners may have requested a veterinarian to investigate the cause of sickness or death in cattle (owners and private veterinarians are required to report to the RLPB any suspect cases of paratuberculosis). Secondly, owners may have voluntarily tested all or some of the herd (using combinations of screening and confirmatory tests) either during an accreditation process or as a participant in a serological survey (here state government or RLPB veterinarians performed follow-up investigations where needed to clarify herd status). Thirdly trace-back investigations following disposal of an infected animal may have revealed additional infected herds. Regardless of the manner in which infected herds were identified, the owners were repeatedly contacted by the veterinarian employed by the RLPB to provide technical assistance to herd owners on control, eradication and regulatory requirements. Through this last mechanism the infection status of herds and application of control measures was monitored. The recorded infection status of all herds in the RLPB was not altered until conditions met the standards prescribed in the National Standard Definitions and Rules for Johne's Disease in Cattle (Anonymous, 2005a). Thus a herd was considered to have eradicated MAP if all susceptible animals at risk of infection had been culled and at least 12 months had elapsed since the removal from the property of any potentially-infected animals over 2 years of age.

From these records it was possible to identify all herds with a history of paratuberculosis infection between 1971 and 2004, the date of initial diagnosis of infection in these herds and the date of eradication (if that occurred). Herd-prevalence estimates and temporal relationships were analysed. A categorical analysis was used to describe the process by which infected herds were detected, the cause of infection being introduced into herds, and the methods used by herd owners to eradicate infection. Confidence limits and statistical hypotheses testing were not produced during analysis because of the near-census nature of the information.

RESULTS

At the end of the study period the prevalence of cattle herds known to be infected with MAP in Casino RLPB was 21.4% (18/84) for dairy herds and 0.71% (18/2460) for beef herds. All commercial dairy herds have had at least one herd test and 64% (54/84) of these are currently enrolled in the Australian Johne's Disease Market Assurance Program ('Cattle MAP'; Anonymous 2005b), an industry wide accreditation scheme involving testing. While 138 beef herds have had at least one whole-herd test, this represents only 5.6% of the 2460 beef herds in the area of interest.

Over the study period 78 herds infected with paratuberculosis were detected in Casino RLPB. Fig. 1 shows that most of these herds were first identified as infected in the period 1990 to 2001, linked in time to major developments in paratuberculosis control in the region. Of the 78 infected herds, 73% were identified as a result of clinical investigations (invariably at the behest of owners), 14% as a result of surveys, 8% as a result of herd-owners' undertaking accreditation tests, and 5% as a result of tracing from known-infected

herds. Introduction of an infected bovine was the probable route of entry of paratuberculosis into 92.3% (72/78) of infected herds. For two herds the most probable source of infection was sharing of cattle handling facilities used by another (infected) herd. The cause of infection being introduced could not be identified for four herds. There was no evidence that herds became infected due to exposure from cattle straying from other infected herds. For infected beef herds, 39 of 42 (92.8%) acquired infection by introducing animals from local herds either in Casino RLPB or the adjacent Tweed-Lismore RLPB. One-third of these introductions were dairy breeds.
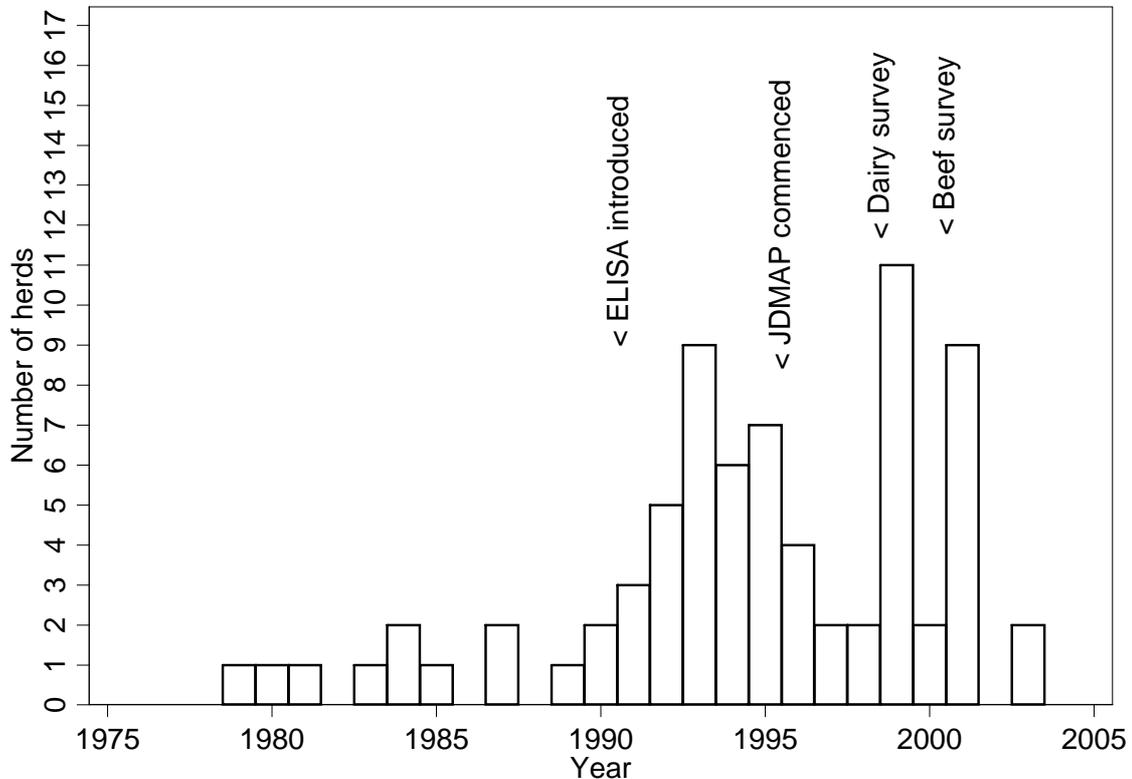
Fig. 1. The number of newly diagnosed infected herds each year in Casino RLPB. The years when the ELISA became available, the Cattle MAP program began, and dairy and beef surveys were initiated are noted.

Eleven of the 32 dairy herds that became infected introduced paratuberculosis with cattle procured from endemic areas outside of New South Wales. Nineteen dairy herds (61%) were classified as having acquired the infection from local herds. Only in two instances were dairy herds classified as having become infected because of animals brought in from low prevalence areas of New South Wales (RLPB's within BJD protected zones). The source of infection for four dairy herds could not be determined.

For the 74 herds where the year of introduction of infection was known the mean duration of the I-D interval was 6.9 years with a range of 0.2 – 28 years. Fig. 2a shows the I-D intervals for each herd sorted by year of introduction of infection and demonstrates a clear trend for a shortening of the I-D intervals over the study period. The time in years from first diagnosis to eradication (D-E) and subsequent status change for the 42 herds that achieved eradication is shown in Fig. 2b, sorted by year of diagnosis. The D-E interval had a mean of 4.4 years and ranged from one to 18 years. A substantial shortening of the D-E interval over the study period is also evident. For the 36 herds infected at the end of the study period the mean time since initial detection of infection was 8.9 years. Herds (both dairy and beef) where transmission of paratuberculosis to homebred animals was detected had much higher mean I-D and D-E intervals compared to herds where transmission had not been detected. Beef herds experiencing within-herd transmission had a mean I-D interval of 9.9 years (compared to 2.2 without transmission), and a mean D-E interval of 10.4 years (compared to 2.2 years without transmission). Dairy herds experiencing within-herd transmission had a mean I-D interval of 11.3 years (compared to 2.1 years without transmission) and a mean D-E interval of 14 years (compared to 3.2 years without transmission).

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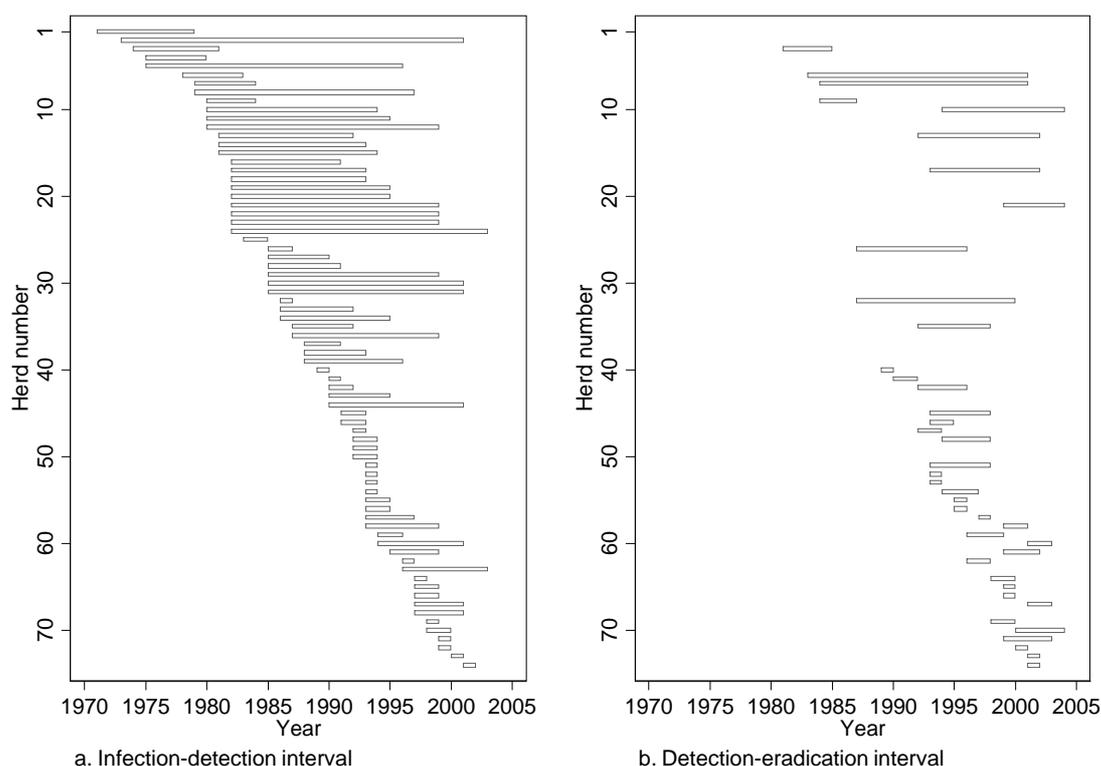
Fig. 2. The infection-detection (I-D) intervals (a) and detection-eradication (D-E) intervals (b) for 74 cattle herds diagnosed with MAP infection in Casino RLPB from 1971-2004 sorted by year of introduction.

For beef herds where no transmission had occurred, 92.3% (24/26) successfully eradicated paratuberculosis compared to 38.9% (7/18) of beef herds with infection detected in cattle born on the property. In the case of dairy herds, 100% (9/9) with no evidence of within-herd transmission eradicated paratuberculosis while only 8% (2/25) of infected herds where within-herd transmission was confirmed had eradicated paratuberculosis by the end of the study period. Total or partial de-stocking were essential elements in all successful eradication programs. 'Test and cull' measures played a supporting role in 42.8% (18/42) of herds achieving eradication.

DISCUSSION

While the 78 case herds in this study represent all the known infected herds in the Casino RLPB over the study period it is likely that paratuberculosis has gone undiagnosed in some herds because of deliberate failure to report (despite regulatory requirements), herds being disbanded before diagnosis, misdiagnosis by owners and veterinarians, and absence of clinical signs. While the magnitude of this error has not been quantified, we consider it likely that the true herd prevalence for paratuberculosis in commercial dairy herds is presently close to the apparent prevalence reported in this study. This is because all commercial dairy herds have had at least one whole herd ELISA test and 60% of the current commercial herds are enrolled in the Cattle MAP and have undergone additional herd testing. Misclassification of the paratuberculosis status of beef herds is likely to be higher. The low assessment rate for beef herds (5.6% of beef herds have had a herd test) and the high prevalence (4/21 or 14.2%) of infected beef herds detected in a recent survey (Anonymous, 2000) suggests that non-detection could be a much greater issue for this group. It is possible that some of these beef herds were infected during their former existence as dairy herds and may still be infected but not recorded as such.

The data shows that introduction of an infected animal is the overwhelming cause of herds becoming infected in the Casino RLPB and this is in agreement with other reports (Sweeney, 1996; Collins, 2003). Much of the rapid increase in infected herd numbers in the 1990's can be attributed to the introduction of dairy cattle from moist-temperate regions in southern Australia (mostly outside of NSW) in the early 1980's when local dairy herds were undergoing expansion. During this period the infection was introduced into 12 herds which in most cases was not detected until the early 1990's (Fig. 1). One of the infected beef herds

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introduced infection by purchasing dairy cows used to foster-rear stud bull calves. While two herds introduced the infection into their herds through sharing common yards for a period, there was no suggestion that contaminated faeces, water or effluent crossed boundaries to initiate infection in a clean herd. Straying occurs in most cattle herds and has been suggested as mechanism for transmission of bovine paratuberculosis (Kennedy and Benedictis, 2001) but was not found to be a cause of new herd infections in this study. Contract rearing of heifers off farm was not identified as a cause of new infections. Wildlife were not evaluated as a source of infection but are considered insignificant as a reservoir of infection in Australia (Kennedy and Allworth, 2001). Spraying of dairy effluent onto pasture, dipping cattle for external parasites in shared facilities, and exposure to contaminated fodder apparently did not cause any herds to become infected. An important caveat is that the above risk factors were assessed using a methodology that may not be sufficiently sensitive to detect small effects. In the future it is possible that the study will be extended to include spatial and temporal analysis techniques to verify the role of some of the above risk factors.

During the study period almost three quarters of the infected herds webecause the herd manager asked for a veterinary investigation. It is probable that this method of detection will decline in usefulness with time because cattle owners are increasingly aware of the adverse social and financial consequences of having paratuberculosis detected in their herds. It was noticeable in this study that very few infected herds were identified by owner notifications after 1997. Financial incentives to meet eradication costs, education and flexible eradication programs appear to be important in encouraging herd owner co-operation in identifying infected herds. Zoning of areas to reflect the risk level for BJD was introduced in 1999 and encouraged herds in the study area to participate in the accreditation program (JDMAP) to retain access to traditional markets. In the process four herds were detected as infected. Misdiagnosis is likely to be a smaller source of error in the classification of herd status than notification failure. For clinical cases serological, microbiological and histological tests all have good sensitivity (over 80%) although in a small number of advanced clinical cases anergy develops and serology is of limited value (Clarke, 1997). Herds with only subclinical infection show non-specific signs (Chiodini et al, 1984) and are unlikely to be identified through owner notification or disease investigation. In this study 27% of the infected herds were identified by tracing, survey or accreditation activities using the abovementioned tests.

Eradication of paratuberculosis in the study area was based on a number of assumptions. These are to do with the age of onset of shedding, the extent of resistance in different age-groups of animals and the duration of survival of MAP in the environment. Although none of these assumptions are likely to be completely valid in all circumstances the findings suggest that in combination they are sufficiently robust for eradication to be achieved in many instances. However, there are some unique features of the location and study population that might have promoted eradication. These include the low rate of within-herd prevalence of clinical cases (usually less than 1% of animals affected per annum) and a low sero-prevalence of infection. The subtropical climate characterized by high summer temperatures and high summer rainfall may also be a factor assisting eradication.

The impact of transmission on eradication success and time taken to achieve eradication was apparent in beef herds and very noticeable in dairy herds. Cattle owners need to become more aware of the benefits of early diagnosis as a way of preventing within-herd transmission and thus making eradication more affordable and feasible. A possible reason for the trend towards a shorter time to eradicate MAP after detection is that herd owners have been granted more options under the regulatory policy. Initially, only purchased steers aged over 12 months could be introduced onto a property after destocking. They could only be grazed on the property for a minimum of 12 months and then sent to slaughter before two years of age. The current policy is that any low risk cattle introduced onto contaminated land when over 12 months of age can remain on the property after the twelve month decontamination period is complete. These animals do not have to be culled for slaughter before a status change can occur. The more flexible policy appears to have been effective and has allowed destocking and restocking to occur simultaneously with the purchases and sales occurring in the same market to avoid price variations. Although it is possible for cattle over 12 months of age to become infected in heavily contaminated environments, they are generally regarded as resistant to infection (Rankin, 1962; Larsen et al, 1975). The emphasis on de-stocking to achieve eradication was particularly attractive (quick and inexpensive) when exposure was confined to specific herd groups only and partial de-stocking could be applied. In dairy herds, young susceptible animals are often reared together and if exposed to MAP widespread infection is possible (presumably the

longer the exposure time the greater the prevalence of infection within the group). The two dairy herds in this study that eradicated MAP in spite of transmission did so by de-stocking and leaving the industry. De-stocking is less affordable for dairy herds due to the prohibitive cost of purchasing replacement milking cows.

Although none of the herds in this study eradicated MAP by testing and calf segregation alone this remains the main method for progressing herd status in dairy herds. If a herd screening test is used regularly to identify and remove infected animals then only asymptomatic animals are likely to remain and intra-uterine spread is less likely. Such programs are lengthy though, requiring repeated rounds of testing and strict monitoring of bio-security to prevent calf exposure (Jubb and Galvin, 2004).

In conclusion, this study demonstrates that despite the complexities and uncertainties associated with bovine paratuberculosis it can be successfully eradicated from many herds in a subtropical environment. Eradication is more easily achieved if within-herd transmission is blocked. Prompt detection of infected herds promotes efficient eradication on a short time scale. Dairy herds with chronic paratuberculosis infection remain the greatest challenge for owners and advisers. Introduction of infected animals is the main method of herd-to-herd spread in the sub tropical environment studied. Further evidence from a spatial analysis of the distribution of infected herds is required to support inferences made here about the causes of herds becoming infected.

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Rabbit to rabbit transmission of *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

Rabbits have been increasingly linked to the persistence of paratuberculosis (Johne's disease) in domestic ruminants in the UK. The aims of this study were to determine the routes of intra-specific transmission of MAP in rabbits in order to gain understanding of the dynamics of MAP in this host. Rabbits were sampled from two sites where MAP had previously been isolated from the livestock and rabbit populations. The overall prevalence of MAP in rabbits was high at both sites studied, 39.7% and 23.0% respectively. MAP was isolated from the testes, uterus, placenta, foetuses and milk suggesting transmission could occur via vertical, pseudo-vertical and horizontal transmission. This is the first time that the bacterium has been isolated from any of these tissues in a non-ruminant wildlife species. The presence of these routes of transmission within natural rabbit populations may contribute to the maintenance of MAP infections within such populations.

Key words: disease dynamics, Johne's disease, *Oryctolagus cunuiculus*, paratuberculosis, routes of transmission

INTRODUCTION

Recent studies have shown that the rabbit (*Oryctolagus cuniculus*) is thought to pose the greatest risk of inter-species transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Beard *et al.* 2001a; 2001b; Greig *et al.* 1997; 1999). This is because of the combination of their high prevalence of MAP infection (up to 63%; Beard *et al.* 2001b; Greig *et al.* 1997; 1999), the high rate of excretion of bacteria in their faeces (up to 10^6 colony forming units per gram of faeces, which could constitute an infective dose for a ruminant; Daniels *et al.* 2001), along with the lack of behavioural avoidance of rabbit faecal pellets by ruminants while grazing (Daniels *et al.* 2001, Judge *et al.* 2005). All of these factors lead to a high potential for interspecies transmission of MAP from rabbits to grazing ruminants. Furthermore, MAP isolated from rabbits is morphologically and genetically indistinguishable from that found in ruminants (Greig *et al.* 1999) and calves experimentally inoculated with the rabbit strain of the organism have subsequently become infected with paratuberculosis (Beard *et al.* 2001c).

There are three main routes through which intra-species transmission of MAP may occur in cattle; vertical, *in utero* (Seitz *et al.* 1989; Sweeney *et al.* 1992), pseudo-vertical, through suckling (Doyle 1956; Taylor *et al.* 1981; Sweeney *et al.* 1992; Streeter *et al.* 1995) and horizontal transmission either sexually (Ayele *et al.* 2004) or via the faecal-oral route, (Sweeney 1996). These routes were used as a focus for the potential routes of transmission in populations of wild rabbits. Determining the dynamics of the disease in rabbits is central to determining if rabbits are a true reservoir for the infection, i.e. can the MAP persist in the rabbit population without input from any other species. The aim of this study was to determine the routes of transmission of MAP in a natural population of rabbits in order to understand the dynamics of MAP in rabbits.

MATERIALS AND METHODS

Collection of samples
Four hundred and eighty-seven rabbits in total were randomly sampled from the population following a standardised sampling regime between April 2002 and May 2004 from two sites in Perthshire, Scotland. MAP has previously been isolated from livestock and rabbits on both sites (Greig *et al.* 1997;1999).

Gross post mortem procedures
Post mortems were carried out within 18 hours of sampling taking place. Rabbits were tagged with an individual identification number and weighed. New sterile disposable scalpels, forceps, pipettes and needles were used for each rabbit and the work area was disinfected after each rabbit to reduce the risk of cross contamination. The post mortems then followed a strict procedure in order to reduce the risk of cross contamination from the gut to any other sample taken.

Histopathology
Sections of the sacculus and appendix from a subset of the sample were taken for histopathological evaluation. The tissues were fixed in 10% formal-saline for a minimum of 24 hours, trimmed, dehydrated through graded alcohols, embedded in paraffin wax and sectioned (5μm). The sections were stained with hematoxylin and eosin for routine histopathological examination and to test for acid fast bacilli (AFB) by the Ziehl-Neelsen method.

Culture
Tissue, faecal, foetal and placental (pool of cotyledons) homogenates were prepared following the protocol in Greig et al. (1997). Foetuses were removed from the placenta and weighed, those weighing less than 3g were processed as a pool of all foetuses from that litter, those above 3g were eviscerated and the liver, gut and stomach processed as a pool for each foetus. Briefly, for all homogenates, 1g of faeces or 0.5cm^3 of finely chopped tissues were homogenised in 5ml of sterile distilled water with a Colworth Stomacher 80 (Seward Medical, London, UK). The homogenates were decontaminated by adding 5ml of 1.5% cetyl pyridinium chloride and allowed to stand overnight at room temperature to allow particulate material to settle. The supernatants were centrifuged at 4000 rpm for 30 minutes and each pellet resuspended in 5 ml of sterile distilled water. The centrifugation step was repeated and each pellet resuspended in 500 μl of sterile distilled water. Two slopes of Middlebrook 7H11 agar supplemented with Selectatabs (amphotericin B, polymixin B, carbenicillin and trimethoprim; code MS24; MAST Laboratories Ltd. Merseyside, United Kingdom), 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment medium (Difco, Surrey, United Kingdom) and 2 μl of mycobactin J (Allied Monitor, Fayette, Mo.) per ml were inoculated with 110 μl of the prepared suspension. The cultures were incubated for up to 16 weeks at 37°C and examined regularly for bacterial growth.

PCR
Mycobacterium avium subsp paratuberculosis was confirmed using the IS900 insertion sequence. Briefly, 100 μl of sterile distilled water was inoculated with a single colony of positive culture. DNA was extracted from the bacterial suspension by heating at 100°C for 10 minutes, then centrifuged at 13000rpm for 2 minutes. 5 μl of the supernatant bacterial suspension was analysed by PCR using electrophoresis in 2% agarose gel in 1XTBE buffer. The gels were stained in ethidium bromide (5 μg/ml) and visualised by UV light transillumination.

Ageing of rabbits
The eye lenses were used to determine the age of rabbits sampled following the protocol described by Wheeler & King (1980). Briefly, the eyeballs were stored in 10% formal saline for a minimum of 14 days before the lenses were removed and oven-dried at 85°C for seven days. The mean dry weight of the lenses was then used to calculate the age of the rabbit using the equation given in Wheeler & King (1980).

Statistical analysis
Generalized Linear Models (GLM) using a negative binomial distribution with a logit link function were used to determine differences in prevalence between age groups (sampling was divided into five age groups; ≤ 3 months – juveniles, > 3 & ≤ 6 months – potentially sexually mature but not likely to be breeding, >6 & ≤ 12

months – likely to be first-time breeders, > 12 & ≤ 24 months – perform majority of breeding, and > 24 months – as ageing is not accurate above 24 months) and sex (divided into 3 groups, male, non-pregnant female and pregnant female).

RESULTS

Of the initial random samples of all rabbit categories, MAP was isolated form the gut pool samples of 100 (39.7%) of 252 from site 1 and 29 (23.0%) of 126 from site 2. None of the animals displayed any visible signs or lesions indicative of paratuberculosis at post mortem examination. The results of culture and PCR for the reproductive organs, faeces and milk from the 100 gut pool positive rabbits at site 1 are given in Table 1.

Table 1. Culture and PCR results from the reproductive organs, faeces and milk from the 100 gut pool positive rabbits at site 1.

	No. sampled	Culture +ve	PCR +ve	%
Testes	52	11	11	21.5
Uterus	47	8	8	17.0
Faeces	8	8	8	100.0
Milk	27	4	4	14.8

The culture and PCR results for all pregnant females' gut pool samples, placentas and litters of foetuses from both sites are shown in table 2. As in some cases the foetuses were too small to be processed individually and were therefore processed as a pool, the results are shown for the litter, not individual foetuses within the litter. Of the pregnant females sampled, 36.8% from site 1 and 53.4% from site 2 were gut pool culture and PCR positive. Of these individuals 7.1% of litters from site 1 were culture, but not PCR, positive for *Mycobacterium* spp. and 3.2% of litters from site 2 were culture and PCR positive. However, the mother of this litter had perforated intestines, therefore cross contamination at collection cannot be ruled out.

The prevalence of MAP varied between age groups at both site 1 (d.r. 4.76, d.f. 4, p<0.001) and site 2 (d.r. 10.16, d.f. 4, p<0.001). Age group 1 (≤ 3 months) had a significantly lower prevalence than any other age group at both sites (11.1% site 1 and 3.2% site 2; Fig. 1).

Table 2. Culture and PCR results of the gut pool samples, placentas and litters of foetuses taken from pregnant females from both sites. Results for placentas and litters are only given for females that were gut pool positive.

	Site 1				Site 2			
	No. sampled	Culture +ve	PCR +ve	%	No. sampled	Culture +ve	PCR +ve	%
Pregnant females	38	14	14	36.8	58	31	31	53.4
Placenta	14	3	3	21.4	31	1	1	3.2
Litters	14	1	0	7.1	31	1	1 [a]	3.2

[a] All 3 foetuses from this litter were individually processed and all 3 were both culture and PCR positive. However, the mother had perforated intestines, therefore cross contamination at collection cannot be ruled out.
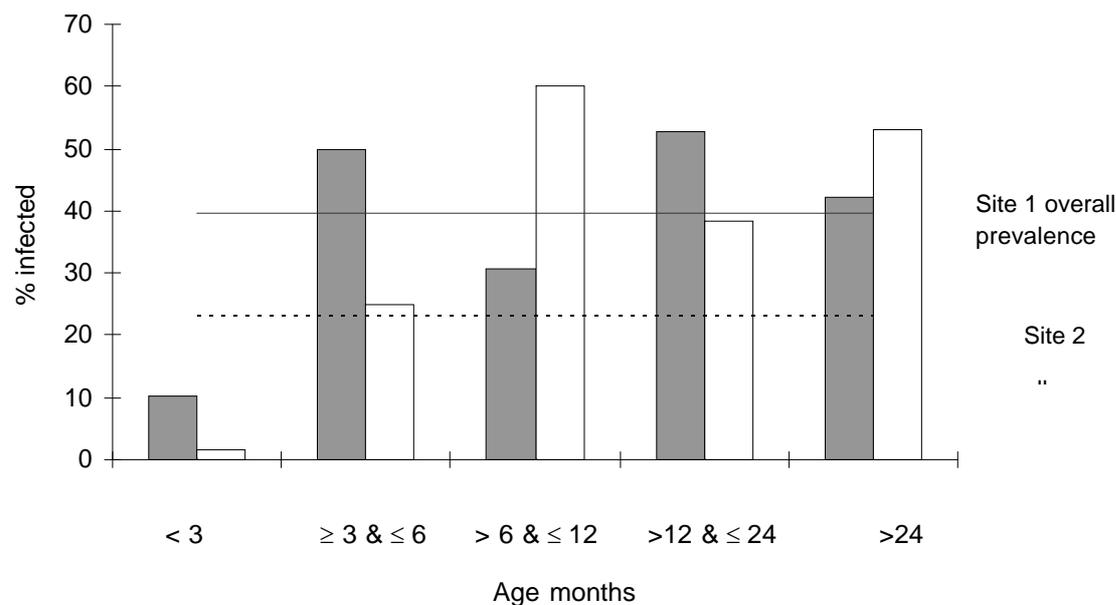
Fig. 1. Prevalence of infection with MAP by age class at both sites. Site 1 = dark shaded bars; Site 2 = light shaded bars

DISCUSSION

Overall the prevalence of MAP in rabbits was high at both sites studied. MAP was isolated from the testes, uterus, foetuses, placenta and milk; this is the first time it has been isolated from these samples in any non-ruminant wildlife species.

As stated earlier, transmission of MAP occurs vertically *in utero* in cattle. Cattle have a syndesmochorial placenta in which the lining epithelium of the uterus is the only maternal tissue that is eroded (Vaughan *et al.* 2000), therefore, there is little contact between the maternal blood and the foetus. In contrast, rabbits have a haemoendothelial placenta, where the endothelium of the capillaries of the chorion come into direct contact with the maternal blood (Vaughan *et al.* 2000). This suggests that there would be much greater potential for vertical transmission of MAP in rabbits than in cattle. However, in this study only 7.1% (1/14) at site 1 and 3.2% (1/31) at site 2 of litters from MAP positive does were culture positive for *Mycobacterium*. Furthermore, the results from the foetal tissues were not conclusive as the culture and PCR positive foetuses from site 2 came from a mother whose intestines had been perforated, therefore cross contamination cannot be ruled out. Also, the culture positive foetus from site 1 was not PCR positive, therefore we cannot be certain that it was MAP and not another mycobacterium. Pseudo-vertical transmission in rabbits may occur through the ingestion of infected milk while suckling and/or the ingestion of infected faecal pellets during the weaning process, as does deposit faecal pellets into the nest in the days prior to weaning for the kits to ingest (Hudson *et al.* 1996).

It is highly likely that in the majority of cases the level of MAP infection in foetuses and young rabbits is very low, due to the slow growing nature of the bacteria. The ability to detect these low levels of infection may be confounded by the under-detection of the bacteria due to the low sensitivity of the culture. The sensitivity of the solid medium culture method used here has been determined to be approximately 30% (McNabb *et al.* 1991; Eamens *et al.* 2000; Whitlock *et al.* 2000) for individuals with low levels of infection, as would be expected in foetuses and juveniles. Consequently, it may be that the methods of detection of MAP at low levels were not adequate and that vertical and pseudo-vertical transmission rates are higher than suggested from the culture results obtained, suggesting that our results are conservative.

Once animals have been weaned, they would only be susceptible to infection via horizontal routes or through inter-species transmission. There are two main routes of horizontal transmission that could be

involved in the transmission of MAP, the faecal-oral route and sexual transmission. It has already been determined that infected rabbits shed a high number of the bacterium in their faeces, therefore there is the potential for horizontal transmission via this route. The recovery of MAP from both the uterus and testes of rabbits suggests that there is the potential for sexual transmission to occur. Further investigation of this route of transmission needs to be undertaken, however the presence of MAP in the semen of bulls (Ayele *et al.* 2004) suggests that sexual transmission may occur in cattle and it is therefore possible that it could also occur in rabbits.

The results provide evidence of the potential for transmission of MAP in rabbit populations via vertical, pseudo-vertical and horizontal routes. The presence of these routes lends further support to the suggestion that rabbits are a significant source of MAP for livestock, as these routes of transmission help maintain MAP infection in rabbit populations.

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Spatial and temporal distribution of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits and the environment

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ABSTRACT

Scotland contains regional hotspots of Johne's disease (caused by *Mycobacterium avium* subsp *paratuberculosis*) in rabbits and there is increasing evidence of a link between paratuberculosis infection in rabbits and cattle. The spatial and temporal dynamics of paratuberculosis in rabbits were studied within a hotspot region with the overall aim of determining environmental patterns of infection and thus risk of inter-species transmission to livestock. The specific aim was to determine if prevalence of paratuberculosis in rabbits varies temporally between seasons or spatially within a hotspot. The overall prevalence of MAP in rabbits was 39.7%; temporal distribution of infection in rabbits followed a cyclical pattern with a peak in Spring of 55.4% and a low in Summer of 19.4%. Spatially, MAP infected rabbits, and thus risk of inter-species transmission, were highly clustered in the environment. However, this is mostly due to the clustered distribution of rabbits.

Key words: Johne's disease, spatial, temporal, disease distribution, rabbits

INTRODUCTION

Scotland is considered a hotspot for paratuberculosis in cattle (VLA 1993). Paratuberculosis is notoriously difficult to control in farmed ruminants and one possible explanation for this could be due to the role of wildlife in the epidemiology of the disease (Daniels *et al.* 2003a). Recent studies have shown that non-ruminant wildlife species carry the infective agent (Beard *et al.* 2001a;b) adding to environmental distribution of disease; of these species the rabbit (*Oryctolagus cuniculus*) is thought to represent the greatest risk of transmission of MAP to cattle.

Rabbits are an asymptomatic carrier of MAP and shed sufficient numbers of bacteria in their faeces so that one faecal pellet may constitute an infective dose for cattle (Daniels *et al.* 2003b). Cattle do not avoid rabbit faeces while grazing (Judge *et al.* 2005), which is unusual. Furthermore, cattle inoculated with rabbit isolates of the bacteria have become infected with the disease (Beard *et al.* 2001c). This leads to a high risk of infection via the faecal-oral route in grazing environments, which is considered to be the main route of MAP transmission (Sweeney 1996).

Therefore, the distribution of the infection in the rabbit population equates to the environmental risk of inter-species disease transmission to grazing livestock as there are no behavioural complexities affecting this risk. Here the overall aim was to determine the temporal and spatial dynamics of MAP within infected rabbit populations, and thus the inter-species environmental risk to livestock.

MATERIALS AND METHODS

Study site
The study was carried out on a 2000ha site situated in Tayside, Scotland, a previously identified regional hotspot of paratuberculosis in rabbits (Greig *et al.* 1999). *Mycobacterium avium* subsp. *paratuberculosis* has previously been isolated from cattle, sheep and rabbits on the study site (Greig *et al.* 1997; 1999). The whole site was surveyed and the position of each rabbit warren (a group of burrows used by a social group of rabbits) was mapped using Global Positioning System (GPS) readings taken on a handheld Garmin GPS12.

Rabbit sampling
A total of 252 rabbits were sampled between April 2002 and May 2003. A standardized sampling regime, using even effort across the whole site on every sampling night, was followed. GPS readings were taken at the position each rabbit was sampled again using a handheld Garmin GPS12. Briefly, the carcasses were weighed, sexed and given a superficial gross examination before post mortem procedures were carried out. Post mortem procedures included taking four sections of the gut (sacculus, appendix, ileum and caecum) along with a mesenteric lymph node, which were pooled for culturing. New instruments were used for each animal. The MAP disease status of each animal was determined by culture on Middlebrook 7H11 solid agar culture media slopes and analysis by PCR using the IS900 insertion sequence, following the protocols previously described (Greig *et al.* 1999).

Histopathological examination
Sections of the sacculus and appendix were taken for histopathological evaluation from a random subset of the sample. The tissues were fixed in 10% formol-saline for a minimum of 24 hours, trimmed, dehydrated through graded alcohols, embedded in parafin wax and sectioned (5μm). The sections were stained with hematoxylin and eosin for routine histopathological examination and to test for acid fast bacilli (AFB) by the Ziehl-Neelsen method.

Statistical analysis
Generalized Linear Models (GLM) using a negative binomial distribution with a logit link function were used to determine differences in prevalence between seasons (sampling was divided into five seasons, Spring 02 covering April and May, Summer - June to August, Autumn – September to November, Winter – December to February and Spring 03 – March to May 2003) and areas (see below). The difference between the observed and the fitted values, in light of the random variation described by the model, is given by the deviance ratio (d.r.). The K-function was used to test for complete spatial randomness (CSR) (Ripley 1976) of the distribution of rabbits, warrens and MAP. The K-function takes an arbitrary point and determines the expected number of points within a given (user defined) distance of this arbitrary point and divides it by the overall intensity of points within the sampling region. If the points conform to CSR:

$$K(r) = \pi r^2$$

where r is the distance. The K-function was plotted against distance in meters, with the sampling region set using the minimum convex polygon containing all points, along with a 95% confidence envelope obtained from 1000 simulations of the K-function (Ripley 1976). If the distribution was completely spatially random the line would follow zero, above zero indicates there is clustering, below regularity. Random labelling was used to 'assess the spatial association between two different types of events in a bivariate process' (Diggle 2003) for the infectious state of the animals sampled i.e. given the locations of the animals, are the infected ones randomly distributed throughout, or is there evidence that the infected animals are more clustered or regular than would be expected under randomness. This method calculates the K-function for each group (i.e. infected and non-infected rabbits) and the difference in the two K-functions was plotted against distance as above providing an indication of the distribution of infection within the rabbit population after accounting for the spatial clustering of the rabbits in the environment.

RESULTS

Culture and PCR
Of the 252 rabbits sampled 100 (39.7%) were culture positive for *Mycobacteria* species, i.e. bacterial colonies grew on the agar slopes. Bacterial colonies from the agar slopes of all 100 culture positive individuals were confirmed as MAP by PCR.

Histopathological examination
Histopathological lesions consistent with MAP infection were found in 16 (28.6%) of the 56 samples sent for examination. No histopathalogical lesions were found in culture negative animals.

Temporal distribution
The prevalence of MAP infection varied between seasons (d.r. 7.38, d.f. 4, p<0.001). The prevalence was significantly lower in Summer and Autumn than in the other three seasons. There was no significant difference between prevalence in Summer and Autumn. The highest prevalence was in Spring 02 falling to a low in Summer, steadily rising again through Autumn and Winter and reaching a similar high prevalence again in Spring 03 (Table 1).

Table 1. Number of rabbits sampled, number of positives (all are culture and PCR positive for MAP) and prevalence in each season

Season	Total sampled	Positives	%
Spring 02	65	36	55.4
Summer	62	12	19.4
Autumn	42	11	26.2
Winter	41	18	43.9
Spring 03	42	23	54.8
Total	252	100	39.7

Table 2. Number of rabbits sampled, number of positives (all are culture and PCR positive for MAP) and prevalence by area

Area	Total sampled	positives	%
A	45	7	15.6
B	39	19	48.7
C	44	15	34.1
D	26	13	50.0
E	93	46	49.5
unknown	5	0	0.0
Total	252	100	39.7

Spatial distribution
The rabbits were clustered in five locations, designated A - E (Fig. 1), and the prevalence of MAP infection in the rabbits was significantly lower in area A than in all other areas (d.r. 4.76, d.f. 4, p<0.001, table 2). The MAP infected rabbits were significantly clustered across the site up to a distance of approximately 700m becoming regularly spaced at around 850m (Fig. 2). That is, when sampling site at a high resolution e.g. with 'quadrats' of between 1 and 700 m radius, infected rabbits appear clustered however when sampling at low spatial resolution, using larger 'quadrats' of radius 850m and greater, the infected rabbits appear regularly distributed in space. All rabbits sampled were also clustered across the site up to a distance of approximately 850m when they also become regularly spaced (Fig. 3). The MAP infected rabbits were regularly spaced within the distribution of all rabbits (i.e. after removing the effect of the clustered rabbit population in the environment in Fig. 3) up to a distance of about 200m, from around 300m they were relatively clustered, after which they become regularly spaced again at around 660m (Fig. 4).
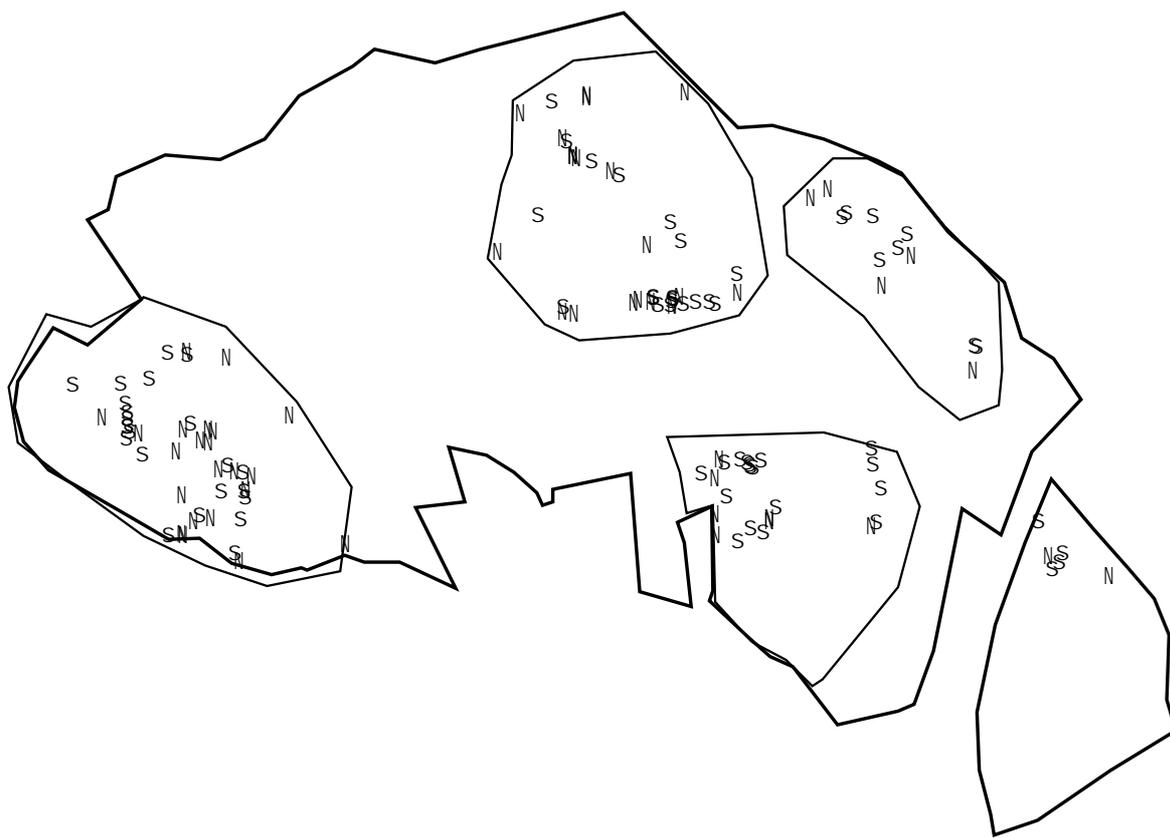
Fig. 1. Distribution of all rabbits sampled divided into the five local populations (μ non-infected rabbits, S infected rabbits)
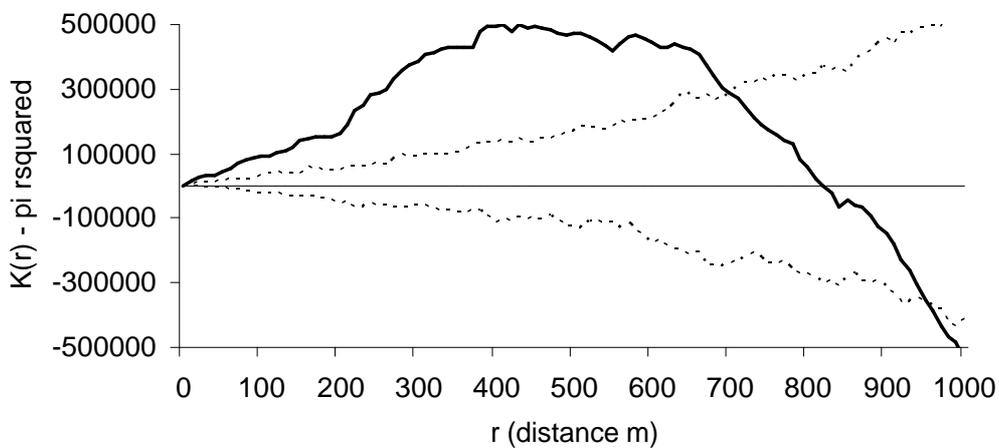
Fig. 2. K- function analysis of the distribution of MAP infected rabbits across the site (--- 95% confidence envelope, — complete spatial randomness (CSR), - M A P distribution). Above CSR indicates the MAP infection is clustered, below indicates it is regularly distributed.
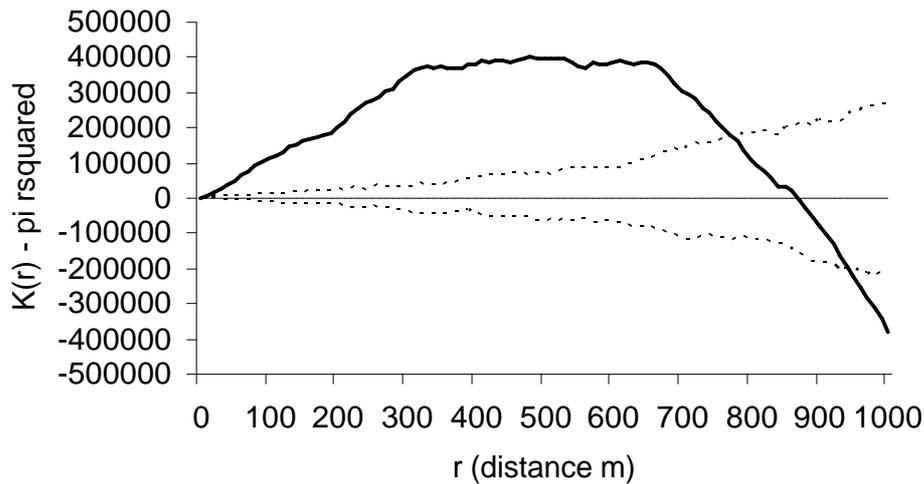
Fig. 3. K-function analysis of the distribution of all rabbits across the site (--- 95% confidence envelope, —
complete spatial randomness (CSR), - rabbit distribution). Above CSR indicates the rabbits are clustered,
below indicates they are regularly distributed.
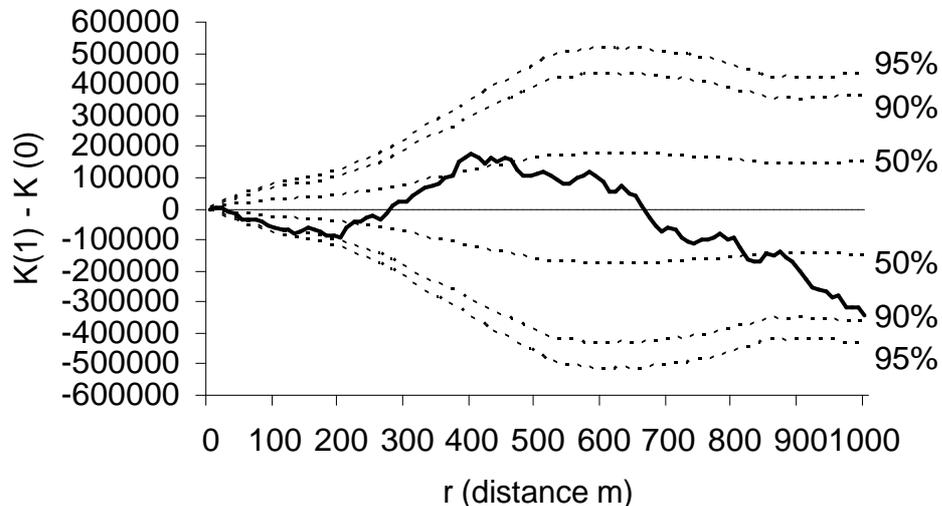
Fig. 4. K-function analysis of the difference in the distribution of infected rabbits (K(1)) and non-infected
(K(0)) rabbits across the site (---confidence envelopes, — complete spatial randomness (CSR), -
difference between K-functions of infected and non-infected rabbits). Above CSR indicates the MAP
infected rabbits are clustered, below indicates they are regularly distributed within the distribution of all
rabbits.

DISCUSSION

There are substantial, apparently cyclical, seasonal variations in the prevalence of MAP in rabbits according
to these data. The temporal variation in prevalence of MAP infection could be seen as important with regard
to the potential rabbit to livestock transmission of MAP as cattle do not avoid rabbit faeces while grazing
(Judge *et al.* 2005). The amount of rabbit faeces ingested is directly proportional to the level of rabbit faecal
contamination of grazing pasture (Judge *et al.* 2005). As the prevalence of MAP in rabbits varies
temporally, the proportion of infected rabbit faecal pellets on the pasture will also vary temporally.
Consequently, the risk of inter-species transmission to cattle, and as such environmental risk of MAP
transmission from rabbits to cattle via the faecal-oral route, will follow the same temporal pattern. In terms
of inter-species transmission, calves are at the greatest risk as they are the most susceptible to MAP
infection (Sweeney 1996). At the study site the main calving period in spring occurs during the season of
highest levels of MAP entering the environment from rabbits in their faeces. Temporally, the peak time of
infection risk to cattle thus coincides with the peak number of most susceptible animals.

MAP infected rabbits were spatially clustered (up to 800m) in the environment, suggesting heterogeneous distribution of inter-specific risk to cattle (Fig. 2). However, this apparent clustering of infection does not take into account the distribution of rabbits, which is similarly clustered (up to 950m; Fig. 3). Therefore the spatial clustering of infected rabbits could be purely an artefact of the distribution of the areas of favourable habitat for the host species. Whilst knowing the actual distribution of the disease in the environment is useful in terms of mapping the risk of rabbit to livestock transmission, to gain an insight into the dynamics of infection in the host population it is necessary to remove any heterogeneous host distribution. The distribution of the infection shown in Fig. 4 takes into account the distribution of rabbits, and so gives a true picture of the distribution of MAP within the host population. Although the disease is obviously highly clustered within the study site, when looked at in relation to the host population, the pattern is quite different. At high spatial resolutions (i.e. <200m) the disease tends towards being regularly spaced within the rabbit population, however at medium resolutions (i.e. >200m <700m) it appears clustered whilst at low resolutions (i.e. >700m) it appears more regularly spaced.

The results of this study show that MAP infection in rabbits varies significantly both temporally and spatially. MAP is highly clustered in the environment, however this is mostly due to the clustered distribution of rabbits. Knowledge of the ecology of the host species, especially its distribution, gives a greater understanding of the mechanisms behind the environmental patterns of disease. Knowing the reasons behind the environmental distribution provides valuable information when determining the risk of transmission of paratuberculosis from rabbits to livestock.

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Risk of inter-species *Mycobacterium avium* subsp. *paratuberculosis* transmission from rabbits to livestock via the faecal-oral route

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ABSTRACT

Two grazing experiments were carried out to quantify the rate of ingestion of rabbit faecal pellets by grazing livestock and therefore the risk of inter-specific paratuberculosis transmission. Ten sheep and ten cattle were each presented with three replicates of nine sward (grassy ground) treatments, created through three sward heights and three densities of rabbit faeces and allowed to graze for short periods. Both sheep and cattle ingested rabbit faecal pellets, the latter showing no behavioural avoidance of rabbit faeces while grazing. Sward characteristics affected the probability and rate of ingestion of faeces and thus parasites. Level of faecal contamination affected the proportion of pellets ingested by sheep, and the probability of cattle ingesting one or more faecal pellets. Sward height affected the proportion of pellets ingested by cattle but not sheep. The results of this study show that rabbits pose a serious risk of inter-specific paratuberculosis transmission to livestock.

Key words: disease transmission, faecal oral route, livestock disease, paratuberculosis, rabbits

INTRODUCTION

This study aimed to quantify the rate of rabbit faecal pellet ingestion and the risk of paratuberculosis (*Mycobacterium avium* subsp. *paratuberculosis* Bergey, 1923) rabbits pose to ruminants. Paratuberculosis was initially thought only to affect ruminants (Daniels *et al.*, 2003a) however, rabbits have recently been shown to be an asymptomatic carrier of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and shed sufficient numbers of bacteria in their faeces such that a single faecal pellet may constitute an infective dose for ruminants (Beard *et al.*, 2001; Daniels *et al.*, 2003a).

Field studies suggest that cattle do not display any avoidance of pastures contaminated with rabbit faeces while grazing (Daniels *et al.*, 2001). If grazing ruminants do not avoid swards (grassy sections of pasture) contaminated with rabbit faecal pellets, the level of contamination and sward height may have an impact on the rate of ingestion of rabbit faeces with involuntary ingestion of non-food items being likely to occur at reduced sward heights (Beresford & Howard, 1991; Thornton & Abrahams, 1983).

MATERIALS AND METHODS

Ten, ten week old Holstein calves were housed as a group but each was individually penned for the presentation of their own sward treatments. Ten Texel x Greyface lambs of 20 weeks old were individually penned for the duration of the experiment. The animals were fed *ad libitum* on high quality pelleted feed and the calves also had *ad libitum* access to hay. The food was removed for the presentation of the treatments. Both sets of animals had been recently weaned and accustomed to grazing from sward trays. For both experiments 270 sward trays, 54 x 54cm for the calves and 36 x 21cm for the lambs, were sown with perennial ryegrass (*Lolium perenne* Linnaeus) in a peat based compost and grown for at least 12 weeks (see Hutchings *et al.*, 1999).

Three sward heights (3, 6 and 12cm) and three contamination levels (35, 70 and 140 rabbit faecal pellets for the calves and 9, 18 and 36 for the lambs, or approximately 120, 240 and 480 rabbit faecal pellets/m^2 respectively) were used for the experiments, creating nine sward treatments. The densities of faecal pellets reflected the range of densities that could naturally be encountered in pasture (Gibb, 1993). Fresh rabbit faeces were collected from an outdoor captive colony of rabbits.

Three replicates of each sward treatment were offered to each animal. To give the animals the possibility of contacting all the faecal pellets on the tray, they were allowed to graze the swards either until they had achieved 60 bites (calves), 90 bites (lambs) or for five minutes. This represents the number of bites that would be needed to graze the entire sward area of the tray if no two bites were taken from the same part of the sward, based on the mean sward area ingested per bite for each species. Each animal was presented with three different treatments in succession on each day. The experimental design was balanced for the random effects, i.e., Animal, Period and Treatment Order.

Statistical analysis
One calf, which was treated for coccidiosis over a three day period during the trial, and one sward tray, which was destroyed by one of the animals, were omitted from the statistical analyses. Residual maximum likelihood (REML) (Patterson & Thompson, 1971) was used to estimate the mean values for grazing behaviour parameters (bite rate and grazing depth). Due to the high number of zero counts a Bernoulli variable within a generalised linear mixed model, iterative reweighted residual maximum likelihood (IRREML), with a logit link transformation (McCullagh & Nelder, 1989) was used to look at the incidence of ingestion and the number of pellets ingested in relation to the level of contamination. The GENSTAT REML (Lawes Agricultural Trust, 1993) option was used to approximate standard errors (SE) and standard errors of the differences (SED). In both the REML and IRREML models Animal, Day, Treatment Order and their relevant interactions were used as the random effects accounting for the repeated measures within the experimental design, Sward height, level of faecal contamination and their interactions were fixed effects. Number of bites was used as the covariate in all models to account for any differences in level of grazing. Wald tests from the REML and IRREML routines were used to determine significant differences ($p < 0.05$) with the wald statistic (w) quoted along with the relevant degrees of freedom (Lawes Agricultural Trust, 1993).

RESULTS

The calves ingested 253 (1.27%) of the 19,845 faecal pellets presented on the 242 sward trays included in the statistical analyses, with individuals ingesting between 16 and 56 pellets in total. Eating of faecal material occurred from 82 (33.9%) sward trays in total, and from up to 17 (62.9%) of the 27 sward trays presented to each animal. When faecal material was ingested, the amount was a mean of 3.07 ± 0.51 faecal pellets (range 1-10).

The level of contamination had a significant effect on the amount of faecal material ingested (w = 17.05, d.f. = 2, P < 0.001), increasing as the level of contamination increased (Table 1). There was no significant effect of the level of contamination on the proportion of pellets ingested (w = 0.65, d.f. = 2, P > 0.5). Calves ingested the equivalent of one faecal pellet every 54 bites, at 35, 70 and 140 pellets per tray, one faecal pellet every 123, 61 and 32 bites respectively. There was no significant effect of the level of contamination on bite rate or grazing depth (w = 1.57, d.f. = 2, P > 0.1; w = 2.39, d.f. = 2, P > 0.1 respectively).

Sward height had no effect on whether pellet intake occurred (w = 1.35, d.f. = 2, P > 0.5), but had a significant effect on the proportion of faecal pellets ingested (w = 7.02, d.f. = 2, P < 0.05) with the greatest proportion ingested at 3cm (Table 1). Sward height also had a significant effect on bite rate (w= 53.14, d.f. = 2, P < 0.001), which increased as sward height increased. Grazing depth was also significantly affected by sward height (w = 790.41, d.f. = 2, P < 0.001); shorter swards were grazed closer to the soil surface. There was no interaction between level of contamination and sward height, except on the proportion of pellets ingested (w = 11.35, d.f. = 4, P < 0.05), however this did not follow any general pattern.

The lambs ingested 27 (0.476%) of the 5670 faecal pellets presented with individuals ingesting between 1 and 6 pellets in total. Ingestion events occurred in 24 (8.89%) of the 270 sward trays in total and from up to 6 (22.2%) of the 27 sward trays presented. When ingestion occurred a mean of 1.12 ± 0.25 faecal pellets were ingested, with a maximum of 2 faecal pellets ingested per event.

Table 1. Ingestion events, pellets ingested, bite rate and grazing depth (cm above soil surface) by calves grazing on swards of different levels and heights.

Level of contamination	Sward height (cm)	Proportion of ingestion events	Proportion of pellets ingested	Bite rate (sec)	Grazing depth (cm)
35	3	0.149 (0.054-0.348)	0.011 (0.006-0.021)	0.4	1.54
	6	0.148 (0.054-0.037)	0.006 (0.003-0.014)	0.50	2.14
	12	0.248 (0.116-0.452)	0.019 (0.012-0.031)	0.54	4.74
70	3	0.354 (0.185-0.570)	0.019 (0.013-0.028)	0.38	1.57
	6	0.291 (0.145-0.500)	0.012 (0.008-0.019)	0.47	2.16
	12	0.320 (0.160-0.527)	0.008 (0.005-0.014)	0.59	4.57
140	3	0.453 (0.260-0.658)	0.015 (0.010-0.021)	0.30	1.45
	6	0.071 (0.033-0.148)	0.010 (0.007-0.014)	0.49	2.37
	12	0.547 (0.346-733)	0.012 (0.009-0.018)	0.56	5.06
SED				0.05	0.21
Significance levels for effects	Sward height	ns	*	***	***
	Level of contamination	***	ns	ns	ns
	Interaction	ns	*	ns	ns

+ results + results of REML: ns=not significant; *=P<0.05; ** P<0.01; *** = P<0.001.

++ back t++ back transformed means, 95% confidence interval in italics

There was no significant effect of the level of contamination on the number of ingestion events (w = 0.61, d.f. = 2, P > 0.5). The proportion of faecal pellets decreased as level of contamination increased ingested (w = 7.09, d.f. = 2, P < 0.05, Table 2). Lambs ingested the equivalent of one faecal pellet every 857 bites, at 9, 18 and 36 faecal pellets per tray, one faecal pellet every 1122, 703 and 839 bites respectively. Level of contamination also had a significant effect on bite rate (w = 13.64, d.f. = 2, P < 0.01), which was faster at 9 pellets per sward tray than at 18 or 36 faecal pellets per sward tray. There was no significant effect of level of contamination on grazing depth (w = 1.26, d.f. = 2, P > 0.5).

Sward height had no significant effect on either ingestion events or proportion of faecal pellets ingested (w = 0.93, d.f. = 2, P > 0.5; w = 0.45, d.f. = 2, P >0.5 respectively). Bite rate was affected by sward height (w = 59.90, d.f. = 2, P < 0.001), being slower at 3 than either 6 or 12cm. Sward height also had a significant effect on grazing depth (w = 44.26, d.f. = 2, P < 0.001), the animals grazing closer to the soil surface as sward height decreased (Table 2). There was no interaction between level of contamination and sward height.

DISCUSSION

This is the first time that the ingestion of faeces by grazing ruminants has been quantified. For calves the probability of swallowing faecal material increased as level of contamination increased and the number of rabbit faecal pellets ingested is directly proportional to the level of contamination suggesting that no behavioural avoidance was displayed. Lambs were better able to avoid rabbit faeces when grazing. Sward height and level of contamination affected rate and probability of ingestion of rabbit faeces in both species and hence the risk of paratuberculosis transmission to livestock.

Table 2. Ingestion events, pellets ingested, bite rate and grazing depth (cm above soil surface) by lambs grazing on swards of different levels and heights.

Level of contamination	Sward height (cm)	Proportion of ingestion events	Proportion of pellets ingested	Bite rate (sec)	Grazing depth (cm)
35	3	0.133 (0.050-0.309)	0.015 (0.006-0.039)	0.69	1.68
	6	0.066 (0.016-0.232)	0.008 (0.002-0.030)	0.92	1.93
	12	0.033 (0.005-0.203)	0.004 (0.001-0.027)	0.82	2.16
70	3	0.050 (0.032-0.270)	0.006 (0.002-0.017)	0.62	1.70
	6	0.133 (0.050-0.309)	0.007 (0.003-0.019)	0.82	1.91
	12	0.010 (0.032-0.271)	0.008 (0.003-0.020)	0.81	2.46
140	3	0.010 (0.031-0.275)	0.004 (0.001-0.010)	0.57	1.64
	6	0.044 (0.016-0.232)	0.004 (0.001-0.008)	0.75	1.94
	12	0.066 (0.017-0.232)	0.003 (0.001-0.009)	0.78	2.25
SED				0.05	0.16
Significance levels for effects	Sward height	ns	ns	***	***
	Level of contamination	ns	*	**	ns
	Interaction	ns	ns	ns	ns

+ results + results of REML: ns=not significant; *=P<0.05; ** P<0.01; *** = P<0.001.

++ back t++ back transformed means, 95% confidence interval in italics
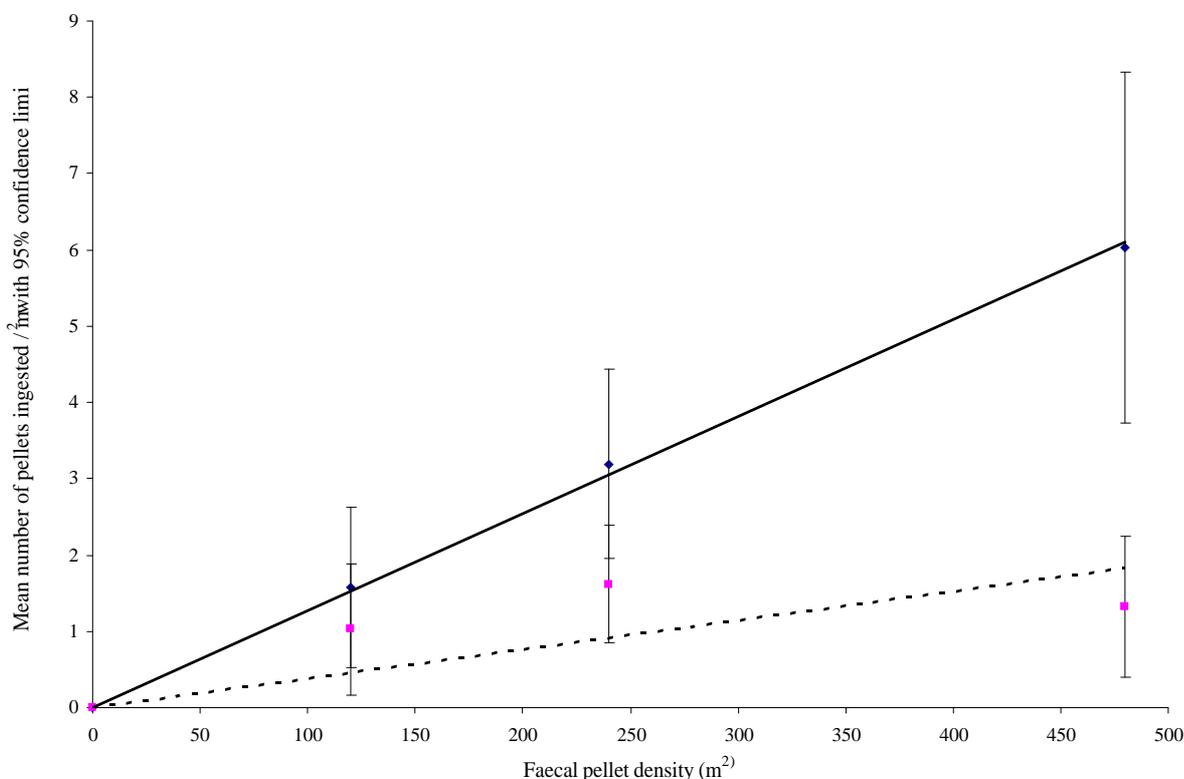
Fig. 1. Mean number of pellets ingested per m^2 at each faecal pellet densities (with 95% confidence limits) for cattle (|) and sheep (|) with simple linear regression (||| = cattle; $y = 0.013x$; $r^2 = 0.999$; -------- = sheep; $y = 0.004x$; $r^2 = 0.271$).

Given a density of 10 – 59 rabbits/ha for Scotland (Kolb, 1994), according to Daniels *et al.* (2003b) the number of rabbit faecal pellets present at any one time on a grazing pasture is between 30,657 – 290,752 pellets/ha. Cattle ingested 1.27% of the faecal pellets or between 389 - 3693 pellets/ha grazed. Daniels *et al.* (2003b) and Greig *et al.* (1997) have shown a wide variation in the percentage of rabbits infected with paratuberculosis, with the average being 36%. As a consequence, one calf could ingest 140 - 1329 infected faecal pellets/ha grazed. Sheep ingested an average of 0.476% of the faecal pellets presented giving the potential to ingest between 146 - 1384 faecal pellets/ha grazed of which 53 - 498 may be infected. Rabbits should, therefore, not be discounted as a potential source of paratuberculosis infection.

The field studies carried out by Daniels *et al.* (2001) suggested that there was no avoidance of rabbit faeces by grazing cattle at a field scale. If no avoidance was displayed, all else being equal, we would expect faeces ingestion proportional to level of contamination, as was the case with the cattle here. Furthermore, bite rate was not reduced with higher levels of faecal contamination nor was grazing depth. As a consequence calves did not show any behavioural avoidance of rabbit faecal pellets.

An increase in the level of contamination did not increase the probability of an ingestion event for lambs and the proportion of faecal pellets ingested decreased markedly with increasing levels of contamination. This suggests avoidance of faecal pellets at higher levels of contamination. Lambs bite rate was slower at higher levels of contamination for the lambs suggesting that they were aware of the presence of rabbit faeces and more selectively grazing. At a field scale sheep, like cattle, did not avoid swards contaminated with rabbit faeces (Daniels *et al.*, unpublished data). However, sheep are highly selective grazers and are capable of great precision when grazing with better ability to avoid non-food items than cattle (Lynch *et al.*, 1992) and while they may not show avoidance at a patch scale, they may display avoidance at a finer scale, i.e. at the bite site. Despite this avoidance lambs still ingested rabbit faecal pellets and are therefore susceptible to infection via the faecal oral route.

The apparent avoidance displayed by sheep towards rabbit faeces leads to a marked difference in the risk of infection via the faecal oral route between cattle and sheep (Fig. 1) especially at high levels of faecal contamination. In cattle the vast majority of the variation in the number of faecal pellets ingested, and thus force of infection, may be accounted for purely by the level of contamination. Therefore, simple relationships may be used to predict the level of cattle exposure to paratuberculosis from rabbits (Fig.1). However, for sheep, the rate of faeces ingestion and thus the faecal-oral route of infection is more difficult to predict. In conclusion, both cattle and sheep ingest rabbit faecal pellets while grazing but the exposure of cattle to paratuberculosis from rabbits via the faecal-oral route is greater.

ACKNOWLEDGEMENTS

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Prevalence of bovine paratuberculosis in the Latium region (Italy)

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ABSTRACT

In Italy there are few studies about the prevalence of bovine paratuberculosis. In the Veneto region a serosurvey on 416 dairy farms recorded a herd seroprevalence of 49% by ELISA and in Tuscany 3.4% of bovine population tested positive by AGID. In order to have a wider evaluation of the infection in cattle population of Latium region we carried out a serological survey on 369 herds (2% of regional herds) uniformly distributed in 5 province districts using an ELISA. A total of 19,627 animals were examined (6.5% of the whole population). ELISA-positives totaled 472 animals (2% ± 0.4% -CI 95%), ranging from one to a maximum of 65 cows per farm. 155 herds were seropositive with at least one positive cow (42% ± 5% -CI 95%). Fecal samples (77) were collected from ELISA-positive herds and from at least one of the seropositive subjects and than examined by Ziehl-Nieelsen (Z-N) stain, bacteriological culture and two PCR techniques. Only in one case was the molecular test negative when culture was positive; in the remaining fecal samples we found a concordance of 99% between the serological and molecular tests. The ELISA is useful for screening herds due to low cost and fast and easy execution. The results obtained confirm a widespread distribution of bovine paratuberculosis in our region, affecting up to at least 42% of cattle farms.

Key words: Latium region (Italy), seroprevalence, bovine, paratuberculosis.

INTRODUCTION

Bovine paratuberculosis is widespread in the world and has been diagnosed in Italy. It was first described in cattle in 1927, in sheep in 1968 and in buffalo in 1996 (Lillini et al., 1999). The prevalence of the infection differs among the countries but generally high. Epidemiological studies on advanced animal husbandry countries show that the average of positive percentage is about 50% and it could reach the 80% within 2020 if surveillance control programmes will be not implemented (Collins, 2002, personal communication). In the Veneto region of north eastern Italy, 49% of 416 herds examined by ELISA were test-positive (Robbi et al., 2002).

In the last 10 years in the Latium region (Central Italy) the infection was found in dairy and beef herds on the basis of a clinical suspicion or on a specific request of breeders.

The aim of the present study was to carry out a survey to determine the seroprevalence of paratuberculosis in Latium region (Central Italy) herds.

MATERIALS AND METHODS

A total number of 2,605 herds with 29 or more cattle are present in the whole Lazio region: Rome 27,5% (n=717), Frosinone 18,1% (N=470), Latina 28,7% (N=747), Viterbo 13% (N=339), Rieti 12,7% (N=332). A stratified sample size was calculated on the basis of herd number for each province and a total of 19,627 bovine sera from 369 dairy and beef herds of the 5 Latium provinces, Rome (100), Frosinone (72), Latina (100), Viterbo (53) and Rieti (44) were tested. This represented 6.5% of the Latium bovine population and 2% of the total herds. An ELISA (commercial IDEXX Antibody ELISA Kit, using the manufacturer's recommended protocol) was used.

Seventy seven follow-up fecal samples (from selected ELISA-positive cows in various herds) were stained with the Z-N TB QUICK STAIN (B.D., USA). The isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was carried out following the National Animal Disease Center protocol (NADC, Ames USA): a) decontamination of fecal specimens with 1% of HPC (w/v); b) growth in solid home-made HEYM additioned with mycobactin J (ALLIED MONITORLABS, USA) and sodium piruvate. The Qia amp DNA Mini® Kit (QIAGEN, USA) was employed for DNA extraction from fecal samples. The DNA amplification of MAP, carried out with the PCR kit ADIAVET PARAtub ® (ADIAGEN, France), is based on amplification of a DNA fragment insertion sequence IS*900* specific for MAP strains (Lillini et al., 2002). The nested PCR was employed by PCR1 and PCR2. The target of PCR1 primers (p90/p91) was 400bp fragment localized within the IS900 insertion element:

- primer   p90:   5' –GAAGGGTGTTCGGGGCCGTCGTTAGG-3'
- primer   p91   5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3'

To generate a specific internal probe sequence, we designed a second pair of oligonucleotide (p25/p26) for a Nested PCR application; these primers amplify a 229 bp fragment using the product of PCR1 as DNA template:

-primer   p25:   5'-CCAGGGACGTCGGGTATGGC -3'
-primer   p26   5'-GGTCGGCCTTACCGGCGTCC –3'

PCR amplification was carried out in 50µl volume reaction (final concentration):

Statistical analyses
The sensitivity and the specificity of the ELISA kit test was required to calculate the sample size of dairy and beef herds to be tested for the bovine paratuberculosis seroprevalence estimation at a population level. Therefore, in order to assure the validity of the investigation and to improve the statistical significance of the seroprevalence estimates a performance evaluation of the ELISA serological test was carried out in a preliminary phase.

ELISA performance
Data related to sensitivity and specificity, plus predictive values of negative and positive sera were deduced from a study carried out in 2002 on 6 herds in Latium region. Five herds were considered infected by MAP and one free of infection on the basis of the following characteristics: during the last five years neither clinical signs of paratuberculosis were observed nor new animals were introduced. Half yearly two faecal culture controls and serological examinations were completed and all gave negative results. During this investigation 633 cows over 2 years old were examined. The faecal culture test was used as gold standard to verify animal infection. Diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of a kit ELISA for antibodies were determined on the basis of results obtained with cultural examinations (Sockett et al., 1992).

The predictive value of negative (PV-) and positive (PV+) sera was calculated on the relative DSn, DSp and on the sero-prevalence value expected by the gold standard (Sweeney et al., 1995).

1° ELISA paratuberculosis test IDEXX
DSn and DSp evaluation were calculated by a contingency 2x2 table, which links the sanitary situation of reference animals with test results obtained. Assay was executed by investigation on 633 cows (Table 1)

Table 1. Evaluation of DSn and DSp

		Reference animals			
		infected			**non infected**
	positive	44	TP	FP	26
test results			**A**	**B**	
			C	**D**	
	negative	13	FN	TN	550
		DSn=A/(A+C)			DSp=D/(D+B)
		0.772			**0.955**

Diagnostic sensitivity ELISA (DSn) = 0.772
Diagnostic specificity ELISA (DSn) = 0.955

Figs 1 and 2 show positive and negative predictive values in infected herds.
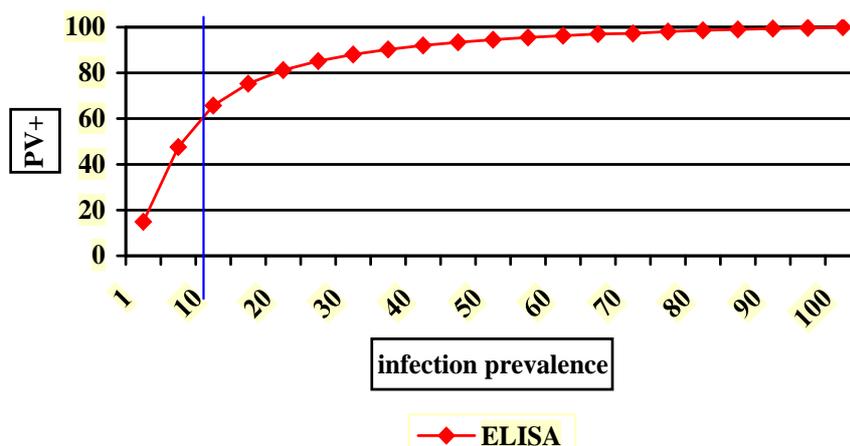
**Fig. 1.** ELISA positive predictive value (PV+)
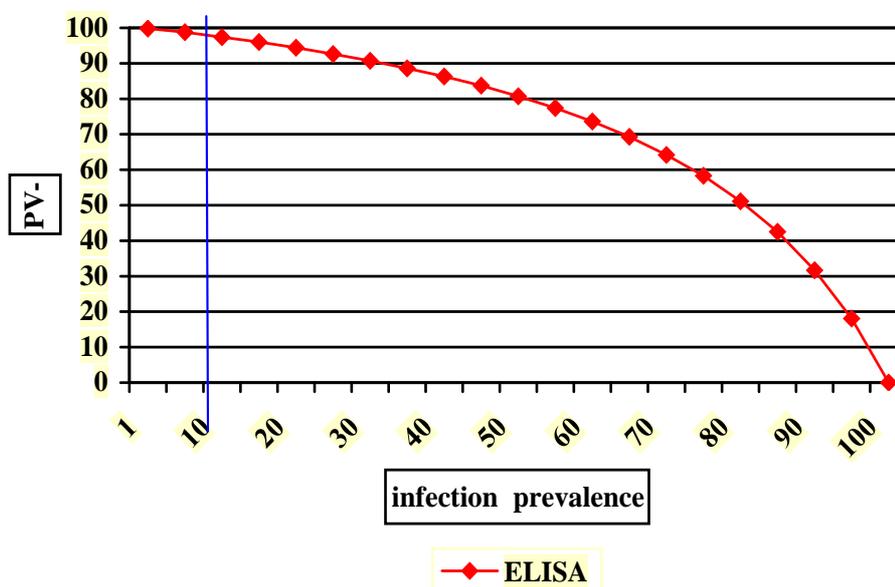
Fig. 2. ELISA negative predictive value (PV-)

Predictive positive value of test calculated on population with 9.93% seroprevalence:
Positive ELISA test result: (PV+) = (PxSE) / [(PxSE)+ (1-P)x(1-SP)] = 0.654

Predictive negative value of test calculated on population with 9.93% seroprevalence:
Negative ELISA test result: (PV-)=(PxSE) / [(PxSE)+(1-P)x(1-SP)] = 0.974; with a confidence interval (CI) of 95%, ELISA sensitivity and specificity range is reported in the following Table 2.

Table 2. ELISA sensitivity and specificity (CI 95%)

	Estimate	**CI 95%**	
Sensitivity	77.193	64.997	- 86.685
Specificity	95.486	93.548	- 96.967

Considering the low positive predictive value of ELISA test IDEXX and using a test-positive threshold value S/P of 0.15, herds were defined as infected if at least 2 results were test-positive; with a cut off of S/P of 0.30 the herd was considered infected if one result was test-positive.

Preliminary data showed that raising the cut-off from 0.15 to 0.30 improved specificity, and consequently the positive predictive value of the test, without decreasing the sensitivity and negative predictive value. So the number of culture tests could be reduced and used only on ELISA positive samples.

Sampling method
Calculation of the number of herds to sample: considering a total of 2,605 herds with herd size greater than 29 animals in the Lazio region, assuming an expected 10% prevalence of infected herds in this category, 3% accuracy and a 95% confidence level, a sample size of at least 335 herds was calculated using Win Episcope 2.0 software. The number of herds to be sampled was then stratified for each province of the Lazio region on the basis of the respective herd number.

Calculation of the number of animals to test: as diagnostic sensitivity of ELISA, on cattle aged >= 24 months, is more than 25% (this value represents the lower value verified in asymptomatic cattle), the probability of finding ELISA positives in infected animals is more than 95% in a herd with a real prevalence of >=2%. So any cattle aged more than 24 months were tested.

RESULTS

Table 3 shows the number of positive herds and cattle for a single Latium province and the respective test-positive percentage. The higher number of positive herds (52%) is in Rieti province while in Frosinone province the percentage is the lowest (28%).

Among 369 examined herds, 155 (42% $\pm$ 5% - CI 95%) tested positive. Of the 19,627 tested sera, 472 (2.4% $\pm$ 0.4% - CI 95%) ELISA-positives were found.

Fig. 3 represents the distribution of ELISA-positive cattle among the infected herds in Latium region.
In Fig. 4 it is shown that in almost 50% of herds one ELISA-positive cow is present; 22% of herds have two positive animals and 16 herds (11%) more than 5 ELISA-positive cattle.

Fig. 5 represents the percentage of positive herds related to the size of the herd. At least one positive cow is found in almost 70% of controlled herds less than 100 head.

Table 3. Total results of bovine PTB prevalence in Latium region.

Province	Examined herds	Positive herds		Examined heads	Positive heads	
		N°	%		N°	%
Frosinone	72	20	28	2315	37	1.6
Latina	100	37	37	4465	111	2.5
Rieti	44	23	52	2516	83	3.3
Roma	100	40	40	6105	162	2.6
Viterbo	53	25	47	4226	79	1.8
Total	369	155	42	19627	472	2.4
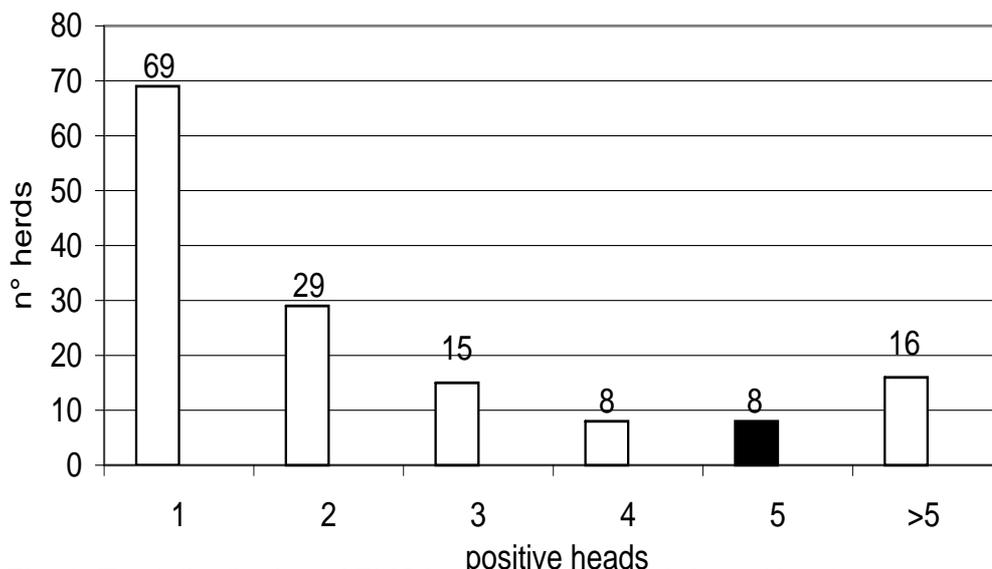
Fig. 3. Total distribution of ELISA-positive cattle in infected herds
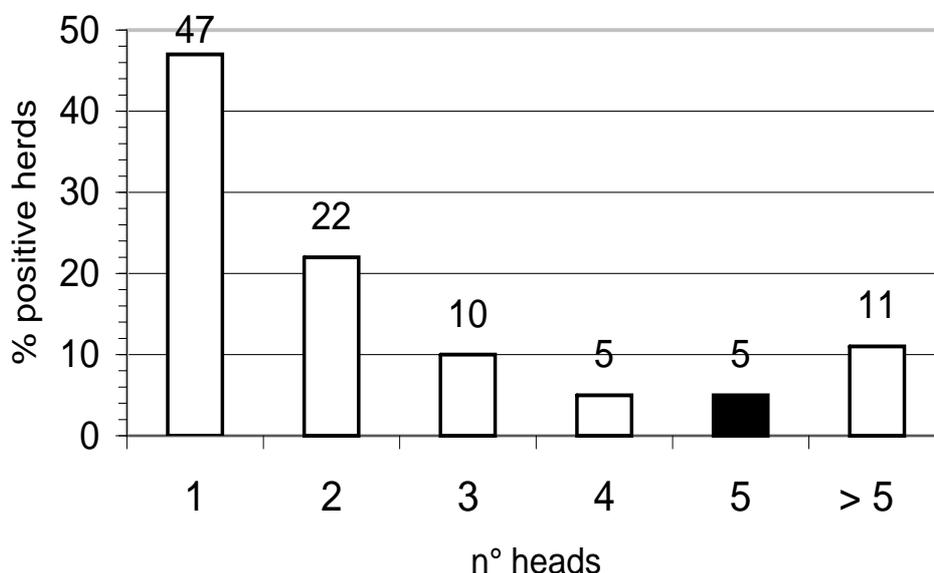
Fig. 4. Percent herds infected on the basis of number of ELISA positives.
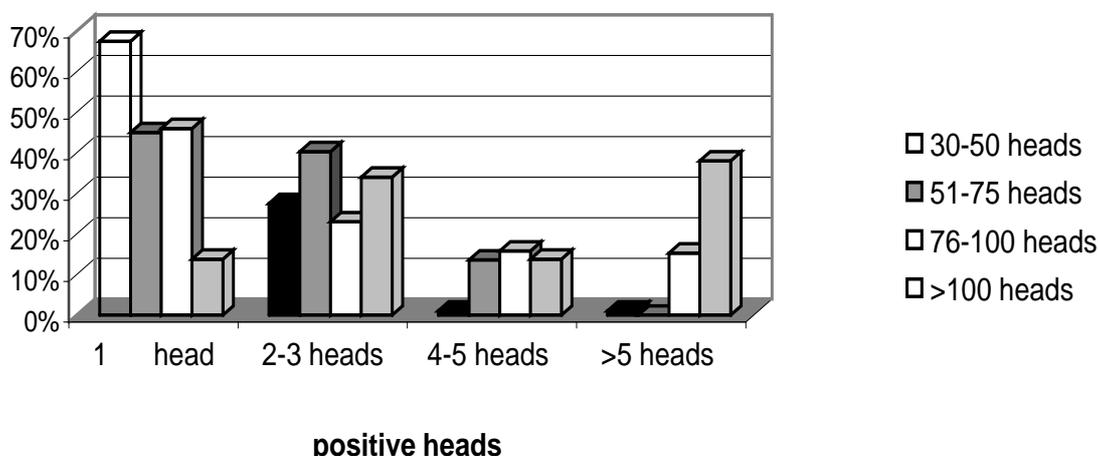
positive heads

Fig. 5. Percent of positive herds on the basis of the herd's size

To confirm the serological results, one or more fecal samples were collected from 77 ELISA-positive cattle in 40 positive herds in Rome province. Culture and slide examination were positive in 9 and 26 herds

respectively; the results obtained by both PCR showed 31 infected herds of a total of 38 examined (81%). Only in one case was the molecular assay negative, even if the bovine was very low shedding, whereas in the remaining 8 herds there was full concordance between the two tests. Fecal shedding of MAP may be intermittent and of few organisms (1-5 colonies). We found one fecal sample positive to MAP culture and microscopic examination for acid fast bacilli but negative to PCR tests.

We found that 34 herds out of 40 sero-positive were confirmed as infected by at least one of the confirmatory tests performed on fecal samples (commercial PCR and/or PCR home-made or bacterial culture or microscopic examination for acid fast bacilli), giving an overall accordance of 84% of test-positive herds.
A more realistic vision on the true situation in Latium region may have been obtained if we had used a seroprevalence calculation with hypergeometric distribution or with other statistical formulas.

CONCLUSION

This epidemiological study could be used to improve control programmes for the eradication of the infection. In our study we found that the elevated number and percentage of infected herds correspond to those obtained in other seroepidemiological surveys carried out in other countries with advanced animal husbandry (Allworth et al., 2002a. Allworth et al., 2002b. Piaggio et al., 2002). In our experience 47% of the examined herds presented only one ELISA-positive animal.

In previous work it was found that in some cases animal trade was the source of infection (Lillini et al., 1986. Lillini et al., 1998).

In conclusion, the prevalence of the infection could rise in future if surveillance control programs are not implemented. Despite its limits, the ELISA remains a suitable method for the identification of the infected herds because it is inexpensive, fast and simple to perform.

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M. avium ss. paratuberculosis strain and dose response trial in red deer

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ABSTRACT

Three groups of 16 red deer weaners, 3 months of age, were orally dosed with high (10^9), medium (10^7) or low (10^3) doses of "bovine" strain *M. paratuberculosis* (MAP) and another group of 16 weaners received medium (10^7) doses of "ovine" strain MAP. There was a fifth control group of 17 undosed weaners. The high bovine and control groups were grazed together, the medium bovine and low bovine groups were grazed together and the medium ovine group was grazed alone in separate small paddocks on a quarantined "Johne's infected" area of the farm.

Five of the 16 animals in the high bovine group developed early signs of Johne's disease 6-10 months after challenge and were euthanized. The surviving deer in the five groups, which were all clinically normal, were killed and necropsied 11 months after challenge. Three animals (two high and one medium "bovine" strain) had small caseous lesions in the intestinal lymph nodes. The remaining animals had no grossly visible lesions. Histological sections of samples of intestines and gut lymph nodes were examined and showed for the "bovine strain" a gradation of lesion severity that was related to the size of the challenge dose of MAP (mean lesion grade = 4.8, 2.9, 0.9 for high, medium and low). The medium "ovine" strain group had a mean score of 0.9, which was the same as the low "bovine" group. The control group, which ran with the high "bovine" strain group, had a mean lesion grade of 2.2 indicating natural transmission of infection.

These results confirm the prediction that the level of challenge in young animals is a major factor determining the clinical outcome in rising yearling red deer and it also suggests that the "ovine" strain is less pathogenic for red deer than "bovine" strain MAP.

Key words: deer, Johne's disease, MAP, dose-response, strain

INTRODUCTION

Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP)*,* occurs in wild and captive deer in a number of countries. It has emerged as a problem on deer farms in New Zealand and overseas (Mackintosh et al 2004). Identified in over 600 farmed deer on 300 properties, the principal surveillance method has been by the examination of suspect 'tuberculous' lesions identified in deer slaughter plants (de Lisle et al 2003). The herd prevalence based on this information is approximately 6%, but the true prevalence is expected to be higher.

Clinical disease in farmed red deer (*Cervus elaphus*) occurs in animals as young as 8 months and outbreaks involving 10-20% of groups of 8-15 month old animals have occurred. Affected animals lose weight and condition and usually develop diarrhoea terminally (Mackintosh et al 2004). Typically they have enlarged mesenteric lymph nodes and granulomatous enteritis. Transmission is presumed to be primarily by the faecal-oral route. Both ovine and bovine strains have been isolated from deer, but the majority of outbreaks of clinical disease appear to be due to the bovine strain. The key epidemiological factors that lead to outbreaks in young deer are largely unknown, although it is likely that they are similar to risk factors in other domestic livestock. These are likely to relate to the age of the animal at the time of challenge, the size of the infective dose, the strain of MAP involved, the genetic makeup of the animal and environmental factors and stressors.

A previous study showed that an experimental challenge model, which used four daily oral doses of approximately 2.5×10^8 cfu of live MAP organisms harvested from the lymph nodes of a clinically affected deer, was able to reproduce typical clinical Johne's disease in 26% of a group of young red deer (Mackintosh et al, 2003). The primary aim of this study was to use this experimental challenge model to examine the relationship between the number of organisms in an oral dose of a "bovine" strain of MAP of deer origin and the severity of the resulting disease in deer. The infectivity and pathogenicity of high, medium and low oral doses of this "bovine" strain was assessed in young red deer. A secondary objective was to compare the pathogenicity of a medium dose of an "ovine" strain of MAP with this "bovine" strain.

MATERIALS AND METHODS

The trial started in March 2003 with 81 newly weaned red deer divided into five groups. The MAP organisms were harvested directly from the tissues of a naturally infected farmed red deer and a merino sheep, both with end-stage Johne's disease. The organisms were subsequently confirmed as MAP "bovine" strain and "ovine" strain respectively, using IS*900* and IS*1311* techniques. Three groups received High (10^9) bacteria, Medium (10^7) or Low (10^3) oral doses of "bovine" strain MAP. One group received Medium (10^7) oral doses of "ovine" strain MAP. The High "bovine", Medium "bovine" and Low "bovine" groups were grazed on one part of the quarantined "Johne's infected" area of the farm and the Medium "ovine" group was grazed in a separate area. The animals were weighed and examined monthly for 3 months, fortnightly for a month and then weekly for 7 months.

Clinically affected animals were euthanased early in the disease and necropsied. All clinically normal animals were killed 11 months after the start of the trial and examined. Samples of jejunum and ileo-caecum and associated lymph nodes were taken fresh for BACTEC culture and fixed for histopathological examination. An overall lesion severity score was assessed for each animal based on an arbitrary scale from 0 (no lesions seen) to a maximum severity of 11 for large areas of granulomatous lesions in intestine or lymph node, submucosal lesions, marked blunting and fusion of villi and mesenteric/pericapsular lymph node granulomas, indicative of severe Johne's disease.

RESULTS

Between six and ten months after dosing (Sep – Dec), 5 of the 16 (31%) animals in the High "bovine" group developed early signs of Johne's disease and were euthanased. The signs included gradual reduction in weight gain over 2-3 weeks, followed by sudden weight loss (2-5% liveweight) over 1-2 weeks, rough coat and soft-watery faeces. There were no clinical cases in any of the other groups.

The surviving deer in the five groups, all apparently healthy reaching good liveweights (~100 kg average), were killed and processed through Otago Venison Deer Slaughter Plant in 2004, 11 months after challenge. Three animals (two High and one Medium "bovine" strain) had small caseous lesions in the intestinal lymph nodes (Table 1). The remaining animals had no grossly visible lesions.

Samples of intestines and gut lymph nodes from all animals were submitted for histopathological examination. The lesions were described and the animal was given an overall severity grade (0-11) where there were large areas of granulomatous lesions in intestine or lymph node, submucosal lesions, marked bluntening and fusion of villi and mesenteric/pericapsular lymph node granulomas, indicative of severe Johne's disease. The histopathology showed a gradation of lesion severity, related to the size of the challenge dose of MAP for the "bovine" strain. The High, Medium and Low groups had mean lesion grades of 5 (range 1-11), 3 (0-6) and 1 (0-2) respectively. The Medium "ovine" strain group had a mean lesion grade of 1 (0-3) which was very similar to the Low "bovine" group (Table 1).

MAP was isolated from tissue from all the High and Medium dose bovine group animals, from 50% of the Low dose bovine and from 69% of the Medium dose ovine group animals (Table 1).

Table 1. Summary of the number of animals with clinical disease, gross lesions and histopathological lesions of paratuberculosis, the group mean histopathological lesion severity score and the number of animals from which MAP was isolated in the five treatment groups.

Group	No.	Clinical Johne's disease	No. with gross lesions at kill	Number with histopath lesions	Mean lesion severity grade†	Number *M. ptb* isolated
High Bovine	16	5 (31%)	2 (13%)	16 (100%)	5	16 (100%)
Medium Bovine	16	0	1 (6%)	14 (87%)	3	16 (100%)
Low Bovine	16	0	0	8 (50%)	1	8 (50%)
Medium Ovine	16	0	0	7 (44%)	1	11 (69%)

† An arbitrary severity scale from 0, being no lesions seen, to a maximum severity of 11

CONCLUSION

This study confirms the prediction that high challenge in young animals is most likely to cause clinical disease in rising yearling deer. The minimum infective oral dose is approximately 10^3 organisms for the "bovine" strain. The results suggest that the "ovine" strain is less virulent for young red deer than the "bovine" strain of MAP.

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A deterministic mathematical model of *Mycobacterium avium* subsp. *paratuberculosis* transmission on commercial US dairy farms

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ABSTRACT

Prevalence of Johne's disease on U.S. dairy farms is estimated at one-fifth of all herds, and larger herds are found to be infected more often. Despite a low prevalence of high-shedding animals, elimination of MAP from herds has proven exceptionally challenging, with few published reports of successful eradication from infected farms. Mathematical modeling may help us understand this apparent contradiction. A deterministic mathematical model of MAP transmission on commercial US dairies was developed. It builds upon and modifies the assumptions in previous work to best reflect the pathobiology of the disease. Of the animals which test positive for MAP, high levels of bacterial shedding are noted in only a small proportion. Transmission was modeled using ordinary differential equations. Calculation of transmission parameters in these models is necessarily non-linear. Previous models of infection utilized linear dynamics only and therefore lack sensitivity to changes in susceptible population size. Animal turnover rates were obtained from the literature and transition from disease states were calculated from retrospective fecal culture data from herds in New York and Pennsylvania. The model shows that aggressive test-and-cull strategies do not result in successful elimination of MAP in a short timeframe. Transmission is relatively insensitive to the presence of high shedding animals. According to the model, multiple levels of contagiousness among infected adult animals and MAP shedding by infected calves explained the maintenance of low prevalence infections in herds. Although previous experimental studies support the potential of infectious transmission among young calves, further research with natural infections is needed to verify the existence of a pool of infectious young animals. If this group of animals is diagnostically identifiable, elimination strategies may need to address this route of transmission.

Key words: Calf, model, transmission, transient shedding, vertical transmission

INTRODUCTION

Johne's disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a slow-growing, Mycobactin-J dependent bacterium. Prevalence of MAP on US dairy farms at a herd level is estimated at 22% of all dairy herds, and 40% of all herds larger than 300 animals (Wells and Wagner, 2000). The high prevalence of infected herds makes transmission of MAP in a farm environment of interest both from animal welfare and economic perspectives. Recent research that considers MAP presence as a risk factor for human Crohn's disease raises transmission of MAP as a potential human health hazard (Shulaw and Larew-Naugle, 2003). Although 2.6% of animals typically test positive for MAP on each infected farm (Groenendaal and Galligan, 1999), high levels of bacterial shedding is noted in only a small proportion of these animals (Whitlock et al., 2000). However, elimination of MAP from herds has proven exceptionally challenging, with few published reports of successful eradication of MAP from infected farms.

Several previous models address not only how MAP is spread on a farm, but also incorporate intervention strategies to determine successful methods of eliminating the infection (Collins and Morgan, 1991;Collins et al., 1991;Groenendaal and Galligan, 1999;Beyerbach et al., 2001a;Beyerbach et al., 2001b;Groenendaal et al., 2002). The transmission of MAP in these models focuses on infection of young calves via MAP in

colostrum or milk, *in utero* infection, or exposure to infected feces. All models assign a shedding status to infected adult animals and base incident infections on the number of infectious adult animals present.

Collins and Morgan developed the first dynamic deterministic model of MAP transmission, which tracked the prevalence of four categories of animals (Collins and Morgan, 1991;Collins et al., 1991;Collins and Morgan, 1992). In this model, young animals remained susceptible up to age one year after which uninfected animals entered a resistant category and infected animals were clinically normal and not infectious. At two years of age, infected animals entered an infectious compartment for the remaining time in the herd. This model assumed homogenous mixing with equal risk of contact between susceptible animals and all herdmates and with calves from infected dams having the same risk of infection as all other calves.

Groenendaal and Galligan's (1999) stochastic model introduced two adult shedding categories (low and high) and an increased risk of infection in selected individual animals via assumptions of non-homogenous mixing. This more complex model allowed for multiple ages at initial infection to better reflect disease dynamics following targeted management changes. Infected animals entered the high shedding category with a probability distribution based on age at infection, and were then retroactively assigned to have entered the low shedding compartment two lactations previously. These two separate infectious categories contributed differently to infection transmission, with the probability of an infectious contact decreasing for low shedding animals. Exposure and infection could occur as a result of vertical transmission and point source exposures (dam→daughter, colostrum→calf, infected milk→calf). Thus high-risk cohorts of animals or individuals existed rather than a constant exposure status among animals of the same age. Farm level risk factors established a base level of exposure as a result of environmental contamination.

Although these models served as the first and most widely recognized transmission models developed for paratuberculosis in a dairy setting, they are not "transmission dynamic". That is, the assumption that halving the transmission / exposure will halve the infection incidence is not necessarily the case, since transmission dynamics are necessarily non-linear and are determined by susceptibility as well as exposure (Edmunds et al., 1999). These models reflect our current knowledge of the biology of MAP transmission, with limited input as to infectious status of calves and age at which animals are no longer susceptible to infection.

The objective of this paper is to develop a series of mathematical models of MAP transmission building upon and modifying the assumptions in previous work to best reflect the pathobiology of infection transmission. In this paper a series of models is used to explore sensitivity of the transmission cycle to a variety of current assumptions. Ultimately the goal of developing this series of models is to aid in determining effective control strategies for eliminating clinical paratuberculosis and MAP infection from the herd.

MATERIALS AND METHODS

We have developed a series of models which seek to mimic herd dynamics and disease process at an increasingly detailed level. Three such models will be developed in this paper. All models calculate a density dependent rate of contact rather than a population size dependent one. This allows models to reflect current data showing that within-herd prevalence is not greater in large herds (Collins et al., 1994;van Schaik et al., 2003). All model parameters are defined in Table 1.

Table 1. Definition of all symbols used in compartmental models. Values used in outputs and sources of data are provided.

| Symbol	Definition	Value	Source
t	Duration of timestep	1 month	1
	force of infection	8	2
	$_1$ = force of infection from Transients (Model C)	8	
	$_2$ = force of infection from Y1 & Y2 (Model C)	8	
	Increased infectiousness of Y2 vs Y1	1-1000	1
μ_d	Universal death rate	0.223 /year	3
μ_1	Death rate Category 1	0.111/year	3
μ_2	Death rate Category 2	0.007/year	3
μ_{3vol}	Voluntary cull rate Category 3	0.285/year	3
μ_{3inv}	Involuntary cull rate Category 3	0.048/year	3
μ_3	Total cull rate Category 3*	0.333/year	3
	Additional death rate from Y/Y2	0.5/year	4
$_i$	Rate of aging Category i	1/year	5
$_i$	Additional cull rate of infected animals	$_1$=0.25/year; $_2$=1/year =average $_1$+ $_2$	1
	Rate of infection at birth given infected dam	H=0.15; Y1=0.15; Y2=0.17	6
	Rate of Exit Tr/Tr$_2$	Mode B =1/year; Model C=1/year	7
	Rate of exit H	Model B = 0.667/year; Model C=1/year	4
	Rate of exit Y1	0.33/year	4

1: user defined; 2:estimated by model sensitivity analysis 3:(2003) 4: (Whitlock et al., 2000; van Schaik et al., 2003) 5: calculated 6: (Benedictus et al, (Sweeney et al., 1992); 7: (Rankin, 1961) *Culling in the adult herd (μ_3) is maintained by decreasing voluntary culls when there are MAP culls (μ_3= μ_{3inv} + μ_{3vol} - $_i$). This calculation reflects turnover in a commercial herd, where increased culling for MAP may result in reduced culling for other voluntary causes. This stable herd turnover maintains constant input of susceptible youngstock.
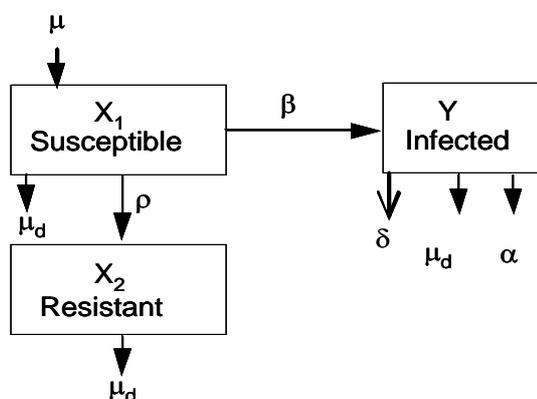
Model A: Most basic state transition model. Three states: Susceptible (X_1), Infected (Y) and Resistant (X_2). Transmission between states is modeled using rates defined in Table 1.

The first, most basic, model utilizes 3 infection states with one infectious stage (Y). All animals enter into a susceptible (X) category and progress to either resistant (X_2) status via aging at a rate ρ or to infected (Y) status at a rate β. Death rate of animals is constant for all stages (μ_d) with the exception of Y, in which there is additional death due to clinical disease (α) and optional additional death from culling (δ). R_0 is defined by a single equation: $R_0 = \beta/(\mu+\alpha)$
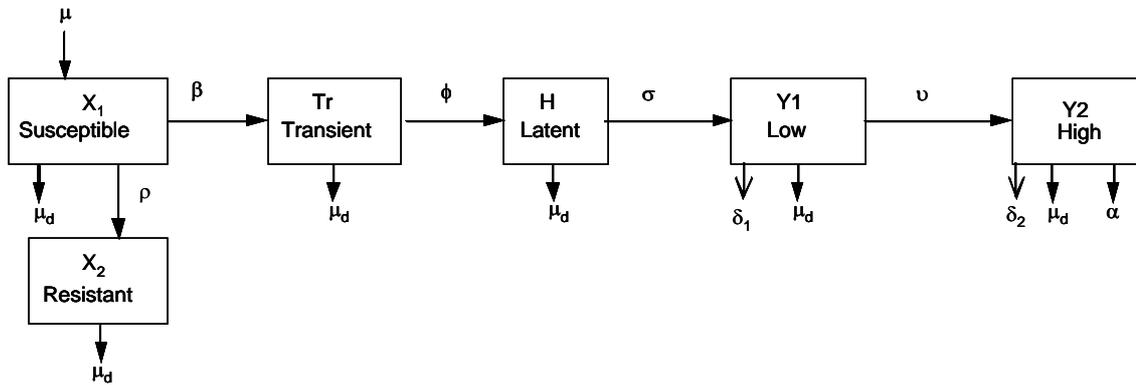
A more developed model maintains a homogenous population of cattle (no age dependent death, homogeneous mixing) but introduces more disease states. Following susceptibility (X_1), infected animals enter a transient shedding compartment (Tr). Animals exit this transient shedding state at a rate ϕ and enter an undetectable shedding or latent stage (H). Latent animals progress to low shedding (Y1) and high shedding (Y2) stages. R_0 is defined by a separate R_0 for each infectious compartment. Overall R_0 is the sum of the three. Homogenous mixing across all disease states requires that the transient shedding group has the same contact rate with X1 as do other infectious compartments (Y1 and Y2).

$R_{0Tr}=\beta_1/(\phi+\mu)$

$R_{0Y1}=\beta_2\phi\sigma/(\phi+\mu)(\sigma+\mu)(\upsilon+\mu)$

$R_{0Y2}=\epsilon\beta_2\phi\sigma\upsilon/(\phi+\mu)(\sigma+\mu)(\upsilon+\mu)(\alpha+\mu)$

Model B: Intermediate state transition model. 5 states: Susceptible (X_1), Transient (Tr), Latent (H), Low Shedding (Y1), High Shedding (Y2) and Resistant (X_2). Transmission between states is modeled using rates defined in Table 1.

Model C: Complex state transition model. Nine states reflect Susceptible youngstock (X_1), Transiently shedding youngstock (Tr_1), Transiently shedding intermediates (Tr_2), Latent intermediates (H_2), Resistant intermediates (X_2), Resistant adults (X_3), Latent adults (H_3), Low shedding adults (Y1) and High shedding adults (Y2). Transition between states is modeled from values in Table 1.

The most developed model in this series incorporates age dependent death which splits several compartments with otherwise similar characteristics (Tr_1/Tr_2, H_1/H_2, X_2/X_3). Animals thus progress through the same disease state in two separate age categories. In addition, this model allows age-dependent contact rates (homogenous mixing within age-category, but not between age categories), with transient

shedding young animals (Tr_1 and Tr_2) having potentially much higher contact with their age-cohort animals (X_1) than low and high shedding adults (Y1 and Y2). The expression of R is tedious due to the age-categories, The basic definition is quite similar to that in the previous model, with a series of equations reflecting the larger number of compartments.

This model has an additional flow of animals entering the system (γ) accounting for calves infected at birth which is evaluated independently in model validation. Calves infected at or before birth enter into the population in the category 1 transient stage (Tr_1) rather than as susceptible young animals (X_1). Input of youngstock from higher or lower risk replacement herds can be modulated to account for different assumptions of open or closed herd status.

Simulation studies
State transition models were constructed in Modelmaker Version 4.0 software (Cherwell Scientific Ltd, Oxford, UK). Simulations were run to determine model output with a range of input parameters to test sensitivity of the model to our assumptions. All simulations follow an average herd during the introduction of MAP at t=0. MAP introduction occurs by the addition of two shedding animals into a naive herd. In Models B and C these are low shedders, while in Model A there is no distinction and all shedding animals are assumed to be equivalence. All herds are modeled as 100 animals appropriately divided into age categories where appropriate; however, as the model is insensitive to herd size, any size herd could be selected with the same output prevalence.
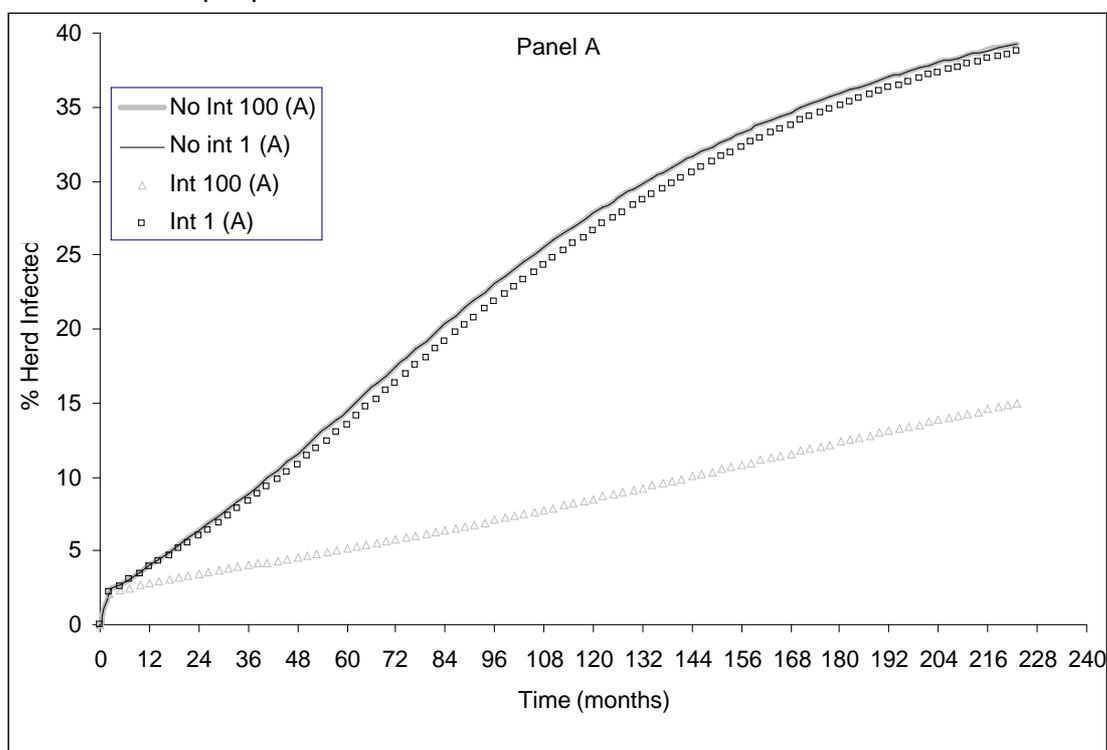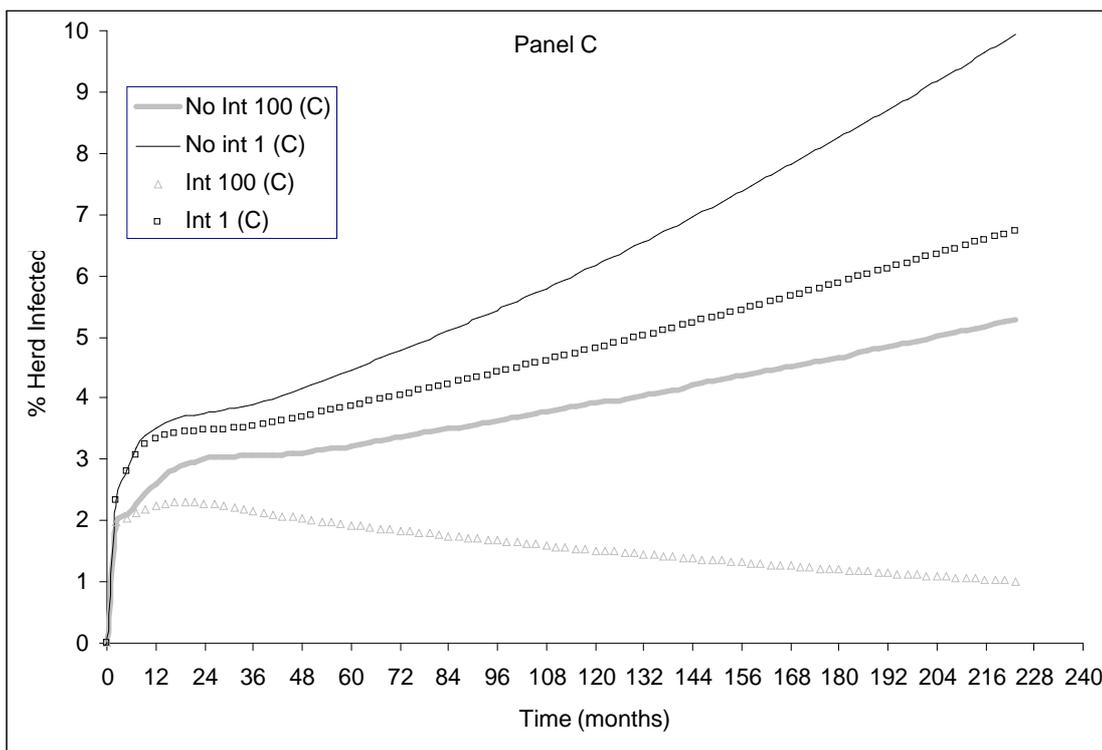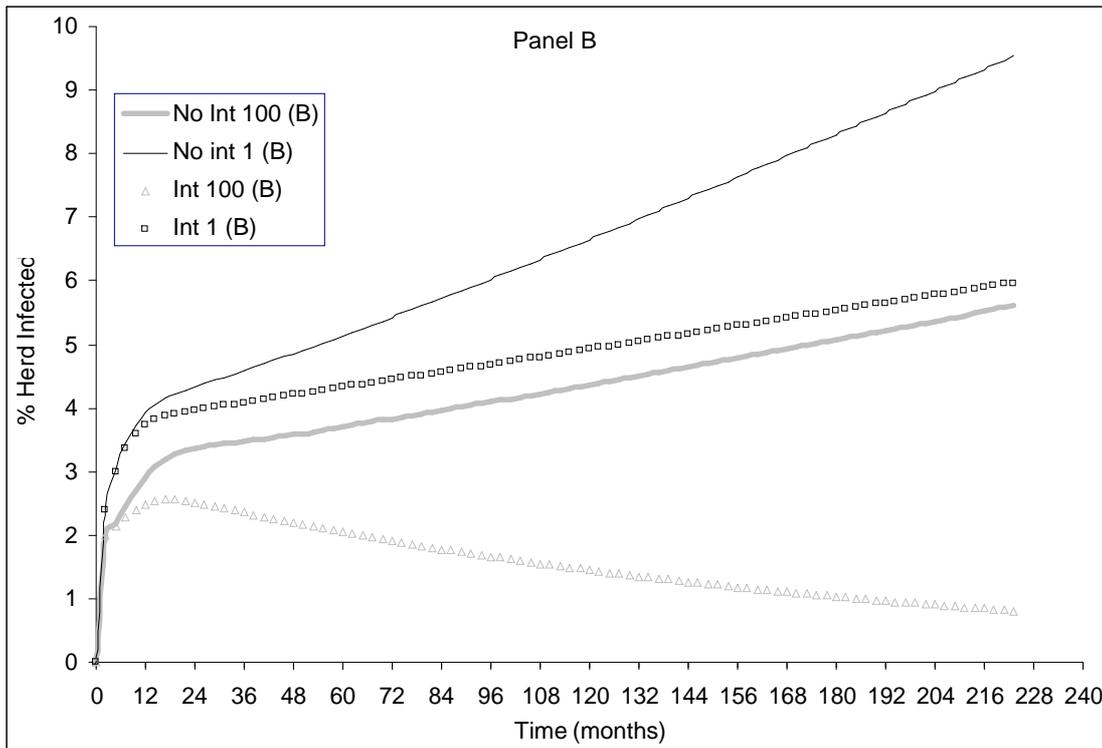
Output 1: Percent of the total herd infected with different ratios of infectiveness (ε) between low (Y1) and high (Y2) shedding adults. In this simulation there is no transient shedding, and all calves are assumed to be born susceptible and uninfected. Output from Models A, B and C when the ratio of infectiousness (ε) is 1 or 100 are shown in Panels A, B and C respectively. Lines are from simulations with no intervention strategies employed (grey:ε=100, black:ε=1). Symbols the same shade reflect disease transmission during a test-and-cull intervention strategy with the values listed in Table 1 (grey triangles: ε=100, black squares: ε=1.)

Output 1 tracks prevalence of MAP infection in herds when the ratio of infectiousness (ε) between Low (Y1) and High (Y2) shedding is 1 or 100. Panel A is output from Model A, which allows disease persistence at both ratios of infectiousness when a test-and-cull intervention is modeled. Panels B and C, which are output

from models B and C respectively, do not allow disease persistence at a ratio of 1:100 during intervention. Results for a ratio of 1:10 produce the same decline in disease under a test-and-cull strategy with parameters outlined in Table 1 (data not shown). Disease prevalence when no intervention is introduced is similar for all ratios of infectiousness for the same model, but there is a large difference in prevalence between models B & C and model A.
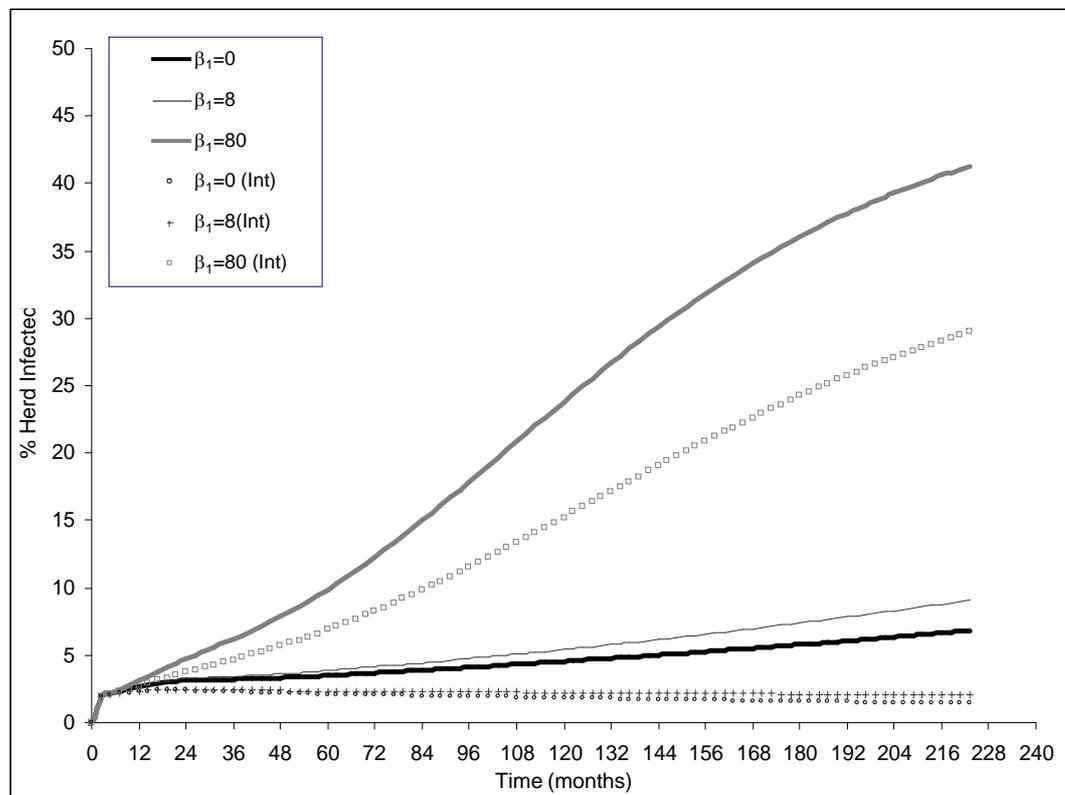
Output 2: Model C transient shedding calves contribute to force of infection (β_1) at a value 0,1 or 10 times that of adult cattle(β_2). Herd level disease prevalence is tracked for all 4 values, with lines representing the simulations without an intervention strategy and symbols representing the same simulation with the addition of an active test-and-cull intervention.

Output 2 shows the impact of a potential infectious contribution (β_1) from transiently shedding (Tr$_1$ & Tr$_2$) juveniles in model C. The base model without transient shedding calves does not maintain disease under a test-and-cull elimination strategy in this model (which utilizes values listed in Table 1 for all parameters, with ε=100). Introduction of β_1 equivalent to β_2 does not prevent successful elimination. Not only does increasing the contact rate of calves with calves to 10 times the contact rate with infectious adults result in higher disease prevalence in a non-intervention setting (β_1=80 assuming equivalent infectiousness of an X_1-Y1 contact and an X_1-Tr contact) but it also results in stable disease prevalence at a low level despite an aggressive intervention.
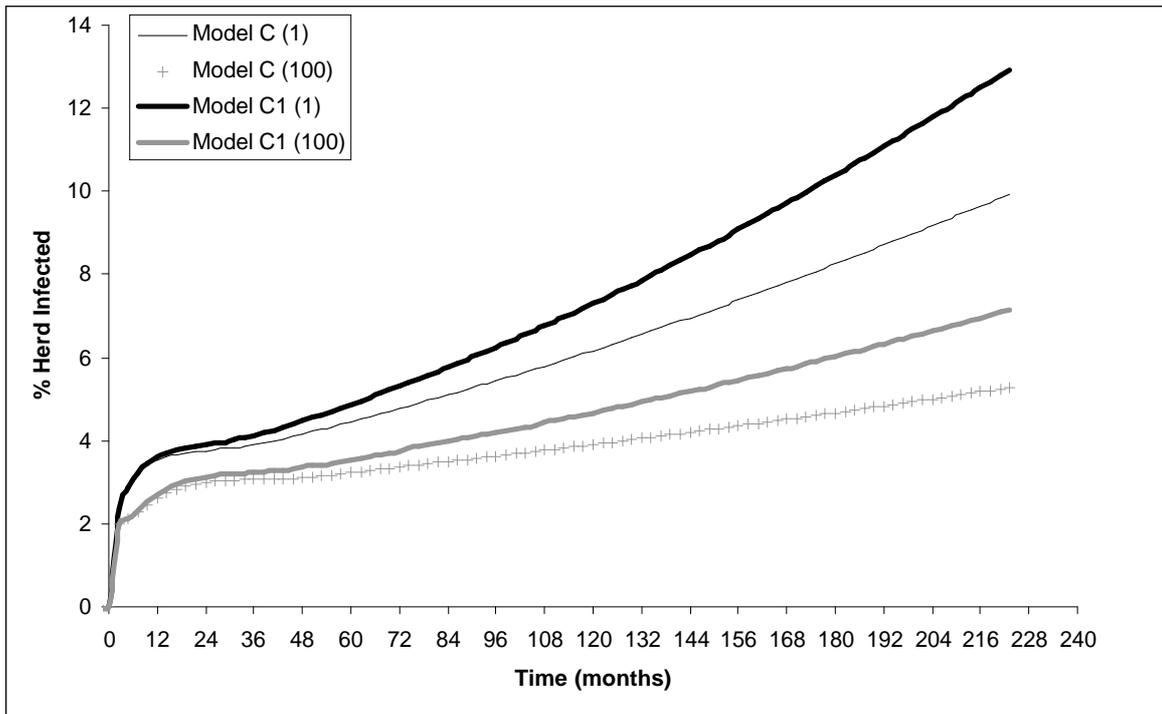
Output 3: Model C and Model C1 are the most complex model with (Model C1) and without (Model C) vertical transmission. Addition of vertical transmission at different ratios of infectiousness (ε) of low and high shedding animals (1 and 100). Black lines (thin = without vertical transmission, bold=with vertical transmission) track herd level prevalence at ε=1, and grey (symbols=without vertical transmission, bold line= with vertical transmission) at ε=100.

The effect of vertical transmission (Output 3, Model C1) is a moderate increase in prevalence of infection. This increase in prevalence occurs at all ratios of infectiousness of low and high shedding animals. Without calf-to-calf transmission however, this increased prevalence does not mean the infection cannot be eliminated (data not shown).

DISCUSSION

The contribution of greater model complexity (B, C vs A) can be evaluated by examining its output. If a complex model produces output equivalent to a simpler version, we would select the simplest model to explore the system. Models B and C offered different results from Model A in the series of panels in Output 1. Based on this and other results (not shown), Model C's output will be discussed.

Supershedders
Recent data examining colony counts of high shedding animals has indicated that there may be a Negative Binomial distribution of shedding levels of MAP among infected adults (Whitlock et al 8ICP). Even prior to exhibiting clinical signs, cows may be shedding in excess of 10^10 bacteria/day. These animals contaminate the environment with massive amounts of bacteria and increase the chances for transmission. Intuitively, one would assume that removing these "super-shedding" animals should result in the virtual elimination of MAP from the farm. If high shedding animals were >100,000x more infectious than low shedders, the input of low shedders should be negligible in controlling infection spread. However, the series of models developed here (Output 1) conclude that these super-shedding animals, while they are responsible for the majority of new infections (potentially greater than 90% on a highly infected farm), are not responsible for all new cases on infection. If model inputs allow super-shedding animals to be even 100 times more contagious than low shedders, all models show that test-and-cull strategies would be an effective mechanism of infection control except the most basic (Output 1:Model A). Eliminating super-shedding animals would drop $R_{effective}$ below 1 and result in decline of MAP and its eradication over the course of several years (results not shown).

This model output indicates that although high shedders are much more infectious than low shedding animals their rate of contact with other animals limits transmission. High shedders are important in a high prevalence environment, where many calves are likely to come into contact with one, but removing high shedders is insufficient to address long term persistence of MAP. To understand sustained transmission in a test-and-cull environment we must look to other sources for potential contributions.

Transient Shedding
Dam-to-calf transmission alone is believed insufficient to maintain infection in a population. This was recently shown for *Neospira caninum* in dairy herds (French et al., 1999). Following contact with cows at birth, calves come into contact with other young animals more often than they do adults for the next twelve to twenty-four months of life. If young animals are excreting bacteria into the calf environment, there is a high risk of exposure and transmission to susceptible animals.

Peer-reviewed literature has described the biologic plausibility of young calves actively shedding MAP. (Taylor, 1953;Rankin, 1959;Collins and Zhao, 1994;Waters et al., 2003) . Rankin (1961) used an experimental infection transmission design to determine whether and when shedding would occur following natural calf-to-calf infection. While these animals may have been exposed to an greater dose of MAP relative to farm levels (maybe even the equivalent of high shedding adult animals), the experiment showed that animals infected from other shedding calves produced culture-positive samples within a short timeframe. Although there are no peer-reviewed studies of young animals with positive culture results due to natural vs. experimental infection, risk factors for MAP at a farm level include an association with group-housing young calves (Wells and Wagner, 2000;USDA, 2005). While other factors associated with group housing may contribute to this finding, infected young animals shedding MAP via pass-through or active shedding would explain this association.

When calf-to-calf transmission is modeled by giving an infectious value to a transient shedding group of young animals (models B and C), model output reaches a stable low prevalence MAP infection at a herd level even with an increased rate of infectiousness of Y2 animals (ε). Herds which previously maintained high levels of disease through the presence of super-shedding adults could sustain infection at a low level with the input of this transient compartment. These transiently shedding young calves may contribute to the persistence of infection in herds when high shedding animals are eliminated.

Dam-daughter transmission
Fetuses from high shedding MAP-infected dams can culture positive from *in utero* culture studies (Seitz et al., 1989;Sweeney et al., 1992). Recent data analysis (Benedictus et al, 8ICP) of a dataset collected by Dr. Robert Whitlock has shown that calves born to low shedding or latent dams also carry a significantly increased risk of being test-positive as adults vs. calves from consistently fecal culture negative dams. This information presents a challenge from an eradication standpoint, as not only does the most recent calf born from a high shedding dam become a high risk animal, but any calf produced throughout her lifespan. Given this model assumes herd size is constant, over the lifespan of a dam one daughter is expected to survive and become a productive member of a herd. Addressing dam-to-daughter transmission requires an elimination strategy which traces an animal's pedigree to cull daughters born early in the adult lifespan of an infected dam, but should not be excessively costly in comparison to other aggressive test-and-cull regimens. If high risk daughters are added to our most developed model (C) using the values calculated based on fetal infection and increased risk of infection with MAP positive dams, vertical transmission is sufficient to increase the stable threshold of infection by a factor of 1.2-1.5 (Output 3) depending on assumptions concerning relative infectiousness of low and high shedding dams. This increased infection prevalence would not be an impediment to control in the long term although it may be detrimental in the short term due to increased immediate culling. If there were no calf-to-calf transmission of infection, these animals could be removed before they had any impact on herd transmission dynamics. However, when the effect of vertical transmission is combined with the presence of infective transiently shedding animals (Tr11, Tr12), then these animals may have a real impact on infection spread.

CONCLUSION

All models demonstrated that "super-shedding" animals can have a significant impact on the prevalence of MAP infected animals in a herd, yet more complex models illustrate that these animals are not predominantly responsible for infection persistence. The maintenance of infection in a low prevalence herd that aggressively tests for and culls shedding animals is caused by other factors. Elimination of infection is a greater challenge in a model which reflects calf-to-calf transmission and dam-to-daughter fetal transmission for dams of unknown status. Further work with this model and field studies will provide opportunity to validate/invalidate the contribution of these sources of MAP to persistence in a dairy environment.

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Effects of *Mycobacterium avium subsp. paratuberculosis* in alpine pasture water ponds on paratuberculosis in dairy cattle

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ABSTRACT

Water contamination has been considered as a risk factor for the spreading of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) among the dairy cattle population in north-eastern Italy, where the herd level prevalence of infection has been estimated to be ~27%. The alpine Monti Lessini area of Verona province contains pastures grazed in the summer season. These 6,290 hectares are divided in 92 portions called "malga", in which cattle from different herds and ages graze from May until September. To estimate the risk of MAP spread through natural and artificial ponds, which are the principal source of water due to the soil structure, a PCR-based survey was carried out in 2003-2004. Water samples were collected at different periods from the same pond: in spring just after the thaw, in summer while cows were present and at the end of the grazing season when the animals left the mountain. A questionnaire was submitted and laboratory results, when present, recorded for each of the 35 herds involved in this study. Descriptive analysis of the herds' health status, ages and structure, geographical information on the pastures, temporal pattern of sample collection and presence of wild animals were considered in relation to water results. The presence of MAP insertion element IS-900 was demonstrated in the water of 8 out of 15 "malga" but only in 11 of the 100 samples. Positive results obtained in the spring season suggested a role of wild ruminants and lagomorphs in the epidemiology of paratuberculosis in the south-eastern Alps.

Key words: environmental MAP contamination, GIS, surface water, IS*900*

INTRODUCTION

The "alpeggio" is a common practice in the alpine mountains that takes advantage of the alpine pastures in summer season. It is valuable for farmers of the mountain area both as a low cost source of feed and for the character it gives to the milk, character that confers distinctive flavors to local cheeses. Moreover, this practice has recently received financial support of the EU for sustainable farming, and summer grazing had increased, even for beef cattle herds. This type of management can however increase the risk of spreading infectious diseases through the direct contact among livestock originating from different cattle herds and with wild animals. This is the case of paratuberculosis, a disease characterized by the oral-fecal transmission of the infection, in which the most important risk factor for infection spread is the aggregation between young cattle and infected cows. The role of wild ruminants and lagomorphs in infection transmission may also be relevant. Furthermore, due to the characteristics of the Venetian alps, the principal source of water for the animals in the "alpeggio" are natural and artificial ponds, in which water quality is generally poor and fecal contamination is common. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) hardiness in the environment is well documented, especially in humid and shaded places where it can survive for many months (Whittington et al., 2004).

Taking into account that the serological prevalence of paratuberculosis at a herd level in the Veneto region was estimated at 27% (Robbi et al., 2002), we intended to examine the possible role of water ponds in MAP persistence and transmission. The aim of this study was to determine if MAP was present in water ponds and whether was associated with paratuberculosis in dairy herds grazing those areas during summer season.

MATERIALS AND METHODS

The territory considered in this study is located in the southern part of the "Monti Lessini" regional park belonging to the province of Verona in north-eastern Italy. Berni et al., 1991 report that in this area are located 92 properties called "malga". Divided by fences or natural dams, the malga cover 6,290 hectares of which 5,034 are pastures. Water sources are in most cases alpine ponds, natural or artificial, and only in rare cases natural springs or rivers. Waterers placed inside and around the "malga" buildings were not considered in this study.

The region was mapped using commercially available software (ESRI ArcGIS 8.2, Redland CA). The elements of the Regional Technical Map, supplied by the Veneto Region, were loaded on a 1:5000 scale. Geographical coordinates of the ponds located in the area were acquired and subsequently 15 "malga" were randomly selected for sampling.

A questionnaire was submitted to each "malga" owner through an interview requesting confirmation of the mapped area, number of ponds or other sources of water, number of animals per herd of origin in 4 categories (cows, heifers, calves < 12 months, bulls) and presence of wild animals. Data on the separation of grazing areas between cattle of different ages were also collected. Furthermore, the database of the paratuberculosis laboratory of Istituto Zooprofilattico Sperimentale delle Venezie was scanned by herd code, seeking any record in the period 1998-2004 for either serological or direct test (fecal smear Ziehl-Neelsen staining, culture and PCR on faeces or ileo-cecal junction) results. When data was missing concerning the paratuberculosis status of the herds, the herds' veterinary practitioners were interviewed.

Among the selected "malga" 25 ponds were sampled at least twice in different seasons (Fig. 2): in spring just after the thaw period, in mid and late summer while cows were present and in autumn at the end of the grazing season. Water samples were collected at the border of the pond at 3 different points and the three aliquots mixed together to obtain a solution of 50 ml.

Samples were divided in 2 parts of 25 ml each and processed within 24 h or frozen at -20°C. At the time of testing, the water of each sample was filtered using filter paper and centrifuged at 10,000 x g for 30 min. The first of the obtained pellets was tested with PCR: DNA extraction was carried out using QIAamp DNA mini kit (Qiagen) and Adiavet Paratb PCR kit (Adiagene) was employed to amplify the IS900 fragment. The amplicon was analyzed by electrophoretic separation on 2% agarose gel. The second pellet was treated as faeces and cultured on Herrold's egg yolk medium (HEYM) as described by Whitlock et al. in 2000.

Statistical analysis was performed using the software Statistica 5.1. Association between herds and ponds were investigated by use of the Fischer exact test and seasonal variation in ponds by the McNemar test.

RESULTS

The average altitude of the "malga" was 1.459 (1.181 - 1.705) m above sea level, 407 ponds were counted, with an average of about 4 (1–10) ponds per "malga". The average number of cattle per "malga" was 71.28 (8–210), with a density of 1.3 cattle per hectare of pasture. A more detailed spatial analysis was performed on the 15 sampled "malga": average pond perimeter was 76.50 (26.05-133.74) m and the surface area 484.93 (50.59–1368.37) m^2. Fig. 1 represents the spatial distribution of the ponds selected for the study; black dots symbolize PCR MAP-positive ponds whilst dark-grey dots indicate PCR MAP-negative ones. Light-grey areas illustrate the other ponds of the territory.

Lactating cows, dry cows, bred heifers and post-weaned heifers over 9 months of age from 35 herds occupied the 15 "malga" selected. One "malga" was occupied by 12 to 24 month heifers from a single paratuberculosis infected herd. Seven more herds were determined according to records or veterinary interview to be infected by MAP. No bulls or young calves were present on any of the 15 properties.
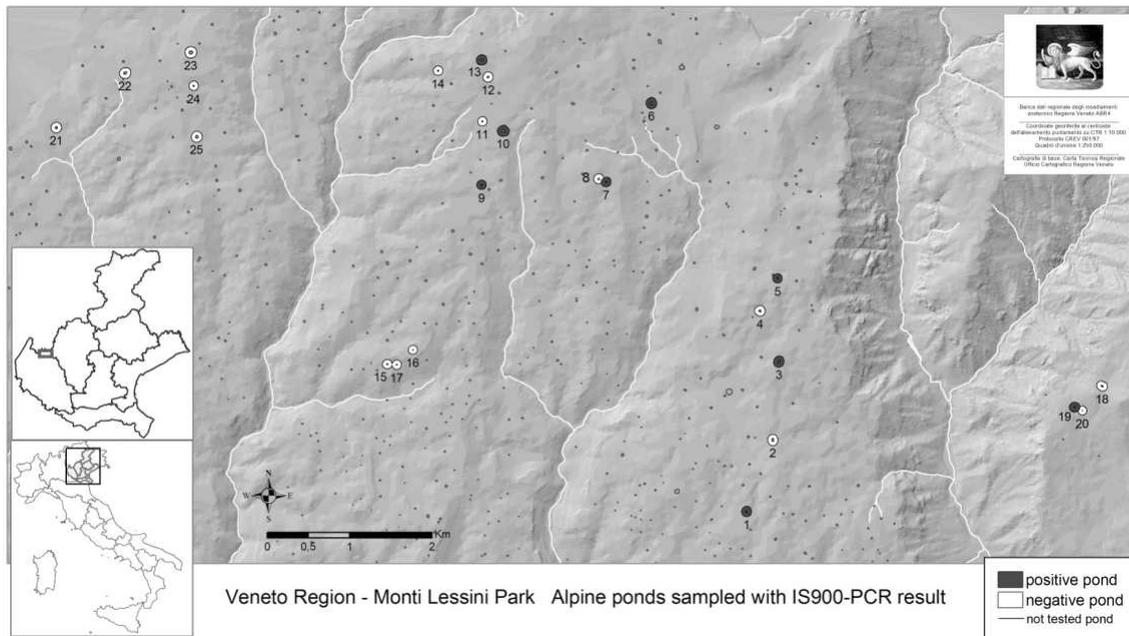
Fig. 1. Spatial distribution of alpine ponds tested for MAP and test results

The presence of wild animals (especially foxes, deer and hares) was described by all the "malga" owners interviewed, but no samples were collected to confirm the presence of MAP infection in these species.

Tests carried out on the 25 water ponds in 4 different periods of the year gave 11 positive results out of 100 samples by PCR. No positive results were obtained by culture after 16 weeks of incubation.

PCR positive results in water ponds were associated in six occasions to the presence of at least one infected herd in the "malga", while in two cases were related to uninfected herds. On the other hand, negative results in water were associated 5 times with negative herds and twice with MAP infected herds. Thus, the prevalence of positive ponds at the "malga" level was higher where MAP infected herds were present (0.75 vs. 0.29 p = 0.1319). Table 1 shows the occurrence of the infection in 8 out of the 35 herds is related to 11 PCR positive outcomes on 100 water samples collected in different seasons.

Table 1. Number of herds with positive paratuberculosis results stratified per "malga" with corresponding water samples and IS*900* PCR positive results. All water samples were negative by culture.

	Herds		Water samples	
Malga	**Total**	**Infected**	**Total**	**PCR positive**
11	3	0	3	0
18	2	0	3	0
27	2	0	3	0
29	2	1	6	0
41	2	0	4	0
42	2	1	14	2
54	2	1	10	2
59	2	0	9	1
63	1	1	15	0
68	3	1	2	1
77	4	1	7	2
78	3	0	2	1
79	3	0	5	0
84	2	1	2	1
92	2	1	15	1
Total	35	8	100	11
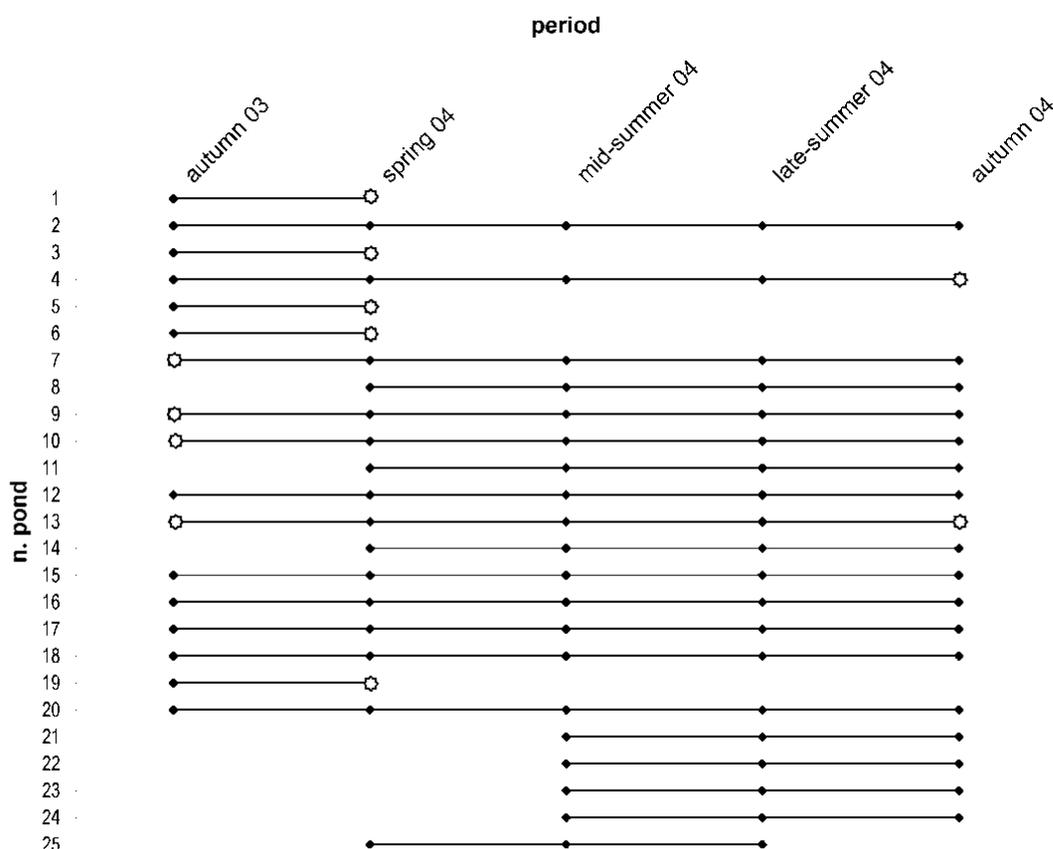
Fig. 2. Samples collected (♦) for each pond during seasons with MAP positive results (⚙).

Fig. 2 illustrates that none of the ponds were found PCR positive in summer, whereas PCR positive results were distributed in autumn and spring. The level of MAP in water ponds was calculated to be notably higher during autumn and spring than in summer (0.1 vs. 0; p<0.001). However the number of tests performed in summer was lower than in other seasons (40 vs. 49). Positive results in the spring (5) were omitted because the ponds were not retested during summer.

DISCUSSION

The ability of MAP to survive in the environment is an important component of the epidemiologic characteristics of paratuberculosis. MAP-contaminated pastures and waters may remain sources of infection for months (Manning et al., 2001). This is particularly true in water samples, in which MAP have been estimated to survive for up to 13 months at ambient temperature (Lovell et al., 1944).

The low die-off rate of MAP and the practice of using manure for fertilizing pastures that may be grazed could lead to increased environmental contamination and an increased risk of infection transmission. This is not the case for alpine pastures where fertilizing does not occur and the primary sources of MAP are infected cattle. Pasture and water ponds contamination with MAP is difficult to control. Nevertheless, the exclusion from the summer pastures of young animals, such as newborn calves and pre-weaning heifers, suggests that this type of practice should be of low risk, considering that older animals are substantially less susceptible to MAP infection.

MAP was never isolated through culture, whilst 11/100 water samples were PCR positive. This suggests that the culture method routinely used for fecal samples may not be sensitive enough for water, whereas by PCR is possible to detect even small amount of MAP genome. Other researchers confirm the presence of MAP in 500 to 1000 ml of water samples by liquid culture (Whittington et al., 2003). In this study the quantity of water used is 20 to 40 times lower and the sensitivity of solid culture against liquid is lower too;

thus, in further studies on water, it would be appropriate to increase the quantity of water sampled in order to raise the sensitivity of the method.

Furthermore, a PCR positive result does not confirm that living bacteria are present in the sample and thus does not demonstrate that the tested water was truly infectious.

The lack of a significant relationship between the prevalence of the disease in the study herds and the presence of MAP genome in water ponds (p = 0.1319) could be related to the low number of "malga" included in the present study. The presence of animals assumed infected was on two occasions associated with negative results on water samples. In one of these "malga" only 12 to 24 months heifers from a single highly infected holding were grazing. It is probable that these young cattle were not shedding or were shedding at low levels. The other case could be explained by the false negative resulting from few identifiable MAP in the water. The same reason could explain the results obtained in pond no. 13 on "malga" no. 42, frequented by 98 animals of which 42 were from a MAP infected holding. Two of five samples were PCR positive from this site.

One of the most intriguing findings on the present study concerned the increased risk associated with the no-grazing seasons versus the summer. There are at least four possible reasons for these observations: manure accumulation, slope effect, presence of infected wild animals and solar irradiation. Firstly, positive outcomes in autumn could be attributable to manure accumulation from infected animals around the pond during the entire grazing season. Secondly, rains and snow melt water drain into ponds that are always located in low lying areas. Thirdly, contamination of ponds from infected wild animals, if any are present, may occur mainly in spring and autumn, when no cattle are present and they can graze undisturbed. The fourth explanation is sunlight reducing the number of viable MAP organisms during the summer. Recent studies reveal that in an unshaded environment the persistence of MAP is reduced from 3 to 7 logs per month (Whittington et al., 2004). The ponds considered in this work were actually located in the middle of the pastures and surrounded by grass only.

No data have been collected about the soil type in this area. This factor has been described as important in determining the levels of MAP in the environment (Reviriego ET a., 2000, Ward et al., 2004)

In some alpine regions there is a significant prevalence of MAP in the deer populations. Typing of MAP strains isolated from red deer confirmed their being the "cattle strain" and thus likely of cattle origin (unpublished data). This observation suggests a risk of transmission of the infection between domestic and wild animals due to pasture and water contamination. Nevertheless the infection of sympatric species of wild ungulates such as roe deer, chamois and ibex, as well as non-ruminant species such as fox and hare, need to be further evaluated in the epidemiology of paratuberculosis in cattle.

Despite the fact that the PCR diagnosis does not differentiate between living and dead microorganisms, the discovery of genomic sequences specific to MAP in the water of alpine ponds emphasize how the practice of "alpeggio" represents a risk factor for the diffusion of MAP within bovine populations. Nevertheless, since the most susceptible age ranges are not present (calves and recently weaned cattle), this practice should be placed in a lower risk category. The "alpeggio" remain a concern however, especially for those farms in which are ongoing programs for control of paratuberculosis. In conclusion this practice should be considered in the planning of certification-and-monitoring programs for the control of MAP infections.

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Longitudinal study of the spread of ovine Johne's disease in a sheep flock in southeastern New South Wales, Australia

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ABSTRACT

On-farm investigation and monitoring for ovine Johne's disease was performed in a flock of approximately 3000 sheep between 1997 and 2002. The study aimed to better understand ovine Johne's disease prevalence, distribution and spread on this recently infected farm and to plan practical disease control and intervention strategies.

The AGID, pooled faecal culture (PFC) and histopathology were used for partial and then whole flock testing using PFC annually three times. Faecal shedding of *Mycobacterium paratuberculosis* (MAP) commenced in home bred sheep around six to seven years after a single introduction of a mob of 410 infected sheep in 1993. For at least seven years there was a clustering of infection and shedding within one or two age groups only. Sheep in these age groups appeared to have been exposed to mycobacterial contamination at an early age (<12 months) and commenced shedding at five years of age or older. Groups that were exposed to contamination as adults did not shed detectable levels of MAP during the study period.

These results provided indirect evidence of the finite duration of survival of MAP on pasture and the influence of age on susceptibility of sheep to developing patent MAP infection.
A further feature of the epidemiology in this flock was the slow transmission of MAP, related to the long incubation period (exposure to shedding interval) of five years and the absence of clinical signs of OJD throughout the study period.

The findings suggest that management practices such as partial flock culling, selective grazing management and vaccination might have reduced mycobacterial contamination on this farm, possibly to a level at which patent MAP infection no longer occured. Better understanding of disease spread within flocks over time through flock profiling using PFC will help devise surveillance strategies (including testing protocols for market assurance testing) that account for clustering of infection as well as very slow transmission of infection through a flock.

Key words: ovine Johne's disease, clustering, whole flock testing, flock profiling, pooled faecal culture testing.

INTRODUCTION

OJD is a chronic wasting disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The major mode of transmission of bacteria is by the faecal-oral route. Once ingested, it usually takes at least 12 months before a patent infection establishes and detectable levels of bacteria are shed via faeces (Chaitaweesub et al, 1999). Appearance of clinical signs of chronic wasting usually takes many years (Whittington et al, 2001). The organism remains viable in the environment for up to about 12 months but infectious doses may be present for a shorter period (Whittington, 2001). Less is known about transmission of MAP in sheep than cattle, but calves are reported to be more susceptible to infection than mature cattle and the same might apply to sheep (Whittington et al, 2001).

There is very little objective information regarding the spread of MAP within an infected flock. It is assumed that most sheep are exposed via pasture, and that all animals in a flock have an equal chance of becoming exposed. Surveillance programs to detect infection entail sampling based on this assumption and ignore potential clustering of infections (Whittington et al, 2001). In addition, recommendations for control of OJD are based on whole flock destocking or quarantine and other trading restrictions to reduce further spread. These make no allowance for the possibility that infection is confined to subgroups or mobs within a flock. The availability of pooled faecal culture (Whittington et al, 2000) has enabled relatively inexpensive testing of whole flocks, to better determine the source and distribution of infection.

The aim of this study was to apply whole flock testing over time to determine the prevalence, distribution and spread of infection in a recently infected flock. This data may support the development of practical plans for farm intervention strategies to control the disease.

MATERIALS AND METHODS

The farm consisted of 1739 hectares of improved pasture located in southeastern New South Wales (NSW), a 600 mm average annual rainfall area. The main enterprise on this farm was sheep breeding, but around 700 beef cattle were also grazed and 250-300 hectares were cropped annually. The sheep flock consists of a commercial Bond breed flock of approximately 3000 breeding ewes and a Bond stud of about 300 ewes. Lambing is in April/May. Wether lambs are sold to slaughter from December onwards each year. Culls are sold to slaughter annually in September/October and usually include the entire oldest age group (six year olds). Prior to their first joining, the ewes are classed and around 50 are selected each year to enter the stud. Sheep are segregated based on age and kept in separate mobs, up to six years old. The commercial and stud ewes are kept separately. The mobs are grazed in a rotational pattern, fitting in with pasture availability and the cropping program. Other than the introduction of some Merino sheep (described below) and a single purchase of five Dorset rams in 1997 no sheep are routinely introduced.

In 1993 the owner purchased 410 mixed age fine wool Merino ewes from farm X, located in what is now recognized as the high prevalence OJD area in NSW. On the study farm the Merino sheep had no direct contact with the Bond flocks. OJD was confirmed on farm X in late 1996.

In early 1996 the owner of the study farm sold 287 of the Merino ewes to another farm (farm F) and the remainder to slaughter. Any remaining progeny from these Merino ewes were sold to slaughter in December 1996.

The 237 merino ewes on farm F were tested with AGID in October 1996 and there were nine positive results. Three of these sheep were examined at post mortem; histology on intestinal tissue showed lesions consistent with multibacillary Johne's disease in all three sheep. The acid fast organisms in the formalin-fixed paraffin-embedded tissues of each sheep were identified as S strain MAP.

In March 2000, OJD was confirmed on the study farm in homebred 5-year old Bond ewes (see results). Whole flock testing of adult sheep by pooled fecal culture (PFC) commenced later in the year and was repeated annually three times in total. Methods for testing for OJD and further details are described in Rast and Whittington, 2005.

RESULTS

The patterns of testing and infection on the study farm are provided in Fig. 1 and Table 1.

Testing prior to 2000
Testing commenced on the study farm in 1997, as potentially infected sheep had been introduced from Farm X in 1993.

- In 1997, 50 homebred 1992-born Bond sheep, which may have had contact with MAP-contaminated land, were tested using the AGID test with negative results.
- In June 1998, 460 homebred Bond sheep, born in 1993 and 1994, were tested using the AGID and PFC, with negative results.
- In March 2000, OJD was finally confirmed in 1995-born homebred Bond ewes, by histopathology following serology (AGID) tests of 450 sheep (Table 1, Fig. 1).

Testing was required on three occasions over a four-year period to confirm infection in homebred sheep. Formalin-fixed paraffin-embedded tissues from one sheep with multibaciliary lesions were examined by PCR and were confirmed to contain S strain of MAP.

Testing in 2000
All sheep older than two years were tested with PFC in June 2000. Shedding of MAP was confirmed in the 1995-born commercial ewes (prevalence 2.5%). This was the age group known to be infected based on AGID tests with subsequent post mortem examination and histopathology testing, performed earlier in the same year. All results from other age groups were negative (Fig. 1 and Table 1).

Testing in 2001
All sheep older than 12 months were sampled for PFC in September 2001. Shedding of MAP was detected in the 1996-born commercial Bond ewes (prevalence 0.8%) and 1999-born stud ewes (prevalence 1.4%) (Fig. 1 and Table 1).

Testing in 2002
 All sheep older than 12 months were tested using PFC in September-October 2002. Shedding was confirmed again in the 1996-born commercial ewes and prevalence had increased from 0.8% to 2.5%. The 1997- born commercial ewes were also positive (prevalence 0.7%). All other results were negative, including the 1999-born stud ewes that had tested positive the previous year (Table 1 and Fig. 1).

Infection management on the study farm.
After notification of infection on Farm X (source of putative infected Merino sheep) but prior to OJD confirmation on the study farm the owner identified the land used to graze the putative infected introduced Merino sheep and their progeny and ceased sheep grazing on this land in 1996. He applied lime at a rate of 2.5 tones per hectare and used the land for cattle grazing or cropping instead.

After OJD confirmation on the study farm in 2000, the first whole flock test by PFC in 2000 established that only the 1995-born sheep were shedding at detectable levels of MAP at the time of sampling. Faecal samples were collected in June, however the serology and histopathology results of multibacillary OJD from a limited number of sheep in that age group in March 2000 made it likely that they were shedding by then. Therefore lambs born to these ewes in April/May 2000 were at high risk of exposure and infection. A recommendation was made in 2000 to cull the entire 1995-born age group, including the lambs born to them. The owner sold all remaining 1995 born ewes (207 ewes) to slaughter in October 2000, and the lambs as they reached market weight between October and December 2000.

At that stage it was considered that the 1999-born sheep were potentially at high risk of infection through contamination from MAP shed by the infected 1995-born sheep in 2000 or earlier. The 1996 and 1997 born sheep were also considered at risk by exposure to contamination from the introduced Merino sheep (Fig. 1). However culling of other age groups considered at risk of infection was not an economically viable option.

It was recommended that land on which the confirmed infected and shedding 1995-born age group had been kept be used for purposes other than sheep grazing. The owner was able to identify and use the paddock these sheep had been in from the beginning of 1999 to late 2000 for cropping and cattle grazing in the following years.

In 2001, use of Gudair$^®$ vaccine was approved on the study farm and a vaccination program began. All ewe lambs born in 2001 and the 1999-born commercial and stud ewes were vaccinated at the time of faecal

sampling. Other age groups were not vaccinated due to the doubtful efficacy of vaccine administered post exposure to adults and the cost.

During 2002, as the test results from the 2001 sampling showed an apparent increase in shedding, all sheep (including adults) considered at risk but not already shedding were vaccinated. These were the 1998-, 2000- and 2002-born ewes and all rams.

DISCUSSION

Observations and test results support the following scenario: 1) Infection was introduced onto the study farm with Merino sheep purchased in 1993 and this source of contamination was removed in 1996; 2) Shedding in infected merino sheep occurred prior to their detection, and infective concentrations of MAP from the Merino sheep were present in the environment on the study farm from 1995 to 1997. This is based on a 12-month survival period on pasture after removal of the infected sheep (Whittington et al, 2004); 3) Only sheep less than 12 months old when exposed to MAP developed patent (progressed to shedding MAP) during the study period; 4) Shedding commenced when sheep were five years or older, a significantly longer time period than the shortest reported 12 months incubation period in sheep (Chaitaweesub et al, 1999).

The source of infection on the study farm was most likely environmental contamination with MAP from some of the 410 Merino sheep and their progeny introduced from Farm X in 1993 and sold in 1996. No other sources of infection are known. Although the Merino sheep were never tested while present on the study farm, these introduced Merino sheep were likely to be shedding MAP because 8 months after their sale to Farm F the prevalence of sero-positive sheep was 3.8%. Estimation of the true prevalence of infection from these results is problematic because of the reported extreme variation in sensitivity of the AGID (Sergeant et al, 2003; Sacks et al 1989). Shedding as early as 1995/1996 by these sheep can be assumed because the seropositive sheep examined on Farm F had multibacillary intestinal lesions, a feature highly correlated with shedding of MAP in faeces (Whittington et al, 1999). Negative test results in the home-bred Bond sheep born in 1994 or earlier (therefore four to six year old when tested) strengthens the assumption that the Merino sheep introduced from Farm X did not shed sufficient numbers of MAP prior to 1994 to contaminate the environment and transmit infection to lambs present on the study farm at that time.

Whole flock testing using PFC of all adult sheep in 2000 confirmed that shedding was limited to the 1995-born age group. It is possible that contamination originating from the Merino sheep was very localized and only the 1995-born commercial Bond ewes were exposed (i.e. by chance). Alternatively, and more likely considering subsequent test results, age susceptibility to infection meant that only lambs exposed to contamination developed a patent infection. If this is the case and if the Merino sheep produced sufficient contamination with MAP from 1995 and lasting until 12 months after their departure from the study farm in 1996, then sheep born in 1995, 1996 and 1997 were at risk of infection, but only the1995-born sheep were old enough to shed at the time of sampling in 2000.

Results from testing in 2001 and 2002 support this hypothesis, and showed that in 2001 shedding was occurring in the 1996-born sheep and in 2002 in 1997-born sheep. Contamination left by the Merino sheep was probably quite low, leading to lower ingested doses of MAP, which would explain the late onset of shedding (5 year old) in the 1995-, 1996- and 1997-born age groups (Whittington et al, 2001). Also these sheep were in good body condition and perhaps other environmental or genetic effects contributed to late onset of shedding. It is also possible that the 1996- and 1997-born sheep became infected as adults from contamination by homebred Bond ewes that were confirmed to shed at the 2000 and 2001 testing (Fig.1). However we consider this unlikely due to insufficient time for development of lesions and shedding and the low prevalence of shedding (Table 1).

At the 2001 testing, shedding was confirmed in the 1999-born stud ewes but not in the commercial ewes of the same age (Table 1). The source of infection of this age group is uncertain. Infection could have been transmitted by chance exposure to high contamination (produced by homebred sheep shedding in 2000, 2001 or earlier). This is unlikely because the prevalence in homebred sheep was low (see table 1). Intrauterine infection may have been the source as

their dams could have been the 1995- or 1996-born infected ewes. However intrauterine transmission is improbable unless sheep are clinically infected, and there were no clinically infected sheep observed on the study farm(Lambeth et al, 2004). The same mob, when retested in 2002 was apparently no longer shedding bacteria. Intermittent shedding may reflect either stage of disease (Whittington et al, 2001), passive shedding of ingested organisms acquired from contaminated pasture or perhaps the effect of vaccination (Eppleston et al, 2003). Alternatively, the sheep being responsible for shedding could have died during the interval between the two tests.

Table 1. The prevalence of OJD infection based on AGID and PFC test results, for groups of samples where either test was positive

Test date	Year of birth	Sample size	Breed	No. sheep/pool	Test	No. pos.	Prevalence of sero-positive or faecal culture positive sheep % (95% C.L.).
Oct 1997	1990/91	239	Merino	na[a]	AGID	9[b]	3.8 (1.7 – 7.0)
Mar 2000	1995	95	Bond	na	AGID	4[b]	4.2 (1.7 - 11.9)
Jun 2000	1995	202 (10 pools)	Bond	20-22	PFC	4	2.5 (0.1 - 5.0)
Sep 2001	1999	100 (2 pools)	Bond	50	PFC	1	1.4 (0 - 4.1)
Oct 2001	1996	306 (6 pools)	Bond	50-56	PFC	2	0.8 (0 - 1.9)
Oct 2002	1996	350 (7 pools)	Bond	50	PFC	5	2.5 (0.2 – 4.8)
Oct 2002	1997	325 (7 pools)	Bond	26(1 pool), 49-50	PFC	1	0.3 (0 - 0.9)

AGID: Agar gel immunodiffusion; PFC: Pooled faecal culture; [a]not applicable; all sheep were tested individually; [b]number of positive individuals

CONCLUSION

The history and extensive testing available on this farm allowed identification of the time period over which mycobacterial contamination occurred by the introduced infected Merino sheep. The study highlights the slow spread of infection within the home bred flock. OJD infection was clustered, remaining limited to one or two age groups for at least seven years after the infection was introduced.

Opportunities for infection control and risks to effective surveillance are apparent retrospectively. On this farm, it may have been possible to stop transmission of OJD through timely implementation of infection management practices such as selective culling, strategic grazing and vaccination. If these disease control strategies had been implemented earlier than was possible on this farm, transmission may have been stopped.

In retrospect, action that could have been taken in 1996/97 that may have led to elimination of the infection from the study farm would include:
- Implementation of an infected flock profile immediately upon identification of the infected introduced sheep
- Culling of these sheep and their progeny
- Culling of home bred sheep born during the preceding and following 12 months, corresponding to the period of contamination, and prior to reaching 12 months of age
- Implementation of a vaccination program of all remaining sheep and annually of lambs at marking time
- Whole flock testing using PFC (infected flock profile) about 5 years after commencement of the program to monitor flock infection status

Vaccination was used on the study farm and the owner plans to vaccinate ewe lambs annually at marking or weaning. Based on recent research results with Gudair ® vaccine under Australian conditions, vaccination on this farm is likely reduce the levels of MAP contamination as well as delay the onset of shedding (Eppleston et al, 2003). It may be that in very low challenge situations such as this, vaccination eventually prevents shedding of detectable levels of MAP. Further testing would be needed to confirm this.

This study demonstrates how difficult effective OJD surveillance may be unless exact history is known and the correct age groups or mobs are tested or unless testing occurs over a long period of time. Clustering of infection reduces the confidence in a negative test outcome if sampling is random. This has significant implications for market assurance program testing (Whittington et al 2001) and surveillance testing undertaken to assess the OJD risk of a flock or mob of sheep intended for sale.
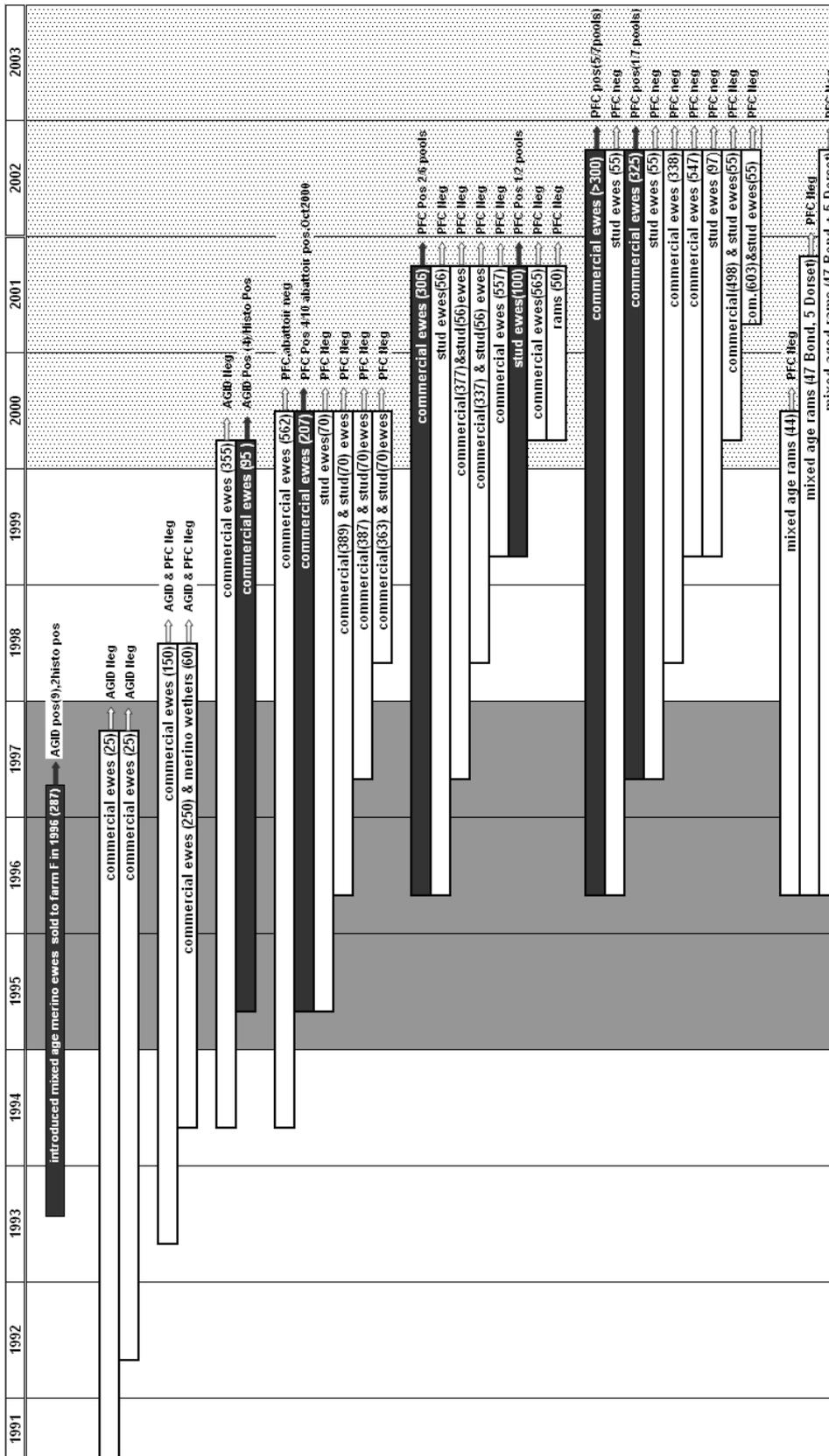
Fig. 1. Representation of MAP spread on the study farm. Sheep mobs are indicated by horizontal bars where the left edge of each bar indicates when sheep were born and the right edge when sheep were tested. Dark bars indicate mobs with positive results in tests for OJD, while white bars indicate mobs that had negative test results. The dark block represents the likely interval of environmental MAP contamination from introduced merino sheep, while light dotted block represents the likely period of environmental MAP contamination from homebred Bond sheep.

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Horizontal transmission experiment of paratuberculosis

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ABSTRACT

In September 2001 two transmission experiments both lasting 3 months were carried out to study the cow-calf transmission of paratuberculosis (Period 1), followed by calf-calf transmission of the infection (Period 2). Every two weeks blood and faecal samples were collected from all animals. After these experiments all 20 calves were housed individually for another 3.5 years to determine the infection status and excretion pattern of each animal. In autumn 2004 the animals were inseminated to observe a possible increase in faecal excretion of MAP shortly before expected calving. One month before calving in 2005, animals were slaughtered and several tissues and organs per cow and unborn calf were sampled.

Results of faecal culture-positive animals will be shown. Horizontal cow-calf transmission was not difficult to achieve (Period 1). At the highest infection pressure (6 shedding cows of which 3 were high shedders in Period 1) all 5 calves excreted MAP in their faeces during Period 1 (shortly after infection), and 4 of 5 shed during Period 2 (when the shedding cows were absent). After that, excretion became less frequent. Horizontal calf-calf transmission did take place (Period 2), as the 4 donor-calves infected 2 receiver-calves. First estimates of the transmission rates (reproduction ratio R) were quantified and are discussed.

Key words: Transmission experiment, *Mycobacterium avium* subsp. *paratuberculosis*, cattle, calf

INTRODUCTION

Paratuberculosis or Johne's disease is an infectious disease that affects mainly ruminants. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and results in a chronic inflammation of the intestines. Infection of calves usually takes place by oral ingestion of the bacterium from contaminated manure, colostrum or milk. It is generally assumed that animals start shedding the bacteria at about two years of age (Chiodini et al., 1984). The study intended to determine whether calves shed and if so, is the shedding level sufficient to infect other calves.

Van Roermund et al. (2002) analysed data of 21 dairy farms where all animals were examined for MAP after slaughter during 10 years. By fitting the data to a transmission model, the transmission rate beta of the infection was estimated. As faecal samples of animals found infected at slaughter were not taken during their life time, the infectious periods of the animals had to be assumed. As a default the classical idea of paratuberculosis was adopted: animals are first latently infected and start shedding the bacteria at 2 years of age. However, when assuming that infected calves were infectious during a certain period immediately after infection, the model fitted much better to the data.

It has been found that calves shed the bacterium shortly after infection, at least after oral inoculation with high infectious doses (Bakker and Zijderveld, unpubl.). Whether this also takes place under farming circumstances without artificial inoculation is not known. To determine if the role of calves in transmitting the infection to other calves is indeed as important as our statistical study suggested, the topic was studied in detail in this study. Cow-calf followed by calf-calf transmission experiments were completed; calves were not artificially inoculated. Each animal was sampled for blood and faeces regularly. After these experiments animals were housed individually for another 3.5 years to be able to detect the infection status and excretion pattern of each animal.

MATERIALS AND METHODS

In September 2001 two groups of 5 unexposed calves were placed between two groups of 6 infected cows in a row for 3 months (Period 1: 1-Sep-2001 until 1-Dec-2001). Calves were one week old at the start and born in certified MAP-free dairy herds, i.e. herds with at least four negative annual herd tests by faecal culture. Cows were artificially infected at one week of age in January 1999 for another experiment (oral inoculation of 5 g faeces of a clinical cow per day during 3 weeks, Bakker and Zijderveld, unpubl.). Each calf was housed in an open box between two cows (to avoid injuries), and their position in the row was randomly changed every two weeks. Calves were not artificially infected. Calf boxes sized 0.70 x 1.50 x 0.95 m (w, l, h). Calves were kept on saw dust and boxes were cleaned weekly with a broom.

During a second period of 3 months (Period 2: 1-Dec-2001 until 1-Mar-2002), each group of 5 calves (now called donor calves) was housed together with 5 new calves (now called receiver calves; one week old at the start) in a separate group-stable of 20 m^2. The calves were kept on straw, and the group-stable was cleaned weekly with a broom.

All calves were fed artificial milk replacer during the first 6-8 weeks of their lives, followed by grass pellets and calf pellets of concentrate. Every two weeks in Period 1 and 2 blood and faecal samples were collected from all animals. After this period, the 20 calves were transferred to a farm (only crops, no other animals) and housed in a row with dividing walls to avoid direct or faecal contact (Period 3: 1-Mar-2002 until slaughter in 2005). Animal feed consisted of grass pellets, hay, dairy cattle pellets of concentrate and mineral pellets of concentrate. Every two months blood and faecal samples were collected from all 20 animals. In autumn 2004 the animals were inseminated. One month before expected calving in April and August 2005, animals were slaughtered and blood, faeces and 10 tissues and organs per cow and unborn calf were sampled for diagnosis (microscopic, culture). Insemination was done to observe a possible increase in faecal excretion of MAP shortly before calving. In this paper faecal culture results of samples until 27-Oct-2004 will be shown, as culture of the bacterium takes 6 months. Results of serum ELISA and gamma-interferon tests are not yet known.

Table 1. Faecal culture (FC) test results of cows of group A during the cow-calf transmission experiment in Period 1 (7 tests at 2 week intervals). Cows were infected ca 2.5 years before. FC+ is 1-10 colonies per tube (0.16 g faeces), FC++ is 10-100 colonies per tube; FC+++ is more than 100 colonies per tube.

Cow number	# FC positive	# FC+++	# FC++	# FC+
1362	6 / 7	1	4	1
1364	7 / 7	7	0	0
1363	7 / 7	7	0	0
1359	5 / 7	0	2	3
1360	2 / 7	0	0	2
1356	2 / 7	0	0	2
Totals	6 infectious cows in Period 1	15 FC+++	6 FC++	8 FC+

Faecal culture
Bacteria were cultured according to a modified method of Jorgenson (1982). Growth of MAP was mycobactin dependent and checked every 4 weeks. If no growth was observed after 6 months of culture, the sample was considered negative. The presence of MAP in positive cultures was confirmed by amplification of the MAP specific IS900 by PCR (Vary et al., 1990).

RESULTS

Group A
Table 1 shows the faecal culture results of the 6 cows of group A during Period 1. All 6 cows shed MAP, and can be considered infectious to the calves. Three of these cows were high shedders. Fifteen samples (of the 29 positives) yielded more than 100 CFU per tube (0.16 g faeces). Compared to the 6 cows of group B (see below) the cows of group A caused the highest infectious pressure.

Table 2. Positive faecal culture test results of calves of group A during cow-calf transmission experiment in Period 1 (7 tests at 2 week intervals); during calf-calf transmission experiment in Period 2 (5 tests at 2 week intervals); and during individual housing in Period 3 (17 tests at 2 months intervals). These calves are called donor-calves in Period 2. Positive results were always FC+, i.e. 1-10 colonies per tube (0.16 g faeces).

Calf number	Cow-calf transmission (Period 1)	Calf-calf transmission (Period 2)	Individual housing (Period 3)
5628	2 / 7	4 / 5	3 / 17
5629	4 / 7	0 / 5	0 / 17
5630	4 / 7	2 / 5	0 / 17
5631	4 / 7	4 / 5	9 / 17
5632	3 / 7	1 / 5	1 / 17
Totals	5 calves infected in Period 1	4 calves infectious in Period 2	

Table 2 shows the faecal culture results of the calves of group A. These calves act as receivers in Period 1, and as donors in Period 2. All five calves were infected by the cows in Period 1, and they all started shedding MAP in their faeces during the same period. At day 4, all five calves were still FC-negative, but at the second sampling date (day 19) three calves were already shedding (not in Table 2). During Period 2 four of these calves were still shedding, whereas the infectious cows were not present anymore. These four calves can thus be considered infectious to the receiver-calves. In Period 3 three of these calves were still shedding.

Fig. 1A shows the number of FC-positive calves of group A over time. Infection took place at 0-3 months (Period 1). A peak in shedding can be seen at 0-6 months of age, afterwards it declined to 0. At 18 months shedding started again in one animal.

Table 3 shows the faecal culture results of the receiver-calves of group A added in Period 2. Two receiver-calves were infected (by the four donor-calves) in Period 2, as can be concluded by the shedding of these two in Period 3. None of the receiver-calves shed MAP during Period 2.

According to Fig. 1C a peak in shedding of the receiver-calves can be seen at 7-9 months of age; it declined to 0 at 12-30 months. After that, shedding started again in one animal. Note that the receiver-calves are born at month 3.

Table 3. Positive faecal culture test results of the receiver-calves of group A during the calf-calf transmission experiment in Period 2 (5 tests at 2 week intervals); and during individual housing in Period 3 (17 tests at 2 months intervals). Positive results were always FC+, i.e. 1-10 colonies per tube (0.16 g faeces).

Calf number	Cow-calf transmission (Period 1)	Calf-calf transmission (Period 2)	Individual housing (Period 3)
6032	-	0 / 5	3 / 17
6033	-	0 / 5	0 / 17
6034	-	0 / 5	2 / 6*
6035	-	0 / 5	0 / 17
6036	-	0 / 5	0 / 17
Totals			2 calves infected in Period 2

*: animal culled in December 2003 because of lameness.
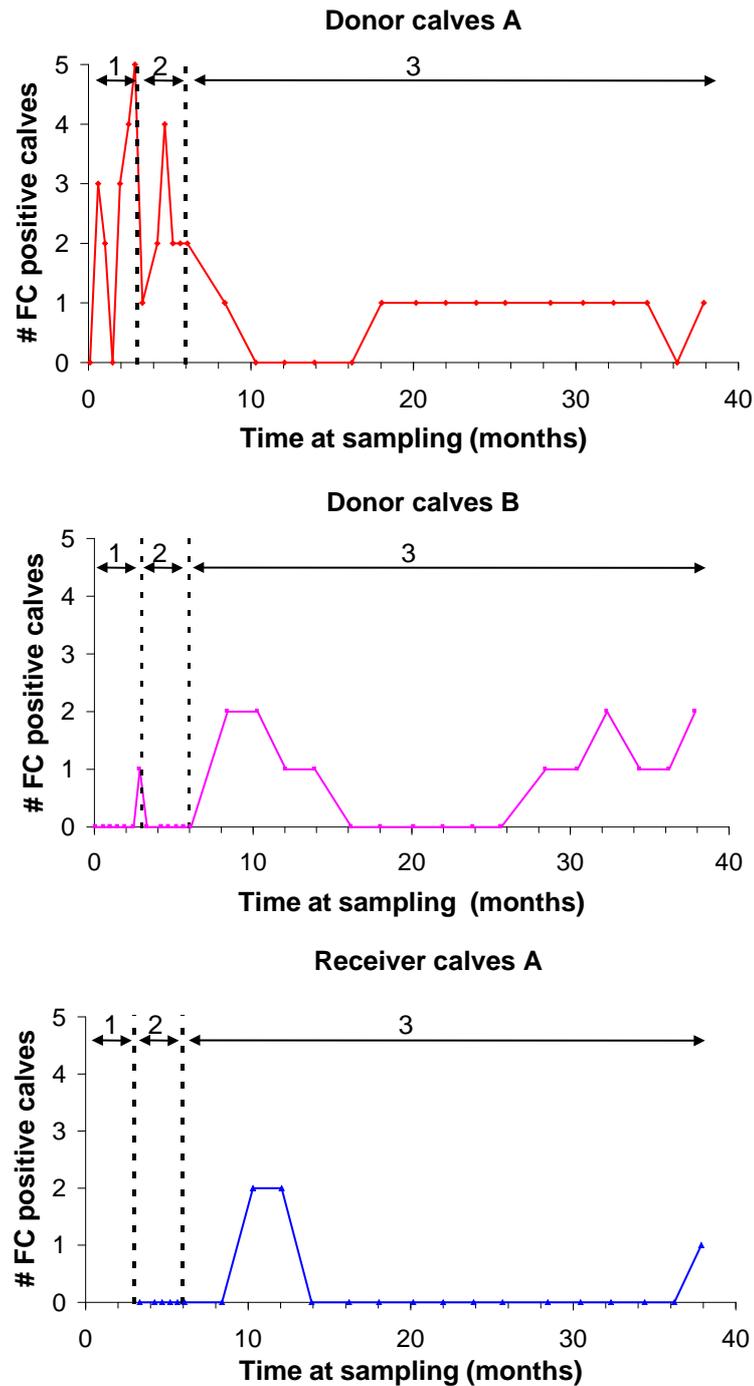
Fig. 1. Number of faecal culture (FC) positive calves over time since the beginning of the first experiment (1 Sep 2001). Donor calves were born at month 0 and infected in Period 1 (0-3 months on X-axis); receiver-calves were born at month 3 and infected in Period 2 (3-6 months on X-axis).

Group B:
The faecal culture results of the six cows of group B are shown in Table 4. Only four of these cows shed MAP in their faeces during Period 1, and can be considered infectious to the calves. The other two cows did shed MAP earlier and later than Period 1, so they were certainly infected, but apparently not infectious during Period 1. Two of the infectious cows were high shedders. Seven samples (of the 20 positives) yielded more than 100 CFU per tube (0.16 g faeces). This is half of that of the cows of group A (see Table 1), so the infectious pressure caused by cows in group B can be considered half of that of group A.

Table 5 shows the faecal culture results of the calves of group B. These calves act as receiver in Period 1, and as donor in Period 2. Four of the five calves were infected by the cows in Period 1, as can be concluded from the shedding results of these four in Period 3. Only one calf started shedding in Period 1; its

FC was positive at the last sampling date in this period. During Period 2 none of the calves were shedding, thus no calves can be considered infectious to the receiver-calves.

Table 4. Faecal culture (FC) test results of cows of group B during the cow-calf transmission experiment in Period 1 (7 tests at 2 week intervals). Cows were infected ca 2.5 years before. FC+ is 1-10 colonies per tube (0.16 g faeces), FC++ is 10-100 colonies per tube; FC+++ is more than 100 colonies per tube.

Cow number	# FC positive	# FC+++	# FC++	# FC+
1349	7 / 7	0	6	1
1350	4 / 7	0	0	4
1357	0 / 7	0	0	0
1355	0 / 7	0	0	0
1367	2 / 7	0	0	2
1351	7 / 7	7	0	0
Totals	4 infectious cows in Period 1	7 FC+++	6 FC++	7 FC+

Fig. 1B shows the number of faecal culture-positive calves of group B over time. Infection took place at 0-3 months (Period 1). A peak in shedding can be seen at 8-12 months of age, where after it declined to 0 at 15-27 months. At 27 months shedding started again.

Table 6 shows the faecal culture results of the receiver-calves of group B, which were added in Period 2. None of the receiver-calves were infected in Period 2 as expected because no infectious donor-calves were present. Also as expected, cross-infection did not occur during the 33 months of separate housing in Period 3, where all 20 animals were kept between dividing walls on the same farm.

Table 5. Positive faecal culture test results of calves of group B during cow-calf transmission experiment in Period 1 (7 tests at 2 week intervals); during calf-calf transmission experiment in Period 2 (5 tests at 2 week intervals); and during individual housing in Period 3 (17 tests at 2 months intervals). These calves are called donor-calves in Period 2. Positive results were always FC+, i.e. 1-10 colonies per tube (0.16 g faeces).

Calf number	Cow-calf transmission (Period 1)	Calf-calf transmission (Period 2)	Individual housing (Period 3)
5623	0 / 7	0 / 5	0 / 17
5624	0 / 7	0 / 5	2 / 17
5625	1 / 7	0 / 5	4 / 17
5626	0 / 7	0 / 5	2 / 17
5627	0 / 7	0 / 5	6 / 17
Totals		0 calves infectious in Period 2	4 calves infected in Period 1

Table 6. Positive faecal culture test results of the receiver-calves of group B during the calf-calf transmission experiment in Period 2 (5 tests at 2 week intervals); and during individual housing in Period 3 (17 tests at 2 months intervals). Positive results were always FC+, i.e. 1-10 colonies per tube (0.16 g faeces).

Calf number	Cow-calf transmission (Period 1)	Calf-calf transmission (Period 2)	Individual housing (Period 3)
6027	-	0 / 5	0 / 17
6028	-	0 / 5	0 / 17
6029	-	0 / 5	0 / 17
6030	-	0 / 5	0 / 17
6031	-	0 / 5	0 / 17
Totals			0 calves infected in Period 2

Estimation of R:
To quantify the transmission during the three months periods of study, we estimated the reproduction ratio R according to the final size method as described in Kroese and de Jong (2001). R is defined here as the number of new infections caused by one infectious animal during three months. The final size approach uses the number of susceptible (S), infectious (I) and 'recovered' animals (R, for animals already infected

but not infectious) at the beginning and at the end of the transmission experiment. More detailed data during the experiment (like positive and negative faecal cultures in between) are ignored for now. It is assumed that the infectious period is equal for all animals in the period of study (that is, an exponential distribution of the infectious period is not assumed here).

For the cow-calf transmission experiment (Period 1):
Group A: At the start: 6 infectious animals (6 I) + 5 susceptible animals (5 S).
               At the end: 5 animals infected, 0 susceptibles left (0 S)
Group B: At the start: 4 infectious animals (4 I) + 5 susceptible animals (5 S) +
                            2 animals already infected but not infectious (2 R).
               At the end: 4 animals infected, 1 susceptible left (1 S)

Using the above data, the MLE estimator for R (during three month) is 2.7 with a 95% CI of [1.1, 6.6]. The p-value for the one sided test H_0: R$\leq$1 is 0.019 so H_0 can be rejected. The p-value for H_0: R$\geq$1 is 0.99 so this H_0 cannot be rejected.

For the calf-calf transmission experiment (Period 2):
Group A: At the start: 4 infectious animals (4 I) + 5 susceptible animals (5 S) +
                            1 animal already infected but not infectious (1 R).
               At the end: 2 animals infected, 3 susceptibles left (3 S).
Group B: At the start: 0 infectious animals (0 I) + 5 susceptible animals (5 S).
               At the end: 0 animals infected, 5 susceptibles left (5 S)

Using the data of group A, the MLE estimator of R (during three months) is 0.9 with a 95% CI of [0.1, 3.2]. The p-value for the one sided test H_0: R$\leq$1 is 0.64 so H_0 cannot be rejected. The p-value for H_0: R$\geq$1 is 0.61 so this H_0 cannot be rejected as well.

DISCUSSION

Infection of calves by shedding cows seems to occur relatively easy when they are housed together. Only one calf (out of 10) escaped infection, and the reproduction ratio R for the period of three months is 2.7, significantly above 1. Since no artificial inoculation of calves was done, and calves were fed milk-replacer, they must have been infected through environmental contamination by the adult cows (oral uptake).

The estimated R of 2.7 is based on an infectious period of cows of three months only. If cows are infectious for a longer period during their lifetime, the reproduction ratio R_0 (number of new infections during the whole infectious period) has to be adapted likewise. The estimated R of 2.7 is also based on mixed housing of calves and cows, leading to many contacts per day as are seen in suckling herds. For dairy herds where cows and calves are separated much earlier, the estimated R should be considered the worst case estimate.

A common pattern in shedding of MAP by infected calves can be observed: a peak shortly after infection, a decline to zero for a relatively long time, and then shedding begins again. The first peak seems to be closer to the moment of infection at higher infectious pressures (compare donor-calves A with donor calves B or receiver-calves A in Fig. 1). Weber et al. (2005, this Proceedings) also found excretion of MAP in the faeces of young stock.

Even more important, it has now been shown that calves can infect other calves shortly after they themselves become infected. Van Roermund et al (2002) found that their transmission model of paratuberculosis fit epidemiologic data much better when they assumed that infected calves were infectious during a certain period immediately after infection. That hypothesis has now been strengthened by the results of this transmission experiment.

Two out of five receiver-calves were infected by the FC-positive donor calves in Period 2, resulting in an R of 0.9. Again, this estimate is based on an infectious period of three months only. According to the shedding pattern of the donor calves of Fig. 1, the total infectious period of calves during their first year of

life (when they are usually housed together) is about twice as long, thus R_0 has to be adapted likewise. However, due to the small lower estimate of the confidence interval of R, it remains unlikely that R_0 then becomes significantly above 1 from the data of this experiment. The broad confidence interval is caused by the unintended loss of one replicate in the calf-calf transmission experiment (no infectious donor calves in group B). This replicate now serves as a control treatment: no cross-infection in Period 3 when the transmission experiments were finished.

As a best case estimate let us assume that R_0 for transmission among calves is indeed 0.9 and significantly below 1. A chain of new infections will always die out and a large outbreak is not possible. However, one infectious calf housed together in a large group of other calves will still cause a number of new infections, which can be estimated by $(1-R_0)^{-1}$ (Diekmann and Heesterbeek, 2000). This means that with $R_0=0.9$, 10 new calf infections will occur on average in a large group of calves.

More results will be shown later: faecal culture results during Period 3 until section of the animals (May-August 2005), ELISA and gamma-interferon tests of blood samples, and the section results of animals and their unborn calves. Quantification of transmission will then be done by estimating beta (number of new infections per infectious animal per day) by GLM when using detailed data of each animal during the transmission experiments.

CONCLUSION

Calves can infect other calves shortly after they themselves become infected. This route of transmission should be considered to improve the effectiveness of MAP infection control programs.

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Evidence for *Mycobacterium avium* subsp. *paratuberculosis* shedding in young cattle

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ABSTRACT

The age at which cattle become faecal culture positive for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) can be used as a proxy parameter for the age at which they become infectious. The commencement of infectivity is an important parameter in the control of MAP in cattle herds. To investigate the age at becoming faecal culture positive, survival analysis methods were applied to asynchronous interval censored data of faecal culture results of samples collected from 37,151 Dutch cattle of dairy breeds in 373 herds between 1996 and 2002. A Weibull proportional hazards model was employed to study risk factors, such as apparent prevalence (AP) within the herd, breed and herd size. The analyses showed that the hazard of becoming faecal culture positive increased with the age of cattle. Cattle became faecal culture positive at younger ages when part of a herd with higher within-herd apparent prevalences. In high prevalence herds (apparent prevalence ≥ 0.10), an estimated 5% to 14% of cattle became culture positive before two years of age, depending on breed and herd size. Our findings indicate that a considerable proportion of young stock was shedding MAP. Therefore, infectious young stock should be a major concern in the control of paratuberculosis, especially in high prevalence herds.

Key words: cattle, young stock, paratuberculosis, faecal culture, age

INTRODUCTION

The aim of this study was to determine the ages at which *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infected cattle become infectious. It is frequently assumed that young stock are not infectious, and that separation of young stock from adult cattle may be sufficient to prevent new cases of infection. Furthermore, the effectiveness of control programmes for MAP infections has been studied using various models that assume a particular age when an animal becomes infective. Quantitative data on infectious ages are lacking however. The distribution of ages at which cattle become infectious can be approximated by the distribution of age at which cattle become faecal culture positive in a longitudinal study. Therefore, the aim of the present study was to determine the ages at which cattle become faecal culture positive, using results of samples submitted to the Dutch Animal Health Service (AHS) in a seven-year period. Survival analysis methods were used to enable the use of censored data. The effect of risk factors on the age distribution was included to improve the fit of the model.

MATERIALS AND METHODS

Data
A database was set up containing culture results, the date of sampling, date of birth, sex and breed of sampled cattle for all samples submitted to the AHS for individual faecal culture (IFC) for MAP. The following inclusion criteria were met: (1) samples submitted between 1st January 1996 and 31st December 2002, (2) at least 20 adult cattle (i.e. 2 years of age) were present in the herd in the quarter of the year of sampling, (3) at least one complete herd examination by IFC was submitted from the herd during the period of study, (4) at least one positive IFC or pooled faecal culture result in the herd during the period of study,

(5) the sample identification could be matched to a unique animal identification, (6) the date of birth, sex and breed could be retrieved from the pedigree records of the Dutch Dairy Cattle Association (NRS), (7) the animal that was sampled was female and classified as a dairy breed (see definition of variables). All samples were assumed to be collected two days before the date of registration at the AHS laboratory. The herd size and unique animal identifications were retrieved from the national identification-and-registration (I&R) system for cattle in the Netherlands (Nielen et al. 1996). A complete herd examination was defined as a submission of samples within a 30-day period, for which the number of samples was $\geq$80% of the recorded number of adults in the same quarter of year. Because herds with at least one positive faecal culture were selected, cattle in these herds were considered at risk of being infected.

Faecal culture
Faecal culture was performed on Löwenstein Jensen (L.J.) medium using the modified Jørgensen method (Kalis et al. 1999, Kalis et al. 2000). In short, four culture tubes were inoculated for each sample and inspected for MAP colonies and contamination at four, eight, 12, 16 and 26 weeks of incubation. Suspect colonies were examined by acid fast staining; acid fast colonies were confirmed to be MAP by IS*900* PCR. A sample was regarded positive if one or more MAP colonies were detected. A sample was regarded as contaminated if three or more tubes were contaminated at 16 weeks of incubation or earlier. A sample was negative if it was not regarded positive or contaminated. During the period of study (1996-2002), the faecal culture method was slightly adapted (inspection at 26 weeks of incubation was terminated; natamycine (1.18 g/l) was added to the LJ-culture media; a sample contaminated at > 8 weeks was regarded negative; acid fast staining on suspect colonies was no longer performed and suspect colonies were directly tested by IS900 PCR). However, the effects of these adaptations on diagnostic sensitivity and specificity were considered of minor importance. Therefore, the IFC results were analysed as if obtained by a single culture method.

Definition of variables
Culture results were classified as positive, negative or missing (for instance due to contamination). Apparent prevalence (AP) of a herd was used as a proxy parameter for within-herd prevalence. AP was defined as the total number of positive IFC results in a herd during the period of study, divided by the total number of positive and negative IFC results in that period. AP was categorized as 'low' (AP < 0.05), 'intermediate' (0.05 $\leq$ AP < 0.1) or 'high' (AP $\geq$ 0.1). Herd size was defined as the average number of adult cattle in the quarter of year of each herd examination during the study period. Herd size was categorised as 'small' (< 50 adult cattle), 'medium' (50 $\leq$ number of adult cattle < 80) or 'large' ($\geq$ 80 adult cattle). Breed of cattle was categorised into four groups: 'HF', 'HF-crossbred', 'other dairy' and 'beef'. Crossbred cattle were categorised according to the breed with the highest proportion in their pedigree. In case of equal proportions, the animal was categorised according to the following steps: (1) if its pedigree was partially unknown, the animal was categorised according to the known part of its pedigree, (2) if its pedigree was partially beef breed, the animal was categorised as 'beef', (3) if its pedigree was partially Meuse-Rhine-Yssel or Frisian-Holland, the animal was categorised as 'other dairy', (4) if its pedigree was partially Holstein-Frisian or Holstein, the animal was categorised as 'HF-crossbred', (5) otherwise, the animal was categorised according to the breed first mentioned in its pedigree. Cattle categorised as 'beef' were excluded from the study.

Data analysis
A survival analysis was performed to determine the age distribut at which cattle start (detectable) shedding of MAP. Let A_{ij} be the unknown age at which individual i in group j started to shed MAP. We assumed that an animal was consistently IFC-negative up to age A_{ij} and remained IFC-positive beyond this age. So, in our survival analysis, the "event" was detectable shedding of MAP and the "time to event" was equal to age A_{ij}. The dataset for analysis was structured according to the description for asynchronous interval censoring by Radke et al. (2003). In short, two variables L_i and U_i were defined as lower and upper bounds of the age interval for A_i. For an interval censored observation (i.e. an animal with a negative sample followed by a positive sample) U_i was set to the age at sampling of the first positive sample of the animal, and L_i to the age at sampling of the last preceding negative sample. For a right censored observation (i.e. an animal without any positive test result) L_i was set to the age at sampling of the last negative sample and U_i was set to missing. For a left censored observation (i.e. the first known test result of an animal is positive) L_i was set to missing and U_i was set to the age at sampling of the positive test.

Survival analysis for asynchronous interval censored data was performed using the PROC LIFEREG command of SAS (1999). A Weibull proportional hazards model was chosen. The general form of the Weibull survival curve in a group of animals j is defined as (Cox and Oakes, 1994):

$$S_j(t) = P(A_{ij} \geq t) = e^{-\left(\frac{t}{scale_j}\right)^{shape}} \qquad \text{(eq. 1)}$$

i.e. the probability that an animal will not shed MAP before age t depends on the scale parameter of group j and all groups in an analysis have a common shape parameter. Differences between groups are modelled by:

$$scale_j = e^{\beta_0 + \sum_{k \varepsilon\, group\, j} \beta_k X_k} \qquad \text{(eq. 2)}$$

where β_0 is the intercept and X_k are factors or covariates to define group j. Then the failure function $F_j(t)$ is the probability of shedding by an individual in group j before age t:

$$F_j(t) = P(A_{ij} < t) = 1 - S_j(t) \qquad \text{(eq. 3)}$$

The density of this failure function is:

$$f_j(t) = \frac{d}{dt}\left[1 - S_j(t)\right] = \frac{shape}{scale_j}\left(\frac{t}{scale_j}\right)^{shape-1} e^{-\left(\frac{t}{scale_j}\right)^{shape}} \qquad \text{(eq. 4)}$$

while the hazard

$$h_j(t) = \lim_{\Delta t \to 0+} \frac{P(t \leq A_{ij} < t + \Delta t \mid A_{ij} \geq t)}{\Delta t} = \frac{f_j(t)}{S_j(t)} = \frac{shape}{scale_j}\left(\frac{t}{scale_j}\right)^{shape-1} \qquad \text{(eq. 5)}$$

is the instantaneous rate of MAP shedding at age t, given that the animal was not shedding up to age t. Using this Weibull proportional hazards model, the effects of various risk factors (AP, herd size and breed) were studied. Risk factors were considered significant at p-values ≤ 0.05 generated by two-sided Wald tests. Two-way interactions were allowed in the model. Non-significant terms were removed in a backward elimination procedure (first interaction terms, then main effects).

RESULTS

Herds and cattle
Complete observations were obtained for 37,151 female cattle classified as dairy breed, including all age groups. These cattle were classified in three breed groups (HF, HF-crossbred and other dairy; Table 1). From these cattle, a total number of 59,575 individual faecal samples were cultured with a positive or negative result (Fig. 1). The number of samples per animal ranged from 1 to 8. The interval between two consecutive samplings from a single animal ranged from 0.0 to 6.8 yr (median 1.0 yr). Individual faecal culture results from female young stock (i.e. <2 years of age) were obtained from 4,661 animals classified as dairy breed in 174 herds (Table 1). The number of herds and cattle in the various classes of herd size and AP are shown in Table 2.

Table 1. Categorisation of female cattle classified as dairy breed.

Breed group	Description	All age groups		Young stock (< 2 yrs)	
		Number of female cattle	Number of herds	Number of female cattle	Number of herds
HF	100% Holstein-Frisian or 100% Holstein	18979	353	3182	149
HF-cross	Holstein-Frisian crossbred or Holstein crossbred	17345	371	1426	126
other dairy	Frisian-Holland, Meuse-Rhine-Yssel, Brown Swiss, Dutch Belted, Groningen, Jersey, Montbeliarde, Swedish Red and White	827	91	53	11
Total		37151	373	4661	174
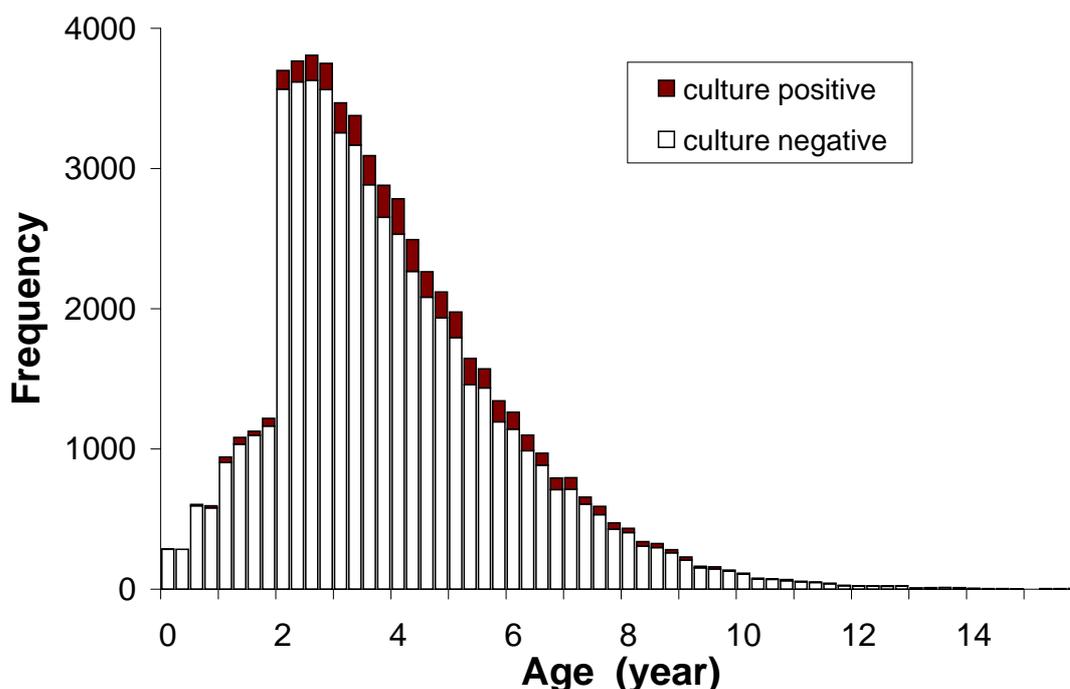
Fig. 1. Distribution of age at sampling and positive or negative result of individual culture for MAP on 59,575 faecal samples collected from 37,151 female cattle classified as dairy breeds.

Survival analysis of faecal culture results of all age groups
Left censored (first observation positive), interval censored (negative observation followed by positive observation) and right censored (all observations negative) observations were made for 2538, 1083 and 33,530 of the 37,151 female cattle respectively. Thus, 34,613 observations of L_i and 3621 observations of U_i were made (Figs. 2A and B).

The shape parameter of the Weibull model was different for the three classes of AP (Tables 3 - 5). Because the shape parameter is required to be the same amongst the various groups in a single analysis, no global analysis was possible and only separate analyses were performed for the three AP classes. Within each AP class, the shape parameters of the herd size classes and breed groups were not significantly different. Therefore, all herd size classes and breed groups were included in a single analysis within each AP class. In each AP class, the hazard $h(t)$ increased with age. With increasing AP, the weight of the failure density curve $f(t)$ shifted to a lower age (Figs. 3 - 5), indicating that the median age at which cattle become culture positive was lower in herds with a higher AP.

Table 2. Number of herds (number of cattle) included in the analysis in each herd size class by apparent prevalence (AP) class.

| Age group	AP class	Herd size class (number of adult cattle)			Total
		<50	50 - 80	>80	
All age groups	AP< 0.05	26 (1409)	75 (5739)	59 (9081)	160 (16166)
	$0.05 \leq AP < 0.1$	14 (711)	45 (4167)	51 (7552)	110 (12430)
	$AP \geq 0.1$	18 (843)	37 (2291)	48 (5421)	103 (8555)
	Total	58 (2963)	157 (12197)	158 (21991)	373 (37151)
Young stock only (< 2 yrs)	AP< 0.05	12 (299)	29 (603)	36 (1609)	77 (2551)
	$0.05 \leq AP < 0.1$	6 (26)	22 (412)	27 (908)	55 (1346)
	$AP \geq 0.1$	7 (59)	17 (232)	18 (513)	42 (804)
	Total	25 (384)	68 (1247)	81 (3030)	174 (4661)
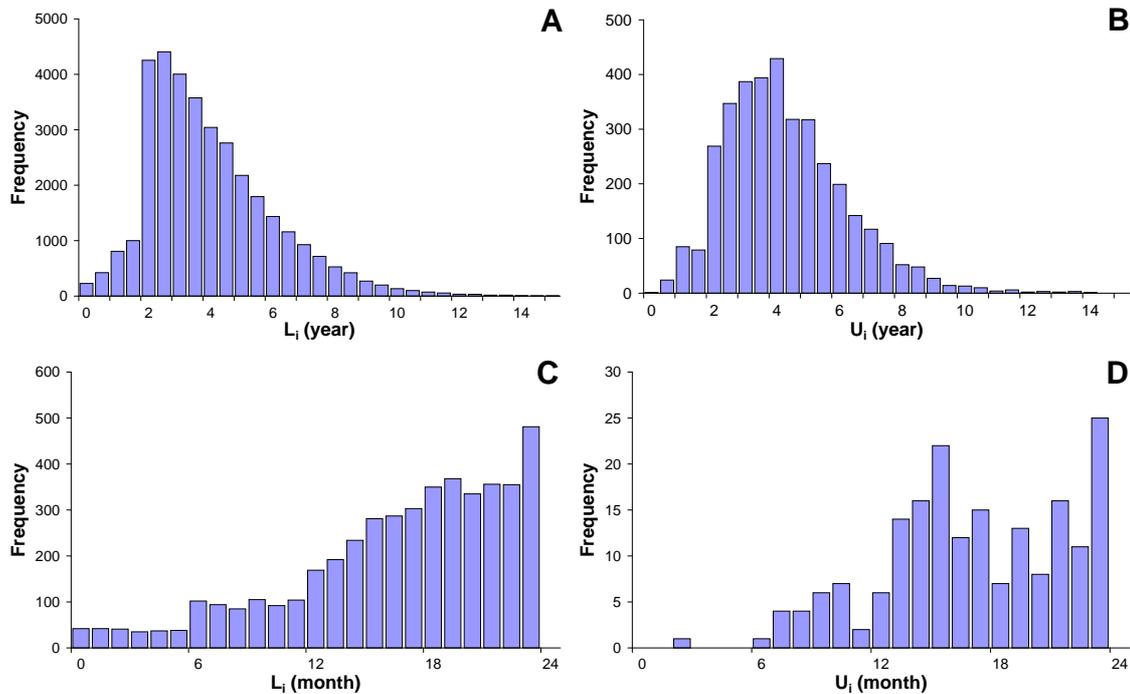
Fig. 2. Age distribution for lower (L_i) and upper (U_i) limits for age A_i at which individual i starts detectable shedding of MAP. (A) L_i of results of samples from all age groups (n=34613); (B) U_i of results from all age groups (n=3621), (C) L_i of results from young stock ($\leq$ 2 yrs) only (n=4528); (D) U_i of results from young stock only (n=190).

In the low-AP class, both breed and herd size effects were significant in the final model, but not their interaction (Table 3). Pair wise comparisons revealed a higher failure density curve in 'HF' compared to 'other dairy breeds' over the range of ages included in our study. This indicates that, on average, 'HF' cattle become culture positive at a lower age than 'other dairy breeds', and that 'HF' cattle are more likely to become culture positive before two years of age. Differences between 'HF' and 'HF cross' and between 'HF cross' and 'other dairy breeds' were not significant. In small herds the failure density curve was higher than in large herds, while the failure density curve was lowest in medium sized herds (Fig. 3). In low-AP herds, an estimated 0.4% to 2% of cattle became faecal culture positive before 2 years of age, depending on breed and herd size (i.e. area under the curve in Fig. 3).

Table 3 Final survival model of "time to faecal culture positive" for 16,166 animals of all age groups in 160 herds with an Apparent Prevalence <0.05

Parameter	($_i$)	estimate	standard error	95% Confidence limits Lower	95% Confidence limits Upper	P-value	Overall P-value
Intercept ($_0$)		3.6787	0.1801	3.3257	4.0316	<0.0001	
Breed	HF	-0.4222	0.1649	-0.7454	-0.0990	0.0105	0.0004
	HF-cross	-0.2600	0.1639	-0.5812	0.0612	0.1127	
	other dairy	0.0000	0.0000	0.0000	0.0000		
Herd size	small	-0.2033	0.0747	-0.3498	-0.0568	0.0065	<0.0001
	medium	0.1411	0.0534	0.0365	0.2457	0.0082	
	large	0.0000	0.0000	0.0000	0.0000		
Shape		1.7392	0.0744	1.5993	1.8914		
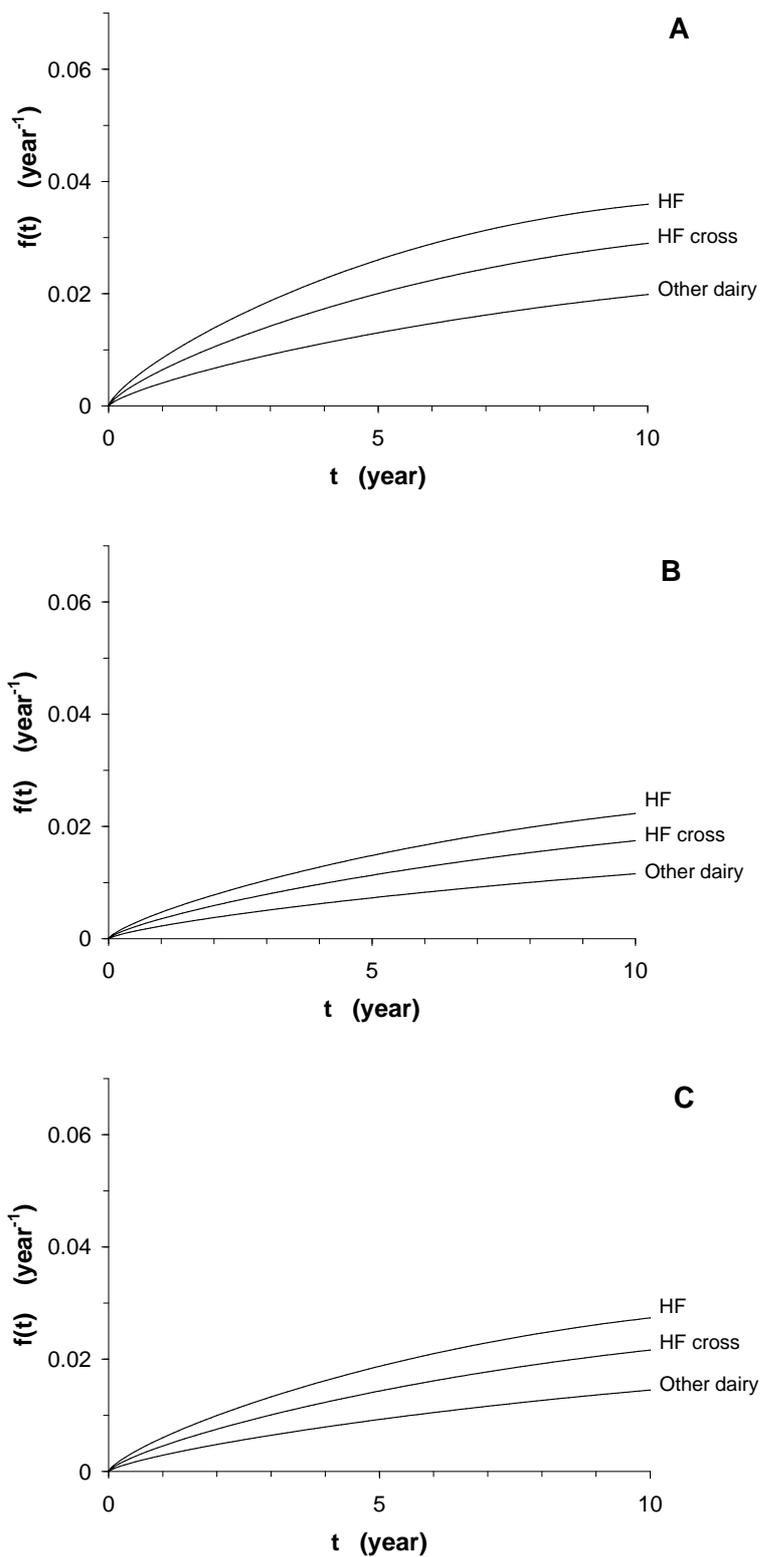
Fig. 3. Fitted failure density $f(t)$ as a function of age t in herds with an apparent prevalence <0.05. (A) small herds (<50 adult cattle); (B) medium sized herds (50 – 80 adult cattle); (C) large herds (≥80 adult cattle).
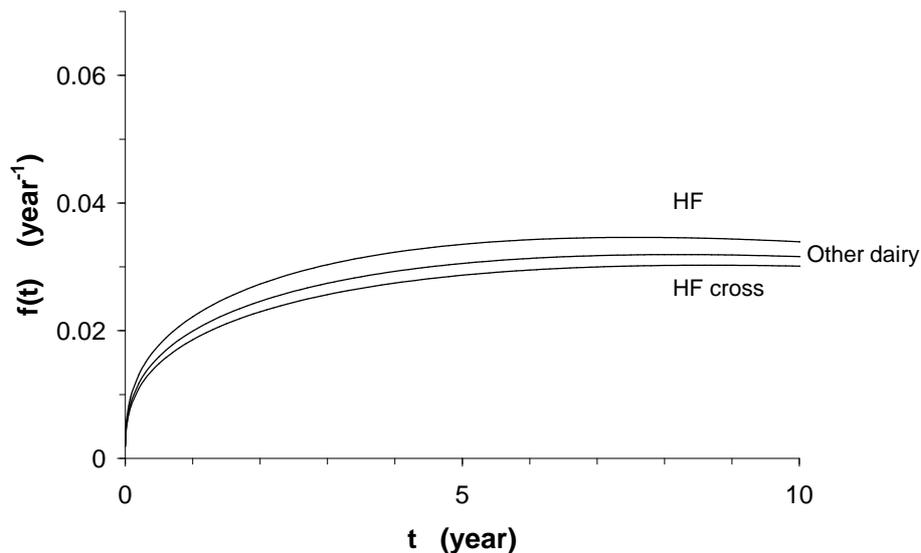
Fig. 4. Fitted failure density *f(t)* as a function of age *t* in herds with an apparent prevalence between 0.05 and <0.10.

Table 4. Final survival model of "time to faecal culture positive" for 12430 animals of all age groups in 110 herds with $0.05 \leq$ Apparent Prevalence < 0.10.

Parameter	($_i$)	estimate	Standard error	95% Confidence limits Lower	95% Confidence limits Upper	P-value	Overall P-value
Intercept ($_0$)		3.1355	0.1382	2.8646	3.4064	<0.0001	
Breed	HF	-0.0812	0.1338	-0.3433	0.1810	0.5441	0.0049
	HF-cross	0.0535	0.1334	-0.2079	0.3149	0.6880	
	Other dairy	0.0000	0.0000	0.0000	0.0000		
Shape		1.3362	0.0441	1.2525	1.4255		

Table 5. Final survival model of "time to faecal culture positive" for 8555 animals of all age groups in 103 herds with an Apparent Prevalence ≥ 0.1.

Parameter	($_i$)	estimate	Standard error	95% Confidence limits Lower	95% Confidence limits Upper	P-value	Overall P-value
Intercept ($_0$)		3.4177	0.1944	3.0367	3.7988	<0.0001	
Breed	HF	-0.5038	0.1829	-0.8623	-0.1454	0.0059	<0.0001
	HF-cross	-0.3233	0.1825	-0.6809	0.0343	0.0764	
	other dairy	0.0000	0.0000	0.0000	0.0000		
Herd size	small	-0.4056	0.0746	-0.5518	-0.2593	<0.0001	<0.0001
	medium	-0.1415	0.0538	-0.2469	-0.0361	0.0085	
	large	0.0000	0.0000	0.0000	0.0000		
Shape		1.0608	0.0410	0.9833	1.1444		

In the intermediate-AP class, only the overall effect of breed was significant (Table 4). In these herds, an estimated 4% of cattle became faecal culture positive before 2 years of age (i.e. area under the curve in Fig. 4).

In our final model for the high-AP class, both breed and herd size were significant, but not their interaction (Table 5). Similar to the low-AP class, pair wise comparisons revealed a higher failure density curve in 'HF' compared to 'other dairy breeds' (Fig. 3). The failure density curve was lower in large herds than in small or medium sized herds. In high-AP herds, an estimated 5% to 14% of cattle became faecal culture positive before 2 years of age, depending on breed and herd size (i.e. area under the curve in Fig. 5).

Survival analysis of faecal culture results of young stock
Left censored, interval censored and right censored observations were made for 133, 57 and 4471 of the 4661 female young stock (< 2 yrs of age) respectively. Thus, observations of L_i were obtained for 4528 cattle and observations of U_i were obtained for 190 cattle (Figs. 2C and D).

The shape parameter of the Weibull model was not significantly different for the three classes of AP. However, because this shape parameter was significantly different for the three AP classes in the analysis of faecal culture results of all age groups, global analysis over all AP classes was not deemed justified in the analysis of culture results of young stock only.

None of the explanatory variables (breed, herd size, season of birth or two-way interactions) were found to be significant in any of the AP classes (Tables 6 – 8). The shape and scale parameters of the failure density curve for young stock did not differ significantly from the parameters of the concomitant failure density curves for all age groups.
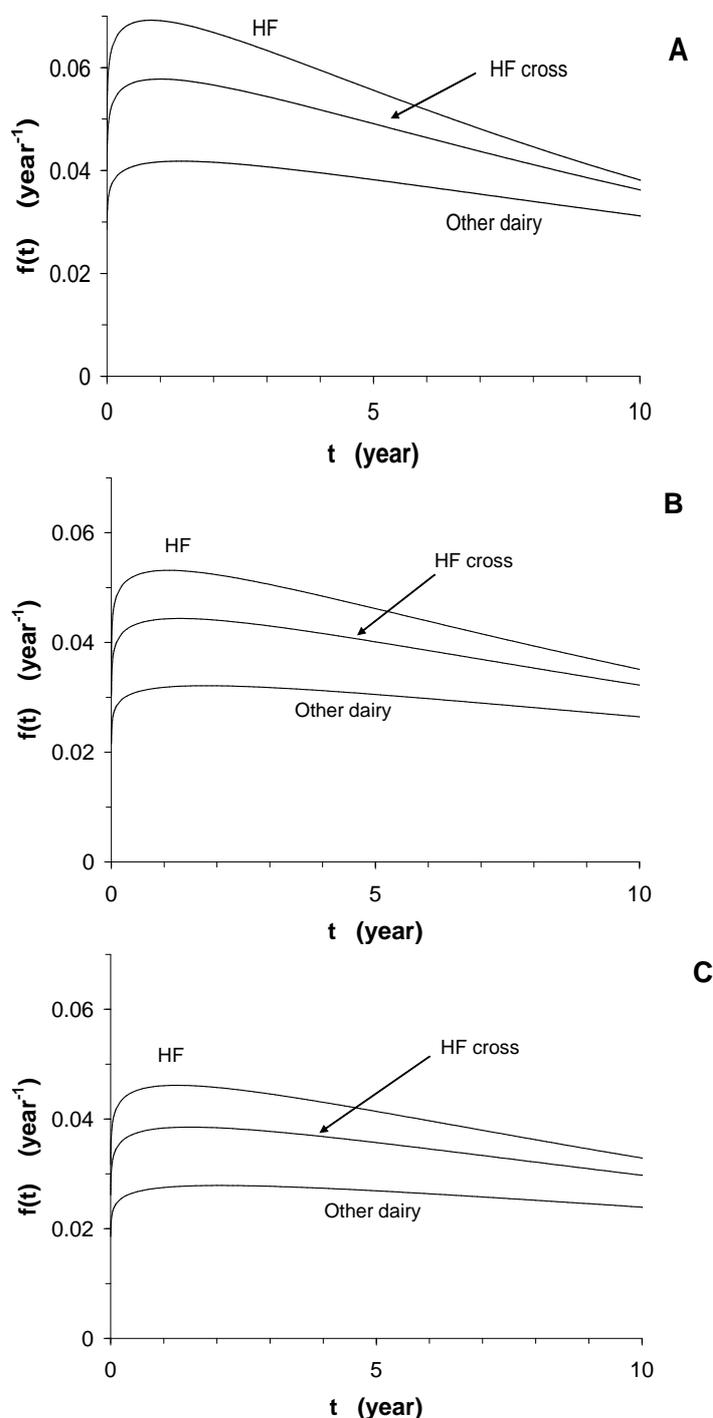
Fig. 5. Fitted failure densify f(t) as a function of age t in herds with an apparent prevalence $\geq$ 0.10.   (A) small herds (<50 adult cattle); (B) medium sized herds (50 – 80 adult cattle); (C) large herds ($\geq$80 adult cattle).

Table 6. Final survival model of "time to faecal culture positive" for young stock (2551 animals $\leq$ 2 years of age) in 77 herds with an Apparent Prevalence <0.05.

Parameter	estimate	standard error	95% Confidence limits		P-value
			Lower	Upper	
Intercept ($_0$)	2.3559	0.4009	1.5701	3.1416	<0.0001
Shape	2.1239	0.4436	1.4105	3.1982	

Table 7. Final survival model of "time to faecal culture positive" for young stock (1346 animals $\leq$ 2 years of age) in 55 herds with 0.05 $\leq$ Apparent Prevalence < 0.10.

Parameter	estimate	standard error	95% Confidence limits		P-value
			Lower	Upper	
Intercept ($_0$)	2.8230	0.4847	1.8731	3.7729	<0.0001
Shape	1.1961	0.2379	0.8100	1.7663	

Table 8. Final survival model of "time to faecal culture positive" for young stock (804 animals $\leq$ 2 years of age) in 42 herds with an Apparent Prevalence $\geq$ 0.1.

Parameter	estimate	standard error	95% Confidence limits		P-value
			Lower	Upper	
Intercept ($_0$)	1.8511	0.2563	1.3487	2.3535	<0.001
Shape	1.4737	0.2522	1.0537	2.0611	

DISCUSSION

The study showed that the distribution of age at which cattle start to shed MAP strongly depends on the within-herd prevalence. In low prevalence herds, the proportion of animals becoming culture positive gradually increased with age during the first 10 years of life (Fig. 3). However, in high prevalence herds, the proportion of animals becoming positive sharply increased during calfhood, and reached a maximum between 9 and 25 months of age (Fig. 5). Our findings indicate that a considerable proportion of young stock became faecal culture positive before 2 years of age, especially in high prevalence herds (5 to 14% in herds with an AP $\geq$ 0.10). Therefore, in high prevalence herds, faecal shedding of MAP in young stock should be a major concern. Shedding of MAP in young stock may result in horizontal transmission of the infection to susceptible calves. This may reduce the effectiveness of control programmes based on the separation of young stock from adult cattle and culling of infectious adult cattle only. In a recent experimental study, horizontal calf to calf transmission was observed indeed (van Roermund and de Jong, 2005, this volume).

Assumptions on the age distribution at becoming infectious have been made in several models developed to study control programmes for MAP (e.g. Groenendaal et al. 2002, van Roermund et al. 2002, Kudahl et al. 2004). This study quantified the age distribution at becoming faecal culture positive (as a proxy parameter for age at becoming infectious) and showed the shape of the failure density curve *f(t)* depended on the within-herd prevalence and that the hazard *h(t)* increased with age. These results may contribute to future modelling studies.

The effect of breed and herd size on age at becoming faecal culture positive was analysed. In all AP classes, breed group was a significant risk factor. In herds with a high (0.10) or low (<0.05) AP, HF cattle became faecal culture positive at, on average, a younger age than 'other dairy' cattle. Possibly, HF cattle are more susceptible to infection or progression of the infection, or the association between breed and time to shedding is confounded by other factors, such as milk yield or herd management. The effect of herd size was less clear. Herd size was a significant risk factor in the high and low AP classes, but not in the intermediate (0.05 to 0.10) AP class. In the high AP class, cattle in small herds (< 50 adults) became faecal culture positive at, on average, a younger age than cattle in medium sized herds (50 to 80 adults). Furthermore, cattle in medium sized herds became positive at, on average, a younger age than cattle in large herds (>80 adults). However, in the low AP class, cattle in small herds became positive at, on average, a younger age than cattle in large herds, which in turn became positive at, on average, a younger

age than cattle in medium sized herds. Moreover, herd management may be a confounder for the effect of herd size. Unfortunately, data on herd management were not available in this study.

Our data were interval censored, animals were sampled asynchronously and a large number of values were missing (i.e. left or right censored observations). This was compensated by the large number of cattle for which data were available. A Weibull hazard function was chosen because it is flexible, can be handled well mathematically, and for which suitable statistical procedures are available to deal with asynchronous interval censored data. A limitation of the Weibull model is that the hazard $h(t)$ can do only one, vs. a combination of, the following: increase, be constant or decrease over time. However, the use of even more flexible hazard functions was not considered because, to our knowledge, no appropriate measures exist to compare the fit of these functions on asynchronous interval censored data with a large proportion of missing values.

Since in most Dutch dairy herds only adult cattle are tested for MAP, the amount of informative data on young stock was relatively sparse. The data from adult cattle may have influenced the estimated survival of young stock in the overall analyses of faecal culture results from all age groups. However, the survival curves were not significantly different in our separate analyses of data from young stock only. Therefore, it is legitimate to interpret the results from the analyses of data of all age groups for young stock as well.

Several sources of bias may have influenced our results. Cattle included in this study were not a random sample of the Dutch dairy population, but were predominantly sampled for certification-, surveillance- and control programmes or research projects. Thus, our results may provide a biased estimate for the general Dutch dairy population. However, it is likely that they provide a good estimate for the type of herds that will take part in control programmes in the near future.

Another potential for bias was the age at which cattle become faecal culture positive was used as proxy parameter for the event of interest: the time at which infected cattle become infectious. Because the diagnostic sensitivity of faecal culture is likely to increase when the disease process progresses, our results overestimate the age at which cattle start shedding MAP. However, sensitivity of faecal culture is relatively high in heavy shedders, animals assumed to contribute most to the transmission of MAP within a herd. Therefore, it was considered reasonable to use detectable faecal shedding as a proxy parameter for infectiousness.

Furthermore, AP and size of a herd were calculated as average values over the seven-year period of study, but in reality may have varied over time. Therefore, some misclassification bias may have influenced the effect of these risk factors. Finally, the study population consisted of cattle in herds known to be infected with MAP, because positive results of faecal culture were obtained in these herds. However, not all cattle in the study population were necessarily infected, and a portion of the infected cattle may have cleared the infection. Therefore, our results effectively apply to cattle in infected herds and are likely to overestimate the age at becoming faecal culture positive of infected cattle.

CONCLUSION

Within-herd prevalence had a considerable influence on the age distribution at which cattle became infectious. Both herd size and breed were associated with the infectious age distribution, but their effect differed between apparent prevalence classes. A considerable proportion of cattle (0.4 to 15%, depending on apparent prevalence, breed and herd size) started shedding MAP before adulthood. Therefore, potential transmission of MAP amongst young stock should be a major concern in the control of MAP, especially in high prevalence herds.

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A survey on paratuberculosis in wildlife in Spain

J Álvarez, L de Juan, A Aranaz, B Romero, J Bezos, A Mateos, L Domínguez

Abstract

Mycobacterium avium subspecies *paratuberculosis* (Map) has been isolated from a crescent number of animal species, including non-ruminant wildlife species, suggesting its host range may be wider than previously noticed. In Spain, there is only one study dealing with the presence of Map in free-ranging fallow deer (Marco and others 2002). A survey in different species of wildlife and peridomestic animals was conducted to clarify the importance of these animals in the epidemiology of paratuberculosis in Spain. Four sampling areas in properties with previously diagnosed paratuberculosis, located in the middle and the south of Spain, were chosen. Several wild animals from other locations were also included in the study. Animals were frozen immediately after catch and sent to the laboratory to be subjected to necropsy, or the necropsy was performed in the field and the frozen samples were submitted to the laboratory. Intestine, mesenteric lymph nodes, liver and spleen were routinely cultured. Grown mycobacteria were identified by PCR and sequencing if necessary. To date 455 animals from 16 species of birds (anseriforms, galliforms, columbiforms, falconiforms and paseriforms) and 13 species of mammals (artiodactyls, carnivores, mustelids, lagomorphs and rodents) were analysed. Two Map isolates were obtained from a wild boar and a fallow deer of the same area. The wild boar isolate was the first obtained from this species in Spain (Álvarez and others 2005). Fourteen different species of rapid growing mycobacteria, some of which are opportunistic pathogen for humans, were also identified. Despite very low frequencies of detection found in the animal species under this study, the epidemiological consequences should be considered with caution. These infected animals represent a source of environmental contamination, where Map is able to persist for long periods of time. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903*

Environmental contamination of Mycobacterium spp. in a goat farm infected with paratuberculosis

J Álvarez, L de Juan, B Romero, J Bezos, A Aranaz, A Mateos, L Domínguez

Abstract

Mycobacterium avium subsp. *paratuberculosis* (Map) is an obligate pathogen and parasite of animals, so in theory it could be eradicated by removal of all infected animals. That is the principle in which the "testing and culling" control programs are based. However, the capacity of Map to survive for long periods of time outside the host allows him to withstand a periodic lack of suitable hosts, remaining in the environment and posing a threat of new infection to other healthy animals. In this context, we conducted a study to determine the environmental burden of Map in a goat farm infected with paratuberculosis and subjected to a control program. Goats in the farm were divided in two groups as a part of the control program: "infected" (not included in the control program) and "free" (under the control program). Environmental samples were taken from the "free" area (where the controlled animals were) and from the milking room; soil, mud, drinking troughs and the water supply system were analysed. These samples were processed for direct extraction of DNA and culture of Map and other mycobacteria. DNA specific fragment for genus Mycobacterium was detected in several places from the free area and from the milking room; but Map specific fragment IS*900* was only detected in the milking room, the only shared area between both "infected" and "free" groups. Atypical mycobacteria were cultured from soil and water, but no Map was isolated. The presence of Map specific fragment IS*900* in the milking area may suggest the cleaning in this zone was not exhaustive enough, and thus animals might become infected there. However, the impossibility of culturing Map raises the question whether they are viable or not. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903.*

Mycobacterium avium subspecies paratuberculosis infection of dairy cows attributable to the infection status of the dam

Sharif S Aly, M C Thurmond

Abstract

The objective of the study was to estimate the extent to which infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) of cows in a large dairy was attributable to the infection status of the dam. Data for 625 dam-daughter pairs of Holstein cows were used in a retrospective longitudinal study design. MAP serologic test results were compared for cows and their dams. Logistic regression was used to assess whether a cow's MAP sero-status was associated with her dam's sero-status. Daughters of seropositive dams were 6.6 times more likely to be seropositive, compared with those of seronegative dams. For seropositive cows born to seropositive dams, 84.6% of infection was attributable to being born to a seropositive dam and 15.4% to other exposures, including exposure as calves to flush water that contained feces of adult cattle. For the herd as a whole, infection in 34% of the seropositive cows was attributable to being born to a seropositive dam. For dairy herds that breed back seropositive cows, subsequent transmission of MAP to their daughters, either congenitally or via exposure to feces and colostrum of the dam soon after birth, can contribute significantly to maintaining prevalence of MAP in a herd. Culling of seropositive, nonclinical cows and their daughters is necessary to reduce MAPinfection attributable to congenital or periparturient transmission from dam to daughter.

Paratuberculosis in Trinidad, West Indies

Lisa Benjamin, G T Fosgate

Abstract

The cattle population in Trinidad is 35,000 head and produces 10 million litres of milk and 800 thousand kilograms of meat annually. Currently, there are fewer water buffaloes, 5,000, and their milk is not marketed. Since dairy cattle have not proved to be a viable option for farmers in Trinidad for reasons related to economies of scale and high prevalence of diseases, the water buffalo (*Bubalus bubalis*) is currently being assessed as a more profitable alternative to dairy cattle. However, there is serological evidence of paratuberculosis in the water buffaloes using ELISA and interferon-gamma. These serological tests still need to be evaluated for use in the water buffalo and compared with relevant results in cattle. The presence of *Mycobacterium avium* subsp. *paratuberculosis* (Map) should be confirmed using culture methods and the prevalent strain identified. The prevalence and incidence of this etiological agent in the water buffalo population should also be determined. Since most farms are mixed, the potential for interspecies transmission and the role of the tropical environment in the survival of this species would provide information with practical implications for the local farmer. Management changes associated with using the water buffalo as a dairy animal, as opposed to the multipurpose role it plays at present, may introduce factors that increase transmission of Map. The impact of these new risk factors, such as increased herd size, needs to be assessed. Paratuberculosis is an under researched topic in Trinidad. Due to the intended increased economic role of water buffaloes, research efforts should be directed towards elucidation of the epidemiology of subclinical paratuberculosis in local water buffaloes population because this disease may be limiting the production capability of this species. The investigation of this etiological agent should lead to identification of cost-effective control options.

Paratuberculosis in Mouflon (Ovis musimon)

A Bernardelli, M Zumárraga, B Alonso, M I Romano, A Gioffré, I Etchechoury, R Sanguinetti, C Zenobi, R Balzano

Abstract

Mycobacterium avium subsp. *paratuberculosis* (Map) is the etiologic agent of a severe gastroenteritis in ruminants. We observed injuries compatible with paratuberculosis in mouflon. Morphology, histopathology, isolation, biochemical typification of strains and the study by molecular biology, are described for the first time in Argentina. The objective was to determine the presence of Map from wildlife species which may open up the possibility of wildlife to domestic ruminant transmission. The samples came from one animal of the province of Buenos Aires, Argentina. Haematoxylin-and-eosin-stained sections of the gross intestine showed hyperplasia in corion and submucosa with diffuse infiltration of epithelioid cells. For bacteriological isolation the fecal specimen was decontaminated by hexadecylpyridium chloride (HPC) technique and cultured on Herrold´s egg yolk medium (HEYM), with and without mycobactin, and in Mycobacterial Growth Indicator Tube (MGIT) with mycobactin. The identification and molecular typing studies were performed at the Institute of Biotechnology-INTA, Castelar, Argentina. A loopfull of bacteria from the culture was taken to perform the PCR. They were resuspended in 100 µl of distilled water and then boiled during 30 min. 10 µl.of the supernadant were used to perform the PCR. The specific insertion sequence IS*900* of Map was used like target. The PCR was carried out producing a DNA fragment of 217 bp. Primary colonies of Map appeared from 4 weeks after inoculation in HEYM with mycobactin and fluorescence in MGIT medium. The isolates were stained by Ziehl-Neelsen's method and examined microscopically for acid-fast organisms that have the morphological characteristics of Map. The strain was positive to IS*900*. Paratuberculosis has been also diagnosed in farmer-deer, alpacas, elk, white tailed deer, mule deer, bighorn sheep, Rocky Mountain goats and bison. Wildlife would appear to the potential reservoirs to Map and this has implications for the control to Paratuberculosis in domestic animals and possible human health.

Biological and financial losses due to ovine Johne's disease in 12 infected flocks in Australia

R D Bush, Peter Windsor, J - Toribio

Abstract

This paper reports on a 3-year observational study that measured the biological and financial impact of ovine Johne's disease (OJD) on 12 farms within the endemic area of Australia, undertaken to address a lack of understanding by producers of the on-farm impacts of the disease. Between 2002 and 2004, 12 OJD-infected flocks from four areas of southern NSW were studied. Each farm was described using property and flock information collected from questionnaires and climatic records. Flocks ranged between 3,500 and 20,000 sheep with some farms also grazing cattle. The importance of cropping varied between areas. Annual mortality rates were estimated from farm records provided by owners. The proportion of OJD mortalities for each year was estimated using information gained from a necropsy study conducted in 2002 where tissues were collected for histological examination from dead and moribund sheep during 5-day inspections in autumn, winter, spring and summer. The costs of deaths attributed to OJD were estimated by 2 methods. The first used a gross margin (GM) analysis over each one-year period. In the second a financial value of the sheep that were necropsied in 2002 was calculated. The average OJD mortality rate for the 12 farms was 6.2% (range 2.1% to 17.5%) in 2002 and 7.8% (range 1.8% to 14.6%) in 2003. Figures for 2004 are currently being compiled. The gross margin for an OJD-infected flock in 2002 was on average $10.90/ha less than if the flock had not been infected. OJD losses accounted on average for two thirds of the total estimated financial loss associated with sheep deaths. The study demonstrated a significant biological and financial impact of OJD on sheep flocks. Producers should be encouraged to undertake OJD control procedures to prevent mortality rates reaching the levels encountered in some flocks during this study.

Identification of risk factors for OJD prevalence level in Australian sheep flocks

N K Dhand, J Eppleston, Richard Whittington, J - Toribio

Abstract

The identification of risk factors that contribute to ovine Johne's disease (OJD) can help producers improve on-farm control of this disease. A cross-sectional study of 92 infected sheep flocks in Australia was conducted during 2004/2005 to identify infection risk factors. Prevalence levels were based on culture of pooled faecal samples collected from 3-4 year old sheep in each flock. Prevalence was calculated using a method for variable pool size and flocks were classified as low (< 2%), medium (2 to 10%) and high (>10%). Information about potential risk factors was gathered from interviews with each producer using a questionnaire (OJD flock history, flock management practices and management of the 3-4 year old cohort sheep) and from analysis of soil samples collected from paddocks grazed by the cohort sheep as lambs, weaners and hoggets or adults (pH, texture and fertility). Univariable and multivariable analyses were conducted to identify risk factors statistically associated with OJD prevalence. The magnitude and direction of associations were quantified after allowing for potential confounding factors, including time since infection, livestock purchases, vaccination and culling practices. The implications for understanding the epidemiology of OJD and on-farm disease control will be discussed.

RFLP identification of Mycobacterium avium subsp. paratuberculosis isolated from small ruminants of Mexico

I Estevez-Denaives, G Chávez-Gris, R Hernández-Castro, I Pavlik, M Bartos

Abstract

Objectives: Establish by RFLP genetic differences of MAP between caprine and ovine isolates, as well as between different geographic areas in several states from Mexico. Material and Methods: Small ruminants with clinical signs of paratuberculosis were detected using ELISA and AGID tests, necropsy of 51 positive animals were performed and samples of animals with multibacilar lesions were collected. Mycobacteria from freezed intestinal samples were concentrated to obtain DNA. RFLP was performed employing BstEII enzyme and IS*900* as probe. Cultures from these samples were developed in Löwenstein Jensen medium. Results: Serum samples were obtained and 13.14% (137/1043) were positive to serological test. 43.14% (22/51) of the animals included in the anatomopathological study presented multibacilar lesions. Enough DNA for RFLP was obtained only from 12 samples. Two new profiles were identified (C33 and I3) in six ovines. MAP DNA obtained from goat intestinal samples were identified as C type. Types C and I were identified in the ovine origin samples. In the different geographic areas, the types most commonly found were C1 and the new type I3. From all the samples of animals with multibacilar lesions, MAP culture was obtained.

Isolation of Mycobacterium avium subspecies paratuberculosis from non-ruminant wildlife in Greece

M Florou, Leonidas Leontides, C Billinis, Poychronis Kostoulas, M Sofia

Abstract

Recent reports of natural paratuberculosis in wildlife species have identified several species as potential carriers and/or shedders of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), thus complicating the epidemiology of the disease in domesticated ruminants. Objective: The aim of this study was to investigate whether MAP was carried by wildlife species interfacing with dairy sheep and goats of endemically infected flocks. Materials and methods: Intensive sampling was carried out on the sheds and in the grazing grounds of 4 dairy sheep and goat flocks, with a history of clinical paratuberculosis. Several tissue samples from 327 wild animals of 11 species were obtained and cultured on two different media, Lowenstein-Jensen and Herrold's Egg Yolk, both supplemented with antibiotics and mycobactin J. All cultures were screened by polymerase chain reaction (PCR) for the presence of the IS*900* insertion sequence. All isolates were differentiated by IS*1311*-PCR between MAP type I and type II strains. Results: Thus far, three house mice (*Mus musculus*), a hare (*Lepus europeus*), a fox (*Vulpes vulpes*) and a black rat (*Rattus rattus*) were found to carry MAP. The isolates from the hare and the fox were type II strains while one mouse isolate was type I strain. Conclusions: To the best of our knowledge, this is the first report of MAP isolation from wild house mice and black rats and of MAP type I strain isolation from wild house mouse. The likely contribution of our preliminary results to the epidemiology of paratuberculosis in small ruminants should be further investigated.

Bayesian modeling of ROC curves for bovine paratuberculosis ELISA tests in the absence of a gold standard

Ian Gardner, Y K Choi, W O Johnson, Michael T. Collins

Abstract

Receiver operating characteristic (ROC) curves plot the sensitivity versus (1-specificity) over all cut-off points of a quantitative diagnostic test and therefore they provide a cut-off independent measure of test accuracy. Estimation of ROC curves (and the associated area under the curve) is straightforward when a gold-standard reference test is available but methods to estimate curves in the absence of a gold standard have been inadequately studied. We developed a Bayesian ROC analysis that can be applied to multiple correlated diagnostic tests with and without a gold standard. Simulation studies showed that the discrimination ability of the no-gold standard (NGS) method was adequate compared with the gold standard (GS) method providing that the overlap between the 2 distributions of ELISA values was not too great. We used the proposed method to analyze results of 2 serum ELISAs and fecal culture for bovine paratuberculosis. Data were available from 449 cattle with a positive fecal culture in paratuberculosis-infected herds and from 393 cattle from paratuberculosis-free herds. Fecal culture results were the averaged values for 3 laboratories, where scores of 1, 2, 3, and 4 represented HEY colony counts of 1-9, 10-49, 50-99 and ^{3} 100 colonies per slant, respectively. Log transformation of ELISA S/P ratios was necessary to achieve bivariate normality, which is a necessary assumption for a parametric analysis. The Parachek ELISA had a greater area under the ROC curve than the HerdChek ELISA by both GS and NGS methods. The NGS method provided adequate discrimination only when the subset of infected cattle with fecal culture scores of ≥ 3 was used. We are investigating semi-parametric extensions of the model to allow for situations where a suitable data transformation cannot be found.

Sample sizes for evaluation of diagnostic tests for bovine paratuberculosis in the absence of a gold standard

Ian Gardner, W O Johnson, A J Branscum, M Georgiadis

Abstract

Test evaluation studies for bovine paratuberculosis are challenging because of the difficulties of correctly defining the true infection status of cows in infected herds. An alternative approach involves methods that don't rely on having gold standard information but instead use test results from 2 conditionally independent tests in 2 or more populations. These populations could be herds or subpopulations within the same herds, such as cows of different parity/lactation number. We have developed frequentist and Bayesian approaches to sample size calculations for studies to estimate sensitivity and specificity with desired precision. For the frequentist approach, we constructed an Excel spreadsheet template (available at http://www.epi.ucdavis.edu/diagnostictests/) to perform the calculations following the Hui and Walter (1980) model that assumes asymptotic normality of ML estimates of parameters. In the Bayesian approach, we determine a sample size that yields high predictive probability with respect to the future study data, of precise estimates of sensitivity and specificity. The method is implemented using the Splus/R library emBedBUGS together with WinBUGS. Comparison of both methods for estimation of the sensitivity and specificity of ELISA and fecal culture tests for bovine paratuberculosis is presented in 2 populations with assumed prevalences of 1% and 15% and where the estimates of the desired interval width for the sensitivity of ELISA and fecal culture are ± 10%, and for the specificity of ELISA and fecal culture are ± 2% and ± 1%, respectively. Findings from a range of other plausible scenarios indicate that large sample sizes ($\geq$ 2000 animals / population) are needed to obtain reasonably precise estimates and these sample size requirements increase as prevalences in the 2 populations become closer to one another. Such large sample sizes might be impractical in many circumstances. The Bayesian approach is more flexible because it avoids limitations in the Hui and Walter model, when one or more estimates is close to 1.

A comparison of different methods of ELISA test validation: Two by two tables, ROC analysis, Logistic regression, Maximum likelihood estimation and Bayesian Inference

F Haro, Gerdien van Schaik, J Kruze

Abstract

Several diagnostics test are available for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map), the ELISA test being the most widely used, because it is simple, fast to do and cheap. The objective of the study was to validate the IDEXX ELISA to detect Map shedding dairy cows in management conditions, prevalence and infection states existing in southern Chile with different statistical approaches. The validation was carried out between September 2003 and July 2004 using 1334 animals from 27 different dairy herds in southern Chile. The herds were selected according to the number of animals per herd, type of management and the expected prevalence of Map in the herd (non infected and infected herds). In order to validate the ELISA test, faecal culture was used as the reference test. We used two-by-two tables, Receiver Operating Characteristics Analysis and Logistic regression as statistical methods. Latent class models such as maximum likelihood methods and Bayesian inference, which do not need a gold-standard test, were also used. The results showed a clear difference in diagnostic sensitivity, specificity and the cutoff values exist among the different methods that were used. The correct selection of the cutoff value should be carried out with a combination of the used methods, taking the ROC analysis as the base and complement it with the logistic regression results. Finally, an important conclusion was that the correct selection of the cutoff values was closely related to the prevalence of Map in the herds. Thus, it is important to have information about the prevalence in a herd in order to make a correct interpretation of the ELISA results. *Grant research supported by FONDO SAG, Ministry of agriculture, Chile*

Prevalence of Mycobacterium avium ssp. paratuberculosis in Austrian cattle 2002/03 in comparison to 1997/98

J L Khol, S Dreier, B Stein, K Fuchs, S Gütler, Walter Baumgartner

Abstract

Paratuberculosis (Johne´s disease) is a chronic infectious disease in ruminants caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP), which is widely distributed throughout the world. Economic losses associated with paratuberculosis due to increased milk production, reduced fertility and premature culling can be high in affected herds (Whitlock, 1996). Cattle slaughtered in Austria in 2002/03 were serological and bacteriological tested for the presence of MAP. Animals were chosen in a way to achieve statistically balanced samples from all parts of Austria. Blood serum was analysed using the Svanovir™-ELISA (Svanova Biotech, Uppsala, Sweden). Faeces, lymph nodes and intestinal samples were tested for MAP by Ziehl-Neelsen staining, polymerase chain reaction (PCR) and bacterial culture. 19% of the samples showed antibodies against MAP. Only four animals were positive for MAP in staining, PCR or culture. In the western part of Austria a higher incidence of antibodies was found than in eastern Austria. Compared with a previous Austrian study of Gasteiner et al. (1999) a highly significant increase in serum antibodies against MAP during the last years in Austria could bee seen. This finding shows for the first time the rapid increase of the incidence of paratuberculosis in a middle European country during the last years and indicates a possibly similar development in other areas. Concerning the increasing role of paratuberculosis for the cattle industry and a possible connection to Morbus Crohn in humans, higher efforts are needed in the future to improve the diagnosis and control of MAP in Europe. <u>References</u> Gasteiner J, Wenzel H, Fuchs K, Jark U, Baumgartner W, 1999. Serological cross-sectional study of paratuberculosis in cattle in Austria. J Vet Med B 46; 457-466. Whitlock R, 1996. Johne´s Disease. In: Smith, B.P. (Ed.): Large Animal Internal Medicine, 899-904. 2nd Ed., Mosby, St Louis, Missouri; 1996.

Paratuberculosis and avian tuberculosis in farmed red deer (Cervus elaphus)

M Kopecna, M Bartos, J Lamka, I Parmova, M Straka, V Ludvik, I Trcka, P Svastova, M , Moravkova, A Horvathova, J Álvarez, I Pavlik

Abstract

In the Czech Republic paratuberculosis has been monitored by faecal and/or tissue cultures in 6 935 wild ruminants originating from wild nature, game parks, and farms since 1997. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was diagnosed in 288 (4.2%) animals from 10 game parks, 10 farms, and from wild nature of 8 regions. The highest prevalence of MAP was found at deer and mouflon farms. In two deer farms the distribution of MAP in the organism of red deer (*Cervus elaphus*) was studied. In farm A 167 animals of different age were slaughtered and 835 tissues (hepatic, mediastinal, and ileocecal lymph nodes, spleen, and ileum) were cultured. Neither tuberculous lesions nor pathognomic signs of paratuberculosis and mycobacteria after the Ziehl-Neelsen staining were detected. MAP was isolated from 71, *M. a. avium* (MAA) from 13, and both pathogens (MAA and MAP) from 18 animals, respectively. Using standardised RFLP methods, three IS*900* RFLP types B-C1, B-C16, and B-C32 of MAP isolates and four IS*901* RFLP types N-B1, N-B3, N-B4 and P-B3 of MAA isolates were identified. On the farm B MAP was cultured from faeces from 27 (34.6) of 78 animals. From ten slaughtered animals 193 tissues were examined (gastrointestinal tract, parenchymatous organs, including genitals, and adjacent lymph nodes). MAP was isolated from all animals from 86.5% tissue samples including supramammary lymph node, mammary gland, milk, uterus, amnion fluid, and testicles. After IS*900* RFLP analysis of 51 isolates RFLP type B-C1 (n=44) predominated; RFLP types B-C5 (n=5) and B-C16 (n=2) occurred sporadically in mixed infected animals. In the faeces of these infected 10 animals and from other faecal shedders and in the environment, MAP isolates of RFLP type B-C1 were found only. In four progenies the infection with the same RFLP type of MAP as in their mothers was documented. *This work was partially supported by the grants from the Czech Republic (No. 0002716201) and EU (No.QLRT-2000-00879). Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903.*

Atypical hosts and vectors of Mycobacterium avium subsp. paratuberculosis

M Kopecna, J Lamka, I Trcka, M Moravkova, A Horvathova, I Literak, M Bartos, I Pavlik

Abstract

While investigating the causative agents of paratuberculosis in domestic and wild ruminants, the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was revealed in different vertebrates and invertebrates. A total of 3,078 vertebrates and 8,489 invertebrates were examined: 40 hares (*Lepus europaeus*) and rabbits (*Oryctolagus cuniculus*), 149 small terrestrial mammals of different species, 554 wild boars (*Sus scrofa*), 77 badgers (*Meles meles*), 3 otters (*Lutra lutra*), 46 martens (*Martes foina*), 78 foxes (*Vulpes vulpes*), 5 wolves (*Canis lupus*), 13 bears (*Ursus arctos*), 2,113 birds of different species, 36 larvae of family Tabanidae and Eristalis tenax, 7 791 adult diptera, 229 adult Coleoptera, and 433 earthworms. MAP was isolated from a hare, a common vole (*Microtus arvalis*), a rat (*Rattus norvegicus*), a lesser white-toothed shrew (*Crocidura suaveolens*), two wild boars, a bear, three larvae of *Eristalis tenax*, four adults of *Diptera* and one earthworm. Isolates of MAP from seven invertebrates, from the hare and the bear were analysed by standardised IS*900* RFLP. MAP isolates from invertebrates were of identical type B-C1 as cattle isolates from infected farms, where invertebrates were collected. RFLP type of MAP isolate from the hare was B-C9 and from the bear E-C1. The bear originated from Slovakia where E-C1 RFLP type of MAP was found in pastured cattle. Our findings suggest that different infected vertebrates as atypical hosts can act as a final host and vector, or even as a reservoir of MAP. Invertebrates can pose as a potential vector by transmitting of MAP not only on their surface, but also in their digestive tracts, as it was documented in our study. *This work was partially supported by the Ministry of Agriculture of the Czech Republic (No. 0002716201) and EC (No. QLRT-2000–00879). Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903.*

Mycobacterium avium subspecies paratuberculosis in free-ranging birds and mammals on livestock premises in the United States

Elizabeth J.B. Manning, J L Corn, S Sreevatsan, J R Fischer

Abstract

Surveys for *M. avium* subsp. *paratuberculosis* (*M. ptb.*) in free-ranging mammals and birds were conducted to evaluate premise contamination (shedding), range of infected species, type of pathology and array of infective *M. ptb.* alleles for two cattle management systems (beef vs. dairy) from two geographically diverse regions. Specimens were collected from 774 animals representing 25 mammalian and 22 avian species from a total of nine dairy and beef cattle farms in Wisconsin and Georgia. Specimens of ileum, liver, intestinal lymph nodes, and feces were harvested from the larger mammals; a liver specimen and the gastrointestinal tract were harvested from birds and small mammals. Cultures were performed using radiometric culture and acid-fast isolates were identified by 16s/IS*900*/IS*1311* PCR and mycobactin dependency characteristics. Thirty-nine samples from 30 animals representing nine mammal and three avian species were test-positive for *M. ptb.* Nine animals were found to be infected in Georgia and 21 in Wisconsin. Shedding, as indicated by the presence of *M. ptb.* in fecal specimens, was documented in seven (0.9%) animals: three raccoons, two armadillos, one opossum, and one feral cat. Isolations of *M. ptb.* were made at least four times from each of the tissue types selected for testing. Strains isolated from wildlife were the same as those obtained from the collection premise's cattle. The use of two highly polymorphic SSR loci for 28 of the 39 isolates identified 10 different alleles. One allelic pattern broadly shared in domestic ruminants ("7,5") appeared in approximately one-third of the wildlife *M. ptb.* isolates studied. Given the few cases of shedding by free-ranging animals, contamination of the farm environment by infected wildlife is negligible when compared to the volume of contaminated manure produced by infected domestic ruminant livestock. Wildlife may however have some epidemiological significance for farms free of *M. ptb.* but located in the geographic vicinity of infected farms.

An investigation of the subclinical and clinical effects of ovine Johne's disease on production parameters in Australian Merinos

Helen McGregor, P Nicholls, Richard Whittington

Abstract

The significance of the impact of ovine Johne's disease as a cause of economic and production loss continues to generate debate. Recent Australian work suggests that on infected properties in a high prevalence area of Australia, annual mortality rates may reach 20%, with a reduction in gross margin on average of 6.4%. OJD mortalities often occur within the first four years of life, when the animal is most productive and the cost of OJD may have been underestimated due to discounting effects on wool production, growth and reproductive performance through the sub-clinical and clinical stages of the disease. As part of an extensive epidemiology trial, serum biochemical parameters, body weight and wool growth and quality parameters were measured over 2 years in 83 sheep naturally exposed to OJD at pasture. Lambs were selected for inclusion in the trial retrospectively based on histopathology lesions and tissue culture results consistent with OJD infection. Control animals were selected randomly from a cohort of unaffected animals. In preliminary univariate analysis of variance or covariance, serum albumin differed significantly between histopathological categories of sheep in two or more periods. Levels were significantly lower in sheep with severe lesions leading to clinical disease. This was apparent before the onset of clinical disease. Mean albumin level in sheep with severe lesions that survived to the end of the trial was significantly lower than that in controls at the last time point ($P<0.01$). There were differences in wool parameters and bodyweight measurements in sheep with severe disease compared to uninfected cohorts at the same time points. The biological significance of these findings will be investigated for correlation between wool and bodyweight changes, serum albumin and severity of disease over time.

Molecular typing of MAP-isolates from cattle and sheep in Germany

P Möbius, G Luyven, H Köhler

Abstract

A detailed analysis of sources and routes of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in different areas of Germany will be performed. The results of these investigations will allow conclusions and recommendations for more effective control measures. MAP isolates from cattle and sheep from different regions and herds in Germany were analysed. Furthermore, isolates from different tissues and organs of the same animals were studied in order to establish whether different MAP strains can be found in one animal. The strains were typed by restriction fragment length polymorphism analysis (RFLP) of the insertion segment IS*900* (Pavlik et al. J Microbiol Meth 1999; 38:155-167) using three different restriction enzymes, and by the amplification of specific "mycobacterial interspaced repetitive units" (MIRU-PCR; Bull et al. Mol Cell Probe 2003; 17:157-164). The diversity of RFLP patterns was different in the herds. A relatively high heterogeneity with eight different RFLP types for 15 MAP strains was found within isolates from cattle of one herd, which had a high entrance of cattle with unknown infection state in the past. A number of new RFLP-types were detected. According to our current results, individual animals were not infected consecutively by different MAP strains. Among the isolates from sheep, two new, unique so called I-types were detected. Furthermore, strains with "cattle-type" RFLP patterns were also found in sheep. Two different MIRU types were found in sheep isolates only, and a third type that was also common in cattle isolates. Trade of sub-clinically infected cattle with unknown infection state is considered the main route for the spread of MAP between herds. Contamination of pasture with MAP by cattle dung is claimed to be a cause of infection of migrating sheep and for the spread of MAP strains originating from cattle in this way. *Attendance to this Congress was sponsored by the EU-funded project SSPE-CT-2004-501903.*

A histopathological study on Johne's disease in cattle in Shahrekord slaughter house

M Pourjafar, I Karimi

Abstract

Introduction: Johne's disease or paratuberculosis is an infectious disease, caused by *Mycobacterium paratuberculosis*, which has been described in numerous ruminant species. In cattle, intractable diarrhea, emaciation and hypoproteinemia in animals older than 19 months characterize the disease. This study was conducted to detect the presence of paratuberculosis in cattle in Shahrekord region from March to August 2001. Methods & Materials: In order to investigate the presence of paratuberculosis in Shahrekord region, 400 cattle that were older than 4 years, examined clinically in Shahrekord slaughterhouse. All cattle were inspected after slaughter and samples from 54 cases that showed thickening of different parts of the intestine and enlargement of mesenteric lymph nodes were obtained for histopathologic examination. Results: At microscopic examination specific histopathological characteristics of paratuberculosis were observed in 9 cases. In 6 cases of them massive infiltration of macrophages and giant cells that contained large numbers of acid-fast organisms and numerous lymphocytes were observed in lamina propria and submucosa of intestine in Ziehl – Neelsen staining. In other cases only macrophages containing acid-fast organism and a little number of lymphocyte were observed. In addition, all cases showed infiltration of macrophages containing bacteria in cortex of mesenteric lymph nodes. Conclusion: The results of this study showed the presence of paratuberculosis in Shahrekord region and the prevalence of the disease in cattle above 4 years old are 2.25 percent.

Presence and trace element analysis in cattle affected by paratuberculosis in Yasouj, Iran

M Pourjafar, K Badiei

Abstract

Introduction: Johne's disease or paratuberculosis is an infectious disease, caused by *Mycobacterium paratuberculosis*, has been described in numerous ruminant species. In cattle, intractable diarrhea, emaciation and hypoproteinemia in animals older than 19 months characterize the disease. This study was conducted to detect the presence of paratuberculosis in cattle in the high altitude region of Yasouj, Iran, from March 2001 to March 2002. Methods & Materials: In order to investigate the presence of paratuberculosis in Yasouj region, from 40 cattle that were older than 4 years and characterized by intractable diarrhea and emaciation, smears from faeces and mucosal scrapings have been prepared and stained by Ziehl–Neelsen staining. Liver biopsies for trace element (copper, cobalt, iron, Manganese) analysis have been done, too. Results: On microscopic examination specific bacilli characteristics of paratuberculosis were observed in 6 cases. The results of this study showed the presence of paratuberculosis in Yasouj region. The prevalence of the disease in suspected cattle above 4 years old was 1.5 percent. Trace element (copper, cobalt, iron, Manganese) analysis on liver biopsy of confirmed clinical cases showed a significantly ($p < 0.05$) lowered level in contrast to non-infected healthy cattle. Conclusion: It seems that, the disease should be carefully monitored in the region and preventive measures be considered.

Prevalence of Mycobacterium avium subsp. paratuberculosis (Bison type) in cattle and buffalo population of Agra region in India

Shri N. Singh, G Sharma, D Yadav, A V Singh, I Sevilla, Ramon A. Juste, V K Gupta

Abstract

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been studied in cattle (milk samples) and buffaloes (tissue samples). Samples were screened using culture and IS*900* PCR (tissues, milk fat and milk sediment), and ELISA (milk and serum). Prevalence of MAP was 78.0% by milk culture (fat – 60.0% and sediment – 50.0%). Milk ELISA was standardized using MAP (Bison type) of goat origin. Milk ELISA detected 32.0% cows as positive from 115 milk samples from dairies. These samples were also screened using MAP (bovine type) antigen procured from USA. Serum samples were also screened using serum ELISA and both the antigens. Molecular characterization revealed that most of the isolates were Bison type and only one isolate was bovine type. High prevalence of MAP in the milk of lactating cows pose direct threat to humans and young calves alike. Raw milk is still consumed as medicine in some parts of rural India. Tissues (mesenteric lymph nodes and large intestine) from 50 slaughtered buffaloes were screened by culture and IS*900* PCR. Of these 48.0% buffaloes were culture positive (MLN – 34.0% and LI – 36.0%). Serum ELISA test was standardized using MAP bison type (native) of goat origin and bovine type (USA), antigens. Of the 167 serum samples 46.7% buffaloes positive in plate ELISA. While none of the buffalo w detected as positive in plate ELISA using MAP bovine type antigen. MAP isolated were typed as MAP bison type on the basis of IS *900* and IS *1311* PCR – REA analysis.

Mycobacterium avium subsp. paratuberculosis infecting Hog deer: A case study

Shri N. Singh, A K Srivastava, A V Singh, V K Gupta

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) was isolated from a Hog deer, died of accident in Mathura district. Necropsy was conducted at Veterinary College Mathura and the tissue samples (Mesenteric lymph nodes and intestine near ileo-cecal junction), and fecal samples were collected for isolation of MAP by direct microscopy, culture. Hog deer was extremely healthy and tissues (MLN and intestine) did not show any gross pathological lesions suggestive of Johne's disease and were normal and not swollen or enlarged. Tissues and fecal samples on screening by culture on HEY medium with mycobactin J showed that only the small intestine tissues collected from ileo caecal junction were positive for MAP bacilli. Direct microscopy of the intestinal tissues revealed abundant acid-fast bacilli similar to MAP. Colonies appeared in all tubes around 36 days of incubation. Colonies were characterized on the basis of slow growth, mycobactin J dependency and acid fastness.

Comparison of methods for estimation of OJD prevalence from pooled faecal samples

J - Toribio, Evan Sergeant

Abstract

Several methods exist to estimate animal-level prevalence of Johne's disease from pooled faecal samples. A recently developed user-friendly computer program – the Pooled Prevalence Calculator - enables ready calculation of animal-level prevalence by seven methods, including methods for imperfect test sensitivity and specificity and for variable pool size. This paper will compare prevalence estimates and confidence intervals between methods and provide comment on the limitations of each method for use in field based research. Prevalence estimates presented in this paper result from analysis of pooled faecal culture (PFC) data from the field trial of a killed vaccine for the control of ovine Johne's disease (OJD), undertaken on three farms in New South Wales, Australia. Estimates for one observation per farm at approximately 30 months post-vaccination were compared between methods and with the results of individual faecal culture. These results illustrate: the trend in prevalence estimates calculated by methods that assume PFC is a perfect test versus an imperfect test the inability to apply frequentist methods that assume an imperfect test when OJD prevalence is high the impact on the Bayesian method of an inappropriate prior estimate of prevalence. To conclude we will provide guidelines on the more appropriate methods for prevalence estimation on data such as that from the vaccination trial and provide comment on further work required to support prevalence estimation from pooled samples in Johne's disease research.

Intra-uterine transmission of paratuberculosis in farmed red deer

H CJ van Kooten, Colin Mackintosh, Adriana P. Koets

Abstract

A trial was undertaken to prove that intra-uterine transmission of infection with *M. paratuberculosis* does occur in farmed red deer in New Zealand. On 4 different farms, 9 clinically affected hinds in the late stages of pregnancy were slaughtered and samples were taken from these hinds and their 10 foetuses. Blood samples were tested with the Paralisa test, a modified IgG_1 ELISA. Tissue samples were cultured using the BACTEC method and fixed samples examined histopathologically. Six hinds were Paralisa-positive, all foetuses were Paralisa-negative. All hinds and 9 of the 10 foetuses were culture-positive. Histopathologically, acid fast organisms were seen in 6 hinds and none were seen in foetal tissues. However, lesions characteristic of Johne's disease were present in all tissues from the hinds and most foetal tissues showed some pathological changes. These results show that there is a considerable risk of transmission of paratuberculosis from clinically affected hinds to their foetuses in late pregnancy. It would therefore be unwise for farmers to delay slaughter of clinically affected hinds in order to keep the fawns.

Estimation of parameters on the vertical transmission of Map in a low-prevalence dairy herd

Robert H. Whitlock, M Widmann, Raymond W. Sweeney, Terry L Fyock, M Linde, A Benedictus, R M Mitchell, Ynte H Schukken

Abstract

Previous studies on modeling Johne's disease assumed that Map-infection on a dairy farm is only spread from infectious adults (>2 yrs) to calves. Preliminary inspection of a longitudinal dataset on full herd culture data casts doubt on this assumption. The Map-prevalence did decline substantially but did not fall to zero on a Pennsylvania dairy farm that had been performing (i) a Johne's test-and-cull program and (ii) very stringent management practices for a period of 20 years. Calf-to-calf transmission might be of substantial influence in maintaining Map-infections on low-prevalence farms. This is in agreement with experimental findings that Map-infected calves can (intermittently) shed low amounts of bacteria in their feces. It is therefore important to find out exactly which calves in a (low-prevalence) herd initially start spreading the disease to their herdmates, i.e. which calves become infected with Map as a result of vertical or horizontal cow-to-calf transmission. In this study we quantitatively estimated parameters on the vertical transmission of Map. The Pennsylvania dataset provides detailed information on pedigrees, calving dates, lactations, culling and sampling results (serology/ fecal culture) of all animals in the herd over the last 20 years. The dataset allows us to look at possible Map-transmission within a dam-daughter relationship, but also at Map-transmission within a given time frame, for instance as a result of temporarily contaminated calving pens. Quantitative estimates of dam to calf transmission will be presented and discussed in light of within-herd transmission dynamics. High quality longitudinal data are essential for further progress in understanding herd infection dynamics.

Summation

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The 8th International Colloquium on Paratuberculosis (ICP) was a great success thanks to the excellent organizational skills and hard work of Dr. Gregers Jungerson, Dr. Søren Nielsen, the Organizing Committee, the high caliber of the scientific presentations, the financial support of the organizations who provided sponsorship, and "wonderful wonderful Copenhagen" as it celebrated the 200th anniversary of the birth of Hans Christian Anderson. It was the largest colloquium in the history of the International Association for Paratuberculosis with 360 registrants. This meeting has shown steady growth in attendance since its inception in 1983 (Fig. 1). Among the seven scientific sessions spanning the four-day meeting there were 65 oral presentations and 207 posters providing state-of-the-art scientific information on every aspect of paratuberculosis.
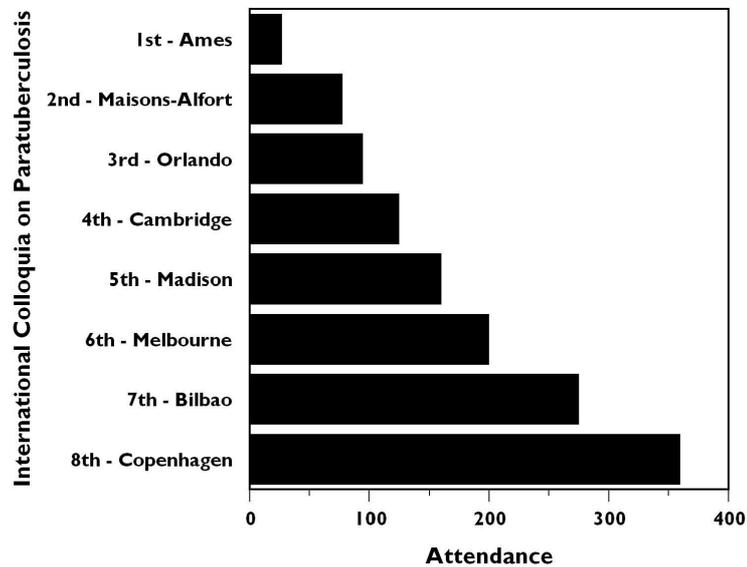
Fig. 1. IAP Colloquia attendance.

The strength of these Colloquia is its very specific focus on a single etiologic agent, *M. paratuberculosis*, and at the same time the breadth of topics related to the disease it causes: everything from molecular biology to macroeconomics was covered at the 8th International Colloquium on Paratuberculosis. The growth of paratuberculosis as a field of scientific investigation forced the first use of parallel sessions at a Colloquium. In addition, the first use of a focused workshop format appeared: these sessions clearly were appreciated by participants but many hoped that future workshops would be run as moderated discussions (instead of watching additional presentations). One of the many valuable aspects of the Colloquia is the synthesis and sparks that occur as we chat with our colleagues - workshops are another way to encourage these discussions on topics of shared interest.

This summation attempts to glean the highlights of important issues from each of the meeting's seven themes. (Failure to mention a specific topic or presentation should not be interpreted to mean it was unimportant!) Capturing 272 papers in a single summation is no small challenge. I have tried to highlight some of the findings presented at the 8th International Colloquium on Paratuberculosis but for the most part I present challenges for the future. The seven sessions of the Colloquium are summarized under three main subject areas.

Control programs
Directly or indirectly, many of the scientific papers at the Colloquium and most of the global financial investment in paratuberculosis has been directed at control of the infection. Paratuberculosis control is attempted at the herd, regional, national and international levels. Methods for control within herds are well-accepted and multiple epidemiological studies reported at the Colloquium affirm that modes of MAP spread within animal populations are fairly well understood. An encouraging and growing body of information provided by field trails confirms that paratuberculosis can be controlled.

More work is needed, however, to document that these programs are cost-effective. Herd owners seem willing to follow recommended changes in herd management practices and use diagnostic testing to find MAP when these control program costs are borne by government or research projects. However, few herd owners adopt such programs when they must pay all of the costs. Defining scientifically _and_ economically justified control programs remains a challenge. Studies like that of our Irish colleagues are important to demonstrate the long-term economic costs of failing to control paratuberculosis (Barrett et al., 2005) vs. the costs for intervention to stem economic losses due to paratuberculosis. We must not just say, but prove, that "prevention pays". For small ruminant herds, with low individual animal economic value, vaccination limits economic losses due to paratuberculosis and are readily accepted by herd or flock owners. Work on the fundamental biology of MAP and the immune response of ruminants promises ways of significantly improving vaccine efficacy.

Methods for controlling the spread of paratuberculosis among herds are also well understood. Infection of herds by MAP can be prevented by closing herds to live animal introductions or by buying herd replacements from herds that are in some sort of formal or informal "certification" program, i.e., herds found test-negative multiple years. National programs striving to promote herd-level control have not been actively adopted by animal industries. A number of countries report that only a small proportion of the dairy, beef, goat or sheep herds or flocks adopt national control programs based on herd certification and animal movement controls. Surveillance data indicate a continuing spread of paratuberculosis at the herd and flock level (Khol et al., 2005). Countries recognize the need to stimulate market forces to promote adoption of national paratuberculosis control programs but are uncertain how this can be accomplished. Animal health researchers and regulatory veterinarians from The Netherlands described a bold new approach to stimulate the dairy industry to adopt more aggressive paratuberculosis control measures based on estimated levels of MAP in bulk milk (van Roermund et al., 2005). Computer modeling indicates it will work. We look forward to the 9th ICP to hear if it works in practice.

There are two other critical issues affecting control of paratuberculosis on a regional or national basis; one based on biology, the other regulatory. The biological issues concerns the host range of MAP. Increasing numbers of studies show that MAP infections can be detected in wildlife. First described by Scottish workers in rabbits at the 5th ICP, recovery of organism from a broad array of wildlife and even invertebrates is now common (Manning et al., 2005; Kopecna et al., 2005). In the Wildlife Workshop, an extensive EU funded study in five countries (Czech Republic, Greece, The Netherlands, Spain, and United Kingdom) presented data that currently MAP infection was very low in wildlife in general, but in the United Kingdom a high prevalence was found in rabbits grazing on MAP-contaminated dairy cattle pastures. Does this mean MAP may become endemic in certain wildlife species? Can infected wildlife serve as reservoirs of MAP infection for domestic animals? Or, are we merely finding the infection more often due to improved diagnostics and more wildlife investigations? Look for answers to these questions to be reported first at future International Colloquia on Paratuberculosis.

The regulatory issue is perhaps of even greater importance. Control programs for paratuberculosis generally involve testing animals to locate those that are most infectious. Culling of these animals as soon as economically possible is usually the subsequent recommendation. Unfortunately, few countries monitor the disposition of culled MAP-infected, clinically normal animals. All too often they move through cattle (or sheep, or goat) markets and end up in another herd (van Weering et al., 2005). Hence, paratuberculosis control in one herd can lead to infection of many others. Until this route of transmission is stopped, regional and national control programs likely will not succeed.

The World Organization for Animal Health (OIE) must strengthen and modernize its requirements for paratuberculosis testing of animals for international trade. The current regulations are based on outdated

diagnostic technology and on individual animal, rather than herd-based, testing. The result is that too many MAP-infected animals move through international trade markets. This particularly affects countries trying to strengthen their animal agriculture industries, i.e., developing countries (Cashman et al., 2005).

Basic Biology
Genomics, proteomics, lipidomics all provide a better understanding of our adversary: MAP. Numerous molecular components of MAP described at the 8ICP present potential for improved diagnostics and vaccines. The methods and supporting data are too voluminous and technical for effective summarization here but they represent major advancements since the 7ICP in the field of paratuberculosis research.

Advances in immunological methods and animal models have improved our understanding of the host response to infection. The interplay between MAP and its host changes over time and is affected by innumerable intrinsic and extrinsic factors. Coupled with studies on the pathogen, immunology and pathogenesis research is bringing us closer to an efficacious vaccine. The work of Ad Koets and others provide tantalizing hints that the genetic make-up of the host also plays a significant role in determining the outcome of the host-pathogen interaction (Koets et al., 2005).

Diagnostics
The field of paratuberculosis diagnostics is a success story illustrating the translation of basic research into commercialized, standardized and practical tools of benefit to animal health. The next challenge is to learn to use these new tools cost-effectively. Use of diagnostics is an added cost of doing business for producers and the benefits must out-weigh the costs. Test evaluations typically report sensitivity and specificity. A higher level of assay evaluation is diagnostic test utility, because a test not used is not a useful test. Whether paratuberculosis testing is paid for by the animal owner or is subsidized by a public or private agency, it is an added cost of animal production. Quantitative interpretation of ELISAs, use of ELISAs on milk samples, pooling of fecal samples, culture of feces collected from the farm environment (i.e., environmental sampling) are all novel approaches to make paratuberculosis diagnostics more affordable and effective. Different methods have different purposes and there is no single "best test". Economic decision systems such as that described by Dorshorst provide useful new tools for economic optimization of paratuberculosis control programs (Dorshorst and Collins, 2005).

Public Health
In 7ICP summation I said:
> *"Candidly speaking, the economic importance of paratuberculosis does not seem to justify the large investments being made by many countries to survey for, control, or subsidize testing and culling of test-positive animals. On the other hand, if M. paratuberculosis can infect humans, and if foods of animal-origin are the major vehicles of exposure for the public to M. paratuberculosis, then paratuberculosis may rank near the top of the list of important zoonotic pathogens needing to be controlled and worthy of investment by animal agriculture, if not society at large. Resolution of this biomedical / food safety question is critical."*

This situation has changed little in the intervening three years nor has my opinion that it is crucial to resolve the zoonotic question.

Several papers at the 8ICP report recovery of MAP from humans with inflammatory bowel disease (Elguezabal et al., 2005). The potential role of MAP in human disease was extended to the Blau syndrome and even diabetes (Dow, 2005a; Dow, 2005b).

The pasteurization efficacy issue remains controversial with some studies reporting survival of MAP in the face of HTST and UHT pasteurization and others not. One group confirmed earlier reports by Hammer (Hammer et al., 2002) that the organism is actually activated by high temperatures (Herman et al., 2005).

Modes of potential human exposure to MAP have broadened to include water (Pickup et al., 2005; Rowe et al., 2005) and beef (Lombard et al., 2005).

The incidence of Crohn's disease is rising globally. A gene conferring susceptibility to Crohn's disease, NOD2, has been described but it does not account for this rising incidence. Epidemiological studies

increasingly incriminate an early childhood environmental trigger for Crohn's disease (Fonager et al., 1997; Bjornsson et al., 1998; Loftus et al., 1998; Kugathasan et al., 2003; Tsironi et al., 2004). *Mycobacterium paratuberculosis* remains the most prevalent bacterial pathogen capable of inducing chronic granulomatous inflammation of the intestine in a diverse array of animal species. Exposure of the general public to MAP via food or water is not only plausible; in some foods in some countries it has been documented to occur, e.g., MAP has been isolated in low levels from retail pasteurized milk in the U.K. the U.S. and the Czech Republic (Grant et al., 2002; Ayele et al., 2005; Ellingson et al., 2005).

Of all scientific questions facing paratuberculosis researchers, we must resolve whether or not MAP is zoonotic. The Colloquia hosted by the International Association for Paratuberculosis offer an ideal venue for scientific debate of this question. We must remain objective and open-minded and not dodge the debate. I hope that we can fully engage this subject at the 9ICP to be held in Tsukuba Japan, November 2007.

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WORKSHOP

The role of wildlife in the epidemiology of paratuberculosis

The objectives of the workshop were to present current information on world-wide prevalence of paratuberculosis infection in wildlife species, and to present the results achieved in the EU project QLRT-2000-00879 "*The role of wildlife in the epidemiology of Mycobacterium avium subspecies paratuberculosis in domestic ruminants in Europe*". Presentations concerning MAP infection in free-ranging wildlife were attended by 47 scientists from 20 countries.

The host range of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) comprises ruminant and non-ruminant species. The impact of this infection is commonly recognised in captive or farmed wild ruminants. Some studies suggest that wild populations could also be an important natural reservoir of MAP since it has also been isolated from a wide variety of wild non-ruminant species, such as carnivores, carrion-eating birds and rodents. Wildlife species may play a role in the epidemiology, maintenance and spread of MAP, and the presence of this pathogen in wildlife may affect the design and implementation of effective programmes of control.

MAP infection in European wildlife
The introductory presentation *Paratuberculosis in wildlife before the EU project* was given by Mike Hutchings [Scottish Agricultural College, Edinburgh, Scotland (UK)]. Natural paratuberculosis in wild ruminants has been well documented and relatively recently the natural occurrence of MAP infection in non-ruminant wildlife has also been described. (Beard *et al*., 2001; Daniels *et al*., 2003; Corn *et al.* 2005).

Intial studies in Scotland make it clear that non-ruminant species can harbour MAP and that the natural host range is far greater than previously thought. In Scotland the combination of high prevalence and level of infection in rabbits and their faeces, high levels of faecal contamination of agricultural pastures by rabbits, and the lack of avoidance by cattle to grazing pastures contaminated with rabbit faeces, suggested that rabbits represented a risk of MAP transmission to grazing domestic livestock.

These Scottish findings led to two main questions: Is Scotland alone in having a significant non-ruminant wildlife host of MAP? and (2) What are the risks to livestock from MAP in rabbits? To address these questions, an EU partnership was created with the overall aim of identifying key wildlife hosts of MAP across Europe and to determine their role in the epidemiology of the disease in domestic livestock. More information about the objectives and tasks of the project can be found at this website (http://www.ucm.es/info/para-tb).

The first part of the workshop was devoted to current MAP infection in European wildlife and the information derived from the EU-funded project "The role of wildlife in the epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* in domestic ruminants in Europe". Alastair Greig [Scottish Agricultural College, Edinburgh, Scotland (UK) and co-ordinator of the project] presented an *Overview of the project*. A study to determine the wildlife species that harbour MAP in Europe has been conducted by the partners in the Czech Republic, Greece, Netherlands, Spain and the United Kingdom. Only the latter has a significant level of MAP infection of wildlife. Overall, tissues were collected in continental EU from 3,051 mammals and 1,719 birds and cultured for MAP. So far, the pathogen has been isolated from 21 animals, including the *artiodactyla* deer, moufflon (*Ovis musimon*) and wild boar (*Sus scrofa*), a fox, hare, black rat (*Rattus rattus*) and house mouse (*Mus musculus*) and a buzzard (some results have been published: Álvarez *et al*., 2005; Florou M *et al*., 2005; Machackova e*t al.* 2003, 2004). In the UK, the rabbit appears to be a true reservoir of MAP (as they have the potential to perpetuate the infection on their own) and a threat to grazing livestock. Overall, 40% of rabbits are found to be infected with MAP. The pathogen is present in faeces, reproduction system (testes, uterus) and milk, therefore there is a potential horizontal, vertical and pseudo-vertical transmission. (Judge J *et al*., 2005a). The risk that infected rabbits pose to livestock is related to the fact that grazing cattle show no avoidance of rabbit faecal pellets while grazing whereas lambs are more selective (Judge J *et al*., 2005b).

Lucia de Juan [Universidad Complutense de Madrid, Spain] presented further data on the *Prevalence of*

MAP in European countries, kindly supplied by partners of the "Veterinary network of laboratories researching into improved diagnosis and epidemiology of mycobacterial diseases" (Co-ordination Action SSPE-CT-2004-501903) through its task "Wildlife reservoirs of mycobacterial infections". In France, MAP has been isolated from a small number of deer, wild boar and moufflon [Agence Francaise de Securité Sanitaire des Aliments (AFSSA), Maisons-Alfort]. In Denmark, the infection has been found in one wild fallow deer (*Dama dama*) from the Western part of Jutland, with no direct contact with domestic animals [Danish Institute for Food and Veterinary Research (DVI), Copenhagen]. In Norway, exhaustive studies on more than 1400 free-ranging red deer, roe deer, moose (*Alces alces*) and reindeer (*Rangifer tarandus*) resulted in 3% postive by ELISA; all histology and bacteriology tests were negative [National Veterinary Institute (NVI), Oslo]. Finland is free from paratuberculosis [National Veterinary and Food Research Institute (EELA), Helsinki]. Large studies have been also carried out in the Czech Republic and Slovakia between years 1997-2004, where 16 red deer, roe deer, fallow deer, moufflon, two wild boar, one bear (*Ursus arctos*), one hare, and three rodents out of 3400 mammals and 2000 birds [Veterinary Research Institute (VRI), Brno, and Slovakia]. In Germany, two of 600 animals, mostly wild cervids were culture positive [Friedrich Loeffler Institut. Federal Research Institute for Animal Health (BFAV). Jena, Germany]. In the southeastern part of Belgium, 29 wild red deer that were found dead were positive by culture [Veterinary and Agrochemical Research Center (VAR) & Laboratory of Applied Molecular technologies (LTMA), Université catholique de Louvain, Brussels]. Wild species in Italy have been tested but all histology, serology, and PCR have been negative [Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia Romagna (IZSLER), Brescia, and Istituto Zooprofilattico Sperimentale Lazio e Toscana (IZS LT), Rome]. Besides the study already cited, MAP has also been infrequently isolated from wildlife, as it was identified by culture in one wild boar out of 327 mammals and 26 birds of ten species in Spain [Instituto Vasco de Instigación y Desarrollo Agrario (NEIKER), Derio]. Four of 166 red deer tested by ELISA were positive [Universidad de Extremadura (UEX), Caceres].

With this information, it can be concluded at this time that MAP can be found, albeit at a very low prevalence, in wildlife in the majority of EU countries, except in the northern countries. This infection is mainly associated with wild ruminant species and some cases with wild boar as well. In general, there is a lack of studies of transmission of MAP from wildlife to domestic animals. This finding indicates a possibility of transmission that should be taken into account. These infected animals represent a source of contamination of the environment that it is not under control programmes.

Karen Stevenson [Moredun Research Institute, Edinburgh, Scotland (UK)] explained the *Results for the three typing methods for MAP*. A total of 68 isolates have been typed by AFLP. However, although AFLP could differentiate MAP from other mycobacterial species, the level of genetic variation detected between MAP isolates was in the same range as the variation in reproducibility of the technique, indicating that AFLP is not suitable for epidemiological studies on paratuberculosis. IS*900*-RFLP was performed according to the standardised method (Pavlik *et al.* 1999) and 101 isolates have been typed detecting 10 different IS*900*-RFLP types of which nine were detected in wildlife species. B-C1 was the most widespread IS*900*-RFLP type except in Scotland where B-C17 predominated. PFGE was performed according to standardised procedures (de Juan *et al.* 2005; Stevenson *et al.* 2002) and 97 isolates have been typed detecting 18 different PFGE multiplex profiles of which eight were detected in wildlife species. Pattern [2-1] was the most widespread PFGE multiplex profile and the greatest genetic diversity was observed among goat isolates. A comparison of the typing methods used revealed that PFGE could detect the greatest number of polymorphisms within this panel of strains and could subdivide the most common IS*900*-RFLP type (B-C1) into 8 subtypes. PFGE and IS*900*-RFLP used together provide the highest level of discrimination for MAP isolates.

MAP infection in wildlife in non-European countries
Paratuberculosis in wildlife in non-European countries was reviewed by invited speakers. In the presentation *Prevalence of Johne's disease in New Zealand wildlife: preliminary surveys and implications for management*, Andrea Byrom [Manaaki Whenua - Landcare Research Lincoln (New Zealand)] outlined the preliminary results from the first formal cross sectional surveys of the prevalence of *M. a paratuberculosis* in wildlife on farms in New Zealand. To date, over 500 animals have been necropsied, mostly small introduced mammal species but also including a few native or introduced birds. The preliminary results suggest the prevalence of MAP is likely to reach high levels (>30%) in wildlife wherever the level of Johne's disease infection in the farmed deer is high. So far, the infected species are feral ferret

(*Mustela furo*), feral cat (*Felis* domesticus), hare, rabbit, European hedgehogs (*Erinaceus europaeus*) and black-backed gull (*Larus dominicanus*), with major differences in prevalence among the studied farms. The next steps are to determine whether infected wild animals are shedding MAP bacilli, and whether the infection is passed between wildlife when infected livestock are absent.

Eran Raizman [Department of Pathobiology, School of Veterinary Medicine, Purdue University, Indiana (USA)] in his presentation *MAP from free ranging deer and rabbits near Minnesota dairy farms* gave results of the prevalence of MAP among deer and rabbits on infected and non-infected Minnesota dairy farms using fecal culture. He also described the frequency of use of farm management practices that could lead to potential transmission of infection between these species. Fecal samples from cows and the cow environment were collected from Minnesota dairy herds, and fecal pellets of free-ranging white-tailed deer and eastern cottontail rabbits were collected from locations surrounding the farms; all samples were tested using mycobacterial culture. The 20% and 25% of the owners of the 114 farms included in the study estimated as daily the probability of physical contact between cattle manure and deer or rabbits, respectively. Possible contact between cattle manure and deer or rabbits was estimated to occur primarily from March through December. The frequency of pasture or drylot use and manure spreading on crop fields may be important risk factors for transmission of MAP among dairy cattle, deer and rabbits. Although the MAP prevalence among rabbits and deer was very low, their role as current reservoirs should be considered.

The presence of MAP in North America in ruminants, non-ruminants and their habitats was also presented by Elizabeth Manning [Johne's Testing Center, School of Veterinary Medicine, University of Wisconsin (USA)]. In the United States and Canada, the organism has been isolated from discrete free-ranging ruminant populations such as tule elk in California, key deer (*Odocoileus virginianus clavium*) in Florida, bison in Montana and Canada, and big horn sheep in Colorado. Direct contact with infected domestic agriculture species has been demonstrated for a majority of these populations and the current direction of infection pressure is believed to be from these domestic species to wildlife. Clinical disease is seen in some, but not all, of these populations. Isolations from non-ruminant species have also been made (starling, armadillo, feral domestic cat, raccoon, etc.) but at a prevalence much lower than what was seen in Scotland.

Management of paratuberculosis in wildlife
Dr. Manning also addressed an essential issue in her presentation: the *Management of MAP infection in wildlife***.** The infection's health impact, persistence and potential for transmission in these populations are all open questions. As she explained, the way IAP researchers manage the currently incomplete data available concerning MAP in wildlife has a great impact on wildlife policy, wildlife management and programs for infection control in both wild and domestic agriculture species. In addition to continuing to expand our knowledge on MAP in wildlife, she recommended that IAP researchers assist wildlife policy makers and managers with interpretation and application of available data as it relates to their particular situation. We can help them consider *relative* risks, encourage them to examine explicitly their level of risk tolerance for the infection, discuss the costs and benefits of investigating and attempting to control the infection and clarify the differences in surveillance and control that exist between a wildlife vs. domestic agriculture setting. For instance, it is helpful to clarify the primary concern: are animals actually sick and is the infection affecting morbidity or herd dynamics? Does the wildlife manager thus see the need to manage the infection based on the health status of the animals?

The workshop attendees then discussed the question of whether wildlife is likely to be a significant reservoir for domestic agriculture infections, i.e. what is the population's *relative* contribution to persistence of infection in the region when compared with infected domestic species?

The design of a wildlife surveillance protocol based on diagnostic testing must take into account (a) an explicit case definition, (b) whether assays chosen can be interpreted for the species in question, (c) an estimate of current prevalence based on herd health in order to choose the optimum assay(s), (d) whether any management changes are actually feasible, (e) the impact of positive results on other programs such as reintroductions or transfers and of course (d) budget. Testing protocols will differ depending on whether the managers' goals are to eradicate disease vs. the infection (not the same thing), or whether the goals are to reduce the number of infectious animals or the level of premise contamination.

More data is needed to establish rational wildlife management MAP infection policies on an informed basis. Meanwhile, experts in Johne's disease can help provide a framework for discussing all the issues pertaining to MAP management for wildlife managers who are less familiar with this challenging infection.

Modelling wildlife and domestic animal infection dynamics
Javier Guitian [Royal Veterinary College, Hertfordshire, England (UK)] presented *Models to analyze the dynamics of MAP infection within and between domestic ruminants and wildlife,* which has two objectives: 1) to assess the potential role of wildlife reservoirs in the within – herd dynamics of MAP and to identify cattle production systems in which wildlife reservoirs are likely to play a role, and 2) to develop a framework for the assessment of the risk posed by wildlife reservoirs in the within farm dynamics of Johne's disease in dairy herds, cow-calf herds and sheep flocks.

Two approaches have been followed to achieve these objectives: 1) a deterministic model of within – farm transmission of MAP in cattle farms, and 2) a model for the qualitative assessment of the risk of introducing a MAP-infected replacement animal.
Two separate models were used, the difference being the way transmission was modelled for dairy vs. beef herds. In the dairy herd model the transmission of MAP is modelled using the S-L-I-R model, where the animals of the herd are divided into: susceptible, latently infected, infectious (shedding the bacterium) and resistant. In this model, the number of new infections per unit time due to MAP contamination from wildlife is assumed to be proportional to 1) the contribution of wildlife to environmental contamination relative to that of an infected cow, 2) the transmission probability, due to environmental contamination, from an infected cow to a susceptible calf, and 3) the number of susceptible calves. The first of these parameters is estimated from 1.1) faecal contamination by wildlife (gr per hectare), 1.2) prevalence of MAP in wildlife and 1.3) infectivity of faeces (cfu per gr of faeces).

In the cattle model the within farm transmission is modelled as one minus the joint probability of escaping the different contamination sources as has been done by Pouillot *et al.* (2004). In this case, these sources are not only clinically and subclinically infected animals but also wildlife reservoirs. Transmission probabilities from clinically and subclinically infected cows were taken from Pouillot et al. (2004) and the number of new infections per unit time due to MAP contamination from wildlife was model in the same way as described before for the dairy herd model.

Based on the standard risk assessment methodology a framework was obtained to assess the risk that wildlife posed to dairy herds, beef suckler herds and sheep farms in a more qualitative fashion than the previous approach. The sources of the agent can be infected cows / ewes and wildlife reservoirs and from these sources, through different routes. The model demonstrated that on-farm sanitation practices had a greater effect on the risk of new cases of infection than did infected wildlife on the premises.

In conclusion, the workshop format was welcomed by the participants and more time for discussion would have been of value. It is hoped that workshops will be part of future Colloquia.

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WORKSHOP

Immunopathogenesis of paratuberculosis

The 8th ICP was in general a one-string conference. By dedicating workshops to particular subjects it was hoped that scientists in that particular field would challenge each other and discuss how progress is best achieved in the coming years. Besides defining areas of interest, the purpose of the workshops was also to introduce scientists working in the same research field to each other.

The Immunopathogenesis workshop attracted more than 40 people for an agenda that included:

- Professor Paul Coussens, Michigan State University presented a stimulating talk on central gaps in our understanding of paratuberculosis survival and pathogenesis. Clearly macrophages play a pivotal role in both hosting the bacteria and possibly driving the immune response to Th1 and/or Th2, which eventually decides the outcome of infection. A number of gaps in our understanding of how macrophages and MAP may interact were presented. Dr. Coussens hypothesized that in progressive subclinical infection a population of suppressor T cells co-exist with the IFN- producing Th1 cells, and that these two cell populations in late stages of infection are dominated by a proliferating cytotoxic T cell response. An obvious question arising from this theory is: how can exploit the increasing knowledge on bovine immunology and immune triggering factors to develop vaccines or therapeutics that can prevent at least clinical disease? A discussion stressed how important it is to develop working hypotheses to explain observations made in the laboratory. This part of project planning is far too often neglected, and thus results in inconclusive study conclusions and overlooked results when results are not what were expected. Remember :

"It is OK to be wrong as long as you are wrong in an interesting way".

- A round of presentations were made by the participants. Some attendees had many years of experience with paratuberculosis research, others were quite new to the field and some were mainly there to listen although not active in immunopathogenesis research. This provided an excellent opportunity for younger scientists to meet more established investigators and to share ideas.

- Dr. Bill Davis, Washington State University presented recent multicolour flow cytometry results on lymphocyte subset distributions in paratuberculosis. Results likely will be published in the near future.

- Following the round of presentations two topics were selected for discussion. It is not possible to give a complete summary of the discussions, but questions that were addressed in more or less detail and which could be investigated in the forthcoming years were:

Possibilities and limitations in studies of MAP and macrophage interactions.
- o Are studies of bacterial survival etc. in monocyte derived macrophages, BoMacs or murine macrophage cell lines representative of what is happening in tissue lesions? What other choices do we have?
- o How old are macrophages in paratuberculosis lesions?
- o How is macrophage dormancy regulated in MAP infections?
- o What is the role of macrophages in adaptive immunity against paratuberculosis e.g. following vaccination

A possible role of regulatory T cells in the modulation of the immune response during progressive paratuberculosis. Is the Th1/Th2 shift fact or fiction?
- o IL-10 upregulation in PBL and in lesions has been shown by some groups, but in other studies this has not been verified. More studies needed.
- o upregulation of TGFβ gene expression in lesions has also been shown and points together with IL-10 expression to Tr cells.
- o If Tr cells are active, will they be only local or also circulating?

   o   Are they of a Tr1 or a Tr3 type? Do these Tr cell types exist in cattle and with the same
       cytokine profile as demonstrated in other species?

No doubt the discussions could have continued several hours more and it would also have been possible to
split the group in smaller working groups, but all in all the workshop was considered very successful by the
organizers.

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Economics of paratuberculosis control

One of the questions posed before this workshop was, '*is there maybe no or very little interest (by researchers, decision-makers, producers, etc.) in the economics of Paratuberculosis control?*' The attendance of 40-50 participants at this workshop showed however that this is certainly not the case among 8ICP attendances.

INTRODUCTION

After quick introductions, the workshop started off with an overview by Huybert Groenendaal of past and possibly future economic research on Johne's disease and different possible methods and techniques that can be used. Methods were illustrated with past studies and a list of advantages and disadvantages of each method was presented.

PRESENTATIONS

Three presentations were given:

1. Dr. A. Braad Kudahl (Danish Institute of Agricultural Science, Denmark): *"The economics of Johne's disease control from a <u>researcher's</u> perspective"*
A detailed presentation was given about the research presented in the PhD thesis titled 'Economic consequences of Paratuberculosis control in dairy cattle herds' undertaken by the presenter, Dr. Anne Braad Kudahl. The results of this detailed simulation study showed that in Denmark, without any control efforts, the Johne's disease prevalence would reach very high prevalence levels and cause great economic losses. Test-and-cull strategies were ineffective and expensive ways to control paratuberculosis. Optimizing management and thereby blocking infection routes to and within infected herds was concluded to be the only effective and economically most attractive way to control MAP infection. These measures are, however, laborious and difficult to implement.

2. Dr. G. Gunn (Scottish Agricultural College, Scotland): "*The economics of Johne's disease control from a <u>veterinarian's</u> perspective"*
Dr. George Gunn stated that under current milk and beef prices in the UK, Johne's disease does not warrant great attention and priority of veterinarians. The losses are small, the prospects of effective control uncertain, and other diseases and issues present a more imminent threat to the economic viability of dairy and beef farms. Dr. Gunn described the results of two studies, performed in 2001, that looked at the economics of Johne's disease. It was calculated that the average losses on infected herds were £16 and £65 per cow per year on beef and dairy herds respectively. Because of the great uncertainty and lack of field data about the current and future true prevalence of Johne's disease, the study did not include any economic analysis of possible Johne's disease control programs. It was concluded that the optimal strategy to deal with Johne's disease greatly depends on the possible link with Crohn's disease.

3. Dr. D. Kennedy (Ausvet Animal Health Services, Australia): *"The economics of Johne's Disease control from a decision-maker's perspective"*
Dr. David Kennedy's presentation focused on the different decision-makers with respect to Paratuberculosis control. He defined decision makers as those who invest in control and prevention of Paratuberculosis (farmers, farmers' and other livestock organizations and government). Dr. Kennedy further emphasized that decision-makers must balance many socio-economic factors (in addition to the direct economic costs and benefits) such as business survival, confidence in the control program, cash flow, market access, land marketability and zoonotic potential. Dr. Kennedy concluded that, while opinions on a number of factors are shared among various decision-makers, differences require recognition and resolution.

DISCUSSION

- *Simulation models:* During the last years, several simulation models have been developed and used to study the economics of Johne's disease control. On the one hand, many valuable insights have been obtained to support decision-making from these studies. On the other hand, they have also generated criticism about their validity. While we are all aware of the fact that "*all models are wrong, but some are useful*", Dr. Gunn added a useful insight in comparing simulation models to diagnostic tests. Both are nothing more than *tools* that can both be very useful in controlling paratuberculosis, as long as the users know about their strengths and limitations.

- *Price-incentives:* The study presented by Mr. M. Weber during the conference showed the price-incentives necessary to motivate Dutch dairy producers to enter a possible voluntary quality assurance program. Price-incentives discriminating between low *MAP* and high *MAP* milk would give many producers enough incentive to enter the program. The price differential would be a signal to producers about where the industry is going in the future. This would be a similar approach to the one taken in the past for milk-price differentiation based on somatic cell-count.

- *Supply-demand analysis:* While the control of paratuberculosis would likely result in an increase of milk production per cow, it may not result in an increased production on an aggregate (herd, regional, national or international) level. This is because, in contrast to increasing overall production, producers could decide to produce milk with fewer animals, thereby attempting to decrease the costs per unit of milk produced.

- *Crohn's disease:* It is often thought and said that if *MAP* is proven to be linked to Crohn's disease in human, this would (possibly suddenly) result in a totally different economic situation in which much greater control efforts would be economically warranted. In the workshop it was argued however that this sudden change may actually not happen, because (1) the potential for a link has long be recognized and (2) there are already many zoonotic diseases in farm animals and *MAP* would just be one more, and (3) Crohn's disease is not a totally new disease such as the case with Creutzfeldt Jakob. Instead, a slowly growing body of evidence could result in little to no change in the economic situation.

- *Communication:* Any economic analysis of paratuberculosis control will not be very useful if the perception of the relevant decision-makers is neither affected nor changed by it. Therefore, it was stressed that effective communication is critically important (and probably underemphasized) in voluntary control programs for paratuberculosis.

- *9ICP, Tsukuba, Japan:* During the workshop, it was not discussed if a separate theme on the economics of paratuberculosis control at the 9ICP would be deemed necessary. Based on the great interest in this post-conference workshop, a new theme on economics, decision-making and effective communication related to the control of paratuberculosis should be considered by the 9ICP organizing committee.

Workshop summary kindly provided by H. Groenendaal, Vose Consulting, Risk Analysis Services, (huybert@risk-modelling.com ; www.risk-modelling.com

WORKSHOP

Vaccination and control of paratuberculosis

INTRODUCTION

The workshop was well attended with more than 50 participants. After two presentations there was a fruitful and active discussion.

Control of paratuberculosis is very complex. Management is considered to be the most useful tool for controlling it. Control programs are based on improvement of the management procedures. Test-and-cull is another policy. But control is facing many problems: inadequate tests, improper implementation of management procedures, a wide range of hosts, persistence of the infection in populations without causing clinical disease, contamination of the environment and natural reservoirs.

In the view of workshop attendees, no eradication program has been proven to be successful. Control programs are often not evidence based, and without proven efficacy. Only parts of control programs are effective when applied under practical circumstances; not the program as a whole. Most of the research on paratuberculosis is focused on improving diagnostic techniques. Vaccination as a tool for the control of paratuberculosis is not widely accepted.

The lack of adoption of vaccination is not quite clear. One of the reasons for this is its interference with tuberculosis diagnosis and the existing control programs on Johne`s disease. The main drawback for vaccination is that it does not completely protect from infection, and therefore vaccination alone cannot lead to eradication of MAP infections on a farm. The experiences in Iceland and Brittany confirm this.

On the other hand, vaccination is successful in reducing the number of clinical cases in a herd or flock. While some are concerned that farmers using vaccination have a false sense of security about transmission and relax their sanitation standards, management procedures to prevent infections with MAP are often not applicable on farms with goats, sheep, deer, beef cattle or very large scale dairy farms. Vaccination can be a cost effective tool in the control of paratuberculosis.

A summary of vaccine studies, conducted in the last decade, shows us that vaccination has the following results:

1) The number of animals with clinical symptoms decreases in cattle, sheep and goats;
2) Fecal shedding is reduced, as well is the number and severity of bacteriological isolations   and histological lesions in the intestines (cattle and sheep);
3) Vaccination elicits an antibody titer (cattle and sheep);
4) Vaccination elicits a cellular immune response (cattle, sheep, goats).

SUMMARY PRESENTATIONS

The first presentation was *"Vaccination and control of paratuberculosis"* by Ad Koets.

First the history of vaccination against paratuberculosis was presented and the pros and cons of whole cell bacterins. A vaccine can either directly prevent infection, by blocking MAP from passing the mucosal barrier, or indirectly by reducing shedding and thus lowering transmission rates. The perfect vaccine has the following qualities:

- it causes minimal tissue reaction;
- there is no interference between tuberculosis and Johne`s disease diagnostics;
- it can discriminate between infected and vaccinated animals;
- it eliminates fecal shedding of bacteria;

- and prevents the occurrence of clinical disease.

Can we control JD without a vaccine? Consider the following:

1) Simulation models suggest that calf hygiene management is the key factor in preventing new infections. Neither test & cull nor current vaccines have a substantial influence on true prevalence.
2) The feasibility of implementing perfect preventive management on a farm is limited.
3) The perfect diagnostic test detects all animals before they become infectious and produces no false positives – there is no, nor will there likely ever be, a perfect test.
4) Realistic (economic, logistically feasible) infection management protocols need to be developed for sheep, bison and deer operations

The observations cited above showed the need for a marker vaccine to control JD.

The second presentation was *"Role of vaccination in Johne`s disease"* by Murray E. Himes.

An optimal JD vaccine prevents infection, disease and fecal shedding; is relatively inexpensive and safe to animals and personnel. It does not interfere with JD or TB diagnostic tests.

There are controversial aspects concerning current Johne's disease vaccination:

- conflicting reports on efficacy of vaccination in reducing disease and or fecal shedding;
- interference with *M. bovis* PPD skin testing;
- often severe tissue reaction at vaccination site;
- potential danger to persons administering vaccine (1 per 1000 doses)

There are different JD vaccines available on the market:

> - MAP strain 18 oil emulsion, this is only used in the USA;
> - Weybridge vaccine, UK;
> - Gudair MAP strain 318F oil emulsion, Pfizer CSL;
> - Aqua Vax Map strain 316F water based;
> - Neoparasec freeze dried live MAP, Merial.

But there are also experimental JD vaccines in development:
> - modified live whole cell MAP vaccines;
> - gene knockout whole live MAP vaccines- GFP labeled relA. pknG;
> - killed whole cell MAP vaccines;
> - killed CWD whole cell vaccines;
> - Subunit vaccines- Hsp 70, 85B, 16,7 kD;

DNA vaccines are not yet available for JD.

There are quite a few studies supporting vaccination for JD:
- Uzonna et al 2003 – reduced colonization with field strain and rIL-12, but not strain 18;
- Begg and Griffin 2005 – protection from clinical disease with Neoparasec, but not AquaVax;
- Emery and Whittington 2004 – live & killed vaccines reduce incidence of clinical disease and fecal shedding; neonatal vaccination has the best prospect;
- Hasvold et al 2002 – live attenuated MAP vaccines reduce lesions;
- Fridriksdottir et al 2000 – vaccination in endemic sheep herds reduced mortality by 94%;
- Gwozdz et al 2000 – experimentally infected sheep, vaccinated 2 weeks later, showed reduced incidence of disease, AFB and + PCR;
- van Schaik et al 1996 – calves vaccinated once showed reduced incidence of clinical disease (with 90%) and subclinical disease; vaccination cost $15 US, the benefit for the farmer is $142 US per animal;

- Wentink et al 1994 – cattle vaccination reduced incidence of disease from 7.8% to 1.8%, histology positives from 11.8% to 5%, but the percentage of infected animals increased from 21.8% to 25.9% (1984-91);
- Kormendy 1994 – cattle vaccination reduced fecal shedding greatly;
- Saxegaard and Fodstad 1985 – 131.000 vaccinated goats showed a reduction in infection rate from 53% to 1% based on 15.000 necropsies since 1967.

But there are also studies that highlight problems with vaccination:
- Muskens et al 2002 - MAP vaccination interferes with TB testing (long lasting), variable vaccine response between animals, marked effect on CMI and humoral responses;
- Kohler et al 2001 – vaccination interferes with TB testing, early humoral response but transient, CMI response was long lasting;
- Kalis et al 2001 – killed vaccine in cattle does not reduce fecal shedding or MAP transmission, the improvement of management procedures is more effective;
- Corpa et al 2000 – detected a wide variation in responses and the persistence thereof, which was related to age of vaccination;
- Wentink et al 1993 – killed vaccine administered 1 month after birth interferes with Johnin skin test (highly significant p=0.008);
- Gilmour and Angus 1974 – found no evidence of immunogenicity of an oral MAP vaccine in sheep.

The literature review led us to the conclusion that more studies are in favor of the use of JD vaccination in multiple species (sheep, goats and cattle). There is wide variation in study results, no doubt a function of differences in the type of vaccine and the protocol used among other factors. Therefore standardization of vaccines and protocols would be helpful.

Goat MAP vaccine study
The preliminary results of a goat study with different vaccines were shown.
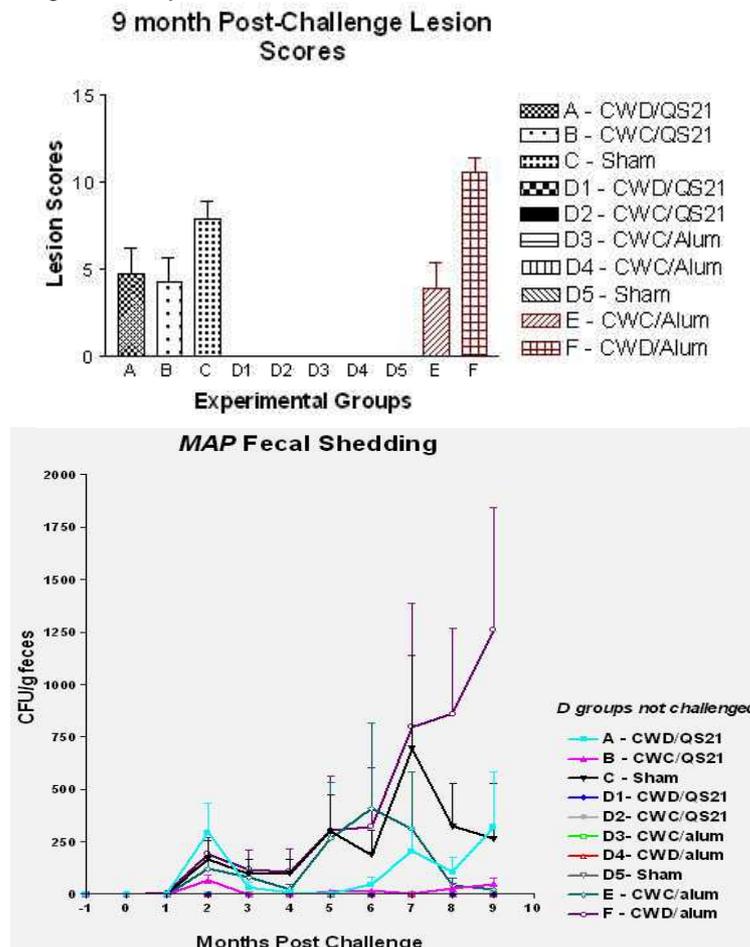
The potential benefits of the use of JD vaccines are the reduction of mortality in endemic herds or regions with high prevalence, and reduction of intra-herd transmission in endemic herds. JD management programs are more difficult to implement and maintain in beef, meat goat and sheep herds, so here the vaccines are a useful tool.

DISCUSSION

There was an exchange of ideas and opinions about the best age for vaccination; when to stop vaccinating in an infected herd; the role of tuberculosis in infected areas and species (like deer); and what went wrong in vaccination control programs like the Icelandic one. The main topic in the ensuing discussion was the evaluation of the efficacy of a vaccine. There is a worldwide need for effective cattle vaccines against JD that do not hinder the control of TB. It was agreed to form a working group to formulate an internationally accepted standardized protocol to measure the efficacy of vaccines. To this end, the knowledge of different countries and companies will be used.

Workshop summary kindly provided by Geart Benedictus, Utrecht University, Faculty of Veterinary Medicine, Department of Farm Animal health, Yalelaan 7, 3584 CL, Utrecht, The Netherlands

Vaccination and control of paratuberculosis

Paratuberculosis control by vaccination

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The 7 ICP in Bilbao saw a review on the current situation of vaccination as a tool for control of paratuberculosis. This is an update of that review.

Current knowledge on paratuberculosis shows that this infection does not follow the standard paradigms of infectious diseases: it is not always possible to experimentally reproduce the disease, there are many individuals with lesions that do not progress, there are herds that are infected but never have clinical cases, there are herds that are apparently free of Map for years according to fecal culture that suddenly become positive, the agent is much more widespread than it has always been assumed, etc. Therefore it appears that Map is a necessary element of paratuberculosis, but not a sufficient one. According to this view it was then necessary to ask several questions regarding the standard approach to control which was eradication of the agent: Is eradication possible? Is eradication necessary? Is eradication profitable? At the 7 ICP those were mainly rhetorical questions that I tried to answer with my presentation. Today, at the end of the 8 ICP it looks like the answers the experts that have met here have, at least hinted, that the answers might be NO, and that the focus of paratuberculosis control has shifted from total eradication of Map from infected herds or regions to control of economic losses.

From this perspective, our knowledge of the immunopathogenesis of paratuberculosis, shows that the main part of infected animals are subclinical infections, and that only around 16, 19, and 38% of sheep, cattle and goats in infected herds progress to develop the disease, and therefore cause a direct economic loss to the farmer. Research on vaccine effects has shown that those are precisely the cases that can be prevented by vaccination. Back in 2002, I showed that out of 20, 14 and 6 reports on vaccination in cattle, sheep and goats, since the first reports of Vallée & Rinjard, 1934, Sigurdsson, 1960. and Saxegaard, 1985, that is for the last 76 years, the vast majority showed a clear effect on clinical cases. The range was 96.02%, 49.98%, and 45.08% in terms of clinical cases or general mortality reduction and 72.70%, 71.73%, and 82.76% (cattle, sheep, goats). Although the main drawback of vaccination is its interference with tuberculosis diagnosis, the most recent vaccines have reduced this effect and our own experience shows that the interference in the comparative IDT is negligible. On the other hand while most of the conclusions drawn of the effects of vaccination were so focused on the eradication that only took into account the persistence of shedders, it should be pointed out that based in a detailed study by van Schaik et al, 1994, and some calculations on shedding, the effect on total contamination was 13 vs. 66% reduction by vaccination in cattle. In sheep, a simulation study also showed that economic positive balance for heavily infected flocks could be reached in just 3 years, with final benefit/cost ratios almost four times greater in 20 years with vaccination than with eradication. These benefits could be quantified at nearly 100 l in sheep after 7 years of vaccination in sheep, and 400 kg for cattle in just a period of two years after vaccination. These are objective data for only a herd in each case, however, farmers in the UK in already in the sixties where as happy with vaccination as to state in almost 90% of the returned questionnaires that they were seeing very clear benefits from vaccination.

In summary, vaccination: is inexpensive, even easier if applied to older animals (1-3 months), and easy to spread: Potentially high overall effect.

In 2002, I postulated the existence of a conspiracy of silence on vaccination as a possible cause for its restricted use. Perhaps today, as the paratuberculosis research community begins to assume the impossibility of eradicating paratuberculosis, and shows a concern for not doing anything clearly effective for controlling what appears as a potential zoonosis, this conspiracy can be considered finally broken. This will allow free discussion on the use of vaccination as an option candidate tool for beginning an effective

control of paratuberculosis like it has been used for other diseases until a low enough prevalence can be reached or new strategies become available.