



Proceedings of the 11th International Colloquium on Paratuberculosis



THE UNIVERSITY OF
SYDNEY

**SYDNEY, AUSTRALIA
5 – 10 FEBRUARY 2012**



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Søren Saxmose Nielsen

Editor-in-Chief to the International Association for Paratuberculosis

Published 20 February 2012

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DIAGNOSTICS AND DETECTION OF *MYCOBACTERIUM* *AVIUM* SUBSP. *PARATUBERCULOSIS*

KEYNOTE ON: DIAGNOSTIC TESTING AND DETECTION OF MAP: TECHNOLOGICAL ADVANCES, DESIGN AND REPORTING GUIDELINES

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Development and validation of diagnostic tests for paratuberculosis is challenging because of the long incubation period, unpredictable disease progression, possible infection with different *MAP* strains, and diverse testing purposes using different specimen types often with low *MAP* loads. The presentation will focus on technical improvements in test methodology and statistical analysis methods since the last ICP, and include discussion of current design and reporting guidelines for test evaluation studies.

Technological advances

The scientific literature as well as abstracts submitted to this ICP meeting demonstrates a concerted effort by many laboratories to identify better antigens for ELISA or IFN-gamma tests. Proteomic studies have provided the backbone for new candidate antigens to test. Secreted or culture filtrate proteins of *MAP* (Lanigan et al., 2007; Shin et al., 2010) were identified because of the diagnostic potential associated with proteins present in those fractions. Likewise, lipid extractions have also been characterized for mostly the same reasons (Eckstein et al., 2006; Eda et al., 2006). A few studies (He and De Buck, 2010; Lanigan et al., 2009) have also been directed at envelope proteins of *MAP*. Once new candidate antigens are discovered, they are tested in a diagnostic platform (Bannantine et al., 2011; Scott et al., 2010).

From a data analysis perspective, numerous authors have acknowledged the need for latent class methods for statistical evaluation of the accuracy of paratuberculosis tests because of the lack of a perfect reference standard. Development of new statistical methods has focused on the use of Bayesian methods for estimation of sensitivity and specificity, and the receiver-operating characteristic (ROC) curve. The motivation for all the new methods has been in the context of paratuberculosis in dairy cows typically using data from 2 conditionally independent tests (ELISA and fecal culture). For cross-sectional data, semiparametric modeling has been shown to improve estimation procedures (Branscum et al., 2008). For longitudinal data, 2 approaches have been used, both based on a change point model that incorporates time to change (Norris et al., 2009) and a second using a Weibull survival model adjusting for covariates and random effects from repeated testing of the same animals (Wang et al., 2011). Although these methods are technically challenging for non-statisticians, many have been implemented in WinBUGS/OpenBUGS and therefore can be implemented by those familiar with these platforms. Bayesian regression models have been developed to estimate diagnostic sensitivity incorporating cow level information such as parity and stage of lactation, and repeated test results (Norton et al., 2011)

Design and reporting guidelines

The World Organisation for Animal Health (OIE) has specified a general pathway for the validation of diagnostic tests for OIE-listed infectious diseases (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.1.04_VALID.pdf). In addition, the OIE has a registry of certified tests (www.oie.int/our-scientific-expertise/certification-of-diagnostic-tests/the-register-of-diagnostic-tests/) but this has had limited acceptance because of the high cost (8000 euro) and lack of agreement that once listed as “certified” that it will be accepted by a country’s veterinary authorities for animal movement testing. The OIE designates 6 possible test purposes which should be considered for the test under evaluation in the context of other available tests. These purposes are not meant to be prescriptive and could be made more specific in the context of a specification application by considering other factors (e.g. dairy vs. beef cattle, commercial vs. breeding herd). Failure to identify a purpose in advance of conducting a study is a likely explanation for the poor quality of many prior test accuracy studies (Nielsen and Toft, 2008). Once the purpose, target condition (e.g. infected or non-infected) and case definition (practical realization of the target condition) are specified, then appropriate study designs can be selected (see Table 1 in Nielsen et al., 2011). Designs should be chosen that minimize the effects of biases that lead to overly optimistic estimates of test performance characteristics such as

sensitivity and specificity. A structured approach to test evaluation has been proposed (Nielsen et al., 2011) which links the application triad (purpose-pathogenesis-target condition) with the evaluation triad (target condition-case definition-study design). This conceptual model is applicable to other chronic infections such as tuberculosis and brucellosis.

Although study design considerations are important, experience indicates that reporting of studies may be inferior regardless of the quality of the original design and its implementation. The Standards for Reporting of Diagnostic Accuracy (STARD) statement was published in 2003 (Bossuyt et al., 2003) and has been widely supported as a guidance document for test evaluation studies (www.stard-statement.org). Because the statement did not address many important considerations in livestock test evaluation studies (e.g. use and interpretation of tests at the herd level, diverse specimen matrices, clustered sampling designs, and possible use of experimental challenge studies), an international initiative was started in 2009 to produce a consensus-based set of reporting standards for paratuberculosis (Gardner et al., 2011). The standards include examples and explanations for each of the 25 checklist items and therefore provide practical guidance as to the key considerations in a peer-reviewed manuscript. It is important to note that these reporting guidelines allow for various designs for a designated purpose and ultimately will be useful for meta-analyses and systematic reviews by encouraging complete and transparent reporting of primary studies.

The aforementioned design and reporting guidelines are informing a Johne's Disease Integrated Program (JDIP) community study involving head-to-head comparison of multiple organism and antibody detection tests. A unique aspect of the JDIP study is use of serum ELISA testing as an initial screening test with the follow-up testing of 3 randomly-selected ELISA-negative controls that are lactation matched to each ELISA-positive case. A Bayesian analysis will adjust for the different sampling fractions of cases and controls and compare results of at least 6 tests on samples from the same cow in the absence of a reference standard. Samples are being banked in a repository at the University of Georgia, Tifton and will be accessible to other investigators at a later time. Progress including initial results of the study will be described in the presentation.

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EXTENSION OF THE STORAGE TIME OF BLOOD IN INTERFERON GAMMA ASSAY TO DIAGNOSE PARATUBERCULOSIS: COMBINATION OF IL-7 AND IL-12 STIMULATION

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Detection of specific interferon gamma (IFN- γ) responses can aid in the diagnosis and control of paratuberculosis. IFN- γ is produced by white blood cells as part of the cell-mediated immune response to MAP infection. IFN- γ detection assays offer the potential to detect more infected animals at an earlier stage of the disease than antibody ELISA, providing an opportunity for control strategies aimed at the removal of young infected animals before they shed bacteria into the environment. A limitation to the widespread application of IFN- γ assays has been logistical difficulties, as the assay needs to be performed within 8hr of blood collection. Earlier research has shown that, for bovine blood samples with a delayed assay setup (24hr), addition of Interleukin (IL)-12 can rescue the Th1 cells that produce IFN- γ . For some countries such as Australia, however, samples may take up to 2 days to reach a laboratory. For this reason an improved protocol, applicable across multiple species, was required to increase white blood cell survival. IL-7 is a survival factor required to maintain resting T cells in cell culture. We hypothesised that IL-7 alone or in combination with IL-12, added at stimulation, could extend blood storage time. The addition of IL-7 and IL-12 in combination had a synergistic effect, giving IFN- γ responses greater than adding IL-12 alone, for sheep blood stored up to 2 days. Better recovery of IFN- γ responses was achieved for animals with low grade and paucibacillary lesions compared to animals with multibacillary disease. From a cohort of naturally infected sheep it was found that the same number of animals could be identified as test positive with blood samples stored for 2 days with addition of IL-7 and IL-12 compared to the same blood samples set up within 8hr of collection without additives. This practical and easily implemented potentiation protocol (IFN- γ Plus assay) extends the permissible transit time of blood samples from farm to laboratory for IFN- γ testing to detect Johne's disease.

USE OF NOVEL RECOMBINANT ANTIGENS IN THE INTERFERON GAMMA ASSAY FOR DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTION IN CATTLE

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Early stage *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection may be detected by measuring antigen specific cell-mediated immune responses by the interferon-gamma (IFN- γ) assay. Available IFN- γ assay use purified protein derivate of Johnin (PPDj) leading to low specificity. The objectives of the study were to evaluate immunogenicity and specificity of 14 novel recombinant antigens for use in the IFN- γ assay and to assess the consistency of IFN- γ responses with repeated samplings. The antigens used were 4 ESAT-6 family members, 4 latency proteins, 4 secreted proteins including Ag85B, 3 other antigens and PPDj. The study included blood samples from 26 heifers of a MAP infected herd, collected three times with 4 and 5 week interval and blood samples from 60 heifers of a MAP non-infected herd collected once. The IFN- γ responses of the non-infected heifers were used to establish cut-off values for each antigen. A case was defined as an animal with ≥ 2 positive tests for ≥ 4 antigens, resulting in 13 cases and 13 non-cases. Based on the case-definition, immunogenicity and specificity of each antigen were calculated. IFN- γ levels against each of the antigens of the infected and non-infected herds were significantly ($P < 0.05$) different and IFN- γ levels against each of the antigens of cases were significantly higher than non-cases ($P < 0.05$ for all antigens). The results of the IFN- γ assay using PPDj did not correlate well with the results using the novel antigens since 5 of the 17 animals that were positive to PPDj were non-cases and one case was negative to PPDj but positive to all other tested antigens. Furthermore, PPDj produced elevated IFN- γ responses in both the infected and non-infected herds and showed low consistency. Immunogenicity was highest for the group of latency proteins (0.65-0.85) which also had high specificity (0.92-1.00). Three latency proteins showed positive IFN- γ tests that correlated highly with the case definition and one of these antigens (LATP-2) had no homologue sequence in the *M. avium* subsp. *avium* or *M. bovis* genome and could be a promising diagnostic antigen. The combination of antigens for use as a cocktail should be further investigated. However, to detect all the animals defined as cases, 8 of the novel antigens and Ag85B would have to be included.

A LONGITUDINAL STUDY TO CHARACTERIZE THE SHEDDING PATTERNS OF *MYCOBACTERIUM AVIUM* SPP. *PARATUBERCULOSIS* IN A NATURALLY INFECTED BREEDING BULL BY POLYMERASE CHAIN REACTION ASSAYS

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ABSTRACT

Although *Mycobacterium avium* spp. *paratuberculosis* (MAP) has already been detected in semen and reproductive organs of bulls, shedding patterns are not well characterized. Our investigation was performed to detect and quantify MAP in feces, semen, and blood samples continuously drawn from a naturally infected 18-month-old German Simmental bull without clinical symptoms over a period of 4.5 years by qualitative and quantitative polymerase chain reaction (PCR) techniques and to correlate time dependent matrix specific contents of MAP. In all matrices, MAP was detected intermittently with MAP-free intervals of at least 5 to 18 weeks using an IS900 semi-nested PCR. The number of MAP positive results in semen and blood was higher than in fecal samples. A quantitative IS900 real-time PCR revealed that the highest amount of MAP was shed in feces (10^3 - 10^6 MAP/g), while the lowest concentrations were found in semen and blood (10^2 - 10^5 MAP/ml). Although a poor relationship was calculated between the presence of MAP in feces and blood, a significant positive agreement between its occurrence in semen and blood was determined ($r = 0.57$, $p < 0.001$, $n = 65$). For identity confirmation and phylogenetic comparisons, the 278 bp PCR amplicon covering nucleotide positions 492 and 769 within the IS900 was cloned into the pCR 2.1-TOPO plasmid vector and sequenced. Nucleotide homologies of 100% were ascertained to the MAP K10 IS900 reference sequence (GenBank: AE16958). Despite the presence of MAP in semen, the quality of semen samples was good with volumes between 3 to 10.5 ml. Morphologically normal spermatozoa varied from 85 to 92%, density was 0.4 to 1.6 million/ μ l, and motility ranged from 65 to 70%. Since 2.5 years, enhanced eosinophilic granulocytes with peaks of up to 55% were noticed intermittently in blood by differential cell counts (granulocytes, lymphocytes, monocytes) using microscopy and flow cytometry. Parasitological examinations were accomplished regularly to exclude helminthic and protozoal infections. The present study highlights the possible risk for MAP transmission during artificial insemination and indicates the need for hygienic measures to prevent the spread of the infection via semen.

INTRODUCTION

It is generally accepted that MAP is mainly transmitted to neonate calves through the fecal-oral route by subclinically or persistently infected cattle in a herd. However, vertical transmission of MAP via the uterus has been investigated before, too. The first report about a bovine fetal infection was published in 1929 [1], and the isolation of MAP from bovine semen was described for the first time in 1948 [2]. Although MAP has been isolated from semen and reproductive organs of infected bulls [3, 4], the pattern of bacterial shedding during different stages of the infection is not well characterized in naturally infected animals. Therefore, the objective of our study was to detect and quantify MAP in feces, semen, and blood samples continuously drawn from a breeding bull candidate naturally infected with MAP showing no clinical symptoms of paratuberculosis by PCR techniques.

MATERIALS AND METHODS

An 18-month-old German Simmental breeding bull candidate (*Bos primigenius taurus*) was kept isolated under quarantine conditions with constant feeding and environmental circumstances over a period of 54 months from June 2007 to November 2011. In total, 101 sample dates were chosen to collect fecal, semen, and blood samples, concurrently.

DNA extraction. DNA was extracted from the different sample matrices (feces, semen, and blood) for PCR analysis using a modified protocol of the QIAamp Blood Kit (Quiagen, Hilden, Germany).

Polymerase chain reaction. A qualitative semi-nested PCR (snPCR) and quantitative real-time PCR (rtPCR) based on the insertion sequence IS900 was performed as described elsewhere [5].

Enzyme-linked immunosorbent assay. During 2007 and 2008, a total of 35 serum samples were tested with the Idexx-ELISA (IDEXX GmbH, Woerrstadt, Germany) according to the producer's manual.

Since 2009, the Idexx-ELISA was no longer available and replaced by the Pourquier-ELISA (Institut Pourquier SAS, Montpellier, France) for testing of 66 sera in 2009, 2010, and 2011.

Statistical analysis. The Pearson's correlation coefficient (r) was calculated to analyze the correlation between the detection of MAP in fecal, semen, and blood samples by snPCR. All analyzes was carried out with Microsoft Office Excel 2003 and Minitab Version 15.0 (Minitab Inc., State College, PA, USA).

RESULTS

During the longitudinal study, the bull's general condition was good and the fecal consistency was normal.

In all matrices, MAP was detected intermittently with MAP-free intervals of at least 5 to 18 weeks by snPCR. The number of MAP positive results in blood (39%) was higher than in semen (35%) and fecal (36%) samples. The rtPCR revealed that the highest amount of MAP was detected in feces (10^3 - 10^6 MAP/g), while the lowest concentrations were found in semen and blood (10^2 - 10^5 MAP/ml). Although a poor relationship was calculated between the presence of MAP in feces and blood, a statistically significant positive agreement between its occurrence in semen and blood was determined ($r = 0.57$, $p < 0.001$, $n = 65$). In 11/35 (32%) serum samples antibody responses were detected by the Idexx-ELISA in 2007 and 2008. No antibody response could be detected at any time in the 66 samples tested by the Pourquier-ELISA in 2009, 2010, and 2011. Despite the presence of MAP in semen, the quality of semen samples was good with volumes between 3 to 10.5 ml. Morphologically normal spermatozoa varied from 85 to 92%, density was 0.4 to 1.6 million/ μ l, and motility ranged from 65 to 70%. Blood differential cell counts revealed eosinophilic granulocytes proportions up to 55%.

DISCUSSION

It is commonly accepted that an infected animal enters a silent phase for two or more years, during which MAP is not detectable in feces. According to our study, MAP in bovine feces and semen was already present at the age of 18 months. This being an unusual event for an animal at such a young age, a recent case study observed fecal shedding of MAP in cattle prior to the age of two years [6]. Therefore, the assumption that young stock is not infectious has to be reconsidered. Over the period of our investigation, MAP was detected intermittently rather than continuously including long periods of MAP absence. MAP was also detected intermittently in blood samples, indicating that viable MAP bacteria in the blood may be responsible for disseminating the infection within the body. There was a statistically significant correlation between MAP occurrence in semen and blood, indicating the association that further colonization occurs via the blood stream. Although PCR verifies only the presence of DNA and does not indicate whether bacteria capable of infection, our results support the hypothesis that raw semen of subclinically infected bulls might contain 10^3 - 10^5 MAP/ml, and can therefore pose a risk for contamination of the bovine uterine environment if MAP survives semen conservation procedures. Further investigations are required in order to determine the likelihood of vertical transmission following natural mating or artificial insemination to make a reliable risk assessment regarding the pathogen MAP in semen. This includes surveys on MAP resistance against antibiotic additives used in diluents for standard semen conservation.

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SPECIFIC VOC PATTERNS IN THE HEADSPACE OVER MAP CULTURES – A NEW DIAGNOSTIC APPROACH TO SPEED UP CULTURAL DETECTION?

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Introduction

Volatile organic compounds (VOC) were analysed in the headspace of MAP-cultures in order to identify early metabolic markers of MAP with the final aim to speed up cultural identification of shedders.

Methods

MAP strain ATCC 19698 and four MAP field strains (three RFLP cattle-type strains and one intermediate strain) were cultivated in four 100-fold dilutions on HEYM with mycobactin and ANV for 8 weeks. Headspace over the cultures and the media (control) was collected by solid phase micro extraction (SPME). VOC were analysed using gas chromatography mass spectrometry (GC-MS).

Results

More than 20 MAP specific VOCs were detected in the headspace of MAP-cultures. These substances were not found in media incubated without MAP for the same time period. Most biomarkers can be detected in all cultures but arise in different concentrations. For most cultures specific concentration changes occurred, related to different colony counts. PCA analysis of selected markers enables separation of different MAP strains.

Conclusions

MAP cultures emit specific pattern of VOCs. These metabolic markers may be used for fast identification of MAP growth.

EVALUATION OF NEWLY DEVELOPED HIGH-EFFICIENCY DNA EXTRACTION METHOD FOR REAL-TIME PCR DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* FROM FAECES IN COMPARISON WITH OTHER DIAGNOSTIC METHODS

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ABSTRACT

In the field of molecular diagnostics, there is a lack of high-yield extraction methods for pure mycobacterial DNA from complex matrices like faeces. Therefore, we have developed a new, high-yield DNA extraction method combined with quantitative real-time PCR (HYDEqPCR) for amplification of insertion sequence IS900 of *Mycobacterium avium* subsp. *paratuberculosis* (Map). Evaluation of HYDEqPCR was carried out versus conventional bacterial culture, milk qPCR, and milk enzyme-linked immunosorbent assay (ELISA).

The detection limit of HYDEqPCR was 90 Map/g Map-spiked faeces, corresponding to 2.4 colony forming units/g Map-spiked faeces, with an estimated efficiency of 85% ($\pm 21\%$). The efficiency of qPCR alone was 97% with the detection limit of 2-10 IS900 DNA copies/ μ l in 67% of the reactions. The qPCR assay was 100% specific, as determined from 50 Map and non-Map strains. When tested on 141 faecal and 91 milk samples collected from dairy cows, heifers and calves without clinical symptoms, pathognomonic for Johne's disease, HYDEqPCR yielded 89% of samples positive for Map, whereas faecal culture, milk qPCR, and milk ELISA detected 19%, 36% and 1%, respectively. Fisher's exact test showed statistical significance ($p \leq 0.05$) for the correlation between HYDEqPCR and faecal culture only. HYDEqPCR could detect low-level Map shedders that go undetected using other herein described methods. It could be applied routinely when screening for Map in a herd or at an animal level.

INTRODUCTION

The choice of tests in diagnosing paratuberculosis in cattle is wide but the necessity for methods with higher sensitivity and specificity for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map), especially in subclinically infected populations, still exists. The infected animals, without clinical symptoms, can shed Map into the environment and are the cause of new transmissions of Map infections within and between herds. Faecal culture, which still is considered to be a gold standard, and enzyme-linked immunosorbent assay (ELISA) have been the most commonly used tests for diagnosing paratuberculosis. However, in recent years, molecular based techniques have vastly improved in the sense of better sensitivity and specificity for Map detection in clinical samples compared to conventional methods.

The aim of this study was to develop a high-yield DNA extraction method from faeces combined with highly sensitive and specific quantitative real-time PCR (HYDEqPCR) for the detection of subclinically Map-infected animals. The usefulness of HYDEqPCR was evaluated in comparison with conventional bacterial culture, milk qPCR, and the milk commercial ELISA test.

MATERIALS AND METHODS

Animals and samples

A total of 141 faecal and 91 milk samples were collected from 141 dairy cows from a free-ranging farm located in the south-west of Slovenia. Among 141 animals, there were 30 heifers and 20 calves. The herd had a past history of clinical and culture-positive paratuberculosis cases. The tested population did not show any clinical symptoms that were pathognomonic for Johne's disease at the time of collection.

HYDEqPCR

DNA extraction

The DNA isolation from the faeces was performed using newly developed SmartHelix™ First DNAid kit (Institute of Physical Biology, Slovenia) of which an integral part is mechanical lysis. Ten grams of each

faecal sample was placed into a sterile 50-ml tube containing 30 ml sterile distilled water, and then mixed for 30 min at 250 rpm. Two ml of the sample was further processed.

To evaluate the efficiency of DNA extraction, faecal samples from a Map-free cow were spiked with prepared dilutions with known amounts of Map cells and the recovery of IS900 was monitored by qPCR. The number of recovered Map cells was calculated from the IS900 copy number.

Quantitative real-time PCR

The IS900 TaqMan based qPCR was performed on a Light Cycler 2.0 real-time PCR instrument (Roche Diagnostics, Switzerland). PCR reactions were carried out in a final volume of 25 µl with Maxima Probe qPCR Master Mix (Fermentas, Lithuania). An internal positive control (Applied Biosystems, USA) was included in the mix to monitor for any inhibition of the amplification reaction. The specificity, amplification efficiency, linear dynamic range, limit of detection (LOD) and limit of quantitation (LOQ) of the IS900 qPCR assay were determined.

Faecal culture

Faecal samples, decontaminated with 0.90% hexadecylpyridinium chloride, were inoculated onto two Herrold's egg yolk agar slants that were supplemented with mycobactin J, amphotericin B, nalidixic acid, and vancomycin (Becton Dickinson, Sparks, USA). The slants were incubated at 37 °C and the resultant colonies were counted weekly over 16 weeks. The slants were incubated at 37 °C and the resultant colonies were counted weekly over 16 weeks. The identities of all of the colonies were additionally confirmed by amplification of IS900 by qPCR.

Milk qPCR and ELISA

DNA from milk samples (50 ml) was isolated as described previously (1, 2). The isolated DNA was tested for IS900 by qPCR. The samples were also tested for the presence of antibodies, using Pourquier ELISA Paratuberculosis kit (Institut Pourquier, France) according to the manufacturer's instructions.

RESULTS

Determined amplification efficiency of the qPCR assay was 97% with a linear dynamic range of 8 log. The LOD corresponded to 2-10 IS900 copies. The LOQ was as low as 20 IS900 copies per reaction. The analytical specificity with the tested reference strains, clinical Map isolates, and non-Map strains was 100%. With the HYDEqPCR we could detect 90 Map cells/g Map-spiked faeces according to the detected number of IS900 copies in 5/6 reactions. The LOD was equivalent to 2.4 colony forming units (CFU)/g Map-spiked faeces. There was also no significant qPCR inhibition by the sample matrix, as seen by a comparison of the Cq values for the internal positive control in samples and in sterile distilled water ($p \geq 0.05$). The mean efficiency of this DNA Map isolation procedure from faeces was 85% ($\pm 21\%$). When HYDEqPCR was tested on field samples, it produced the highest number of positive results (89%), followed by milk qPCR (36%), faecal culture (19%) and milk ELISA (1%). Fisher's exact test showed statistical significance ($p \leq 0.05$) for the correlation between HYDEqPCR and faecal culture only. All of the culture positive samples were also positive by qPCR.

CONCLUSION

HYDEqPCR proved to be fast, highly efficient, sensitive and specific test for diagnosis of Map in cattle faeces. The results also suggest that the proportion of Map shedders in an animal population is underestimated at present and should be looked at more closely.

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TEST COMPARISONS FOR MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTIONS IN LOW PREVALENCE HERDS

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Introduction

Most of the studies evaluating the performance of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) antibody ELISAs have been conducted in high prevalence herds. Those results are not necessarily valid for low prevalence farms, because estimated sensitivities are lower in low prevalence herds (Tiwari et al., 2006). Additionally, the sensitivity of MAP ELISAs is higher when used in serum than in milk (Hendrick et al., 2005; Lombard et al., 2006). Consequently, the objectives of this study were to evaluate a MAP antibody ELISA in low prevalence herds using serum and milk samples and to determine whether cow characteristics would explain a difference between test characteristics of the used ELISA test assays.

Material and Methods

Cows located at 24 southern Alberta dairy farms were sampled twice within a two year interval. During each visit, fecal samples and blood samples were collected from every cow older than 36 months. Milk samples (with Bronopol preservative) were acquired from Canwest DHI who also provided the individual cow production data recorded at the milk sampling date. Until processing, fecal samples were stored at -80°C, while serum and milk samples were stored at -20°C. Fecal samples were pooled in groups of five and processed using TREK ESP® culture system II (TREK diagnostic systems, Cleveland, Ohio, USA) combined with IS900 PCR as a confirmation. Samples from cows in positive pools were retested individually to identify culture-positive cows. Serum and milk samples were processed according to manufacturer's instructions using commercial ELISA kits. While samples collected in 2009 were analysed using the Pourquier ELISA™ (Institute Pourquier, Montpellier, France), samples from 2010-2011 were processed using the new IDEXX ELISA (IDEXX Laboratories, Westbrook, United States). Sensitivity, specificity, positive- and negative predictive value estimates were reported for the dichotomous ELISA results using fecal culture as a gold standard. Multilevel linear regression on farm and cow level was performed to analyse how cow characteristics influence the relationship between serum and milk ELISA results.

Results

The serum ELISA had a sensitivity of 33.3% and a specificity of 98.6% compared to fecal culture (FC) (Table 1). The milk ELISA had a sensitivity of 15.4% and a specificity of 99.0% (Table 2). Performance estimates with corresponding confidence intervals are illustrated in Table 3.

Table 1: Results of the Pourquier serum ELISA

	FC+	FC-	Total
Serum ELISA+	6	22	28
Serum ELISA-	12	1517	1529
Total	18	1539	1557

Table 2: Results of the Pourquier milk ELISA

	FC+	FC-	Total
Milk ELISA+	2	11	13
Milk ELISA-	11	1037	1048
Total	13	1048	1061

1939 matched milk and serum Pourquier or IDEXX ELISA sample pairs were considered for the multilevel analyses. Of those, 1860 had matched lactation records. In the univariate analyses the serum ELISA result was associated with fat- and protein content of the milk sample as well as the age of the cow and the stage of lactation. The same variables plus the milk yield and the somatic cell count were associated with the milk ELISA result. Those variables were considered for the multivariate analysis. Milk yield and milk protein content modified the relationship between the serum and milk ELISA.

Table 3: Characteristics of the Pourquier ELISA used in serum and milk samples compared to fecal culture

	Serum ELISA (95% CI)	Milk ELISA (95% CI)
Prevalence of infectious animals	1.2 (0.6-1.7)	1.2 (0.6-1.9)
Sensitivity	33.3 (13.3-59)	15.4 (1.92-45.4)
Specificity	98.6 (97.8-99.1)	99 (98.1-99.5)
Positive predictive value	21.4 (8.3-41)	15.4 (1.92-45.4)
Negative predictive value	99.2 (98.6-99.6)	99 (98.1-99.5)
Area under the roc curve	0.66 (0.55-0.77)	0.57 (0.36-0.77)

Discussion

As expected, the milk ELISA detected a lower number of MAP culture-positive cows than the serum ELISA (Hendrick et al., 2005; Lombard et al., 2006). The wide and overlapping confidence intervals could be due to the low number of culture-positive cows in the study. The results also show that a single ELISA test result is not sufficient to detect MAP shedders in low prevalence herds.

Several previous studies have reported that immunoglobulin concentrations and resulting ELISA results are influenced by cow characteristics such as age or stage of lactation (Guidry et al., 1980; Lombard et al., 2006; Nielsen et al., 2002). In this study, the concentration of MAP antibodies in milk depended on the antibody concentration in serum. Additionally, it was positively influenced by the milk protein content and was diluted with increasing milk yield. We therefore conclude that milk yield and protein corrected interpretation of milk ELISA results should be considered.

Acknowledgement

The authors would like to thank field and lab- technicians for sample collection and processing. Funding for this project was provided by Alberta Milk and Alberta Livestock and Meat Agency (ALMA). This presentation received the Johne's Disease Integrated Program (JDIP) USDA-NIFA Award (No. 2008-55620-18710) and an award of the University of Calgary (University Research Grant Committee). A big thank you goes to the producers for their participation in the project.

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NEW AND IMPROVED DIRECT FAECAL PCR TEST FOR JOHNE'S DISEASE

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The development of a sensitive and robust direct faecal PCR (DPCR) test for both bovine JD and ovine JD has been a goal in Johne's disease research since the mid 1990s. We have developed a new high throughput DPCR test (termed the HT-J test), suitable for faecal samples from both cattle and sheep. The HT-J test is based on a highly sensitive DPCR test that was applicable mainly in research applications because of its complexity and labour intensity (Kawaji et al., 2007). To validate the HT-J test, 1329 bovine faecal samples, representing 31 submissions from both unexposed (n=8) and exposed herds (n=23), were tested in two laboratories. Of the submissions representing exposed herds, faecal culture positive samples were identified in only seven. All seven of these herds were also identified by the HT-J test, however, positive samples were identified in a further eight submissions by the HT-J test. Validation was also undertaken on 596 ovine faecal samples, representing 18 individual submissions/flocks. Culture positive samples were identified in 14 submissions, all of which were identified by the HT-J test. The HT-J was developed to suite Australian conditions and during the course of the study it became apparent that local optimisation is paramount to the success of the test. Furthermore, sample storage prior to testing was identified as a critical issue. Here we report the development, optimisation and performance of a new high throughput DPCR test for the detection Johne's disease in cattle and sheep. The new test has demonstrated sensitivity and specificity equal to or greater than faecal culture and is suitable for use in routine diagnostic laboratories.

Materials and methods

Faecal samples

Faecal samples were obtained from 1329 cattle and 596 sheep representing both exposed (cattle n=870, sheep n=507) and unexposed (cattle n=459, sheep n= 89) herds and flocks from throughout Australia. Where there were sufficient faeces in each faecal collection container, the sample was mixed thoroughly by hand using a wooden applicator stick and two aliquots were removed and placed in sterile containers to enable testing at both laboratory 1 and laboratory 2. At each laboratory each sample was processed to yield a single DNA extract which was tested in duplicate (replicate 1 and replicate 2).

HT-J faecal extraction method

DNA was extracted from cattle and sheep faeces using an automated magnetic bead isolation method incorporating the BioSprint 96 One-For-All Vet kit (Qiagen) and a magnetic particle processor (MagMax Express 96, Applied Biosystems or Kingfisher Flex, Thermo Scientific). Following extraction, eluted DNA samples were frozen at -80°C and thawed prior to quantification of *Mptb*-specific DNA using an IS900 quantitative PCR (qPCR) assay as described by Kawaji et al., (2007) on a MX3000p Multiplex Quantitative PCR system (Stratagene) for laboratory 1 and 7500 Fast (Applied Biosystems) for laboratory 2.

Interpretation of PCR results

Replicate DNA extracts were prepared from each faecal sample. A positive sample result was reported if both replicates returned a positive result, otherwise a negative sample result was reported. To accept the qPCR result for each sample, several criteria had to be met: (1) amplification efficiency for the qPCR run had to be between 90.0 and 110.0%, (2) the sample result had to produce a T_m of 89.4±1.5°C (Stratagene platform) or 87.8±1.5°C (AB platform) and have the DNA quantity ≥ cut-point of 0.0005 pg as determined by a standard curve derived from each qPCR run.

Results

Of the 1329 cattle samples tested at laboratory 1, only 1298 samples were tested at laboratory 2 due to insufficient sample. The 596 sheep samples were tested only at laboratory 1. Sensitivity and specificity data comparing HT-J and faecal culture results for individual animals, cattle and sheep, for each laboratory are presented in Table 1. Sensitivity ranged from 63.06% to 85.59% compared to culture and specificity ranged from 98.88% to 99.35%.

Table 1. Sensitivity and specificity of HT-J relative to faecal culture for individual animals.

Species	Laboratory 1		Laboratory 2	
	Sensitivity	Specificity	Sensitivity	Specificity
Cattle	63.06 N=1329	99.35 N=459	70.64 N=1298	99.33 N=448
Sheep	85.59 N=596	98.88 N=89	NOT TESTED	

* both replicates must be positive to call a positive result

Within exposed herds and flocks, the HT-J test identified a greater number of positive animals than faecal culture. This was observed in both laboratories for the cattle samples and at laboratory 1 for the sheep samples. Thus, the apparent sensitivity of the HT-J test was greater than faecal culture at the herd/flock level. To demonstrate this, results are shown in Table 2 for the exposed cattle tested at laboratory 2. Firstly all HT-J and faecal culture positive results are evaluated with the respect to each other. HT-J identified 73.39% (80/109) compared to faecal culture whereas only 44.94% of the total HT-J positives (80/178) were identified by faecal culture. When the HT-J and faecal culture positives were individually compared to the total number of all tested positive from both tests (n=207), faecal culture identified 109 (52.65%) and HT-J identified 178 (85.99%).

Table 2. HT-J results compared to faecal culture results for exposed cattle tested at laboratory 2. Note: Three times as many animals were HT-J positive/faecal culture negative (n=98) compared to faecal culture positive/HT-J negative (n=29).

HT-J	Faecal Culture		
	Positive	Negative	Total
Positive	80	98	178
Negative	29	643	672
Total	109	741	850

Discussion

The sensitivity of HT-J appeared to be greater than faecal culture in that it detected more animals from infected herds. However, the true infection status of these animals was not able to be determined in this project. The results suggest that the HT-J test will detect a set of animals that overlaps with those detected by faecal culture on an infected property. A comparison of the HT-J test with faecal culture, tissue culture and histopathology is required to better understand the nature of HT-J positive results that were faecal culture negative. It is already known that many animals with positive tissue culture results are negative in faecal culture because of stage of infection (Whittington and Sergeant, 2001).

The HT-J test provides results on a continuous scale, making it possible to set different positive negative cut-offs. There is an option to use different positive-negative cut-off points for different testing purposes. The selection of the cut-point affects both sensitivity and specificity and may need to be evaluated at individual laboratories to account for differences in equipment, such as real time PCR platform.

Overall, this study has demonstrated that the HT-J test is as sensitive as faecal culture; neither test will detect all infected cattle and both tests may detect overlapping subsets infected cattle. Furthermore, as both tests work best at herd level, sample sizes will need be chosen to suit the level of assurance required.

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PASSIVE MAP FECAL SHEDDING ATTRIBUTABLE TO SUPER-SHEDDER COWS

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Introduction

The detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in fecal samples has long been acknowledged as the gold standard diagnostic test of Johne's disease. With the recognition of super-shedder cows excreting up to 50 billion MAP organisms in their feces daily, we hypothesized that in some cows, culture-positive fecal samples could be attributed to the ingestion of MAP-contaminated feces from super-shedder cows, and result in passive shedding of MAP in the herd-mates.

Methods

The current report is based on a prospective longitudinal study of 3 dairy herds from the northeastern US states. Semi-annual individual fecal cultures and annual ELISA were performed on a total of 556 cows (Herd A: 325 cows, Herd B: 106 cow, Herd C: 125 cows) for a total of 4 years. Additionally, tissue samples (ileum, IC valve, and 2 ileo-cecal lymph nodes) from selected fecal-positive cows followed to slaughter were cultured for MAP to determine the extent of MAP tissue infection. For the purpose of this study, "passive shedders" were defined as cows with low to moderate numbers of MAP CFU/g feces on their initial HEYM fecal culture; with at least two subsequent negative fecal cultures; and a negative ELISA test.

Results

A total of 2,299 fecal samples were tested during the 4-year study period. Of the 556 cows tested, 78 were positive by fecal culture. Of those 78 positive cows, 15 were classified as "super-shedders" (> 1,000 MAP CFU/g feces), and 40 cows were classified as "passive shedders". Twelve of the 40 "passive shedder" cows were followed to slaughter. Of those 12, 4 had positive tissue cultures.

Discussion

Low level MAP passive shedding, attributable to the presence of one or multiple MAP super-shedders is more common than realized in many herds. Exposure to high levels of MAP (as evidenced by passive shedding) resulted in eventual MAP infection in a number of cows.

GAMMA INTERFERON RESPONSES TO PROTEOME-DETERMINED SPECIFIC RECOMBINANT PROTEINS: POTENTIAL AS DIAGNOSTIC MARKERS FOR PARATUBERCULOSIS

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Diagnosis of Johne's disease is problematic and no test can reliably detect both clinical and sub-clinical disease. It is thought that Th1 responses to *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) are the first to be activated with a later switch to a Th2 response and concomitant progression to clinical disease. Thus detection of a cell-mediated response, as indicated by measuring the levels of interferon gamma (IFN- γ) produced in response to mycobacterial antigens, may give an early indication of subclinical infection. Until fairly recently, crude extracts of *Map* (PPDj) have been used to detect the cell mediated response, however more specific, quantifiable antigens used as reagents would improve the specificity and reproducibility of the test.

A number of *Map*-specific proteins (as determined by proteome analysis) were screened for their ability to raise a cell-mediated immune response in subclinically infected ovine paratuberculosis. Thirty five *Map* proteins were expressed and used in the IFN- γ release assay of peripheral blood mononuclear cells isolated from subclinically infected animals. From this initial screen four proteins were selected and tested using peripheral blood of six subclinical animals and four animals from a paratuberculosis-free flock. Three of the four proteins selected had higher mean IFN- γ release in the subclinical animal group than the paratuberculosis-free control group. Thus these proteins have the ability to discriminate groups of infected and uninfected animals and as such have potential as novel reagents for the diagnosis of ovine paratuberculosis.

PARATUBERCULOSIS AND ROUTE IN CATTLE IN THE THREE (3) NORTHERN REGIONS OF CAMEROON

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From our recent research study, the prevalence of *mycobacterium avium* subsp. *Paratuberculosis* MAP in Cameroon is high in cattle herds in Cameroon imported from France and concentrated in the Three (3) Northern regions of Cameroon which are surrounded by Nigeria, Chad, and the Central African Republic. Many diagnostic tests were performed. In a survey, serology with an absorbed ELISA and faecal culture were performed on each imported animal. A large number of tests were also carried out for tracing on and back from known infected herds. Cattle with serological reactions in ELISA were tested with faecal culture or sent to slaughter and cultured from ileum and the ileocaecal lymph node. When *Mycobacterium paratuberculosis* was isolated from an animal, the whole herd was stamped out and ileum and ileocaecal lymph nodes were collected from a number of cattle at the abattoir for culture and histopathological examination. During 2007 - 2010 approximately 4000 animals were cultured and 8200 blood samples were tested in ELISA. *M. paratuberculosis* was isolated from 102 animals in 36 herds. Seventy-five of these animals were also tested serologically, 09 of which proved ELISA positive. Blood samples from a total of 132 animals were positive in ELISA. Thirty-Four of these animals were slaughtered and samples from ileum and the ileocaecal lymph node were cultured and histopathologically examined. *M. paratuberculosis* was isolated from Nine of these cases. Histopathological lesions were only found in cases with a strong serological reaction. The low sensitivity of the absorbed ELISA in this situation can be explained by an early stage of infection in most cases. Our observations demonstrates the difficulties in detecting early cases of paratuberculosis with the absorbed ELISA and the short comings to obtain exact results using diagnostic techniques to trace infected cattle.

A NOVEL ELISA TEST FOR DIAGNOSIS OF JOHNES DISEASE

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The lack of a reliable, sensitive, and rapid test for the detection of *Mycobacterium avium* ssp. *paratuberculosis* (MAP), the causative agent of Johne's disease (JD), is a major barrier impeding the effective control of this economically devastating disease in dairy cows. The diagnosis of MAP is difficult because of the organism's fastidious slow growth *in vitro* and the lack of a specific diagnostic test that is sensitive enough to detect most subclinical yet infected cattle that are intermittently shedding pathogen. Currently, the cattle industry does not have a rapid nor reliable JD test for cattle¹, and the gold standard test, fecal culture, has a long turnaround time (up to 16 weeks). Due to its relatively short turnaround and low cost, the most commonly used diagnostic test for MAP is ELISA, which is used to detect the presence of MAP-specific antibodies. However, its sensitivity is very low (15%-30%) in subclinically infected low fecal-shedding cows² with specificity of less than 100%^{3,4}. Therefore, it is crucial to develop a screening test for cattle that is rapid, highly specific, and sensitive.

The objective of this study was to identify the test parameters, particularly sensitivity and specificity, for a novel ELISA using novel antigens from MAP.

MAP resides, survives, and multiplies within macrophages⁵ as an intracellular parasite circumventing all the efforts of macrophages to kill the engulfed microorganism. Within macrophages the bacillus has to modulate complex immunological pathways and response mechanisms in the host cell.

This interference is carried out solely through the secretion of virulence factors within the cytosol of the macrophage. We have reported that the Protein Tyrosine Phosphatase A (PtpA) of MAP is actively secreted within macrophages in the first 24 hours post-infection⁶. Additionally, PtpA dephosphorylates the host vacuolar sorting protein VPS33B necessary for phagosome-lysosome maturation⁷. Therefore, our central hypothesis is that antibodies against PtpA will be found in the animal serum because the protein has to be constantly secreted within macrophages.

To test our hypothesis, the recombinant antigen (PtpA) was produced and used to create the ELISA. Sera from 269 cows were used for evaluation. To establish specificity, 230 cows from long-term test negative herds were considered true negatives after fecal culture evaluation. To examine sensitivity, sera from 39 adult cows known to be positive by conventional serum ELISA, subsequent fecal culture, and fecal PCR were used. Using this sample structure, the relative sensitivity of the novel ELISA compared to conventional ELISA and culture was established. Preliminary evaluation indicated that there was an extremely high level of agreement between the methods. Using the fecal culture results as gold standard, the novel antigen ELISA showed a high sensitivity and specificity. A Receiver Operating Characteristic (ROC) was used to analyse the ELISA results.

An area under the ROC curve of 0.9506 was calculated (p value <0.0001) (Figure 1), with a cut-off of 0.067 (0.95 confidence). Statistical analyses using ROC calculated a sensitivity of 0.97 (95% CI- 89.16%-99.62%) and a specificity of 0.9807 (95%CI- 13%-99.47%).

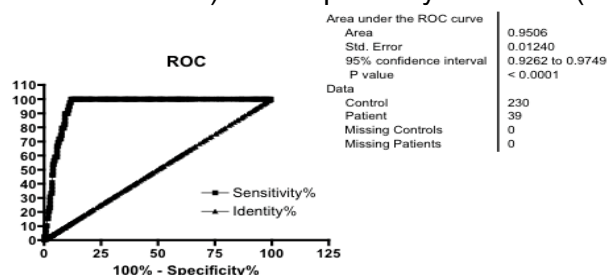


Figure 1. Calculation of sensitivity and specificity of the novel ELISA using ROC analysis.

To analyse whether this novel ELISA can be used for early MAP detection we randomly selected 9 samples that showed "negative" results according to the ELISA commercial kit, but showing "positive" reading as recorded with our assay (cut-off >0.067). These

Results demonstrate that 78% (7 out of 9 samples) of animals testing negative using the commercial ELISA are positive for MAP PtpA protein and positive to suspect on PCR of fecal samples (Table 1). These

results suggest that antibodies specific to proteins secreted at an early stage of MAP infection can be used for detecting subclinical cattle.

Table 1. Fecal PCR analysis in JD negative animals (commercial kit) vs. PtpA JD positive animals.

Sample ID	Commercial ELISA* (OD)	PtpA ELISA (OD)	PCR on fecal samples [#]
93	0.063	0.304	Negative
218	0.06	0.336	Suspect
228	0.05	0.268	Suspect
793	0.071	0.2775	Negative
1016	0.059	0.236	Suspect
1075	0.059	0.214	Positive
1465	0.071	0.246	Positive
1632	0.05	0.214	Suspect
2515	0.057	0.260	Positive
FCS (negative control)	-	0.062	-

*Optical density of < 0.100 is referred as negative. [#]Samples were run in duplicates-PCR termination cycle number =42. Positive: both samples positive; Negative: both samples negative; Suspect: one sample negative, the other appearing late (~cycle 38), perhaps indicating a single cell. Test specificity = 100%. Ct < 38 for ABI and BioRad series of real-time PCR thermocyclers.

To further validate our results, selected animals were followed up. Results shown in Table 2 indicate that MAP was detected before that commercial ELISA and fecal PCR tests.

Table 2. ELISA and fecal PCR performed at different times in selected animals.

Sample	Date	ELISA (IDEXX)*	PtpA ELISA**	Fecal PCR	Early detection (months)
1922	06/25/08	0.064	0.1265	NP	22
	06/24/09	0.045	0.0895	NP	
	12/01/09	0.067	0.116	Negative	
	04/01/10	0.057	0.1035	Positive	
2233	04/30/08	0.081	0.100	Negative	12
	04/08/09	0.423	0.0915	Positive	
2514	04/09/08	0.058	0.1065	Negative	24
	06/04/09	0.069	0.096	Negative	
	04/01/10	0.072	0.099	Positive	
276	01/06/09	0.056	0.043	NP	Negative
	05/06/09	0.049	0.052	NP	
	12/01/09	Negative	0.059	Negative	
580	07/12/08	0.288 (Positive)	0.065	NP	Negative
	05/06/09	0.485 (Positive)	0.059	NP	
	12/01/09	Negative	0.062	Negative	
	04/01/10	Negative	0.064	Negative	
1941	12/01/09	Negative	0.059	Negative	Negative
	04/01/10	Negative	0.065	Negative	

*Optical density (<0.100-negative), **Optical density (average of triplicate). NP=not performed. Ct < 42 for Cepheid, Roche and Stratagene real-time PCR thermocyclers

In conclusion, the use of this novel ELISA for the detection of MAP serum antibodies is significantly more sensitive than commercially available serum ELISA tests and can detect antibodies against MAP prior to substantial fecal shedding of the organism. This will provide improved control of JD by rapid identification and elimination of infected animals from the herd. A bigger cohort of animals is necessary to validate the test.

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THE HOLY GRAIL OF MAP DETECTION: A SPECIFIC ANTIBODY

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Due to a close genetic relatedness, there is no known antibody that detects *M. avium* subspecies *paratuberculosis* (MAP), which causes Johne's disease in cattle and sheep, and does not cross-react with other *M. avium* subspecies. In the present study, a monoclonal antibody was identified from mice immunized with a cell membrane fraction of MAP strain K-10. This antibody is 100% specific as it detected a 25-kDa protein in all 30 MAP whole cell lysates, but did not bind to any of the 30 non-paratuberculosis strains tested in immunoblot assays. However, the antibody revealed variable reactivity levels in MAP strains as it detected higher levels in bovine isolates but comparably lower levels in ovine isolates of MAP. In order to identify the target binding protein, a lambda phage expression library of MAP genomic fragments was screened with the monoclonal antibody. Four reactive clones were identified, sequenced and all shown to be overlapping. Further analysis revealed all four clones expressed an unknown protein encoded by a sequence that is not annotated in the K-10 genome and overlapped with MAP3422c on the opposing DNA strand. This putative ORF was termed UP1, but its arrangement and size suggested it was not expressed. The antibody epitope on UP1 was precisely defined to 7 amino acids and was used to query the K-10 genome. Similarity searches revealed another protein, encoded by MAP1025, possessed a similar epitope (1-amino acid mismatch) that also reacted strongly to the antibody. A single nucleotide polymorphism (SNP) in MAP1025 was then identified by comparative sequence analysis, which results in a Pro28His change at residue 28, the first amino acid within the epitope. This SNP is present in all MAP strains but absent in all non-MAP strains and accounts for the specificity of the antibody. This new antibody is the first ever isolated that binds only to the paratuberculosis subspecies of *M. avium* and opens new possibilities for the specific detection of this significant ruminant pathogen.

THE DEVELOPMENT AND EVALUATION OF NEW RAPID METHODS FOR DETECTING VIABLE MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS FROM DAIRY PRODUCTS

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Mycobacterium avium subsp. *paratuberculosis* (MAP) may have a role in the development of Crohn's disease in humans. The presence of MAP in dairy products has been demonstrated using both conventional culture and rapid DNA detection-based methods and therefore this poses a potential health risk to humans. Recently we have successfully developed a new combined bacteriophage-PCR method for detecting MAP in milk samples. Here we describe the development of a new protocol that also allows the use of this assay for the identification of MAP from cheese. The phage-based methods have the advantage over PCR-based methods that they only detect the presence of viable MAP cells. Both bacteriophage-PCR assay formats were tested on milk and cheese samples. Results from the testing of bulk milk samples suggested that this new method is far more sensitive than the conventional culture as the method removes the need for heavy chemical decontamination of samples prior to testing known to destroy a large proportion of the MAP cells. In these raw milk samples the prevalence results gained were very similar to those achieved using a PCR-based detection method alone, and both methods gave a much higher prevalence rate than conventional culture. In contrast in cheese samples, where the MAP cells present in the milk may be inactivated, the PCR-based method appeared to give a higher prevalence than the phage-based method, showing the benefit of using a method that can discriminate between live and dead cells when sampling processed foods. This new way of detecting MAP bacteria from food matrixes using natural bacteriophages is a very promising tool in food that can rapidly identify the presence of viable MAP in less than 48 h with an increased sensitivity. When combined with PCR-based identification tests the method also has very high specificity

COMPARATIVE DIAGNOSTIC POTENTIAL OF MICROSCOPY, CULTURE AND DIRECT IS900 PCR FOR THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN FECAL SAMPLES OF DOMESTIC LIVESTOCK SPECIES IN INDIA

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Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) adversely affects animal productivity has been found endemic in herds of domestic livestock in most of the countries, including India. Effective control of JD in resource limited countries has been hampered by the lack of effective and cost effective diagnostic tool. Therefore, present study was aimed to evaluate the diagnostic potential of microscopy, culture and direct IS900 PCR for the detection of MAP in fecal samples of domestic livestock. A total of 66 fecal samples (32 from Cattle, 11 from Buffalo and 23 from Goat) were collected from domestic livestock species and screened for the presence of MAP using microscopy, culture on Herrold's egg yolk Medium and direct IS900 PCR. Performance of diagnostic methods were compared by calculating Kappa Scores (0 < poor; 0.0-0.20- slight; 0.21-0.40- fair; 0.41-0.60- moderate; 0.61-0.80- substantial and 0.81-100- almost perfect). Of the 66 fecal samples, 31 (46.9%), 6 (9.0%) and 16 (24.2%) samples were positive for the presence of MAP using microscopy, culture and direct IS900 PCR, respectively. Species-wise, 40.0, 9.3, 28.1%; 54.5, 0.0, 18.1% and 52.1, 13.0, 21.7% animals were positive for MAP using microscopy, culture and direct IS900 PCR from cattle, buffaloes and goat, respectively. Out of 66 samples, 3.0% (2/66) and 50.0% (33/66) samples were positive and negative in all the three tests, respectively. There was an agreement of 53.0% and mismatch of 46.9%, in these three methods. Independently, 19.6, 1.5 and 1.5% samples were detected as positive by microscopy, culture and direct IS900 PCR, respectively. In combination higher number of positives were detected by microscopy & direct IS900 PCR (22.7%) as compare to microscopy & culture (9.0%), and culture & direct IS900 PCR (3.0%). Statically, moderate (Kappa value- 0.4682), fair (Kappa value-0.2444) and slight (Kappa value-0.0571) agreement was found between microscopy & IS900 PCR, microscopy & culture and culture & direct IS900 PCR combinations, respectively. Present study indicated that 'multiple tests' based diagnostics are necessary for the accurate diagnosis of JD in animal herds, however, the combination of microscopic examination and direct IS900 PCR can be used for the screening of domestic livestock species in resource limited countries, where cost is limiting factor. Acknowledgement: Authors are thankful to Indian Council of Agricultural Research, New Delhi for financial support (Grant No F N 14(1) 2009-2010).

VALIDATION OF THE ID SCREEN INTERFERON GAMMA CAPTURE ELISA: INTRODUCTION OF A STANDARD REFERENCE CONTROL TO IMPROVE RESULT INTERPRETATION

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Detection of Interferon gamma (IFN-g) by capture ELISA is widely used to detect the cellular response to pathogens such as *Mycobacterium avium* subsp *paratuberculosis* by measuring the difference between activated and inactivated whole blood or peripheral Blood Mononuclear Cells (PBMC) IFN-g signals. Results, expressed as the difference between raw optical densities, are not generally linked to a stable reference control.

IDVET has developed an Interferon Gamma Capture ELISA, which contrary to other commercial ELISAs, expresses the level of IFN-g with respect to a standardised, freeze-dried positive reference control. This relative expression of the measured quantity of IFN-g guarantees the standardisation of results between runs and kit batches. Results are expressed as sample / positive reference control (S/P) ratios. Kit validation data will be presented, including: - “specificity” S/P results obtained for inactivated plasma and serum populations - analytical sensitivity for samples activated by both specific and non-specific antigens, and comparisons with other commercial kits. - results obtained with the IDVET kit from a Map-infected goat herd further to sample activation by different antigens.

The new IDVET ELISA, which detects bovine, ovine and caprine IFN-g, has improved upon current IFN-g ELISA technology by incorporating a standard positive reference control for better result interpretation.

DETECTION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN BACTRIAN CAMEL (CAMELUS BACTRIANUS) BY POLYMERASE CHAIN REACTION IN IRAN

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Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease in domestic and wild ruminants like, cattle, sheep, goats, deer, antelope and bison worldwide (Stabel, 1999). Johne's disease (JD), also called paratuberculosis, is one of the most economically important diseases of dairy cattle, costing over \$250 per cow annually in highly infected herds (Ott et al., 1999). Johne's disease affects camels worldwide causing characteristic clinical illness of severe diarrhea ending in death (Manefield and Tinson, 1997; Wernery and Kaaden, 2002). The course of disease is often more rapid than that in cattle (Higgins 1986). There are no published reports of JD in camels in Iran. It is unlikely to exist within the Ardebil camel herd. Long incubation period is the main characteristic feature of MAP infection (Stabel, 1999). To reduce the infection rate in a herd, the test and cull strategy of JD control programs require sensitive and specific diagnostic techniques. Fecal culture is considered as the gold standard for the diagnosis of MAP infected animals but requires 12–16 weeks (Stabel and Whitlock 2001); therefore the development of a rapid, sensitive and specific diagnostic method for the detection of Map is essential in the control of Johne's disease in economically important animals (Vansnick et al., 2007). The IS900 element is an insertion sequence considered to be a MAP-specific gene with 15–20 copies per genome and is a target for rapid detection of MAP by PCR (Collins et al., 1989; Ikonomopoulos et al., 2004). The aim of this study was to detect MAP in apparently healthy Bactrian camels (*Camelus bactrianus*) by PCR and acid fast staining of faecal samples. A total of 26 faecal samples from Bactrian camels at different ages from Ardebil province, Iran were taken, and stored at -20 °C until use. For Ziehl-Neelsen staining, fecal smears were stained for 1 hour with TB carbol fuchsin Ziehl-Neelsen acid-fast stain (Quinn et al., 1994). DNA was extracted from all fecal samples as previous protocol (Stabel et al., 2004). IS900 PCR was conducted as described by Corti and Stephan (2002) with the primers P90, 5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3' and P91, 5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3'. Using the Ziehl-Neelsen acid-fast staining technique, *M. paratuberculosis*-infected fecal samples were identified by the red staining of bacteria. Acid fast staining results showed that only 4/26 (15.3%) samples were suspected to MAP while PCR analysis showed no bands corresponding to MAP in all samples.

Mycobacterium paratuberculosis has been detected in dairy herds throughout Iran (Anzaby et al., 2006; Pourjafar and Badiei, 2005; Kasravi and Nowrouzian, 2004; Khodakaram Tafti and Rashidi, 2000). In this study, we used a rapid and simple DNA-extraction methods for the detection of MAP in fecal samples were taken from *Camelus bactrianus* (Stabel et al., 2004). Acid-fast staining of suspect tissues is rapid and requires little optimization. However, Ziehl-Neelsen staining has been reported to falsely identify *Nocardia* and *Corynebacteria* and cannot differentiate among the various mycobacterial species. Previous reports have determined the sensitivity of Ziehl-Neelsen to be 36.4 % (Zimmer et al., 1999). Although we showed presence of MAP infection in the Ardebil province, Iran 15.3% based on acid fast staining but acid fast staining could not clear cut the presence of MAP completely. To the best of our knowledge, this study is the first description of a work of this kind performed in Iran. Traditionally, fecal culture for MAP is considered as the gold standard for diagnosis. However, fecal culture is time-consuming and detects only 38–50% of cows infected (Stabel, 1998; Whitlock et al., 2000). Evidence showed that the PCR was found to be more sensitive than the bacterial culture in detection of organisms in water buffalo. Use of PCR methods in contrast to culture and serological tests, allowed to detect nonviable as well as viable micro-organisms and would be a more sensitive detection method (Sivakumar et al. 2005; Stabel et al., 2002; Englund et al., 1999; Djonje et al., 2003). Therefore, in comparison with serologic or culture methods, detection of MAP directly from fecal samples by IS900 PCR could be considered a valuable test for presence of MAP in fecal samples. Our results showed no bands on faecal samples by IS900 PCR, so we could conclude that the all *Camelus bactrianus* for our region are free of infection but further research is required.

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EVALUATION OF AN ELISA FOR THE DETECTION OF ANTIBODIES AGAINST *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

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Introduction

The objective of this study was to evaluate the diagnostic performance of SERELISA M. ParatB Mono Indirect (Synbiotics Corp., Lyon, France) in serum samples from cattle and goats.

Material and Methods

750 blood samples from clinically unsuspecting cattle were selected randomly. Additionally blood samples

from 99 clinically suspicious cattle and 13 goats suffering from diarrhea and emaciation were chosen.

These samples were sent to the IVET Linz as part of the Austrian paratuberculosis control programme.

All sera were examined with SERELISA M.ParatB Ab Mono Indirect and Paratuberculosis Screening ELISA (Institute Pourquier, Montpellier, France).

Adiavet Paratb Realtime PCR Kit (Adiagene, Saint Brieuc, France) for the detection of MAP in faeces respectively tissue samples was used for testing 106 clinically suspicious animals as well.

Results

744 samples of the clinically unsuspecting cattle tested negative, 1 doubtful and 5 positive with the Synbiotics ELISA. The Pourquier ELISA identified 744 samples as negative, 4 as doubtful and 2 as positive.

The two tests agreed on 739 samples. 92 of 112 samples from clinically suspicious animals were classified

as positive and 20 were classified negative by Synbiotics ELISA. The Pourquier ELISA identified 91 samples as positive and 21 as negative. The results of both ELISA agreed on 107 of 112 tested samples. Table 1 shows the comparison of results from Synbiotics ELISA and Real-time PCR.

Tab. 1: Comparison of Synbiotics ELISA and Real-time PCR

	Synbiotics ELISA neg	Synbiotics ELISA pos	Σ
MAP PCR neg	6	2	8
MAP PCR pos	12	86	98
Σ	18	88	106

Conclusion

The sensitivity detecting MAP PCR positive animals by Synbiotics ELISA was 87.8% whereas the specificity in the study group of 750 clinically unsuspecting cattle was 99.2 %. Assay agreement between both tested ELISA was relatively high. Therefore the Synbiotics ELISA can be recommended for the detection of MAP specific antibodies in routine laboratory diagnosis.

PARA-LP-01 AS A DIAGNOSTIC TOOL FOR A CELLULAR IMMUNE ASSAY TO DETECT JOHNE'S DISEASE

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Johne's disease (JD) is caused by *Mycobacterium avium* subsp. *paratuberculosis*, a lipid-rich pathogen. JD is a chronic granulomatous enteritis in domestic and wild ruminants. JD poses a significant problem in animal health, and this is underscored by its extremely high prevalence in US dairy herds, with 95% average for large dairy herds and an average prevalence of 68.1% for all dairy operations.

Most diagnostic approaches are particular of serological diagnostics and rely on crude antigen extracts (as in existing diagnostic tests) or focus on immunogenic proteins as new diagnostic tools. Although mycobacteria consist of at least 40% lipids, more than any other bacteria, lipids are widely ignored as to their ability to induce specific immune responses and their potential use for diagnostics is in its infancy. It is widely accepted that mycobacterial diseases induce primarily cellular immune responses and only late during infection humoral immune responses can be used as tools for diagnostics.

Previously, we identified a major cell wall immunogenic lipid (Para-LP-01) that could be used as a diagnostic tool for a serological diagnostic test. The newly designed Lipid-ELISA yielded in an improved sensitivity and specificity when compared with commercially available test, but the sensitivity is still not in the same range as the specificity data. Diagnostic tests based on the cellular immune responses are clearly needed to support herd management at an early time point.

Here we show the use of Para-LP-01 as a diagnostic tool for a cellular immune assay measuring induced interferon gamma in an ELISpot assay for PBMCs.

Clinical examination of the herd identified at least five out of 15 animals that have Johne's disease. While the serology identified only one infected animal from a small local herd of nubian goats, the IFN γ ELISpot identified all five animals. Further evaluation with defined animals is needed.

PARASAFE – A NEW LIPID-BASED ELISA FOR DIAGNOSING CATTLE WITH JOHNES DISEASE

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Johne's disease (JD) posed an increasing problem within the dairy industry not only in the US but also in other developed countries. It currently ranks as one of the most costly infectious diseases of dairy cattle in the USA. Infection with MAP usually occurs after birth followed by a long incubation period of two to five years in which the animals are presumably healthy, non-shedding, and without any symptoms hinting toward the development of JD. Following the incubation period, MAP-infected cattle develop disease with the characteristic symptoms of chronic diarrhea (with shedding) and weight-loss, loss of milk production, and finally death.

There are no specific tools for the prevention or treatment of JD. The only effective control measures are culling the infected animals and/or instituting good herd management practices. Although the key immune response of infected animals is a cellular immune response, due to financial restrictions of dairy farms within the US, serology of JD is still the most common diagnostic approach. The currently available ELISAs in the US are based on a mixture of crude aqueous antigens and require preabsorption and comparison with positive control sera.

Here we present our new diagnostic ELISA for JD based on a lipid analogue that avoids additional steps including preabsorption. Several hundred defined positive and negative bovine sera were analysed. In addition, large local dairy herds were analysed for their disease management.

The new ELISA has high reproducibility and increased sensitivity with a nearly 100% specificity. The new diagnostic test detects especially animals in early stages of Johne's disease and thus is an excellent diagnostic test for controlling the chronic disease within dairy herds.

EVALUATION OF REAL-TIME PCR FOR THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN ALPACAS AND THE PREVALENCE OF JOHNE'S DISEASE IN ALPACAS PRESENTED TO FOUR VETERINARY TEACHING HOSPITALS IN THE UNITED STATES

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INTRODUCTION

The prevalence of Johne's disease in alpacas in the United States is unknown. Real-time polymerase chain reaction (RT-PCR) has been used in cattle to quickly and accurately identify MAP in fecal specimens. The limits of detection of PCR in alpaca feces have not been determined. The objectives of this study were to validate the PCR test for use in alpaca feces, and define the limits of MAP detection in these samples; and to estimate the prevalence of MAP fecal shedding in alpacas presented to 4 veterinary teaching hospitals in the US.

MATERIALS AND METHODS

Ten dilutions of a wild MAP strain were added to negative alpaca feces and processed for MAP detection using a commercial RT-PCR assay, and cultured on Herrold's Egg Yolk Medium (HEYM) and MGIT liquid broth. The limits of detection for each method were determined. Fecal samples from alpacas presenting to 4 US veterinary teaching hospitals from November 2009 through February 2011 were processed for MAP via PCR and HEYM.

RESULTS

The lowest MAP dilution detectable via PCR was 243 MAP CFU/g of feces. At that concentration, MAP growth was detectable on HEYM. The lowest dilution of MAP to trigger a TTD in MGIT culture medium was 1,216 CFU/g of feces. Ten (6%) of the 180 fecal samples collected from hospitalized alpacas were positive on PCR. The 95% confidence interval was between 3% and 9%.

CONCLUSIONS

These results indicate that PCR can provide an accurate and rapid detection of MAP fecal shedding in alpacas; and the prevalence of MAP fecal shedding in hospitalized alpacas in US veterinary teaching hospitals is low.

MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN WOOD BISON: DIAGNOSTIC OPTIMIZATION AND HERD-LEVEL OCCURRENCE

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Introduction

Wood bison (*Bison bison athabasca*), one of two subspecies of North American bison, are listed as threatened by the Committee on the Status of Endangered Wildlife in Canada.¹ Although wood bison once ranged in the hundreds of thousands across most of the northwestern corner of the continent, today only 11000 wood bison remain in the wild. The translocation of bison between existing herds or their introduction to new locations has been an essential part of wood bison conservation, resulting in the creation of conservation herds and leading to an increase in population size. However, there are certain risks involved in translocating animals, including exposing naïve animals to new pathogens, or inadvertently introducing infectious pathogens such as MAP to new areas or populations.² Initial research found unexpectedly high MAP prevalence in some herds using direct faecal polymerase chain reaction (PCR).³ However, attempts to culture the organism were unsuccessful. Since the strain(s) infecting wood bison in northern Canada have not been investigated in detail, it is unknown whether a genotypically distinct form of MAP with different culture requirements is present. It has been shown that small variations in culture protocols can alter the sensitivity of an assay, and that growth requirements may differ depending on the strain.^{4,5} We hypothesize that it may be possible to cultivate MAP from wood bison samples by varying the culture conditions used. The objectives of this study are 1) to validate a direct faecal PCR protocol to screen for infected animals, and 2) to optimize culture conditions for detecting MAP in bison faecal and tissue samples from animals that tested positive by direct PCR, with the overall aim of describing the infection status of MAP among Canadian wood bison herds in order to facilitate informed conservation management.

Materials and Methods

Sample Collection

Samples were collected between 2008-2011 by the authors and by various wildlife partners from the governments of the Northwest Territories (NWT), Alberta, and British Columbia. Faecal samples were collected fresh from the ground or per rectum using a new glove for each collection. Tissue samples (n=60) were collected from hunted animals or from animals culled for herd health monitoring. Samples collected were terminal ileum and ileo-caecal lymph node (2008-2010), and in 2011 additionally jejunum and mesenteric lymph nodes. Samples were stored at -20°C until shipped to the University of Calgary where they were stored at -80°C until processed.

Direct Faecal PCR

DNA was extracted from faecal samples using the MagMAX extraction kit as per manufacturer's instructions. qPCR was performed using two separate duplex reactions for IS900 and F57 targets that include an internal amplification control as previously described by Kralik et al.⁶

Culture Panel set-up

In order to maximize the possibility of successfully culturing MAP from bison samples, a panel of different culture conditions was used (Table 1). Faecal samples from wood bison (n=2) positive on direct faecal PCR were tested, along with positive and negative bovine faecal samples from the USDA MAP proficiency panel as controls. Four different culture media were included: 1) Middlebrook 7H9 based media used in the TREK ESP liquid culture system, which is our current laboratory standard, 2) Herrold's Egg Yolk (HEY) medium with mycobactin, which has long been the standard medium used for MAP culture in many laboratories, 3) Lowenstein Jensen (LJ) medium with mycobactin, and 4) Middlebrook 7H11 with mycobactin, the latter two which have been shown to allow for maximum recovery of Type I and III MAP strains when used in combination.⁴ Double incubation was used as the standard MAP concentration protocol (protocol a),⁷ while the double centrifugation method⁸ was performed on a subset of each sample prior to inoculation onto each of the different media (b). In the standard double incubation method, samples are decontaminated overnight in an antibiotic cocktail containing vancomycin, amphotericin B, and naladixic acid (VAN). However, since vancomycin has been shown to

be inhibitory to sheep strains,⁹ and even to particular isolates,¹⁰ a variation on the antibiotics used at this step (PANTA) was performed for each of the media (c). Finally, different supplements were included in or omitted from the standard protocols. Pyruvate was added to a subset of the LJ and 7H11 slants and liquid 7H9 media (d), since it has been shown in some cases to improve culture sensitivity⁴ or alternatively inhibit the growth of certain strains.¹¹ Other variations made to the 7H9 liquid culture were to omit the addition of egg yolk supplement (e), to add heat-inactivated fetal bovine serum which has been previously shown to enhance the growth of *Mycobacterium tuberculosis* (f),¹² or to add filter-sterilized culture supernatant from actively growing MAP culture, which we hypothesize may contain compounds which could enhance the growth of MAP (g). All four faecal samples were inoculated onto the 18 different culture variations, using duplicates for all solid media. Samples will be cultured for a total of 6 months, with observations made of solid media every two weeks. Any growth will be confirmed by PCR and acid-fast staining of isolates. A similar panel of culture conditions will be run using tissue samples from these same animals.

Table 1: A total of 18 different culture conditions were tested for their ability to grow MAP from bison faecal samples positive by direct PCR (n=2), using positive and negative cattle faecal samples as controls.

Medium	a)Standard	b) Double centrifugation	c) PANTA	d) Pyruvate	e) Egg yolk omitted	f) Fetal bovine serum	g) Culture supernatant
1. 7H9	X	X	X	X	X	X	X
2. HEY	X	X	X				
3. LJ	X	X	X	X			
4. 7H11	X	X	X	X			

Discussion

Once culture conditions have been optimized, samples from eight wood bison herds will be tested to determine the herd-level distribution of MAP. Genotyping of isolates will also be pursued in order to investigate possible genetic differences associated with differing growth requirements. Understanding the distribution and genetic diversity of MAP among Canadian wood bison herds will help facilitate informed conservation management for this threatened species.

Acknowledgements

Funding for this study was provided by the Alberta Conservation Association Grants in Biodiversity, the Natural Sciences and Engineering Research Council of Canada, and the Arctic Institute of North America. We thank the many collaborators who contributed to sample collection, including Terry Armstrong, Karl Cox, and Nic Larter of the Government of NWT, Jane Harms of the University of Saskatchewan, Helen Schwantje of the Government of British Columbia, Lyle Fullerton and Mark Ball of the Government of Alberta, and Rhona Kindopp and Archie Handel of Parks Canada.

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IMMUNOGENICITY EVALUATION OF *IN SILICO* IDENTIFIED MAP RECOMBINANT PROTEINS THAT WERE UPREGULATED UNDER STRESS CONDITIONS

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Mycobacterium avium subsp *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD) in ruminants. It is known to enter a dormant phase outside the host typically on soil (Whittington et al., 2004). Survival inside the host macrophage is a hallmark of MAP infection and dormancy may play a role in this survival. *In vitro* experiments have reported regulation of certain MAP proteins when exposed to stressors similar to dormancy (Gumber and Whittington, 2009). It is believed that *in vivo* regulation of dormancy genes and associated proteins by MAP may play an important role in evading the host defence mechanisms and the host may also mount an immune response against these dormancy related proteins. Evaluation of such proteins may provide insight to host-pathogen interaction during the course of MAP infection. A group of dormancy genes upregulated under stress conditions were examined using *in silico* analysis to identify B and T-cell epitopes. Five potential candidate genes based on epitope prediction results were selected and cloned: three hypothetical proteins and two proteins involved in fatty acid metabolism. Recombinant proteins were produced, purified and evaluated for their immunogenicity using a panel of sera from sheep with a spectrum of JD and sheep free of MAP infection, by detection of host specific antibodies in ELISA. Individually, the five proteins were found to have the ability to partially discriminate between sera from sheep unexposed and exposed to MAP infections. *In silico* analysis of genes is a rapid approach for functional characterisation and discovery of novel antigens for MAP diagnosis.

CORRELATION BETWEEN A COMMERCIAL REAL-TIME PCR ASSAY AND HERROLD'S EGG YOLK MEDIUM CULTURE FOR MAP IN BOVINE FAECAL SAMPLES

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Abstract: Disease control programmes for MAP rely on accurate and sensitive tools for the detection of infected animals. Culture based detection of MAP takes many weeks whereas PCR enables rapid detection. Several commercial and many user designed real-time PCR assays exist for the detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in bovine faecal samples. We selected one commercial assay, the VetMAX™ MAP Real-Time PCR Screening Kit (Life Technologies), and calculated the correlation between real-time PCR threshold cycle (Ct) values and colony-forming units (CFU) on Herrold egg yolk medium (HEYM) culture, using different nucleic acid extraction kits. Results of HEYM culture of 40 faecal samples were negatively (inversely) correlated with their respective real-time PCR results. The Spearman's rank correlation between Ct and CFU ranged from good (0.67) to excellent (0.93), depending on which nucleic acid extraction kit was used. The MagMAX™ Total Nucleic Acid Isolation Kit (Life Technologies) and the InviMag® Stool DNA Kit (Invitek) produced the best correlations with HEYM culture. These results suggest that this real-time PCR assay is a useful alternative to culture on HEYM.

Introduction: Numerous studies have been conducted to evaluate the accuracy (sensitivity and specificity) of the tests available to detect MAP.¹ Culture on HEYM has for a long time been considered the gold standard test for MAP because it is highly specific.¹ However culture is a very slow process with results taking 6 weeks or more. Recent studies have shown that real time PCR is a highly accurate alternative to HEYM for the detection of MAP in bovine faeces.^{2,3} The VetMAX™ MAP Real-Time PCR Screening Kit is a complete set of reagents for a simple real-time PCR assay. This assay is intended for use in the rapid, *in vitro* detection of MAP DNA purified from bovine feces. The assay targets a sequence element in the MAP genome to provide highly sensitive and specific results. This study looked at comparing the performance of different nucleic acid extraction kits when coupled with this screening kit to traditional HEYM culture for the detection of MAP in bovine faeces.

Materials and Methods: Forty bovine faecal samples were supplied by the Oregon Department of Agriculture Animal Health Laboratory. The MAP culture status of these samples had been determined in the Oregon laboratory using HEYM. Faecal samples were transported to Austin and stored at -20°C. For sample preparation, the following DNA isolation kits were used: MagMAX™ Total nucleic acid purification kit (Life Technologies), InviMag® Stool DNA Mini Kit (Invitek) and the QIAamp® DNA Stool Mini Kit (Qiagen). The kits were used according to the manufacturers' instructions. The MagMAX™ purifications were performed manually and using an automated platform, the MagMAX™™ Express - MME24 (Life Technologies). The InviMag purifications were performed on an automated platform, the KingFisher 96 instrument (Thermo Electron). The Qiagen purifications were performed manually.

In order to monitor extraction efficiency and to enable detection of PCR inhibitors, 1 µL of Xeno™ DNA Control (5,000 copies/µL) was added per isolation to the lysis solution. Presence of PCR inhibitors would be seen by failure of both MAP Control DNA and Xeno™DNA Control to amplify. The real-time PCR was set up, according to the manufacturer's instructions. Briefly, 25 µL reactions were prepared, and the assay was run on the Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies).

In the second part of this study, data gathered from the USDA Johne's disease proficiency panels from 2008 – 2011 was collated. The panels were assessed in the year of their issue using the MagMAX™ Total nucleic acid purification kit and the VetMAX™ MAP Real-Time PCR Screening Kit. Results of the testing, including the average CFU/tube for each sample which was determined by the NVSL using HEYM, were collated. For statistical analyses, JMP® software was used. We used Spearman's rank correlation to assess the relationship between CT value and colony count. This statistic

is a non-parametric measure of the strength and direction of association that exists between two variables measured. It has been used in the literature for similar comparisons.³

Results: There were 20 negative samples and 20 positive samples with colony counts ranging from 8 CFU/ml to 1000 CFU/ml. As a measure of extraction efficiency, Xeno™ DNA detection in samples was assessed. All extraction kits and samples produced positive Xeno™ DNA results except for Sample 35 which is the only sample to give a negative Xeno™ result on the MagMAX™ Manual extraction. It was negative for MAP DNA across all of the extraction kits and platforms. The detection of Xeno™ DNA also provides a monitor for PCR inhibition.

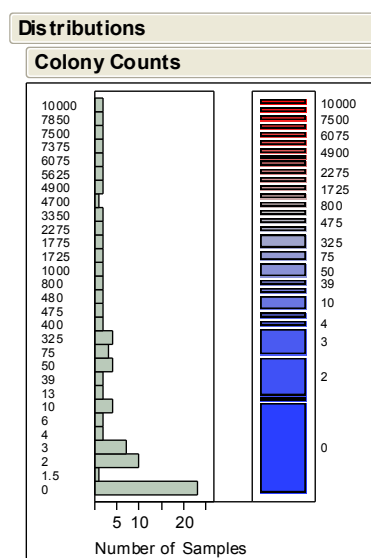
Table 1: Spearman's rank correlation for colony count (CFU) and threshold cycle (CT) values of 40 samples tested for MAP using culture on HEYM and the VetMAX™ MAP Real-Time PCR Screening Kit, utilising different extraction kits. The criteria for interpreting the Spearman's rank correlation which ranges from -1 to +1 were as follows: greater than absolute value of 0.75 as excellent, less than absolute value of 0.40 as poor, and for absolute values between 0.40 and 0.75 as fair to good correlation.⁴ (* all p values < 0.001)

extraction kit	Spearman's rank correlation*	Interpretation
Qiagen	- 0.676	good correlation between tests
Invitek	-0.918	excellent correlation between tests
MagMAX™ - manual	-0.931	excellent correlation between tests
MagMAX™ – MME24	-0.924	excellent correlation between tests

Figure 1: Histogram of the colony counts (CFU/tube) for 97 bovine faecal samples tested as part of the USDA Johne's disease proficiency panels from 2008 – 2011. There were 23 MAP negative samples and 64 samples with colony counts ranging from 1.5 CFU/tube to 10,000 CFU/tube. Spearman's rank correlation between colony count and CT was -0.913 (p < 0.001).

Conclusions: Results of the current study show that the correlation between quantitative MAP results from real-time qPCR (CT) and culture on HEYM (CFU) was excellent when using either the MagMAX™ or Invitek extraction kits, and good if using the Qiagen kit. The excellent correlation between colony count and CT was illustrated again when analysing USDA proficiency testing results from 2008 – 2011 where the VetMAX™ kit was used following extraction by the MagMAX™ kit. It is probable that the magnetic bead based extraction kits outperformed the column based kit because they were better suited to disrupting the tough bacterial cell wall of MAP. PCR inhibition was not an issue as the internal control (Xeno™ DNA) was reliably detected in all MAP positive samples.

This study shows that the VetMAX™ MAP Real-Time PCR Screening Kit for the detection of MAP in bovine faeces is comparable to traditional culture methods, and gives rapid turn around time. For a complete workflow this kit can be coupled with either the MagMAX™ Total Nucleic Acid Isolation Kit or the InviMag® Stool DNA Mini Kit for superior results.



Acknowledgements

Darcy Myers and Angela Burrell, Life Technologies, Austin for performing the USDA proficiency testing and Lee Effinger, Oregon Department of Agriculture, for the supply of bovine faecal samples

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DETECTION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN CAPRINE FECES; VALIDATION USING ADIAVET® PARATB REAL TIME KIT

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Abstract: A commercial real time PCR kit for detection of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) was tested for use in goat feces under Norwegian conditions. The study was initiated as a part of a project; "Healthier Goats, project for eradication of CAE, CLA and Johnes disease in Norwegian goats", aiming to eradicate diseases of great importance for the goat industry, including paratuberculosis. For eradication of the disease in a herd, rapid diagnosis of paratuberculosis is essential. PCR was performed with Adiavet® Paratb real time kit (Adiagène) using the company's new protocol for DNA isolation, where 3-10 g of feces is analyzed.

The results showed that the real time PCR was rapid, reliable and more sensitive than culture. None of the negative control samples were positive for PCR, confirming the specificity of the test. In the future, analysis with the Adiavet® Paratb real time kit might be implemented as a standard test in the Norwegian disease control program.

Introduction: *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the causal agent of paratuberculosis or Johnes disease (JD), a chronic granulomatous enteritis affecting ruminants. In Norway, the disease has been a problem in the goat industry. The clinical picture in goats is different from what is seen in cattle, as diarrhoea usually is not prominent. The typical picture is emaciation and reduced production yield[1].

The study was initiated as a part of a project; "Healthier Goats, project for eradication of CAE, CLA and Johnes disease in Norwegian goats", aiming to eradicate diseases of great importance for the goat industry, including paratuberculosis (<http://geithelse.tine.no/English>). The project is based on "snatching" goat kids, that is to remove the kids from the mother and the rest of the herd directly after birth. The newborn kids are then fed cows colostrum, housed separately and regularly tested for paratuberculosis. A problem for the follow-up investigation the long time needed for culture of *Map*, there is therefore a need for rapid diagnosis. The aim of this study was to validate a commercial real time PCR kit for detection of *Map* in goat feces under Norwegian conditions.

Material and methods: Faecal samples from goats were analysed by real time PCR and culture. Twenty samples collected over five days from four goats naturally infected with *Map* and 60 samples from goats in paratuberculosis free areas of Norway were sampled. Additionally, stored fecal samples from 24 previously culture positive and 18 previously culture negative samples were analysed by real time PCR. The stored samples originated from 2008 to 2010.

PCR was performed with Adiavet® Paratb real time kit (Adiagène, Saint-Brieuc, France), using the company's new protocol for DNA isolation, where 3-10 g of feces can be analysed, increasing the sensitivity of the method. In short, 3 ± 0,2 g of feces was suspended in 20 mL sterile distilled water using a stomacher and left overnight for rehydration as recommended by Adiagène. The supernatant was filtrated using chemfilter (Adiafilter, Adiagène) for removal of PCR inhibiting substances, the pellet redissolved and the bacterial cells mechanically disrupted by bead-beating. DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Real time PCR was performed with the Adiavet® Paratb real time kit (Adiagène) as recommended using the EPC-Extraction internal control. The PARA positive control supplied with the kit was run for quality control of each assay, and Milli-q water was run as negative control. Real-time PCR was performed using Stratagene Mx3005P (Stratagene, La Jolla, CA, USA).

Results and discussion: The real time PCR from Adia-gène worked well for analysis of goat feces in Norway (Table 1). The specificity was confirmed as 60 samples from goats in known paratuberculosis free areas were negative for both culture and PCR. The real time PCR was able to detect Map in all 20 samples from the four naturally infected goats. By culture, only 15 of these samples were positive (Table 1). The real time PCR was able to detect Map in 22 out of 24 previously culture positive samples. These samples had been stored at -20°C in up to three years, something that can explain the lower sensitivity compared to culture for these samples. 18 previously culture negative samples from positive herds were also analyzed, and Map was detected in three samples.

Table 1: Real time PCR and culture of 122 caprine fecal samples.

Material	PCR		Culture		Total
	+	PCR -	+	Culture -	
Naturally infected goats*	20	0	15	5	20
Goats from paratuberculosis free areas	0	60	0	60	60
Previously culture positive samples	22	2	24	0	24
Previously culture negative samples, known positive herds**	3	15	0	18	18

*Four goats naturally infected with Map, sampled five times each.

**Stored at -20°C, originating from 2008-2010.

Conclusions: The Adiavet®Paratb real time kit (Adia-gène) worked well for analysis of goat feces under Norwegian conditions. The sensitivity was better than culture in the tested material, but more testing is required for statistical interpretation. None of the samples from paratuberculosis free herds were positive in the real time PCR, confirming the specificity. Even though culture still is the gold standard for diagnosis of Map, real time PCR decreases the time of diagnosis in goats from 12-16 weeks to two days. This is of great importance in an eradication programme like “Healthier Goats.”

Acknowledgements: Funded by: Adia-gène, member of the AES Laboratory Group, and “Healthier Goats, project for eradication of CAE, CLA and Johne’s disease in Norwegian goats” and the Norwegian Veterinary Institute.

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MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN WILD BOARDS FROM KOREA

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Mycobacterium avium subspecies *paratuberculosis* (MAP) causes a chronic infectious enteritis in various domestic and wild animals. It is widely distributed globally in animals and also inter-species transmission has been frequently reported. We investigated the presence of Map from December 2010 to March 2011 via collection of blood and feces from 222 hunter killed wild boars. One hundred ninety-seven serum and 180 fecal samples were collected and examined by PCR and enzyme-linked immunosorbent assay (ELISA). In this study was carried out to investigate the status of MAP infection and genotypes of MAP present in the wild boar population of Korea using IS900 PCR and IS1311-REA typing. By PCR, 18 animals were positive for MAP and 5 sera showed a good humoral response to MAP. The PCR product was purified and sequenced and the genetic analysis revealed a 99% identity match between the sequences IS900 deposited in the GeneBank. PCR positive DNA samples were genotyped as 'Cattle type' and 'Bison type', which is a major MAP genotype infecting domestic species in Korea. Our study provide new information on the presence of mycobacterial infection among wild boars and suggest that a more effective program should be developed to monitor mycobacterial infections in wild animal population.

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CONTROL OF PARATUBERCULOSIS IN THE CLOSED HERD OF CATTLE BY REAL TIME PCR: DOES PASSIVE SHEDDING REALLY EXISTS?

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Introduction

The aim of this study was to investigate, whether it is possible to control paratuberculosis by quantitative real time PCR (qPCR) in the closed cattle herd with known history of paratuberculosis and to determine general rules of the qPCR results interpretation. For this purpose, a closed herd of Limousine cattle monitored for 2 years (6 collections) for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in faeces by culture and IS900qPCR.

Materials and methods

Herd status and sampling

- » 40 cows and 1 breeding bull of Limousine beef cattle included in the Paratuberculosis control programme based on faecal culture examination.
- » 3 last culture examinations were negative, the 4th (Month 0) was done in parallel with IS900qPCR.
- » The herd is stabled in a shed during winter and unsheltered on the pasture during summer. Calves remained with their dams until the age of 8 to 9 months, when heifers were moved to another location to prevent inbreeding and young bulls were transferred to feedlot for the fattening.
- » Individual faecal sample were collected directly from the rectum using a disposable glove.
- » Tissue samples (intestine or mesenteric lymph nodes) from slaughtered animals were collected where possible and served as the final confirmation of infection.

Sample analysis

- » For culture, 5 g of faeces or 1 g of tissue (mesenteric lymph nodes) was decontaminated in HPC, seeded on HEYM slants with 2 µg/ml of Mycobactin J and incubated at 37°C for 3 months (Pavlik et al., 2000).
- » DNA from faeces was isolated by modified protocol of QIAamp DNA Stool Mini Kit (Kralik et al., 2011). The detection and quantification of MAP was performed by IS900qPCR with internal amplification control. Quantification was done according to the plasmid gradient (Slana et al., 2008).
- » Blood sera were investigated for the presence of antibodies against MAP by ID Screen Paratuberculosis Indirect ELISA kit (ID Vet, Montpellier, France).

Results

- » In the first collection two cows (4.9%) were culture positive and 25 (61.0%) were positive by IS900qPCR. Two cows with more than 10⁴ MAP cells in 1 g of faeces were culled. When after 4 months the examination was repeated portion of positive animals dropped to 42.1% (16 out of 38) with no culture positive individuals. After additional 5 months, only 6.1% (only 2 cows out of 33 remaining) were positive by IS900qPCR. This status remained more or less unchanged until the end of the monitoring.
- » During the course of monitoring 14 cows were slaughtered for different reasons. IS900qPCR found ten of them at least once positive in faeces. Only 2 of them were positive by faecal culture and 4 by culture of their tissues. Both cows positive in faeces by culture were low shedders.

Conclusion

- » Analysis of the closed herd revealed that MAP is shed by animals in very low amounts undetectable by culture.
- » Data confirmed previous study (Kralik et al., 2011) that the detection limit of culture is approx. 10³ MAP cells per g of faeces.
- » Removal of two high shedders at the beginning of experiment led to the statistically highly significant decrease of IS900qPCR positivity within 9 months.

- » Animal shedding low amounts of MAP in faeces in more than two consecutive samples should be considered as highly suspected.
- » The data suggest that passive shedding likely does not exist on the level of culture examination of faeces (Pradhan et al., 2011), but it is likely that it exists on the level of qPCR.
- » Sporadic occurrence of animals slightly positive for MAP in faeces by qPCR is probably unavoidable in herds with history of paratuberculosis.

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This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic “AdmireVet” (CZ 1.05/2.1.00/01.0006; ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716202, QI101A094 and QH81065).

ENUMERATION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* BY QUANTITATIVE REAL TIME PCR, CULTURE ON SOLID MEDIA AND OPTICAL DENSITOMETRY

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Introduction

Nowadays, there are many different approaches towards determining the number of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cells in a suspension. The majority of them are based upon culture (determination of CFU) or visual/instrumental direct counting of MAP cells.

In this study, we have compared the culture method with a previously published F57 based quantitative real time PCR (F57qPCR) method, to determine their relative abilities to count the number of three different MAP isolates in suspensions with the same optical densities (OD). In accordance with routine laboratory practice, McFarland turbidity standards were prepared, their exact OD were determined and the theoretical amount of bacterial cells was compared with F57 qPCR and culture results to assess its applicability for the enumeration of MAP.

Materials and methods

Experimental design

- » A single colony from each of MAP field isolates 8819, 8672 and 12146 was resuspended in M7H9 with Mycobactin J and then cultured for up to 5 weeks at 37°C to avoid excessive cell clumping.
- » In order to remove clumps from the suspension, the broth culture was vortexed at full speed for 10 s with 1 mm zirconia silica beads and centrifuged at 100 × g for 30 s. The presence of MAP clumps in each MAP suspension was checked using Ziehl-Neelsen staining and optical microscopy.
- » Each suspension of MAP isolate was 2-fold serially diluted in M7H9 medium in seven succeeding steps to ensure that zero OD was reached. Each dilution was processed in biological triplicates per 500 µl and in portion of each of them (60 µl) absorbance at 600 nm was recorded.

Determination of MAP numbers by F57qPCR

- » In 200 µl aliquots of biological triplicates from the 2-fold diluted MAP suspensions the medium was replaced by the identical volume of TE buffer supplemented with Fish Sperm DNA at a concentration of 50 ng/µl.
- » After the addition of 350 mg of 0.1 mm zirconia silica beads the MAP cells were mechanically lysed.
- » The lysed MAP cells were centrifuged at 18000 × g for 5 min and the supernatant then used as the template for qPCR, amplifying the single copy fragment F57 (Slana et al., 2008).
- » The absolute quantity of MAP cells was determined according to the calibration curve, derived from 10-fold dilutions of plasmid standards containing the F57 qPCR product insert.

Determination of MAP counts by culture on solid media

- » Aliquots from biological triplicates of each serial two-fold dilution were immediately after OD determination diluted 1:100 in M7H9 in two consecutive steps for the purpose of CFU number determination by culture.
- » One hundred micro litres of the undiluted and diluted solutions (1:100 and 1:10 000) were precisely spread on HEYM with Mycobactin J and antibiotics and incubated at 37°C for 3 months.

McFarland turbidity standards preparation

- » McFarland turbidity standards of 0.5, 1 and 2 McFarland were included prepared and their optical density was measured and paired with an estimated bacterial cell density (number of CFU of *E. coli*) according to the following approximations: McFarland 0.5 = 1.5×10^8 CFU/ml, McFarland 1 = 3.0×10^8 CFU/ml and McFarland 2 = 6.0×10^8 CFU/ml (Forbes et al., 2002).

Data processing and statistical analysis

- » Mean OD values of each isolate suspension were calculated from the triplicate aliquots prepared for the determination of absorbance.
- » Student's t-test was used to statistically evaluate data from F57qPCR and culture for the relevant dilution and isolate. P-values lower than 0.05 were considered statistically significant.
- » The differences in logs of absolute counts between F57qPCR and culture data were expressed as logarithm of quotient of mean absolute counts from F57qPCR and mean CFU counts from culture.

Results

- » Despite all attempts to reduce the number of MAP clumps, in all three MAP isolates there were still several or even tens of small clumps present, visible after staining with Ziehl-Neelsen and optical microscopy.
- » The absolute numbers of MAP determined by F57qPCR were approximately 2 log₁₀ greater than the CFU counts from culture at respective ODs and a highly significant statistical difference ($P < 0.0001$) between all the compared samples was observed.
- » No isolate specific pattern was observed.
- » The dependence of OD and F57qPCR or CFU numbers was shown to be linear and all R² coefficients were close to 1.
- » The limit of detection for OD MAP enumeration by F57qPCR was similar for all isolates and reached approximately 4×10^6 MAP cells per ml, whereas for culture it was lower at 3×10^4 CFU/ml.
- » Almost perfect fit of F57qPCR MAP numbers and theoretical CFUs of *E. coli* according to McFarland standards were observed.

Conclusion

- » It is very difficult to get rid of all MAP clumps in a suspension making data from culture and qPCR incomparable.
- » qPCR provide higher absolute numbers of MAP compared to CFU counts using the culture method.
- » This discrepancy could be explained by the by the formation of clumps and/or viability of MAP cells in the suspension.
- » Uniform pattern of all three MAP isolates used in the study allows excluding any possible differences in the *in vitro* viability of the cells at the isolate level.
- » Concordance of F57qPCR with theoretical numbers arising from McFarland standards strengthens the reliability of F57qPCR enumeration and highlights the problems with quantification of MAP by culture.
- » No enumeration method can be recommended as golden standard! MAP enumeration strategy should be considered with respect to the experimental design.

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This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic "AdmireVet" (CZ 1.05/2.1.00/01.0006; ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716202 and QH81065).

SHEDDING PATTERNS OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN FECES, MILK, AND COLOSTRUM OF DAIRY COWS

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative organism of Johne's disease. Dairy Farmers of Canada lists Johne's disease as one of the top two animal health priorities of the Canadian dairy industry.¹ It is a production limiting disease of adult dairy cows, but infection in newborn calves from ingestion of infected feces, colostrum, or milk is considered the main route of transmission and a major concern in a herd. As an intracellular bacterium (within the host's macrophages), MAP is noted for evading the host body's defense mechanisms.² Being a macrophage-rich environment, colostrum becomes a primary risk to calves if it contains these MAP-infected cells. Current diagnostics rely on identification of the bacterium in feces through culture and molecular tests or identification of the antibodies in milk or serum via enzyme-linked immunosorbent assays (ELISA).² However, these tests are inadequate to meet our current needs for herd biosecurity and environmental transmission control of MAP, especially during the peripartum period and subclinical stages. Milk ELISA has poor predictive values due to imperfect sensitivity (Se) and specificity (Sp) combined with low prevalence.³ Furthermore, although fecal cultures are currently the gold standard diagnostic, the long incubation times, costs, and intermittent shedding of MAP hinder efficient screening programs.^{3,4} This study will assess how shedding patterns of MAP in feces, milk, and colostrum vary with lactation stage and season. Accurate knowledge of these patterns is vital for effective herd management of shedder cows to reduce MAP transmission risks and for development of improved diagnostic and screening protocols.

MATERIALS AND METHODS

For this study, 54 confirmed MAP positive cows from 7 dairy farms (located in Prince Edward Island and New Brunswick, Canada) were purposively selected from data collected for a companion project. These cows were confirmed positive by fecal broth culture (TREK ESP), acid fast stain (AFS), and real-time PCR (Tetracore). Samples of feces and milk were collected monthly as per the stage of lactation for 12 months starting in July 2010 and ending in December 2011, depending on the first month of farm participation, and for as long as the cow remained in the herd. The farmers were not blinded to the MAP status of the cows; therefore due to culling, only 26 cows were present in the study by December 2011. Colostrum samples were also collected within 24 hours of calving. For ELISA analysis, a control group of 54 confirmed test-negative herd mates (MAP negative on fecal broth culture and fecal PCR, milk ELISA and serum ELISA) were selected from the same companion project. Selection criteria for the control group required that these herd mates were matched as closely as possible by age, lactation, days in milk (DIM), and reproductive status to the test group. Cows chosen for the study were primarily Holstein and had an average lactation number of 2 (range 1 to >7). Farms were mainly free-stall, and ranged from 83-490 cows per herd. All samples collected were subsequently frozen at -80°C and stored until processed in the laboratory. Fecal, milk, and colostrum samples from the test group were processed via broth culture, solid culture, and direct PCR. Milk samples from both the study and control groups were also tested with the Paracheck 2 ELISA kit (Prionics AG, Switzerland), following the kit protocol. Broth culture protocols used the ESP culture system II (TREK Diagnostic System). Direct real-time PCR methods followed the procedures listed for the Tetracore VetAlert Johne's Real-Time PCR kit, using the IS900 insert. Milk and colostrum direct PCR followed the procedures of Gao et al⁵ with some modifications along with the Tetracore kit methods. Solid cultures were performed on Herrold's egg-yolk medium, supplemented with mycobactin J (Becton -Dickinson). Procedures used for the solid cultures followed methods described by Stabel⁶ for feces, Donaghy et al⁷ for milk, and Godden et al⁸ for colostrum. All broth and solid culture results were confirmed by AFS, and any positive results were further confirmed with PCR. The solid culture results will be used to quantify shedding amounts and thereby infection risk through a colony forming unit (CFU) count. Statistical analysis is pending completion of laboratory test for all samples.

RESULTS

Due to the time requirements for processing the samples, only data from July 2010 through June 2011 is presented in this report. Results for feces and milk samples from the test group of known positive cows are reported in Table 1 and Table 2. MAP was also detected in colostrum by culture and PCR but insufficient data is available for publication at this time.

Table 1. Percent positive results from MAP positive cows per sampling month

		jul-oct_10	nov_10	dec_10	jan_11	feb_11	mar_11	apr_11	may_11	jun_11
T ^a	f ^b	64.3 (28) ^c	64.9 (37)	57.4 (47)	73.7 (38)	73.3 (15)	51.5 (33)	52.2 (23)	48.1 (27)	35.0 (20)
	M	14.3 (14)	0.0 (4)	0.0 (17)	28.0 (25)	0.0 (13)	10.7 (28)	28.6 (21)	14.8 (27)	18.2 (22)
H	F	64.0 (25)	63.6 (22)	67.6 (34)	56.3 (32)	63.6 (11)	60.9 (23)	47.1 (17)	34.6 (26)	40.0 (15)
	M	5.3 (19)	10.5 (19)	12.9 (31)	14.7 (34)	0.0 (13)	3.7 (27)	19.0 (21)	14.8 (27)	23.8 (21)
P	F	60.7 (28)	67.6 (37)	71.7 (46)	81.6 (38)	93.3 (15)	90.9 (33)	69.6 (23)	88.9 (27)	95.0 (20)
	M	21.1 (19)	0.0 (13)	3.4 (29)	15.2 (33)	7.7 (13)	35.7 (28)	9.5 (21)	19.2 (26)	18.2 (22)
E	M	42.9 (21)	50.0 (18)	29.4 (34)	44.1 (34)	38.5 (13)	23.2 (30)	14.3 (21)	29.6 (27)	27.3 (22)

Table 2. Percent positive results from MAP positive cows per lactation months

		dry	fresh ^d	1	2	3	4	5	6	7	8	9
T ^a	f ^b	87.5 (16) ^c	46.2 (13)	70.0(20)	69.6(23)	62.5(24)	58.3(17)	58.8(17)	47.6(21)	50.0(14)	53.8(13)	61.1(18)
	m			14.3(14)	5.6(18)	11.8(17)	6.3(16)	21.4(14)	11.8(17)	25.0(12)	23.1(13)	14.3 (7)
H	f	90.9 (11)	60.0 (10)	78.6(14)	61.1(18)	42.9(21)	56.3(16)	57.1(14)	57.9(19)	50.0(10)	66.7(9)	70.0(10)
	m			9.5 (21)	10.0(20)	19.0(21)	0.0(19)	13.3(15)	10.5(19)	21.4(14)	25.0(12)	20.0(10)
P	f	86.7 (15)	76.9 (13)	85.0(20)	78.3(23)	79.2(24)	83.3(24)	82.4(17)	81.0(21)	85.7(14)	92.3(13)	73.7(19)
	m			15.0(20)	9.1(22)	21.1(19)	17.6(17)	6.7(15)	21.1(19)	28.6(14)	15.4(13)	22.2 (9)
E	m			40.9(22)	42.9(21)	18.2(22)	33.3(18)	23.5(17)	35.0(20)	28.6(14)	46.2(13)	30.0(10)

^a T=TREK ESP culture, H= HEYM-j culture, P=direct PCR, E=Paracheck ELISA

^b f=feces sample, m=milk sample

^c value=percent positive samples (total number of positive and negative samples for that period)

^d lactation months refer to 30 days-in-milk (dim) periods, e.g. fresh=0dim, 1=1-30dim, 2=31-60dim, etc.

DISCUSSION AND CONCLUSION

Current Johne's tests are inadequate to meet the dairy industry's needs for herd biosecurity and environmental transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Understanding patterns in shedding, as well as patterns in milk antibody ELISA readings for MAP can lead to efficient testing of animals for enhanced control programs. In a study performed by Nielsen et al, milk ELISA increased in sensitivity for MAP at the beginning of lactation.⁹ In our study, the raw data also suggested an increase in ELISA Se at beginning of lactation, but statistical evaluation for this and a possible increase at late lactation is still pending. Although ELISA's typically indicate persistent infections, antibodies may occur prior to shedding of MAP in feces, therefore a positive ELISA without concurrent fecal shedding may be a true result, and can only be properly assessed by subsequent fecal testing over the next few months.² For feces, Whitlock et al reports that liquid cultures tend to have increased sensitivity over solid cultures.¹⁰ Preliminary results for our study indicate a slight difference between the two culture types, but a more noticeable increase in Se was observed with real-time PCR. For culture-based fecal tests, although sample sizes were small (mean = 14), higher sensitivities in the raw data were observed for the dry period than other lactation stages. In summary, knowledge of MAP shedding patterns and milk ELISA patterns could be important for effective herd management to reduce MAP transmission risks and for development of more time-efficient, cost-efficient, and effective diagnostic protocols for critical control and biosecurity programs.

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EVALUATION OF THREE COMMERCIAL ELISA KITS FOR THE DETERMINATION OF HERD STATUS FOR JOHNE'S DISEASE ON ATLANTIC CANADIAN DAIRY FARMS

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Introduction

Results from individual cow milk ELISAs can be used to determine herd status for *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Compared to fecal culture, milk ELISA samples are less invasive to collect, and the results can be obtained earlier and at a lesser expense. The objective of this study was to evaluate the test characteristics of milk ELISA for determination of herd MAP status in Atlantic Canadian dairy herds.

Materials and Methods

A total of 34 purposively selected herds from the Maritime provinces participated in this 2 year project. Median herd size was 66 milking cows (range: 30 to 220). Individual fecal and milk samples were collected from all milking cows biannually. All manure samples were processed by the Maritime Quality Milk Laboratory, Atlantic Veterinary College, Prince Edward Island, Canada. Individual cow fecal cultures were pooled by age into pools of five. Fecal samples were cultured in the ESP® Culture System II (TREK Diagnostic Systems, Inc., Cleveland, Ohio, USA). Samples were prepared and cultured following manufacturer's instructions. After culture, all broth samples were examined microscopically for the presence of MAP using an acid-fast stain. Confirmatory PCR for detection of IS900 was performed on all samples positive by the culture system and/or microscopic visualization using the VetAlert™ Johne's Real-Time PCR kit (Tetracore, Inc., Rockville, Maryland, USA). Milk samples were processed following manufacturer's instructions. Throughout this paper, the kits are referred to as: ELISA A: Parachek2 *Mycobacterium Paratuberculosis* Test Kit® (Prionics); ELISA B: *Mycobacterium Paratuberculosis* Antibody Test Kit® (IDEXX); ELISA C: Paratuberculosis Indirect® (IDVet Innovative Diagnostics). Statistical analysis to estimate test characteristics was based on GEE logistic models with exchangeable correlation structures, which accounted for the repeated measures data. Agreement was described using kappa statistics.

Results and Discussion

Apparent Prevalence of Study Herds: A herd was considered positive by fecal culture if there was at least one positive fecal pool, and positive by ELISA if there were at least 2 positive milk ELISA results in the herd. As displayed in Table 1, compared to herd prevalence estimated by fecal culture, herd prevalence for ELISA A was higher, ELISA C was slightly higher, and ELISA B was slightly lower. Within-herd prevalence was underestimated by all three ELISAs compared to fecal culture.

Table 1. Apparent herd prevalence and within-herd prevalence of positive herds based on fecal culture and three commercial milk ELISAs.

Test	Herd Prevalence	Within Herd Prevalence (Pos Herds)
Fecal Culture	41.2%	6.3%
ELISA A	55.9%	2.7%
ELISA B	35.3%	3.3%
ELISA C	47.0%	3.4%

Diagnostic Sensitivity and Specificity:

As seen in Table 2, if two positive milk ELISAs designated a herd MAP positive, the sensitivity (Se) for all 3 ELISAs ranged from 50.5%-60.0%, and specificity (Sp) from 88.3%-96.1%.

Table 2. Diagnostic sensitivity and specificity of three commercial milk ELISAs.

	Se (CI)	Sp (CI)
ELISA A	54.5% (33.5-74.0%)	88.3% (80.4-92.8%)
ELISA B	50.5% (29.4-71.5%)	96.1% (89.4-98.6%)
ELISA C	60.0% (38.6-78.1%)	94.8% (88.0-97.9%)

Agreement Between Milk ELISAs and Fecal Culture: Table 3 below demonstrates the agreement (kappa) between fecal culture and 3 commercial milk ELISAs (ME). Agreement ranged from moderate (0.41-0.6) to substantial (0.61-0.8). In general, the best agreement between milk ELISAs and fecal culture occurred when 2 positive milk ELISAs were required to define a herd as MAP positive. Due to the repeated measures within the data, standard methods to calculate confidence intervals cannot be used. The kappa statistic, however, is still useful as a descriptive statistic to compare agreement between the various tests.

Table 3. Kappa values for herd status agreement between fecal culture and three commercial milk ELISAs, with increasing number of positive ELISAs required to designate a herd positive.

	Pos Herd= ≥ 1 ME+	Pos Herd= ≥ 2 ME+	Pos Herd= ≥ 3 ME+
ELISA A	0.435	0.525	0.449
ELISA B	0.637	0.618	0.476
ELISA C	0.640	0.711	0.516

Agreement Between Commercial Milk ELISAs: As can be seen in Table 4, agreement (kappa) was greater between ELISA B and C than between ELISA A and ELISA B or C.

Table 4. Kappa values for herd status agreement between three commercial milk ELISA kits.

	ELISA B	ELISA C
ELISA A	0.618	0.598
ELISA B		0.869

Note: 2 or more positive results designated a herd as MAP positive.

Conclusion

As a herd diagnostic tool for MAP, the milk ELISA has moderate to substantial agreement with herd fecal culture. Sensitivity ranges from 50-60% and specificity from 88-96%.

EVALUATION OF ENVIRONMENTAL CULTURE TO DETERMINE HERD STATUS FOR JOHNE'S DISEASE ON ATLANTIC CANADIAN DAIRY FARMS

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Introduction

Environmental culture (EC) is a non-invasive and relatively inexpensive method to detect the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in a herd. Research into the test characteristics of EC has generally focused on large, MAP positive herds. This study was designed to evaluate the test characteristics of EC within typical Atlantic Canadian dairy herds. These herds tend to be smaller in size and exist within colder climatic conditions than herds that have been used in previous EC studies. As well, many of the herds are suspected to be test negative or have low MAP prevalence.

Materials and Methods

A total of 34 purposively selected herds from the Maritime provinces participated in this 2 year project. Median herd size was 66 milking cows (range: 30 to 220). Individual fecal samples were collected from all milking cows biannually. Environmental samples were collected quarterly following a protocol adapted from the Voluntary Bovine Johne's Disease Control Program (USDA-APHIS, 2010). Two samples were collected from the manure storage areas and four samples were collected from the mature cow manure concentration areas. All manure samples were processed by the Maritime Quality Milk Laboratory, Atlantic Veterinary College, Prince Edward Island, Canada. Individual cow fecal cultures were pooled by age into pools of five. Fecal samples were cultured in the ESP® Culture System II (TREK Diagnostic Systems, Inc., Cleveland, Ohio, USA). Samples were prepared and cultured following manufacturer's instructions. After culture, all broth samples were examined microscopically for the presence of MAP using an acid-fast stain. Confirmatory PCR for detection of IS900 was performed on all samples positive by the culture system and/or microscopic visualization using the VetAlert™ Johne's Real-Time PCR kit (Tetracore, Inc., Rockville, Maryland, USA). Statistical analysis was based on GEE logistic models with exchangeable correlation structures, which accounted for the repeated measures data.

Results and Discussion

Diagnostic Sensitivity and Specificity: Based on the results of null logistic GEE models (Table 1), Sensitivity (Se) of EC was 71.4% (CI 49.2-86.5%) and specificity (Sp) was 98.6% (CI 94.8-99.6%).

Predictors Affecting Sensitivity: Season of EC collection, herd management system and within herd fecal culture test prevalence were entered into the null model for Se. Only fecal culture test prevalence was found to be significant. Results of the final logistic GEE model are shown in Table 1. Using this multivariable model with test prevalence as a predictor, the Se of EC can be estimated at various prevalence levels and is displayed in Figure 1.

Table 1. Final logistic GEE models

Model	Estimate	95% CI-lower	95% CI-upper	P-value
Null logistic model of Dx- herds (Sp)				
(Intercept)	-4.29	-5.67	-2.91	0.000
Null logistic model of Dx+ herds (Se)				
(Intercept)	0.91	-0.03	1.86	0.058
Multivariable model predicting Se				
(Intercept)	-1.53	-2.40	-0.65	0.001
Fecal culture test prevalence	0.78	0.42	1.15	0.000
Model predicting within-herd prevalence				
(Intercept)	-2.22	-2.37	-2.07	0.000
Log of Proportion Positive Ind. EC	1.98	1.62	2.34	0.000

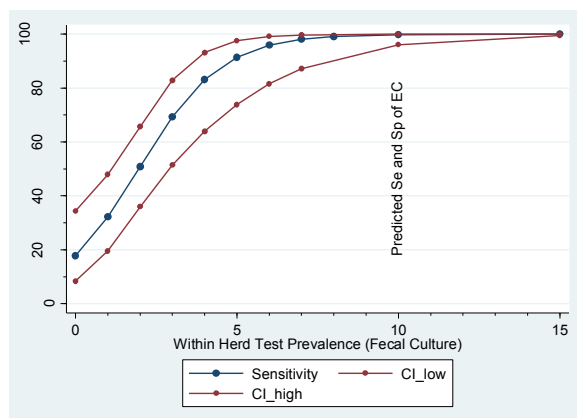


Figure 1. Sensitivity of environmental culture by within herd test prevalence based on fecal culture.

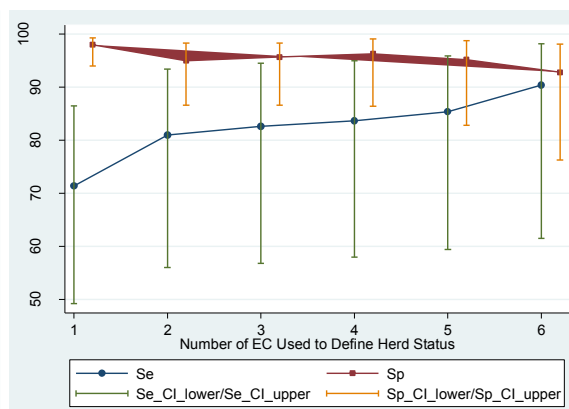


Figure 2. Predicted sensitivity and specificity of environmental culture, with increasing number of sample sets used to determine herd status.

Repeated Environmental Sampling: Se and Sp of EC using stepwise combinations of 2, 3, 4, 5 and 6 EC results were derived from separate null logistic regression models (not shown). Graphical representation of the estimated Se and Sp values is displayed in Figure 2.

Prediction of Within-Herd Prevalence: The model coefficients for prediction of within-herd prevalence are shown in Table 1. The best fit of the predictor was found with a log transformation, after a small value was added to the predictor to allow for log transformation of the zero values. Interpretation of the back-transformed predictor is displayed graphically in Figure 3, showing the estimated within-herd fecal culture prevalence as the proportion of positive individual EC increases.

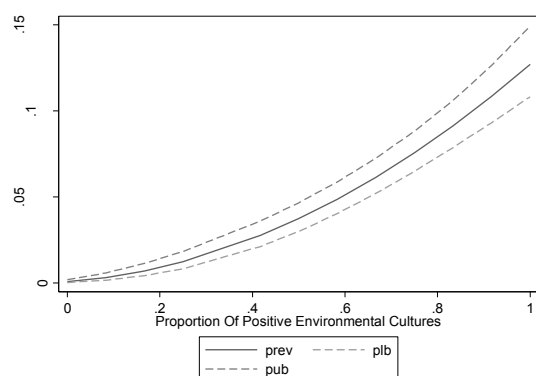


Figure 3. The effect of proportion positive individual environmental cultures on the within-herd fecal culture prevalence.

Conclusion

The sensitivity and specificity estimates from this research indicate that environmental culture is an acceptable tool for herd diagnosis of MAP in Atlantic Canadian dairy herds. Sensitivity of environmental culture improves as within-herd fecal culture prevalence increases. There was also a numeric increase in the sensitivity when two samples were used compared to one but no apparent increase with a frequency of greater than two samples.

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EFFICIENT DNA EXTRACTION AND PURIFICATION FOR SENSITIVE DETECTION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN BOVINE FAECES AND TISSUES

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Bacterial culture is one of the most important diagnostic methods for Johne's disease (JD), however, the extremely slow growth of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) have hampered the rapid and accurate diagnosis of JD. The detection of MAP by quantitative real-time PCR (qPCR) has been expected to overcome these issues, and efficient DNA extraction from faecal or tissue samples is crucial for sensitive and specific detection of MAP in downstream qPCR test. In the present study, we have developed a new DNA extraction and purification method, named 'Johne-Spin', which combined the DNA extract solutions without chloroform or chelating material and spin-column purification using optimal DNA binding material to MAP DNA. The Johne-Spin method was applied to 117 faecal samples collected from MAP-infected cattle herds and produced qPCR positive rates of 98.6% for 75 faecal samples with MAP culture positive. In contrast, 93.3% of the same samples were positive with a conventional method using chloroform and isopropanol-precipitation, thus the Johne-Spin method indicated higher sensitivity. This method was further evaluated by qPCR using tissue samples from intestine and mesenteric lymph nodes of infected cattle. The cycle-threshold (Ct) of qPCR using DNA extracted by the Johne-Spin method was decreased more than 6 cycles compared to that of a conventional method, and the calculated concentration of MAP DNA from the Johne-Spin treated samples, as the result, 10 to 100 times increased. This increase of MAP DNA concentration in the samples suggests that the method was highly efficient for DNA extraction and removal of PCR inhibitors. Thus the Johne-Spin method is highly useful, and demonstrated an advantage over conventional DNA extraction methods, resulting in higher DNA yields, lower PCR inhibitors and increased sensitivity for the qPCR test of JD.

A LONGITUDINAL STUDY TO CHARACTERIZE THE SHEDDING PATTERNS OF *MYCOBACTERIUM AVIUM* SPP. *PARATUBERCULOSIS* IN A NATURALLY INFECTED BREEDING BULL BY POLYMERASE CHAIN REACTION ASSAYS

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ABSTRACT

Although *Mycobacterium avium* spp. *paratuberculosis* (MAP) has already been detected in semen and reproductive organs of bulls, shedding patterns are not well characterized. Our investigation was performed to detect and quantify MAP in feces, semen, and blood samples continuously drawn from a naturally infected 18-month-old German Simmental bull without clinical symptoms over a period of 4.5 years by qualitative and quantitative polymerase chain reaction (PCR) techniques and to correlate time dependent matrix specific contents of MAP. In all matrices, MAP was detected intermittently with MAP-free intervals of at least 5 to 18 weeks using an IS900 semi-nested PCR. The number of MAP positive results in semen and blood was higher than in fecal samples. A quantitative IS900 real-time PCR revealed that the highest amount of MAP was shed in feces (10^3 - 10^6 MAP/g), while the lowest concentrations were found in semen and blood (10^2 - 10^5 MAP/ml). Although a poor relationship was calculated between the presence of MAP in feces and blood, a significant positive agreement between its occurrence in semen and blood was determined ($r = 0.57$, $p < 0.001$, $n = 65$). For identity confirmation and phylogenetic comparisons, the 278 bp PCR amplicon covering nucleotide positions 492 and 769 within the IS900 was cloned into the pCR 2.1-TOPO plasmid vector and sequenced. Nucleotide homologies of 100% were ascertained to the MAP K10 IS900 reference sequence (GenBank: AE16958). Despite the presence of MAP in semen, the quality of semen samples was good with volumes between 3 to 10.5 ml. Morphologically normal spermatozoa varied from 85 to 92%, density was 0.4 to 1.6 million/ μ l, and motility ranged from 65 to 70%. Since 2.5 years, enhanced eosinophilic granulocytes with peaks of up to 55% were noticed intermittently in blood by differential cell counts (granulocytes, lymphocytes, monocytes) using microscopy and flow cytometry. Parasitological examinations were accomplished regularly to exclude helminthic and protozoal infections. The present study highlights the possible risk for MAP transmission during artificial insemination and indicates the need for hygienic measures to prevent the spread of the infection via semen.

INTRODUCTION

It is generally accepted that MAP is mainly transmitted to neonate calves through the fecal-oral route by subclinically or persistently infected cattle in a herd. However, vertical transmission of MAP via the uterus has been investigated before, too. The first report about a bovine fetal infection was published in 1929 [1], and the isolation of MAP from bovine semen was described for the first time in 1948 [2]. Although MAP has been isolated from semen and reproductive organs of infected bulls [3, 4], the pattern of bacterial shedding during different stages of the infection is not well characterized in naturally infected animals. Therefore, the objective of our study was to detect and quantify MAP in feces, semen, and blood samples continuously drawn from a breeding bull candidate naturally infected with MAP showing no clinical symptoms of paratuberculosis by PCR techniques.

MATERIALS AND METHODS

An 18-month-old German Simmental breeding bull candidate (*Bos primigenius taurus*) was kept isolated under quarantine conditions with constant feeding and environmental circumstances over a period of 54 months from June 2007 to November 2011. In total, 101 sample dates were chosen to collect fecal, semen, and blood samples, concurrently.

DNA extraction. DNA was extracted from the different sample matrices (feces, semen, and blood) for PCR analysis using a modified protocol of the QIAamp Blood Kit (Quiagen, Hilden, Germany).

Polymerase chain reaction. A qualitative semi-nested PCR (snPCR) and quantitative real-time PCR (rtPCR) based on the insertion sequence IS900 was performed as described elsewhere [5].

Enzyme-linked immunosorbent assay. During 2007 and 2008, a total of 35 serum samples were tested with the Idexx-ELISA (IDEXX GmbH, Woerrstadt, Germany) according to the producer's manual. Since 2009, the Idexx-ELISA was no longer available and replaced by the Pourquier-ELISA (Institut Pourquier SAS, Montpellier, France) for testing of 66 sera in 2009, 2010, and 2011.

Statistical analysis. The Pearson's correlation coefficient (r) was calculated to analyze the correlation between the detection of MAP in fecal, semen, and blood samples by snPCR. All analyzes was carried out with Microsoft Office Excel 2003 and Minitab Version 15.0 (Minitab Inc., State College, PA, USA).

RESULTS

During the longitudinal study, the bull's general condition was good and the fecal consistency was normal.

In all matrices, MAP was detected intermittently with MAP-free intervals of at least 5 to 18 weeks by snPCR. The number of MAP positive results in blood (39%) was higher than in semen (35%) and fecal (36%) samples. The rtPCR revealed that the highest amount of MAP was detected in feces (10^3 - 10^6 MAP/g), while the lowest concentrations were found in semen and blood (10^2 - 10^5 MAP/ml). Although a poor relationship was calculated between the presence of MAP in feces and blood, a statistically significant positive agreement between its occurrence in semen and blood was determined ($r = 0.57$, $p < 0.001$, $n = 65$). In 11/35 (32%) serum samples antibody responses were detected by the Idexx-ELISA in 2007 and 2008. No antibody response could be detected at any time in the 66 samples tested by the Pourquier-ELISA in 2009, 2010, and 2011. Despite the presence of MAP in semen, the quality of semen samples was good with volumes between 3 to 10.5 ml. Morphologically normal spermatozoa varied from 85 to 92%, density was 0.4 to 1.6 million/ μ l, and motility ranged from 65 to 70%. Blood differential cell counts revealed eosinophilic granulocytes proportions up to 55%.

DISCUSSION

It is commonly accepted that an infected animal enters a silent phase for two or more years, during which MAP is not detectable in feces. According to our study, MAP in bovine feces and semen was already present at the age of 18 months. This being an unusual event for an animal at such a young age, a recent case study observed fecal shedding of MAP in cattle prior to the age of two years [6]. Therefore, the assumption that young stock is not infectious has to be reconsidered. Over the period of our investigation, MAP was detected intermittently rather than continuously including long periods of MAP absence. MAP was also detected intermittently in blood samples, indicating that viable MAP bacteria in the blood may be responsible for disseminating the infection within the body. There was a statistically significant correlation between MAP occurrence in semen and blood, indicating the association that further colonization occurs via the blood stream. Although PCR verifies only the presence of DNA and does not indicate whether bacteria capable of infection, our results support the hypothesis that raw semen of subclinically infected bulls might contain 10^3 - 10^5 MAP/ml, and can therefore pose a risk for contamination of the bovine uterine environment if MAP survives semen conservation procedures. Further investigations are required in order to determine the likelihood of vertical transmission following natural mating or artificial insemination to make a reliable risk assessment regarding the pathogen MAP in semen. This includes surveys on MAP resistance against antibiotic additives used in diluents for standard semen conservation.

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TEMPORAL DEVELOPMENT OF ANTIBODIES IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTED CATTLE

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Background

Appropriate test-strategies are required for surveillance and control of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Testing using milk antibody ELISA may be a cost-effective tool, but interpretation still needs improvement. Milk ELISA primarily detects IgG1-type antibodies, which indicate occurrence of humoral immune (HI) responses. The HI responses indicates occurrence of detrimental infection processes, while cell-mediated immune responses (CMI) indicates that the animal has a beneficial immune status.

Stressors such as calving and moving animals have been hypothesised to affect the transition from CMI to HI

Objectives

The objectives of this study were to: (1) investigate effect of days in milk (DIM) and milk yield on odds of testing positive in milk ELISA; (2) determine the effect of the potential stressors calving and animal movement on the risk of converting from CMI to HI; and (3) estimate specificity (SP), age-specific sensitivity (SE) and age-specific proportions of animals with CMI and HI

Materials and Methods

Data from approximately 200,000 Danish dairy cows tested in the Danish MAP control programme using MAP ID-Screen® (ID-Vet) milk antibody ELISA were used for these studies. For the first objective, data were used from 222774 Danish Holstein cows. Cows were categorised as test-positive (E+) or test-negative (E-) as suggested by the manufacturer, and assessed using logistic regression

$$\text{logit}(E+) = \text{Parity} + \text{DIM}(\text{Parity}) + \text{Milk Yield}$$

where Parity was parity 1, parity 2 or parity >2, DIM(Parity) was DIM nested in Parity, and Milk yield was the effect of milk yield grouped into 10 equally sized groups. DIM was initially included as a categorical factor, but because the effect from 6 to 350 and from 350 to 500 days in milk seemed piece-wise linear, DIM was included as linear effect in each of those two intervals and retained as a categorical variable from 1 to 6 DIM.

Data from 190,978 cows that had been tested repeatedly were used for the second objective. These animals had been tested repeatedly in period of up to 2.5 years, and a Kaplan-Meier estimator was used to estimate the time to becoming test-positive for cows that had calved compared to those that had not, and for cows that had been moved between farms compared to those that had not in the testing period.

For the last objective, specificity was estimated among 96,138 cows that had a minimum of 5 test results, and where 4 last tests were negative. A cow with the last 4 tests negative was considered a non-case, and the specificity was estimated based on samples obtained before these four test-results. Age-specific sensitivities were estimated among 12,174 cows whose last test was positive. Only test-results prior to these results were used. The proportion q of cows with HI was then estimated as

$$q = \frac{P(HI|Age_i)}{P(HI|Age_i) + P(CMI|Age_i)} = \frac{\max(P(E)) - P(E|Age_i)}{\max(P(E)) - (1 - \text{Specificity})}$$

where $p(E|Age_i)$ was the sensitivity at Age i .

Results

The effect of DIM was large at 1 to 3 DIM, with OR of 27, 9 and 4 at 1, 2 and 3 DIM, respectively, compared to other DIMs. Correction for milk yield resulted in almost linear effects of DIM through remaining lactation, corresponding infection progression (Figure 1).

Calving and movement did not appear to be stressors affecting the risks of progression from CMI to HI, except that animals that had been moved initially appeared to have a lower risk of progression to HI (Figure 2).

The specificity was estimated to 0.985. Age-specific sensitivities increased from 0.22 at 2 years of age to 0.58 at 3 years of age, 0.74 at 4 years of age 0.8 at 5 years of age and 0.83 as the maximum sensitivity. This resulted in q-values of 0.25 among 2-year old animals and 96% for 5-year old cows.

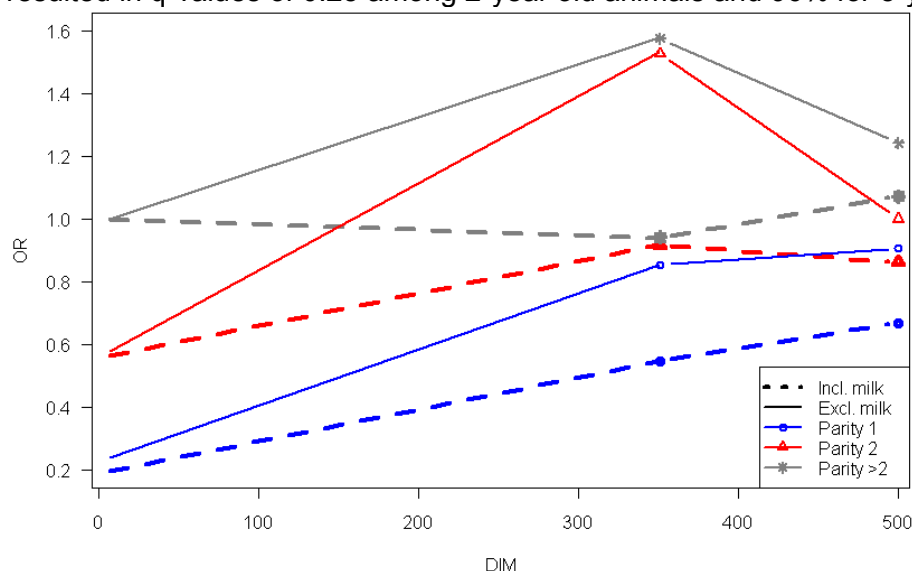


Figure 1. Effect of DIM from DIM 6 to DIM 500 with and without correction for milk yield.

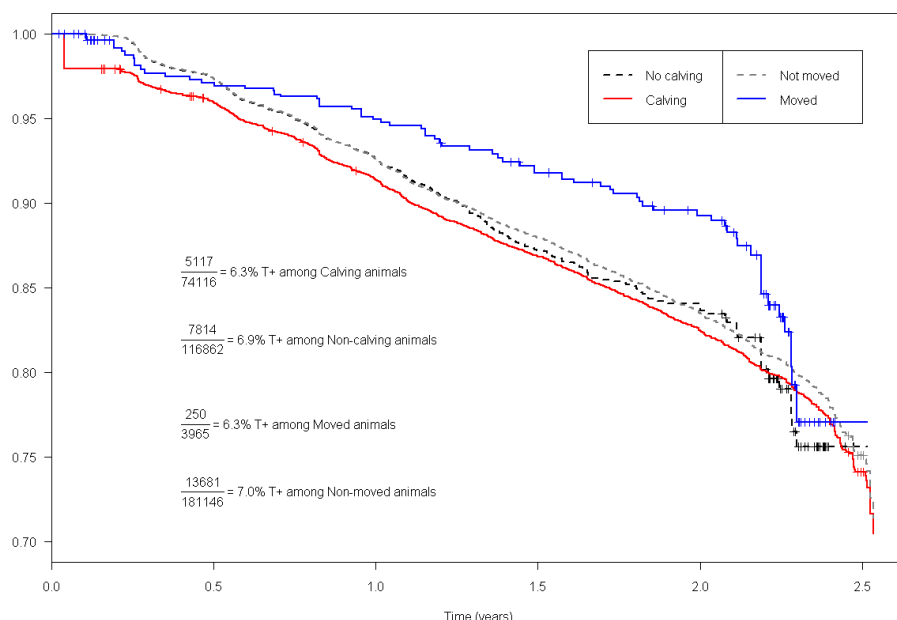


Figure 2. Kaplan-Meier survival plots illustrating the effect of calving and movement.

Discussion and Conclusions

Milk samples obtained 1 to 5 DIM should not be used for diagnostic purposes until their role is elucidated. The reason for the higher odds of testing positive in early lactation is likely the high concentration of non-specific IgG in colostrum, which results in non-specific binding of protein to ELISA-plates.

After 5 DIM, correction for milk yield improved the predictive value of milk ELISA significantly, resulting in an almost linear effect of DIM within each parity group. Without the correction, the pattern was less smooth across lactation (Figure 1).

Contrary to common beliefs, the potential stressors calving and movement of animals did not seem to affect the risk of progression from CMI to HI. Factors affecting this progression are still unknown. The proportion of MAP infected cows with HI can be predicted for specific ages. Knowledge of the age-distribution in a given population would for example allow modelling of infection dynamics. Age-specific sensitivities can be very useful for age-specific predictions of diagnostic test-results, where positive and negative predictive values for different ages can become much more specific at specific prevalences than if average results are used.

USE OF DIFFERENT DIAGNOSTIC TESTS USED TO SCREEN NATURALLY INFECTED PARATUBERCULOSIS CATTLE

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In Tamilnadu different organized cattle farms were selected for screening of naturally infected paratuberculosis animals. The initial screening tests employed were AF examination of faecal samples, IS900 PCR on both faecal and milk (ShrimpeX DNA extraction kit, India), Single intradermal test using Johnin PPD, Gamma interferon assay (Bovigam, USA) and cattle type absorbed ELISA (labor Diagnostik, Leipzig, Germany). There was no significant correlation among the tests used (Data not shown). Hence the IS900 milk and faecal PCR animals were selected as true positives and to examine the progression of the disease the positive 34 white dairy cattle were humanly slaughtered. The gross pathological findings in all 34 animals were emaciated carcass and serous atrophy of fat with thickening and corrugation of intestinal mucosa and mesenteric lymph node enlargement with calcification. Lesions were predominant in the ileum, colon and ileo-cecal junction. Histological lesions were microgranulomas in the intestine and large granuloma in MLN with Acid fast bacilli. The DNA was extracted from intestinal and MLN using Qiagen Dnase (Synergy, Germany) tissue extraction kit. The IS900 PCR was performed on 34 tissue samples from slaughtered cattle collected on Post-mortem and all 34 were positives. 34 cattle which gave positive in IS900 PCR were subjected to DMC-PCR and all 34 samples gave amplicon size of 162 bp specific to sheep type. All 34 smears made from MLN and intestines showed acid fast bacilli suggestive of *Mycobacterium avium* subsp. *paratuberculosis* by ZN staining. On culture in MB7H9 5 isolates from 34 mesenteric lymph nodes and intestinal tissue section samples showed growth in the form of mild turbidity and granularity. On ZN staining and IS900 PCR the growth was confirmed as MAP. From the study it was observed that IS900 PCR of both milk and faecal samples may be used as an initial screening test in commercial herds.

COMPARISON BETWEEN SINGLE INTRA DERMAL TESTS AND GAMMA INTERFERON ASSAY IN ORGANIZED FARMS TO DIAGNOSE BOVINE PARATUBERCULOSIS

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For diagnosis of paratuberculosis single intradermal test was most popular and reasonably accurate in individual animals. The gamma interferon assay has good sensitivity and specificity in detecting paratuberculosis in all age group of animals. To compare the two cell mediated immunity tests 77 healthy dairy cattle from organized farms were selected. The Single Intradermal test (SID) was employed to screen 77 healthy dairy cattle using 0.1 ml of Johnin PPD (IVRI, Izatnagar). Out of 77 cattle aged between 2 and 4 years tested 11 (14.28 per cent) were found to react with Johnin PPD after 72 hours post inoculation in the mid cervical area. The same 77 animals were tested by gamma interferon assay (Bovigam, USA) and 42 animals gave positive with a percentage of 56.75. The diagnostic sensitivity and specificity of γ -IFN over SID was 38.10 per cent, 88.57 per cent respectively. The concordance between these two tests was 61.04. There was highly significant difference between these two tests (7.036**, $P < 0.01$). Based on economic calculation of these two tests the SID test requires two visits to the farm but single visit is enough for gamma interferon assay moreover the sensitivity and specificity of gamma interferon assay is far better than conventional SID. From the above study it was observed that the cell mediated immunity based test gamma interferon assay can better detect early infection in clinically healthy animals.

SEARCH FOR MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) ANTIGENS FOR THE DIAGNOSIS OF PARATUBERCULOSIS (PTB)

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Introduction

Since the sequencing and analysis of the entire *Mycobacterium avium* subsp. *paratuberculosis* (MAP) genome was obtained, several specific proteins have been detected and their immunoreactivity evaluated. However, individual antigens have been able to identify only a subset of PTB-infected animals. Then, a mix of antigens could be a good candidate for serological diagnosis. Several studies have attempted a high-throughput production of MAP recombinant proteins. The present study aimed to evaluate the antigenic capacity of 54 recombinant proteins of MAP to be recognized specifically by sera from animals with PTB and then develop a cocktail of selected antigens to be evaluated by ELISA.

Results

The 54 recombinant proteins included in the present study were characterized *in silico*. According to PSORTb prediction, the recombinant protein set contains 28 cytoplasmic proteins, 7 cytoplasmic and membrane proteins, 6 extracellular proteins, and 13 proteins with uncertain localization.

The 54 proteins were applied in line onto nitrocellulose membranes. Macroarrays of recombinant proteins of MAP were generated and evaluated with sera from animals with PTB. In order to identify potentially cross-reactive epitopes, the macroarrays were also used to probe against sera from healthy animals and from animals experimentally infected with *M. bovis*. Macroarrays were subjected to densitometry analysis to provide quantification for reactivity at each spot. Serum samples with density values higher than the mean obtained with the control (PBS) were considered positive and the number of animals reactive to each protein was obtained.

The antigens selected were those that showed stronger intensity with sera from MAP-infected animals than with sera from non-MAP-infected animals. These antigens were MAP 0038, MAP 0210c, MAP 1272, MAP 1693c, MAP 0209, MAP 2020 and MAP 2513.

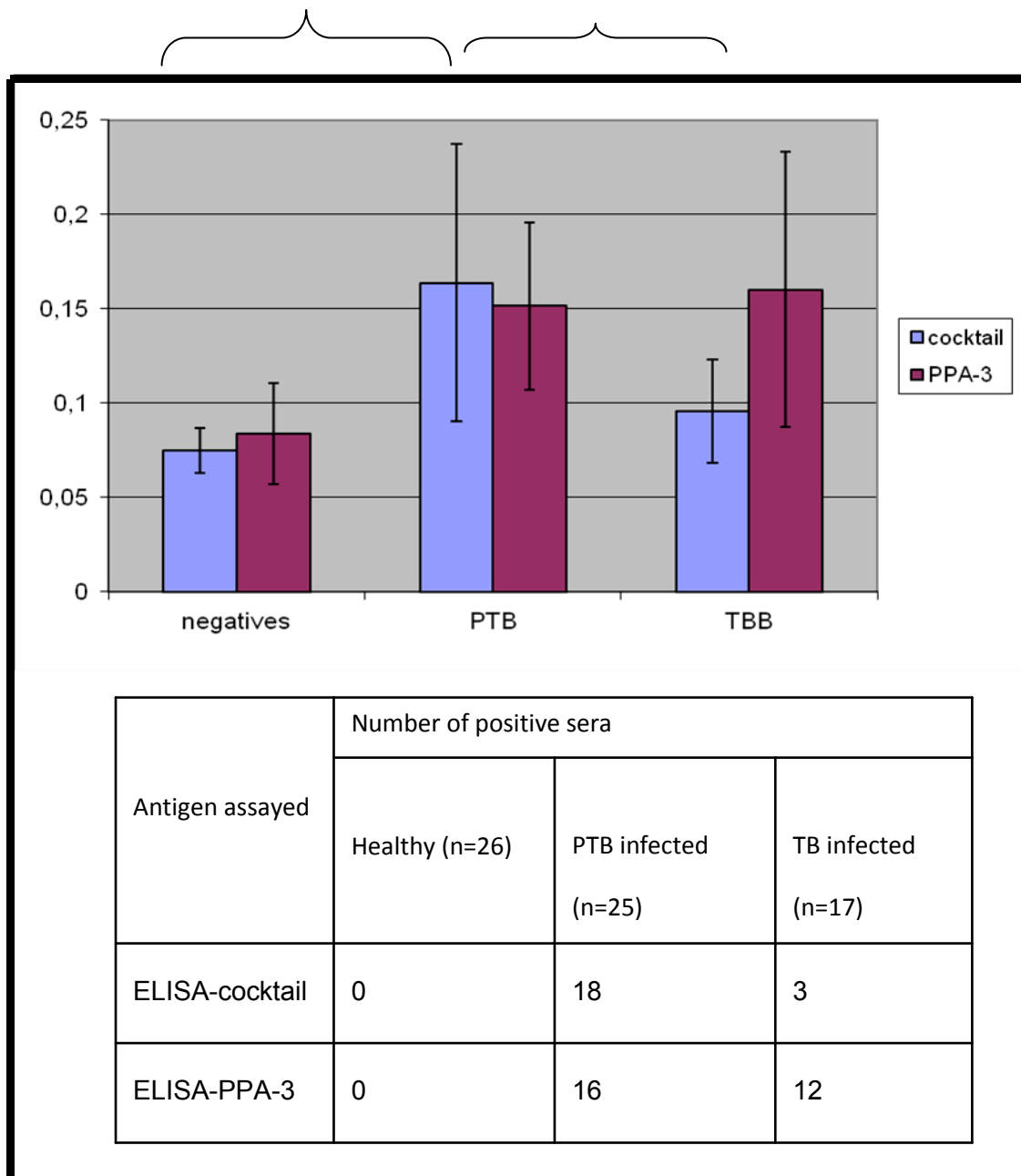
These results contributed to the development of an antigen mixture with the seven antigens, which was used to evaluate whether it was able to enhance the detection of humoral response in animals with PTB. The ORF of MAP 1272 codes for a protein that possesses an NLP/P60 domain of unknown function that is found in several lipoproteins. MAP 0210 codes for a protein similar to the P36/Erp protein of *M. bovis*. The PSORTB analysis software predicted that MAP 2513 is localized in the cytoplasm, that MAP 0210c is localized in the cytoplasm-membrane and that MAP 1272 and MAP 0209c are extracellular. The remaining proteins selected were of unknown localization. All seven of these proteins had not been previously evaluated, except for MAP 0210c.

ELISAs with PPA-3 and with the cocktail with the seven antigens were evaluated with sera from animals with PTB (n=25), healthy animals (n=26) and animals experimentally infected with *M. bovis* (n=17).

The ELISA-PPA-3 test recognized 16 of the 25 animals with PTB but also 12 of the 17 animals experimentally infected with *M. bovis*, while the ELISA-cocktail detected 18 of the 25 animals with PTB and 3 of the 17 animals experimentally infected with *M. bovis*.

$P < 0.0001$

$p < 0.0002$



ASSOCIATION BETWEEN HERD INFECTION LEVEL AND THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) IN BULK TANK MILK USING REAL-TIME PCR IN SMALL HOLDER DAIRY FARMS IN SOUTHERN CHILE

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In southern Chile, a social important spectrum of dairy producers is categorized as subsistence farmers and most of their cows are fed with direct milk consumption from their dams for at least six months. Since variable *Map* quantities have been informed in milk of cattle affected both clinically and subclinically (Giese and Ahrens, 2000), direct detection of the bacterium in milk represents a diagnostic opportunity and a sensitive PCR using milk samples could be an attractive alternative.

The study was carried out in 132 small dairy herds in southern Chile. To determine herd infection level, individual fecal samples were collected from 2,385 lactating cows. In the laboratory, the individual fecal samples were pooled by 5 for *Map* culture detection using the BACTEC MGIT 960 system. In parallel, milk for PCR testing was sampled from the milk buckets or bulk milk tank from each herd. The *Map* DNA extraction procedure for PCR on milk consisted in centrifugation, pooling of pellet and cream fractions to be subjected to enzymatic digestion plus the use of a commercial DNA extraction kit based on mechanical disruption, proteinase K, and column purification. The procedure was followed by real-time PCR. *Map* was culture-detected in 25% of the herds.

Within the infected herds, distribution of pool test-positive results varied by herd ranged between 70-100% (high herd infection level), and 30-69% (moderate), and 12-29% (low). All herds categorized as high and medium herd infection level showed positive milk PCR results (Table 1).

Table 1. Real time PCR results with milk samples related with herd infection status

HERD	N° ANIMALS	POOLS	POSITIVE/TOTAL	INFECTIOUS STATUS	INFECTION LEVEL ESTIMATION	REAL TIME PCR RESULTS
1	40	8	3/8	INFECTED	MODERATE	POSITIVE
74	35	7	7/7	INFECTED	HIGH	POSITIVE
84	18	3	2/3	INFECTED	MODERATE	POSITIVE
88	6	1	1/1	INFECTED	HIGH	POSITIVE
126	23	5	4/5	INFECTED	HIGH	POSITIVE

The results of the study are consistent with what has been informed in the literature. In this regard, sick clinical cows could shed about 100 CFU/mL in milk; meanwhile subclinical animals could shed between 2 to 8 CFU/50 mL or even less (Giese and Ahrens, 2000). However, the fecal contamination of the teats could represent the most important presence in milk, which depends on the amount of *Map* shed by fecal material, as well as the hygiene management (Herthnek, 2008). Although the dilution effect should be taken in mind, the ability of the real-time PCR using milk samples to detect *Map* genome in bulk tank is directly related to the level of *Map* herd infection. More epidemiological studies are needed in this regard. Milk PCR could become a practical sample to identify the most infectious animals in a herd.

Milk samples for Real Time PCR could represent a practical type of sample for identification of those herds with a high level of infection and the most infected animals within them.

ACKNOWLEDGMENTS

This study has been funded by an UACH-COLUN agreement.

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ACCURATE ESTIMATION OF THE ANALYTICAL SENSITIVITY OF AN AUTOMATED BROTH CULTURE AND A REAL-TIME PCR SYSTEM ON THE DETECTION AND CONFIRMATION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP)

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The pathobiology of *Map*-infection is characterized by a very slow progress and late immune response, which make difficult an accurate diagnose.

Liquid culture of *Map* has shown advantages over culture on solid media to detect *Map* infection. Real-time PCR represents a rapid and efficient means of confirming *Map* in broth culture. For *Map* studies, where quantification is needed in order to determine either diagnostic or analytical sensitivity, CFU has been the mostly used unit. Besides, Real-Time PCR represents a faster and more analytically sensitive diagnostic tool in comparison with the conventional PCR systems, to confirm *Map* presence. However, in order to account with a higher analytical sensitivity of the latter it is mandatory to standardize the whole system. This new diagnostic information should give a better evaluation and surveillance of the level of infection.

In order to assess a more accurate estimation of the analytical sensitivity of the above mentioned system, a suspension virtually free of aggregated bacteria was obtained by sonication and filtration of a rich *Map* suspension. The organisms in the suspension were visually quantified by microscopic counting. Thereafter, MGIT tubes and 7H9 agar plates were spiked with serial dilutions of the bacterial suspension for time to detection (TTD) in the BACTEC-MGIT machine and CFU estimation, respectively. Besides, the same serial bacterial dilutions were suspended in PBS buffer and immediately processed for DNA extraction method and tested with real-time PCR to measure the level of inhibition of the commercial MGIT medium to the real-time PCR system.

The analytical sensitivity obtained for a positive MGIT tube with a TTD of 45 days, was just over 9 organisms/ml liquid medium (Table 1), meanwhile the equivalent CFU/plate ranged between 0.5-1. Regarding the Real-Time PCR system (ROCHE), the cycle positive (CP) for a positive results (< 40 CP) was calculated over 10 bacteria (Table 2).

Table 1. Analytical sensitivity of the BACTEC-MGIT 960 system

CFU	Cells	TTD
600	85000	12.08
600	85000	12
600	85000	11.88
60	8500	14.13
60	8500	14.29
60	8500	14.16
6	850	17.33
6	850	18.63
6	850	19.17
0.6	85	25.96
0.6	85	42.5
0.6	8.5	45

Table 2. Analytical sensitivity of the Real Time PCR system

Células	Log células	CP
1040000000	9.017033339	16.42
1040000000	9.017033339	17.6
1040000000	8.017033339	24.95
1040000000	8.017033339	24.73
104000000	7.017033339	S/I
104000000	7.017033339	S/I
10400000	6.017033339	27.42
10400000	6.017033339	30.71
104000	5.017033339	31.83
104000	5.017033339	32.81
10400	4.017033339	35.25
10400	4.017033339	36.61
1040	3.017033339	36.3
1040	3.017033339	38.36
104	2.017033339	38.80
104	2.017033339	39.20
10.4	1.017033339	39.50
10.4	1.017033339	39.80

An inhibitory effect of the MGIT components on the PCR performance was observed, where positive MGIT tubes showed on average 5.6 more CP. The analytical sensitivity according to the estimations of the number of *Map* CFU underestimated the real amount of organisms, hence leading to and overestimation of the analytical sensitivity. The separation of the bacterium from aggregates and the *Map* counting visually improved the estimation of the analytical sensitivity.

The bacterium separation from clusters and its visual quantification is a key element to correctly estimate the analytical sensitivity in the context of paratuberculosis diagnosis. From these results, new studies could be proposed to improve our knowledge of this infection, such as *Map* environmental survival, resistance to drugs and disinfectants, etc.

ACKNOWLEDGEMENTS

This study has been funded by FONDECYT, project N° 11100200.

VALIDATION OF EASY TO USE FIELD LEVEL 'FECAL MICROSCOPY TEST' AND COMPARISON WITH BLOOD PCR FOR THE SCREENING OF LIVESTOCK POPULATION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS

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Along with many developed countries, John's disease (JD) is endemic in livestock population of India and some other poor resource countries. In India, JD control program is hampered majorly due to high cost of advanced and sensitive tests and low priority to JD. In this view screening of animals by fecal microscopic examination seems as a cost effective, easy to perform and sensitive tests for JD surveillance program in low income countries. In present study, the diagnostic potential of fecal microscopic examination (Ziehl Neelsen staining of fecal smear) was compared with blood PCR test on 252 animals (39 goat, 133 sheep and 80 cattle). Of 252 animals 39.3 and 13.1% animals were detected as positive by fecal microscopy and blood PCR tests, respectively. Proportional agreement between fecal microscopy and blood PCR was 72% indicating substantial agreement. Further, microscopy examination test was adopted for the screening of 1262 fecal samples (collected from 281 goat, 602 sheep, 309 cattle and 70 buffaloes of different states of country during 2009-2010). A total of 41.3 % samples were found positive for the presence of characteristic acid-fast bacilli. The prevalence was higher in cattle (61.8%) followed by goat (41.3%), sheep (33.4%) and buffalo (15.7%). Overall results showed that fecal microscopy may be used as a promising cost effective test in large scale screening of livestock population.

DETECTION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS BY 251 GENE LOCUS POLYMERASE CHAIN REACTION IN THE TISSUES OF NATURALLY INFECTED SHEEP

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Abstract

In the present study, a PCR targeting 251 gene locus of MAP was developed and applied on suspected and healthy tissue samples, and the results were compared with microscopic examination of Ziehl Neelsen's (ZN) stained smears. Of 15 suspected tissue samples, 13 (87%) were positive by 251 gene locus PCR and 12 (80%) were positive by smear examination. The higher sensitivity of PCR assay in comparison to ZN method suggested that 251 gene locus PCR assay could be used as a rapid and confirmatory test for Johne's disease investigation.

Introduction

Diagnosis of paratuberculosis (Johne's disease) is difficult especially in early and sub-clinical stages. Among the various diagnostic tests, bacterial culture is considered a definitive test for paratuberculosis, but it is time consuming³. The polymerase chain reaction (PCR) based assays especially IS900 gene has been extensively used for diagnosis of MAP infection on clinical samples including faeces, blood, milk and fresh and fixed tissues^{3,7}. However, IS900 like gene sequence has been reported in other mycobacterial species, therefore, it is imperative to examine potential of other specific gene such as 251 gene⁶. Despite being present in a single copy, the sensitivity of 251 gene locus PCR was found to be comparable to the IS900 PCR⁶ but was rarely employed for diagnosis of MAP infection. In the present study, a 251 gene locus PCR was developed for the detection of MAP in tissues and compared with microscopic smear examination.

Materials and Methods

A total of 15 naturally died sheep received for post mortem examination at Division of Animal Health were thoroughly examined for the gross lesions of paratuberculosis during necropsy. Five healthy adult sheep tissues (intestines and lymph nodes) were collected from LPT section of the institute and used in the study as uninfected control animals. The sections were also collected for histopathology in 10% neutral buffer formalin.

One gram of tissue was suspended in 3 ml sterile distilled water in a pestle and mortar and homogenized. Smears prepared from pellet after centrifugation were heat fixed and stained with Ziehl Neelsen's (ZN) staining procedure.

DNA was extracted from the tissue homogenate using HiPura kit following manufacturer instruction with slight modification (HiMedia, India). The primers (251-F-5'GCAAGACGTTTCATGGGAAGT3' and 251-R-5'GCGTAACTCAGCGAACAACA3') flanking a region of 203 bp of 251 gene locus were designed commercially⁶. The PCR reaction mixture (50 µl) contained 1X PCR buffer, 2 mM MgCl₂, 200 µM dATP, dGTP, dCPT and dTTP, 0.5U of Taq DNA polymerase and 1 µM of primers and 2 µl of template DNA. The PCR amplification was carried out in an automated thermal cycler according to the following programme: initial denaturation at 94°C for 5 min, followed by 40 cycles each of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min and synthesis at 72°C for 1 min, and final elongation at 72°C for 1 min. For each round of PCR, positive DNA (from culture of MAP) and negative DNA control were included. The PCR products were analysed by visualization of desired size of DNA band in the ethidium bromide stained agarose gel (1.5%).

Results and Discussion

In India, paratuberculosis has been reported in all domestic ruminants since 1930's and considered a problem more in animals of the organized farms than in animals reared in villages and semi intensive system. In the present study, majority of the sheep suspected for the JD (n=12) exhibited deterioration in the body condition, emaciation, bony prominence and chronic intermittent diarrhoea. In some cases oedematous fluid in subcutaneous tissues and gelatinization of visceral fat was also noted. The small intestinal lesions varied from mild velvety thickening to corrugation of mucosa, which was more prominent from mild jejunum to the distal ileum. The ileocaecal valves (ICV) were consistently thickened.

The cut surfaces of MLN bulged out and distinction between cortex and medulla was generally lost. In a few sheep, thickening and corrugation extended to large intestines especially caecum and proximal part of the colon. Microscopically, 10 sheep showed paratuberculosis lesions such as multiple granulomas, diffuse sheets of epithelioid cells and a few giant cells in the intestine and lymph nodes. Another three sheep showed focal or multiple granulomas, diffuse infiltration with lymphoid cells and macrophages, giant cell formation in the intestine and lymph nodes. Similar clinical signs, gross and microscopic lesions have been reported previously in ovine paratuberculosis^{1,7}. In two sheep, intestinal and MLN lesions were not prominent and diagnosed to have been died due to other etiologies such as ruminal impaction and pneumonia. All uninfected control animals (n=5) had healthy ileum and mesenteric lymph nodes with no lesions of Johne's disease and were paratuberculosis negative by ZN staining and PCR. Microscopic smear examination method is simple, quick, and economic and widely used in the preliminary diagnosis of JD. In the present study, 12 (80 %) out of 15 sheep were found to be positive for AFB. Generally, the sensitivity of the ZN staining have been reported to be lower (30-60 %) by previous workers⁵, however, in the present study it was quite higher (80 %) which was probably due to the fact that most sheep tested were in clinical forms of disease and the sensitivity of ZN method is always better in clinical forms than the subclinical form of the disease^{5,3}.

The 251 gene locus PCR was reported to be 100% sensitive and specific when tested on MAP and non-MAP isolates, but has not been widely tested on clinical samples. In the present study, 13 (87%) out of 15 sheep were found positive by 251 gene locus PCR. All the sheep suspected for JD during necropsy were found to be positive for MAP genome. Similar sensitivity of 251 gene locus PCR has been reported previously in multibacillary sheep (93 %) in naturally occurring paratuberculosis⁷. In free ranging bison faecal samples 251 gene nested PCR detected 3.1% (26/835) as positive². When the results were compared, 251 gene locus PCR was found to be superior (87 %) than ZN staining (80 %) in the detection of MAP. One sheep in this study diagnosed as pneumonia at necropsy, was negative for AFB but was positive in the PCR, which demonstrates the potential of this PCR assay in detecting subclinical infection in tissues. A sheep showed gross lesions similar to the JD, but was negative by ZN staining and positive by PCR. The negative result of ZN staining might be due to the fact that ZN identifies only intact microorganisms⁴.

The results of the present study suggest the importance of 251 gene as a potential target of diagnostic value for postmortem confirmation of paratuberculosis cases.

Acknowledgement

The study was funded by an institute and national fund project to carry out this research work.

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OPTIMIZATION OF METHODS FOR THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN MILK AND COLOSTRUM OF NATURALLY INFECTED DAIRY COWS

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Mycobacterium avium subsp. *paratuberculosis* (MAP) is primarily shed into the feces but it has also been isolated from the milk and colostrum of cows. Because of this, there exists concern about transfer of the organism from dam to calf and about the prevalence of MAP in the milk supply. The prevalence of MAP in milk is not well defined because of the complexity of the milk matrix. The objective of this study is to optimize the decontamination of whole raw milk for the isolation of viable MAP and compare recovery rates in liquid and solid culture mediums. The efficacy of two liquid culture mediums, TREK-ESP and BD Bactec 12B were compared for recovery thresholds, speed of recovery, incidence of contamination and reproducibility of results. Milk collected from a non-infected cow was spiked with MAP (10^2 to 10^8 cfu/ml). Variables investigated included concentration of hexadecylpyridinium chloride (HPC) as the primary decontaminant, temperature of decontamination, centrifugation speed, and time of incubation. It was found that neither length of exposure to HPC or concentration of HPC had significant effects on the recovery of MAP from milk. Because of known lethal effects of HPC on MAP, the most efficient decontamination parameters with the highest recovery rates of MAP were 0.75% HPC exposed for 5 hr at room temperature. Since increased temperature ($>25^\circ\text{C}$) enhanced growth of microbial contaminants, room temperature was the optimal choice. In comparing the two liquid culture mediums, Bactec 12B was superior in recovery thresholds and speed of recovery of viable MAP. TREK-ESP culture demonstrated an increased incidence in false positive and false negative results that were not observed in Bactec 12B medium. Future decontamination studies will evaluate the efficacy of N-acetyl-L-cysteine (NALC)-sodium hydroxide. Optimized methods will be used to assess the frequency and level of MAP shed into milk during a complete lactation period of naturally infected dams.

INTRODUCTION

The primary route of exposure of neonates to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is fecal-oral, however, MAP is also shed into the milk and calves can be exposed to this pathogen by suckling the dam or being fed colostrum or waste milk from infected cows. There is little information in the literature to document the shedding of MAP into the colostrum and milk of infected dams, particularly, the bacterial load and how this relates to the infection status of the dam and the stage of lactation. This is due in part to the difficulty in culturing the organism from a complex moiety such as milk. Yet if producers could understand the association of disease with bacterial load in the milk they might be willing to make critical management decisions to further prevent dissemination of infection within the herd.

In order to obtain this information, an effective culture protocol must be established. Because of the presence of nonspecific microorganisms found naturally in milk, a decontamination protocol must be determined to inhibit the growth of the non-target microorganisms while minimally affecting the viability of MAP. In addition, various culture mediums, including liquid and solid mediums, must be investigated for their efficacy to determine the superior medium. Together, the decontaminating protocol and the efficacy of the mediums will be combined to ensure that the bacteria detection thresholds are as low as possible.

MATERIALS AND METHODS

Milk collected from a non-infected cow was inoculated with live MAP (strain 167 from a clinical cow) to achieve final concentrations of 10^8 , 10^6 , 10^4 , 10^2 cfu/ml. Non-inoculated milk was used for a negative control and 10^6 cfu/ml in PBS was used as a positive control. Milk samples were separated by centrifugation and the whey layer was discarded leaving the pellet and the cream. Two different decontaminating chemicals were investigated: hexadecylpyridinium chloride (HPC) and N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH). Milk was treated with either HPC at concentrations of 0.75, 1.00, 1.25, and 1.50% for 5, 24, and 48 hours, or with NALC-NaOH solution consisting of 0.25% NALC,

0.725% sodium citrate, and 0.5, 1.0, 1.5, and 2.0% NaOH for 5, 15, and 30 minutes. After each chemical treatment samples were centrifuged again, discarding the chemical layers and retaining the cream and the pellet. The cream and pellet were resuspended in PBS for media inoculation. Each decontaminated milk sample was inoculated into three mediums: Bactec 12B, Trek-ESP *para*-JEM, and Herrold's Egg Yolk media (HEYM) each supplemented, incubated, and monitored with their own specifications.

RESULTS AND DISCUSSION

Results suggest that the concentration or time of exposure to HPC does not influence the level of non-target microorganisms in milk samples and recoveries of MAP in the milk were also not affected. However, high concentrations of HPC (1.25%) and time of exposure (48 hours) may reduce the recovery of MAP. Therefore, optimal decontamination conditions for milk samples were determined to be 0.75% HPC for 5 hours. Experiments to determine optimal conditions for treatment of samples with NALC-NaOH demonstrated that increased length of exposure and increasing concentration of NaOH did decrease the level of contaminating microorganisms. In contrast to HPC, increasing concentrations of NaOH did not appear to influence the recovery of MAP. Optimal decontamination conditions for NALC-NaOH were determined to be 1.5% NaOH for 15 minutes. HPC was superior at reducing fungal contamination and NALC-NaOH was superior at reducing bacterial contamination. The NALC-NaOH decontamination protocol also appears to result in more rapid recovery of MAP than the HPC decontamination protocol (Fig. 1). A comparison of MAP recovery from the two liquid medium systems showed that Bactec 12B media was superior to Trek-ESP *para*-JEM in sensitivity of MAP detection and the speed of recovery from inoculated milk samples (Fig. 1). HEYM is inferior to both liquid medias in respect to MAP recovery. The liquid media each have their own strengths with regard to the abilities to suppress the growth of non-target microorganisms. The Bactec 12B media was superior in anti-bacterial properties and the Trek-ESP *para*-JEM was superior in anti-fungal properties.

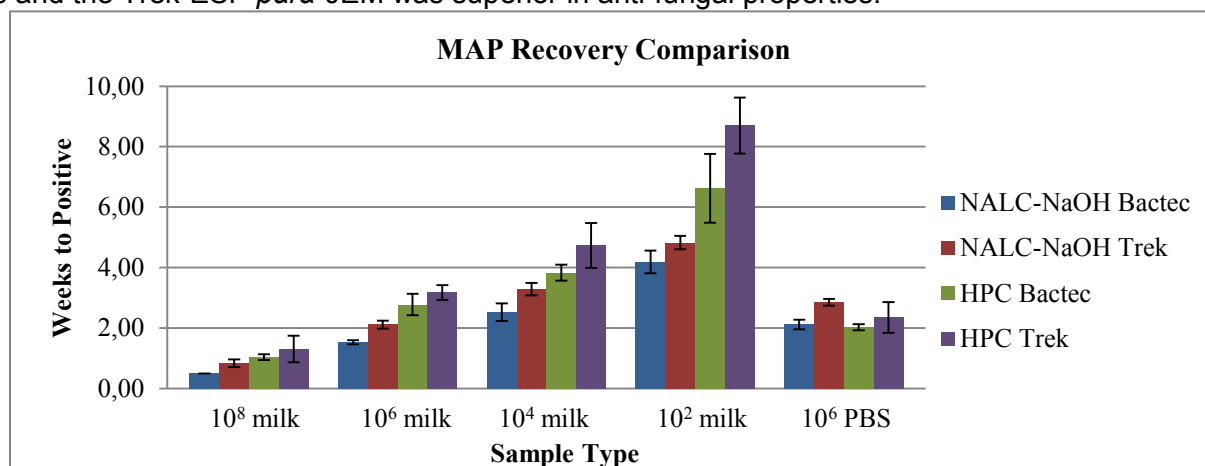


Figure 1. Comparison of decontamination protocols and liquid culture mediums on the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from milk

CONCLUSION

NALC-NaOH is the superior chemical for reducing the level of non-target microorganisms at 1.5% NaOH for 15 minutes, while minimizing the kill of MAP. Bactec 12B media is the superior media for the lowest detection threshold and fastest time to detection. Each liquid medium has its own strengths with regard to suppressing the growth of non-target microorganisms. Both liquid mediums have more sensitive detection thresholds than HEYM.

ACKNOWLEDGMENTS

We would like to acknowledge and thank the support staffs at the National Animal Disease Center and the National Veterinary Services Laboratories in Ames, IA for their excellent technical assistance.

BAYESIAN ESTIMATION OF THE SENSITIVITY AND SPECIFICITY OF INDIVIDUAL FAECAL CULTURE AND PARALISA™ TO DETECTED MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTION IN YOUNG FARMED DEER

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The aim of this study was to estimate the sensitivity and specificity of individual faecal culture (IFC) and an IgG1 serum enzyme-linked immunosorbent assay, the Paralisa™, to identify young (12-24 month old) deer infected with *Mycobacterium avium* subsp. *paratuberculosis*. The unit of analysis was the individual, and the primary objective was to assess test accuracy for the purpose of herd classification or “freedom from infection” sampling.

Paired faecal and serum samples were collected from 20 individual yearling deer from 20 herds in the South Island and 18 herds in the North Island of New Zealand, and subjected to the candidate tests. A priori, the two islands have differing infection prevalence, and a two-test two-population Bayesian latent class model was developed, incorporating a zero-inflated random effect logistic model to allow zero-infection herd status, as well as capturing variation in within-herd prevalence. The estimate of IFC sensitivity was 77% (95% CI: 61-92%) with specificity 99% (95% CI: 99-99.7%), while the Paralisa™ sensitivity estimate was 19% (95% CI: 10-30%), with specificity 94% (95% CI: 93-96%). All estimates were robust to variation of priors and assumptions tested in a sensitivity analysis.

These are the first independent estimates of test performance in a sub-clinically infected young deer population, and may be used to inform the use of the tests to determine infection status at the individual and herd level.

SENSITIVE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* FROM COW'S BLOOD WITHIN 48 H

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Existing methods for the routine detection of MAP are problematic due to low or variable sensitivity. Although culture remains the Gold standard for MAP detection, the need for chemical decontamination of samples reduces sensitivity, requires lengthy incubation of samples and PCR identification of isolates. Hence routine diagnoses of Johne's disease in the UK relies on either blood or milk ELISA tests but these are reported to have variable sensitivity, especially in sub-clinically infected animals, making multiple repeat tests necessary. PCR-based methods of detection have been developed, however the sensitivity is limited by DNA extraction efficiency and most tests do not provide live/dead differentiation. The *FASTPlaqueTB*TM assay uses a mycobacteriophage (D29) to detect *Mycobacterium tuberculosis* in human sputum samples. We have successfully adapted this test to detect viable MAP in milk within 48 h (Stanley et al 2007; Botsaris et al., 2010).

In this work we have been investigating whether this assay can be used to detect low numbers of MAP in bovine blood samples. Experiments were performed to optimise bacterial recovery from blood and to determine the limit of detection of the assay. The optimised blood assay and *FASTPlaqueTB*TM kit reagents were then used to test blood samples from cows that had been identified as suffering from Johne's disease using repeated milk ELISA tests. Viable MAP cells (approximately 20 pfu/ml) were detected in blood taken from all nine of the infected animals within 2 days and results were confirmed by PCR. This test shows promise as a new rapid diagnostic that can be applied to all animals in a herd and removes the need for multiple testing for accurate diagnosis.

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DETECTION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) IN ALCOHOL-FIXED TISSUES OF SHEEP BY ISMAV2 GENE PCR AND ITS COMPARISON WITH HISTOPATHOLOGY, BACTERIAL CULTURE AND IS900 PCR

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Abstract

Tissue samples preserved in 70% alcohol for 6-8 months from 23 naturally infected paratuberculous sheep and 7 healthy sheep were used for DNA extraction. In PCR amplification targeting ISMav2 gene of MAP, 19 (82.6 %) were found to be positive. Bacterial culture, ZN and fresh tissue IS900 PCR detected 65%, 100%, and 95% cases, respectively. It was concluded that alcohol could be an alternative fixative for transportation of tissues for molecular detection of MAP genome in tissues by ISMav2 PCR, which compared well with fresh tissue IS900 PCR for the diagnosis of paratuberculosis in sheep. This may be useful in tropical countries, where shipment of fresh tissues for molecular diagnosis may be expensive proposition and most of the times facilities for maintaining cold chain are not available.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the cause of chronic granulomatous enteritis of domestic and wild ruminants and responsible for huge economic losses world-wide. The confirmative diagnosis of MAP infection is achieved by bacterial culture, which is cumbersome and time consuming. Nucleic acid based detection methods are rapid and specifically detect MAP genome in a variety of biological samples reducing the time of detection to 2-3 days. The ISMav2 gene, present in at least 3 copies, has been used in the conventional as well as real-time (RT) PCR as a sensitive and specific method for detection of MAP in faeces and milk [6, 8]. Reports have shown that PCR can be used to detect MAP genome in formalin-fixed paraffin embedded tissue sections [7]. However, formalin adversely affects the quality of mycobacterial DNA [5, 9]. Tissues fixed in alcohol and other fixatives such as methacarn, acetone, and OmniFix used for DNA extraction and subsequent PCR amplification have shown superior performance over formalin-fixed tissues [2, 9]

Therefore, the purpose of this study was to determine the efficacy of an ISMav2 PCR, a less frequently used target sequence for diagnosis MAP infection on DNA extracted from alcohol-fixed tissues of naturally infected paratuberculous sheep and its comparison with pathology, bacterial culture and fresh tissue IS900 PCR.

Materials and Methods

Twenty three adult sheep of either sex, originating from known paratuberculosis infected flocks in a semi-arid district of Rajasthan were used in the study. Breeds included were Avikalin, Chokla and Garole. All 23 animals were diagnosed as MAP positive by regular clinical and fecal smear examination. These animals culled or brought dead to the division were subjected to detailed necropsy examination. The representative tissue sections from the small intestines (duodenum, jejunum, ileum and ileo-caecal valve) and associated mesenteric lymph nodes (MLN) were collected in 10% buffered neutral formalin for histopathology. Three adjacent portions of the ileum were collected in sterile vials: one for culture and another for IS900 PCR were brought on ice to the laboratory and stored at -20° C until used. The third portion was collected in 70% alcohol and kept at room temperature for 6-8 months before subjected to DNA extraction and PCR analysis. Comparable tissue samples from seven healthy sheep slaughtered at small animal slaughterhouse, Bareilly, were also collected for histopathology, culture, and fresh tissue and alcohol-fixed tissue PCRs. Bacterial culture and confirmation were carried out as described by Sivakumar et al. [4].

Extraction of DNA from fresh tissue sample was performed as per the method described previously [4]. Briefly, 1 g of alcohol-fixed ileum from each animal was cleaned, washed three times with sterile distilled water (DW). After homogenization in 4 ml sterile distilled water, the suspension was allowed to settle. A

quantity of 200 µl of homogenate was taken into a sterile microcentrifuge tube and added with 500 µl of TE buffer and kept undisturbed for 15-20 min. The mixture was then centrifuged at 13000 g for 10 min and supernatant was discarded. The DNA was extracted using a commercial kit with slight modification (Himedia Laboratories). The collected sediments after addition of 180 µl of lysis solution and 20 µl of proteinase K (20 mg/ml) was incubated at 56°C overnight. The subsequent procedures were followed as per manufacturer's instructions. ISMav2 PCR on alcohol-fixed tissue DNA [6] and IS900 PCR on fresh tissue DNA were performed [1].

Results and Discussion

Histopathology is the oldest and time-tested method for diagnosis of paratuberculosis in animals. But due to possible involvement of other mycobacteria in causation of granulomatous enteritis and lymphadenitis, laboratory confirmation by specific detection of MAP or its genome is required. In most of the developing countries especially tropical ones, shipment of fresh biological samples is practically not feasible, and therefore, alternative methods are required to be developed. Gross and histopathological features in MAP infected group showed typical lesions of paratuberculosis. Out of 23 cases, 19 (82.6 %) were positive in ISMav2 PCR. All animals in control group were PCR negative. The IS900 PCR carried out on fresh tissue samples on these sheep was positive in 95% cases. The bacterial culture on HEY medium detected 65% of all positive cases tested. None of the tissues from healthy control sheep was positive in any of the tests.

During necropsy of sheep, tissue samples are routinely collected in 10% formalin for histopathology. While these tissues are good enough for histopathology, they are not suitable for nucleic acid detection as formalin has deteriorating effect on the quality of DNA and, therefore, on the sensitivity of PCR detection methods [6] (Srinivasan et al., 2002). Our procedure of DNA extraction and ISMav2 PCR on alcohol-fixed tissues could detect 82.6% of all cases tested which had demonstrable AF bacilli in tissue sections. The results were comparable to IS900 PCR that was carried out on the freshly collected tissue samples. Despite the wide difference between the copy number of these two genes: IS900 (approximately 17 copies) and ISMav2 (3 copies), non-significant differences in the detection rate of two PCRs suggest the utility of alcohol-fixed tissue PCR. The sequence analysis of the PCR products (GU045498) showed 98.1 per cent homology with published sequences in the NCBI database. The specificity of the primers used in the study needs further confirmation in light of a recent report [3]. Thus, based on the previous reports and our results, alcohol appears to be a superior fixative for PCR analysis, but it is generally not recommended for histologic analysis because of excessive shrinkage and reproduction of tissue architecture. Therefore, if PCR analysis is anticipated for laboratory confirmation of MAP infection, tissue samples would be split into two halves; one half to be fixed in 10% formalin and other half in 70% alcohol. Though this aspect was not investigated in the study and could be a subject for further research, it is expected that tissue specimen fixed in alcohol could provide opportunity for detailed analysis of DNA for molecular typing or other purposes especially for the organism like MAP which is fastidious and slow grower.

From our study it is observed that ISMav2 PCR on alcohol-fixed tissues is a sensitive method in comparison to culture, and comparable to IS900 PCR on fresh tissue samples. The test offers an alternative or an additional method for the rapid detection of MAP in the biological samples from suspect animals.

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MOLECULAR DETECTION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* ENTERITIS FROM PARATUBERCULOUS AND NON-PARATUBERCULOUS ENTERITIS OF SHEEP IN INDIA

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Abstract

Tissue samples were collected from 50 sheep showing gross abnormality in the intestine and associated lymph nodes following examination of over 500 sheep post-slaughter for histopathology, bacterial culture and PCR. Many of these animals had poor body conditions and soiled perineum. Eighteen sheep showed mild to severe histological lesions characteristic to early (paucibacillary, 12) and advanced (multibacillary n= 6) cases of paratuberculosis. The remaining 32 cases had lesions of nongranulomatous chronic enteritis, subacute enteritis and parasitic enteritis. Six (33.3%) of 18 histologically identified paratuberculosis cases were positive in the bacterial culture, while none was positive in non-paratuberculous cases. The conventional ISMav2 PCR detected 44.4% (8) and 28.1% (9) and the real-time PCR detected 66.7% (12) and 40.6% (13) cases positive from histologically positive and negative cases, respectively. Taking all cases together, detection rates for ZN staining, culture, conventional and RT-PCR were 20%, 12%, 34%, and 50%, respectively.

Introduction

Paratuberculosis is widely distributed in most countries of the world including India (Stabel, 2000; Tripathi et al., 2006). In India, paratuberculosis is generally considered a problem of organised herd/flock than at unorganised farm, where incidence is largely unknown. It is generally difficult to detect MAP during subclinical and carrier stages, therefore, clinical detection of MAP infection is regarded as an underestimate of actual infection rates in the animals. However, the detection of MAP and associated tissue reaction could be an indicator for the prevalence of the infection. In the present study, we collected tissue materials from sheep slaughterhouse and analysed for the MAP infection by bacterial culture, histopathology and conventional and quantitative PCR.

Materials and Methods

For pathological, bacteriological and molecular studies, tissue samples (duodenum, ileum, mesenteric and ileo-caecal lymph nodes) were collected from Bareilly slaughterhouse (n =500). Tissue samples from 10 healthy adult sheep were also collected from the slaughterhouse and used as a control. Detailed necropsy examination were performed on all animals. For routine histopathology samples were collected in 10 per cent formalin and for molecular studies, samples were collected in sterile tubes on ice. Formalin-fixed tissues were processed conventionally for preparation of H&E and ZN stained slides. Bacterial culture was carried out as described previously (Tripathi et al., 2006).

Extracted tissue DNA was subjected to ISMav2 PCR using oligonucleotide forward ISMav1- 5'GTA TCA GGC CGT GAT GGC GG3' and reverse ISMav2- 5'CCG CAC CAG CGC TCG ATA CA3' primers flanking 313 bp nucleotides of ISMav2 gene of MAP (Stratmann et al., 2002). The PCR products were analysed by agarose gel electrophoresis. PCR product of two test samples (BDR-16, S-23) and a positive control (IVRI/C-132) were sent for sequencing for the confirmation of amplified products of ISMav2 gene. Quantitative real-time PCR (q-PCR) using same primers was performed (SYBR Green Master Mix (Invitrogen, USA). The standard curve was created by using known standard MAP DNA, measuring the concentration of DNA by Nanodrop. This was converted to genome copy numbers using mass of DNA per copy of the gene. The mass of the MAP genome was calculated by using the formula : $m = \{n\} \{1.096e^{-21} \text{ g/bp}\}$, Where, n = Genome size ((MAP genome size is 4829780 bp), m = Mass of the genome, $e^{-21} = 1 \times 10^{-21}$. The mass of genomic DNA per copy of gene of interest was worked out in the standard DNA sample and then serially diluted in tenfold dilution to achieve copies of 3×10^6 to 3×10^1 . The standard curve in terms of a regression line equation was drawn by plotting the known gene copies in the dilutions against the threshold values ("Ct"). Finally, the MAP ISMav2 gene copies in the clinical samples were estimated based on the regression line equation of standard curve with the help of intercalated DNA template calculator displayed on the screen.

Results and Discussion

Most of these animals had poor body condition and soiled perineum. In bacterial culture, 4 samples were positive for MAP after 16 weeks of incubation. On gross pathology 50 sheep showed perceptible thickening or corrugation of intestinal mucosa (ileum and ICV) and enlargement of lymph nodes whereas, samples from 40 sheep were either had acute enteritis or had no grossly visible thickening. Most of the animals showed lesions on the distal small intestine and ileo-caecal valve (ICV). Out of 50 animals, 6 (12%) showed variable degree of thickening and corrugations of the intestinal mucosa. Eighteen sheep showed mild to severe histological lesions characteristic to early (paucibacillary, 12) and advanced (multibacillary n= 6) cases of paratuberculosis. The remaining 32 cases had lesions of nongranulomatous chronic enteritis, subacute enteritis and parasitic enteritis. Six (33.3%) of 18 histologically identified paratuberculosis cases were positive in the bacterial culture, while none was positive in non-paratuberculous cases. In all histopathology positive cases the lesions were characteristics of MAP infection.

The conventional ISMav2 PCR detected 44.4% (8) and 28.1% (9) and the real-time PCR detected 66.7% (12) and 40.6% (13) cases positive from histologically positive and negative cases, respectively. The number of ISMav 2 gene copies detected ranged from 7.82×10^1 to 9.016×10^5 and 1.45×10^2 to 1.523×10^4 in histologically positive and negative cases, respectively. Taking all cases together, detection rates for ZN staining, culture, conventional and RT-PCR were 20%, 12%, 34%, and 50%, respectively. Sequences of PCR products analysed from a sample showed 98-99% homology with published sequences available in the NCBI database.

At a number of occasions, it has been observed that in spite of presence of characteristic lesions of paratuberculosis, AFB were not demonstrated by ZN staining and bacterial culture. The negative bacterial culture in most of the cases and non- demonstration of AFB in tissue sections of PCR positive sheep would accord with previous observations that bacteria were difficult to demonstrate in early lesions (Corpa *et al.*, 2000). In this study, we could observe that the non- granulomatous chronic enteritis cases, which were negative by histopathology, were positive for MAP genome. Demonstration/detection of MAP genome (ISMav2 gene) in some cases of chronic (41.6%), subacute (50%) and parasitic enteritis (25%) without granuloma and demonstrable AFB suggest that these sheep might have been infected during early life, but had resisted infection and kept the infection under control without completely eliminating them. Their benign presence could be a source of mild irritation resulting in mild to moderate inflammatory changes. Such animals could serve as carrier of infection. It has been earlier reported that some animals remained carrier through out their life without being apparently ill. Though, we confirm the PCR products by sequencing, in view of a recent report of non-specific results obtained with this primer (Mobius *et al.*, 2007), the diagnostic value of this PCR needs re-evaluation.

It was concluded that all granulomatous enteritis cases indistinguishable from paratuberculosis lesions in sheep couldn't be confirmed as paratuberculosis even by qPCR. On the other hand detection of MAP genome in many cases without detectable characteristic histological lesions and positive bacterial culture suggest that a sizeable population of sheep may be infected but never become clinical, possibly influenced by a genetic immune mechanism.

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DEVELOPMENT OF A LAB-ON-A-CHIP IMMUNOASSAY FOR DIAGNOSIS OF JOHNE'S DISEASE

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INTRODUCTION: Johne's disease (JD) is caused by infection of mostly ruminants (including dairy cattle) with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and is responsible for significant economic losses to the global dairy industry. Diagnosis of JD is currently conducted in diagnostic laboratories, creating costly expenses to dairy farmers for veterinary service, sample handling, and shipping. An automated on-site diagnostic device for JD would reduce these diagnosis-related costs. Lab-on-a-Chip (LOC) technology has been used in various analytical processes and is offering opportunities for the development of on-site diagnostic devices. In this report, we developed and tested a LOC immunoassay system based on AC electrothermal (ACET) effect for detection of JD-specific antibodies in bovine serum samples.

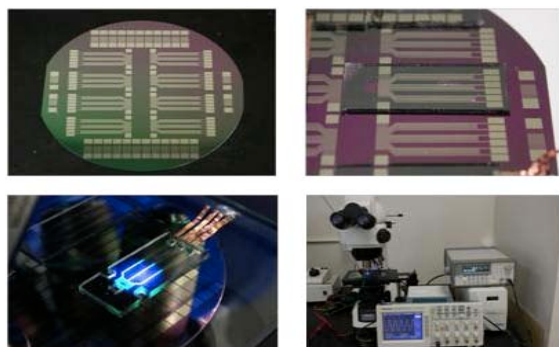


Figure 1. Experimental set of the Lab-on-a-chip device. Electrodes were fabricated with one-mask photolithography and lift-off process. Microchannels are fabricated using PDMS (polydimethylsiloxane) soft lithography. The optical detection set up included a LED with 490 nm wavelength (Aqua 15 5mm LED, LED Supply, Randolph, VT), a LED mounts (S1LEDM, Thorlabs, Newton, NJ), a shortpass filter with cut-off wavelength at 500 nm (FES0500, Thorlabs, Newton, NJ) and aspheric lenses.

METHODS: The LOC used in this study was composed of poly-dimethylsiloxane (PDMS) microchannels sealed over an ACET electrode chip (**Figure 1**). MAP antigens were extracted from the bacteria with 80% ethanol [1] and the surface of the ACET electrode chip was coated with the antigen and, after blocking uncoated surface, reacted sequentially with bovine serum sample and fluorescently (DyLight488) - labeled secondary antibody. Liquid flow was electrically controlled by ACET micropumping effect. The level of antibody binding was then measured by using a LED-induced fluorescence with a low cost mini-spectrometer. JD-positive and JD-negative serum samples were tested with this LOC immunoassay system. Further, the limit of detection of the LOC system was determined by measuring binding of fluorescently (DyLight488)-labeled anti-bovine IgG molecule to bovine IgG whole molecules immobilized on the microchip. □

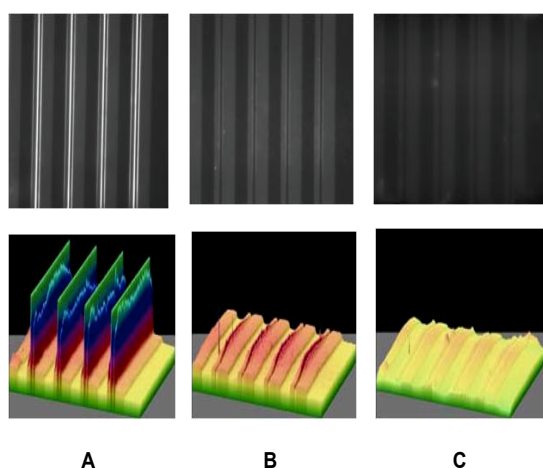


Figure 2 (A, B and C) . Preliminary results with optimized conditions. Using various optimized conditions we tested positive (A) and negative (B) serum sample and compared it with a control (C) group.

RESULTS: The ACET - LOC immunoassay experiments were conducted using PDMS microchannels sealed over an ACET electrode chip. Our preliminary studies have shown that ACET - LOC sped up the binding process and achieved accelerated detection [2]. A numerical simulation revealed that the acceleration is due to the ACET effects – generation of net pumping effect and vortices to guide target molecules towards the reaction site. The results in **Figure 2** are from the experiment which was conducted to observe and evaluate the antigen-antibody binding process on the ACET microchip. **Figure 2** shows fluorescence images of MAP-antigen-coated microelectrodes after incubations with serum samples (JD-positive serum [A], JD-negative serum [B], and no serum [C]) and fluorescently-labeled secondary antibody. A clear differentiation between the JD-positive and JD-negative serum samples was observed in this experiment. Further experiment was carried out to study the spectral responses from the accelerated immunoassay using LED-IF (induced fluorescence) detection for 6 serum samples (3

JD-positive and 3 JD-negative samples). A significant difference in the intensity of the spectral response

among the JD-positive and JD-negative samples was observed in the experiment. In the following experiment, time course of antibody binding in JD-positive and JD-negative samples was evaluated (**Figure 3**). Binding of secondary antibody to microchip treated with a JD-positive sample occurred rapidly and reached saturation at 50 seconds after loading the secondary antibody (positive-10Vpp). In contrast, much lower fluorescence intensity was observed when the chip was treated with JD-negative samples (negative-10Vpp) or no serum (blocking-10Vpp). Also, a low level of antibody binding was observed on the chip treated with JD-positive serum when electric voltage was not applied after loading secondary antibody (positive-0Vpp). This indicates that AC signal is essential for rapid antibody binding. Further experiments were conducted to estimate the limit of detection of our system. A significant antibody binding was observed with 1 μ g/ml and 10 μ g/ml of fluorescently-labeled anti-bovine IgG

antibody. However, there was no significant antibody binding in 0.1 μ g/ml and 0.01 μ g/ml of the antibody. Based on this result, limit of detection of this system was estimated to be between 0.1 μ g/ml and 1 μ g/ml.

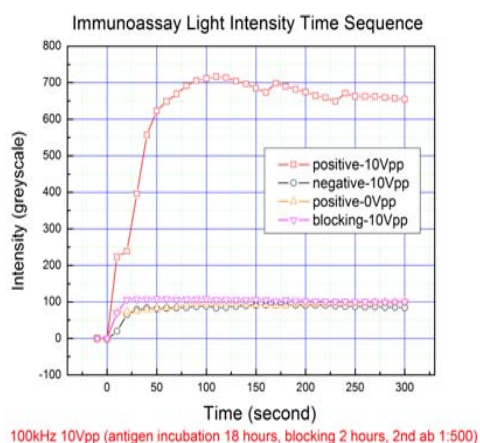


Figure 3. Immunoassay light intensity time sequence. The fluorescence intensity vs time for positive, negative and control (blocking) samples, with and without AC signal were tested.

detection method in its simple device design and no requirement of labeled secondary antibody in addition to the shorter process time.

ACKNOWLEDGEMENT: This work was supported by the following grants to SE: USDA/NRI, JDIP, University of Tennessee research foundation and University of Tennessee M-CERV. We also acknowledge funding to AW in the form of NIMBioS graduate student assistantship.

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COMPARISON OF FECAL DNA EXTRACTION KITS FOR THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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Abstract

Fecal culture is considered the gold standard for the diagnosis of paratuberculosis, however, PCR for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in fecal material is widely used today, having demonstrated great sensitivity and specificity. To insure the most efficient and reproducible PCR assay, there are many obstacles that a DNA extraction method needs to overcome, including the presence of inhibitors in feces and the thick waxy cell wall of MAP. In this study, we compared six commercial fecal DNA extraction kits for their ability to extract DNA from fecal samples of animals shedding MAP. Samples obtained from 24 animals shedding different levels of bacteria as characterized by fecal culture were extracted blindly in duplicate. Real-time PCR was done for the insertion sequences IS900 and ISMap02, and DNA purity and yield were measured by spectrophotometry. The kits evaluated were: MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems™), PowerSoil® DNA Isolation Kit (MO BIO Laboratories), ZR Fecal DNA MiniPrep™ (Zymo Research), ExtractMaster™ Fecal DNA Extraction Kit (Epicenter® Biotechnologies), Tetracore® MAP Extraction System (Tetracore®) and QIAamp® Stool DNA Mini Kit (Quiagen). The kits evaluated showed significant differences amongst each other in the purity and yield of DNA obtained, as well as different sensitivities in identifying MAP DNA in animals shedding the bacteria. All of the kits had good reproducibility between the duplicate samples. The best results were observed with the ZR Fecal DNA MiniPrep kit and the MagMax™ Total Nucleic Acid Isolation Kit, having identified 16/17 (94%) and 13/17 (76%) of the positive samples, respectively. This study demonstrates the importance of choosing the correct methodology for the most accurate diagnosis of paratuberculosis through fecal PCR.

Introduction

The diagnosis of paratuberculosis is hampered by the several factors. The slow progression of the disease with intermittent and low shedding of MAP in feces by subclinical animals can make isolation of the organism difficult (Collins, 1996), and a cellular immune response in the early stages of the disease makes the sensitivity of conventional serologic diagnostic methods low (Stabel, 2007; Nielsen, 2008). Feces are considered to be one of the most important materials for the diagnosis of paratuberculosis since it is possible to identify subclinical and clinical animals by the shedding of MAP. The gold standard for the diagnosis of Johne's disease is fecal culture, however, this method has disadvantages of being labor-intensive and taking up to 16 weeks for results (Collins, 1996). In recent years, PCR has become widely used for the diagnosis of paratuberculosis, demonstrating sensitivity and specificity comparable to or greater than fecal culture (Bogli-Stuber et al., 2005; Douarre et al., 2010). PCR has an advantage over culture as results are obtained more rapidly (2-3 days). However, the application of PCR to fecal samples for the diagnosis of MAP presents some obstacles that different methodologies need overcome, including the presence of inhibitors in feces (Thorton & Passen, 2004) and the thick and waxy cell wall of MAP which makes the extraction of DNA difficult. The objective of this study was to evaluate the performance of different commercial fecal DNA extraction kits, including the ones currently being used for the diagnostics of paratuberculosis, and identify new potential commercial kits for the diagnosis of the disease.

Materials and Methods

Six commercial fecal DNA extraction kits were evaluated in this study: MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems™), PowerSoil® DNA Isolation Kit (MO BIO Laboratories), ZR Fecal DNA MiniPrep™ (Zymo Research), ExtractMaster™ Fecal DNA Extraction Kit (Epicenter® Biotechnologies), Tetracore® MAP Extraction System (Tetracore®) and QIAamp® Stool DNA Mini Kit (Quiagen). For each kit the manufacturer's protocol was used and preliminary extractions were performed with bovine fecal samples of cows from the National Animal Disease Center (NADC) to adapt the kit to the laboratory and standardize results. After consistent results were confirmed by analysis of

DNA yield and purity, extraction was done on the samples used for its evaluation. For the evaluation of each kit, extraction was done with 24 samples for laboratory certification provided from the National Veterinary Service Laboratories (NVSL), all samples were kept at -70°C until used. These samples were blinded and extracted in duplicate. The samples comprised of seven low shedders (<10 cfu/g), one moderate shedder (10 to 100 cfu/g), nine high shedders (>100 cfu/g) and seven negative samples. All samples were blinded and extracted in duplicates.

The IS900 sequence was amplified based upon a method described by Kim et al., 2002. A standard curve was made from the MAP strain 19698 DNA, with the dilutions of 10^{-1} to 10^{-3} ng/μl. This served to compare each PCR reaction performed on the samples extracted by each kit. A positive control (known positive sample) was run in all IS900 reactions, and stayed within the same cycle threshold (Ct) in all runs. For the detection of the ISMap02 sequence, a nested PCR was performed as described by Stabel and Bannantine, 2005, with the use of TaqMan technology. The purity and yield of MAP DNA obtained from fecal samples was compared across each kit using the NanoDrop ND 1000®. For both IS900 and ISMap02 PCR reactions, a sample was considered positive if it had a Ct value of 40 or less in both duplicate samples.

Results

Using IS900 and ISMap02 qPCR, significant differences were observed comparing the different extraction kits in their ability to identify fecal samples containing different quantities of MAP. IS900 gave the highest number of positive samples per kit (Table 1). The kits also varied greatly in their protocols: using different methods of chemical or physical techniques for cell lysis, different quantities of starting material for extraction, and yielded different amounts and purity of the DNA extracted (Table 2).

Table 1: Relative identification of positive and negative samples by the different kits.

PCR		MagMax™	Power Soil®	QIAamp®	E. Master™	ZR Fecal™	Tetracore®
IS900	Positive	13/17 (76.5%)	10/17 (58.8%)	9/17 (52.9%)	3/17 (17.6%)	16/17 (94.1%)	6/17 (35.3%)
	Negative	7/7	7/7	7/7	7/7	7/7	7/7
ISMap02	Positive	12/17 (70.6%)	7/17 (41.2%)	8/17 (47.1%)	3/17 (17.6%)	13/17 (76.5%)	10/17 (58.8%)
	Negative	5/7	7/7	6/7	7/7	7/7	7/7

Table 2: Comparison of kits for method of cell lysis, quantity of sample used, average DNA yield and average DNA purity. Standard deviation is included in parentheses.

Kit	Method of cell lysis	Sample size used in kit (mg)	Average DNA yield (ng/100mg)	Average DNA Purity (Abs ₂₆₀ /Abs ₂₈₀)
MagMax™	Physical	300	7.5 (4.24)	2.25 (0.44)
PowerSoil®	Physical	250	4.4 (1.01)	1.44 (0.24)
ZR Fecal™	Physical	150	22.8 (8.99)	1.05 (0.20)
ExtractMaster™	Chemical	50	5.0 (2.08)	1.36 (0.40)
Tetracore®	Physical	2000	38.8 (25.84)	2.03 (0.10)
QIAamp®	Chemical	200	2.2 (1.29)	1.67 (0.57)

Discussion and Conclusion:

The great variation observed between kits demonstrates the difficulty in the extraction of MAP DNA from fecal samples and suggests that not all protocols can be followed for this purpose. The ZR Fecal DNA MiniPrep™ was found to perform the best for the fecal DNA extraction of MAP and diagnosis of paratuberculosis in animals using real-time PCR, detecting 16 of 17 known positive samples for a sensitivity of 94.1%. The MagMax™ Total Nucleic Acid Isolation Kit followed with a sensitivity of 76.5%. The PowerSoil, QIAamp, and Tetracore DNA extraction methods yielded comparable results with sensitivities ranging from 58.8 to 35.3%, and the ExtractMaster kit performed the most poorly with a sensitivity of detection at 17.6%. In all of the kits that performed poorly, only high shedders were identified, with the exception of the Tetracore® kit which identified one low and one moderate shedder. This study demonstrates how choosing different diagnostic kits can impact the accuracy of identifying animals shedding MAP, and that not all kits currently being used by diagnostic laboratories perform the same. Studying the kits that performed best also gives insight for the development of new protocols including in-house methods.

DETECTION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN INFANT FORMULAS BY CULTURE, PCR AND COMBINED PHAGE-PCR

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Mycobacterium avium subspecies *paratuberculosis* (MAP), the causative agent of Johnes disease in cattle and other ruminants, may have a role in the development of Crohn's disease in humans. The presence of MAP in infant powder milk has been demonstrated in the past by both culture and PCR based methods and can be due to process contamination or the survival of the organism in the powder matrix during the manufacturing process. MAP can form clumps, making it more heat resistant and given also the coating with milk proteins and fat, viable cells could escape besides heating efficacy. The objective of this study was to test different infant milk-based formulas for the presence of MAP by culture and PCR and also using a combined phage-PCR method which is rapid, sensitive and can detect and indentify viable MAP in milk samples. A total of 35 samples from a total of 11 different producers were analyzed. Following reconstitution and decontamination all samples were cultured for MAP onto Herrold's Egg Yolk Agar supplemented with Amphotericin, Nalidixic Acid, Vancomycin and Mycobactin J and then incubated for a period of 6 months. Samples were also tested using an IS900 PCR assay to detect the presence - or verify the absence of - MAP DNA. Finally, reconstituted milk samples were tested using the phage amplification assay and DNA extracted from plaques to allow PCR identification of the cell detected. The presence of MAP in infant formulas highlights the need to decrease the risk of exposure for infants and young children by assuring that skim milk intended for the manufacture of formulas is be from MAP-free herds.

PERSPECTIVES ON JD DIAGNOSTICS: A LOOK TO THE PAST, A LOOK TO THE FUTURE

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In summarizing the 46 diagnostics and detection abstracts submitted to this Colloquium, 10 reported on serology, 6 on cellular assays such as IFN-gamma, and 13 focused on a PCR or real-time PCR assay. IS900 was the most common target used among the PCR studies, although locus 251, ISMav2 and F57 were other reported targets. Hosts examined included cattle, sheep, goats, camels, alpacas, bulls and even wild boars. The search for Johne's disease continues to become more global as geographical regions surveyed included Australia, Cameroon, Europe, India, Iran, Korea and the United States. The most novel studies reported the use of a phage-based PCR to detect MAP in cheese, a MAP-specific antibody, and detection of volatile organics from MAP culture.

In order to obtain a larger perspective, past ICP meeting abstracts were considered. When comparing the total diagnostic abstracts submitted to the three most recent Colloquiums (Tsukuba-2007, Minneapolis-2009, and Sydney-2012) one noteworthy trend is the emergence of studies on improved DNA extraction. Three DNA extraction abstracts were submitted to the Sydney Colloquium whereas only one study was submitted at previous Colloquiums. This suggests the continued emergence of PCR-based assays and the need to obtain quality DNA from complex samples such as feces, milk or tissue samples. The spike in studies aimed to improve the IFN-gamma test at this Colloquium also suggests the recognized potential of this test. However, there was surprising similarity in the content of abstracts between the Minneapolis-2009 and Sydney-2012 meetings, indicating that research in this area may be somewhat static.

HOST RESPONSE AND IMMUNOLOGY

KEYNOTE ON: DISSECTING PROTECTIVE IMMUNE RESPONSE TO MYCOBACTERIA IN CATTLE

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Defining protective immune mechanisms against *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* will underpin targeted vaccine design and may influence the development of new diagnostic tests. These pathogens cause economically important diseases: bovine tuberculosis and Johne's disease. For both diseases there are no effective vaccines currently used, and diagnostic tests are not sensitive, or specific enough to detect all affected animals. As a result the incidence of these diseases is increasing. We have performed detailed studies of cellular immunology in cattle, focussing particularly on innate immune cell functions. Alongside this, the development of immunological tools, reagents and assays will facilitate dissection of the protective immune mechanisms induced by vaccination or infection. These will enable the definition of immunological correlates of protection. *Mycobacterium bovis* is the causative agent of bovine tuberculosis. In a number of countries, including the UK, New Zealand and the USA, bovine TB affects a significant number of cattle, with associated economic and animal welfare concerns. Current control strategies include the skin test and slaughter policy, whereby animals responding positively to antigens derived from *M. bovis* in the tuberculin test are culled from affected herds. Ancillary blood tests, where antigen-specific IFN secretion is measured may be used alongside skin test and slaughter to increase sensitivity and specificity of diagnosis. However, additional measures are required and vaccination is likely to be a cornerstone of disease control. The attenuated form of *M. bovis*: Bacille Calmette Guérin is used worldwide as a vaccine for TB control in humans. When administered to human infants, BCG vaccination affords good protection. Similarly, vaccination of neonatal calves with BCG induces significant protection from *M. bovis* challenge [1-2]. The enhanced efficacy of BCG vaccination in neonatal calves, compared with adult cattle, may reflect appropriate priming of the immune response prior to exposure to environmental mycobacteria. In addition, there are important differences in the composition of peripheral immune compartments in neonatal cattle that can influence vaccine efficacy. Notably, neonatal calves have high numbers of circulating innate effector cells, natural killer (NK) cells and gamma-delta T cell receptor bearing lymphocytes which co-express the WC1 scavenger receptor. WC1+ $\gamma\delta$ TCR+ T cells can comprise greater than 60%, and NK cells up to 10%, of the peripheral blood mononuclear cells in young calves – representing a significant proportion of the potentially responsive immune population. Each of these innate cell populations has been shown capable of high level IFN γ secretion which could influence the bias of downstream CD4+ and CD8+ T lymphocyte responses. We therefore hypothesised that the enhanced protection observed following BCG vaccination of neonates is associated with increased numbers, and differential functionality, of WC1+ $\gamma\delta$ T cells and NK cells, and that appropriate immune response induction is orchestrated by complex interactions between these innate lymphocytes and dendritic cells (DC). This could then facilitate optimal activation of CD4+ and CD8+ T lymphocytes for protective immunity.

Dendritic cells are the only antigen presenting cells capable of stimulating naive T cells and are pivotal in the induction of immune responses. The effective control of mycobacterial infection is reliant upon transport of antigen by migratory DC to draining lymph nodes to effectively prime CD4+ T cells and subsequently polarise Th1 biased immune responses. In order to become fully active, DC require additional signals from the innate immune system, in addition to the signals derived directly through pathogen sensing. Reciprocal interactions may occur between DC and other innate immune cells whereby activation of DC enhances their capacity to stimulate T lymphocyte responses, and increased secretion of IFN γ by innate effectors contributes to Th1 polarisation. These interactions are likely to significantly affect anti-mycobacterial immunity.

Natural killer (NK) cells may be a critical link between the innate and adaptive immune systems with reciprocal interactions influencing not only the innate response but also the adaptive immune response. Subsets of NK cells have been defined in cattle based upon differential expression levels of CD2: these subsets have divergent functional capabilities [3]. Bovine NK cells are cytolytic, expressing important mediators such as perforin and granulysin which have in other systems been shown to substantially reduce the viability of intracellular mycobacteria. NK cell derived IFN γ is believed to be implicit to the Th1 polarisation of the immune response and may therefore be pivotal in defining protective immune responses. Reciprocal activation of NK cells and DC occurs and this is an important early event post-vaccination or infectious challenge. NK cells from immunologically naive neonatal calves responded to BCG-infected DC by proliferating and secreting IFN γ . The NK cell proliferation observed was much more rapid in calves aged one day when compared to older calves and was associated with increased secretion of IL-12 and expression of IL-18 by DC. Further studies to assess the cross-talk and reciprocal effects on NK cell subsets and DC in the context of *M. bovis* and BCG have identified significant effects on both DC and NK cell subsets which could substantially influence the adaptive immune response to mycobacterial infections in cattle. BCG vaccination induced NK cell recruitment *in vivo* with increased numbers of NKp46+ cells found within draining lymph nodes following intranasal inoculation of BCG. The majority of the NK cells present were CD2-, the key IFN γ secreting subset. In addition, neonatal BCG vaccination was associated with significant increases in the percentage of NK cells within the peripheral blood. These were elevated at week 2 and significantly different to pre-vaccination levels, and to naive controls, at week 4 post-vaccination. Significant antigen specific IFN γ responses in whole blood are induced in neonatal calves from week 2 post-BCG vaccination [2] and we hypothesise that increased numbers of NKp46+ cells may contribute to this early secretion, and to the optimal priming of adaptive immune responses through interactions with DC at early time-points post-vaccination. Studies in humans and cattle have demonstrated key roles for $\gamma\delta$ TCR+ T cells in anti-mycobacterial immune responses. We demonstrated significant infiltration of WC1+ $\gamma\delta$ T cells into tissues of the head and lungs following intranasal delivery of BCG to calves. These $\gamma\delta$ T cells were co-localised with DC, and expressed significant levels of IFN γ . Following *in vitro* stimulation with IL-12, WC1+ $\gamma\delta$ T cells from neonatal calves produced significantly higher levels of IFN γ than those from adult animals, indicating an inherent capacity for pro-inflammatory behaviour [4]. In addition to cytokine-mediated interactions, reciprocal molecular interactions between DC and WC1+ $\gamma\delta$ T cells are likely to form a critical part of the immune response to mycobacteria in cattle. Neonatal WC1+ $\gamma\delta$ T cells and DC interacted in the presence of *M. bovis*, leading to significantly enhanced IFN γ secretion by WC1+ $\gamma\delta$ T cells, concomitant with up-regulation of MHC II and CD25 [5]. Reciprocal enhancement of IL-12 secretion by the DC was also observed; this was shown to be contact dependent. We also found evidence for an early, transient signal between the WC1+ $\gamma\delta$ T cells and the DC, required to promote the synthesis of biologically active IL-12. This in turn induced the secretion of IFN γ and the up-regulation of cell surface-expressed molecules by the WC1+ $\gamma\delta$ T cells. Taken together our data demonstrate significant interactions between innate lymphocytes and DC that are likely to influence both the early response to infection or vaccination, but which may also significantly affect the downstream adaptive immune response. These observations are relevant to studies of *Mycobacterium avium paratuberculosis*.

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THE ROLE OF INDOLEAMINE 2,3-DIOXYGENASE (IDO) AND TRYPTOPHAN CATABOLISM IN PARATUBERCULOSIS DISEASE PROGRESSION

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This study examined immune regulatory pathways involved in paratuberculosis disease progression. Indoleamine 2,3-dioxygenase (IDO) is an enzyme that regulates tryptophan metabolism and was originally reported to have a role in intracellular pathogen killing. It has since been shown to be a potent immunoregulatory molecule, particularly in chronic immune diseases. MAP infection is slowly progressing with a latent or subclinical phase that leads to clinical disease in a proportion of cases. Using a well characterised experimental infection model, we were able to study changes that occurred throughout the disease process and follow these through subclinical to clinical disease. This was at the level of gene expression using quantitative PCR, protein localisation by immunohistochemistry, and also functional effects by HPLC determination of plasma tryptophan levels. In this study, IDO gene expression was shown to be increased in peripheral blood cells of MAP-exposed sheep and cattle and this expression was highest in animals with severe clinical disease. IDO mRNA levels were significantly increased in MAP-infected monocytic cells and both IDO gene and protein expression were significantly increased within the tissues of affected sheep. This was particularly evident at the site of primary infection (ileum) of animals with severe multibacillary disease, and lesion severity was correlated with the level of IDO gene expression. IDO breaks down tryptophan and the increases in IDO gene and protein expression were functional as shown by decreases in plasma tryptophan levels that correlated with the onset of clinical signs, a stage well known to be associated with Th1 immunosuppression. A novel pathway in mycobacterial infections by which the pathogen may harness host immune regulatory pathways to aid survival has been described, involving IDO production and tryptophan catabolism. These findings raise new questions about the host:mycobacteria interactions in the progression from latent to clinical disease. MAP infection in the natural host may be a useful tool that leads to important findings

THE SEARCH FOR CANDIDATE GENES THAT CHARACTERISE SUSCEPTIBILITY OR RESISTANCE TO *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

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Introduction

Resistance and susceptibility to infectious diseases is well-documented in a variety of host species and while many factors contribute to resistance/susceptibility dynamics, a major component is the genotype of the host and the way in which it is expressed. In terms of mycobacterial diseases, the immune response of the host macrophage is crucial in determining the fate of the infection – whether an appropriate immune response is activated and the microbe is destroyed or whether the immune response is subverted and allows the microbe to persist and establish a chronic infection.

At the Disease Research Laboratory (DRL), red deer are the host species that are studied in terms of immune response and there is unique access and opportunity to obtain samples from deer of breed lines exhibiting an extreme resistant or susceptibility phenotype. The results presented here show differences between the “R” and “S” animals in terms of the macrophage immune response to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection.

Methods

Twenty yearling red deer were selected for this study by examining Johnes's disease susceptibility as indicated by Johnes's Breed Values (JBV). Blood samples were taken from these animals and the monocytes were separated by Ficoll gradient centrifugation. The monocytes were cultured for 7 days *in vitro* by which time they had taken on the phenotypic characteristics of macrophages. These cells were infected with MAP for 24 hours and then the gene expression analysed by quantitative PCR. Specific molecules assayed included iNOS, IL-1 α , TNF- α , IL-3p19, IL-12p35 and IL-10.

Macrophages from susceptible and resistant animals were also examined microscopically after infection with MAP. Fluorescent staining was used to detect both apoptosis and the microbe allowing any differences in cell death rate and phagocytosis to be measured.

Finally, two animals were selected to represent the susceptible group and resistant group (the selection was based on the extreme candidate gene expression and JBV of the animals). Macrophages from these animals were infected with MAP and the resulting transcriptome of the cells was analysed by Next-Generation Sequencing. This technology allows the identification of novel transcripts regulated in response to MAP infection and differentially regulated between the two groups of animals.

Results

Candidate gene expression results, as assayed by quantitative PCR, are presented in Table 1. Of note, the inflammatory molecules, iNOS, IL-1 α and IL-23p19 as well as TNF- α to a lesser extent, are shown to be expressed to a significantly higher level by the macrophages from susceptible than resistant animals after MAP infection.

No differences were seen in terms of uptake of MAP by macrophages *in vitro*, with approximately 50% of cells phagocytosing the microorganism after the incubation period. There was a difference in the rate of apoptosis detection at 24 hours after MAP infection of macrophages – approximately 40% of resistant macrophages were apoptotic compared to approximately 10% susceptible macrophages. However, due to the small sample size, this was not significant.

Finally, following the transcriptome sequencing of macrophages from two resistant and two susceptible red deer, a considerable number of genes were found to be differentially expressed between the two groups and in response to MAP infection. Several candidate genes were selected from this set to be confirmed by quantitative PCR in macrophages from the original 20 resistant or susceptible animals. These included Aquaporin 3, RNase6, synapsin II (downregulated in response to MAP), C type lectin,

RSAD2, EIF4E (upregulated in response to MAP), CXCL13, G0S2, ISG20 (upregulated in “R” animals compared to “S” animals), CXCL9, IL2RA and MMP1 (upregulated in “R” animals compared to “S” animals).

Table 1: Gene expression profiles of 10 resistant and 10 susceptible animals shown as mean expression of gene in macrophages after MAP infection relative to uninfected macrophages $\pm$ standard error of the mean (* p value < 0.01).

	Resistant	Susceptible
iNOS *	4.03 $\pm$ 1.48	56.2 $\pm$ 41.17
IL-1 α *	5.49 $\pm$ 2.10	137.9 $\pm$ 88.40
TNF- α *	2.17 $\pm$ 0.53	5.26 $\pm$ 1.49
IL23p19 *	1.71 $\pm$ 0.44	24.4 $\pm$ 9.59
IL10	0.78 $\pm$ 0.08	0.86 $\pm$ 0.15
IL-12p35	3.55 $\pm$ 0.96	10.56 $\pm$ 3.74

Discussion and Conclusions

These results indicate that there are gene expression differences between genetically resistant and susceptible animals which are not MAP-infected. A higher level of inflammatory gene expression was observed in S macrophages relative to that in R macrophages. This may indicate a dysregulated inflammatory response to MAP that may contribute to some of the pathology of Johne’s disease. Furthermore, there is a trend in macrophages from resistant animals to become apoptotic once infected compared to the macrophages from susceptible macrophages which do not. Pathogenic mycobacteria are generally thought to suppress macrophage apoptosis in favour of the more inflammatory necrosis pathway which aids them in infecting neighbouring cells. If macrophages from resistant animals are able to undergo apoptosis more readily than those from susceptible animals, the spread of MAP may be limited in the resistant host.

The transcriptome sequencing analysis is ongoing and has yielded a considerable amount of data in terms of genes that are up- or down-regulated in response to MAP infection and those that are differentially expressed between the resistant and susceptible groups. It is hoped that analysis of the host immune response in resistant and susceptible macrophages may provide a platform on which animals could be selected for their phenotype as well as providing correlates for protection against mycobacterial pathogens in vaccine studies.

GENE EXPRESSION IN RED DEER RESISTANT OR SUSCEPTIBLE TO MAP

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Introduction

Paratuberculosis (Johne's disease) results in serious losses of farmed red deer (*Cervus elaphus*) in New Zealand. Young deer are particularly susceptible, especially to heavy challenge with the bovine strain of MAP, and clinical disease occurs in deer as young as 8 months. Field data suggests that some breed-lines of red deer display heritable resistance to paratuberculosis. This paper presents results of a study of gene expression in jejunal lymph nodes (JJLN) of red deer that were either resistant (R) or susceptible (S) to paratuberculosis after heavy oral challenge with MAP.

Method

Two challenge studies were carried out in 2008 and 2009. In 2008, 18 offspring were bred from two unrelated stags by AI across unselected red hinds. In 2009, semen from two stags designated R or S, based on the outcome of natural MAP field challenge of their offspring, were used across unselected red hinds to produce 9 offspring of each. In both years the offspring received heavy oral challenge with a bovine strain MAP (4 daily doses of 10⁹ cfu) extracted from JJLNs of clinically affected deer. Samples of posterior JJLN were surgically biopsied at Weeks 4 and 12/13 post challenge (pc). JJLN samples were also collected at euthanasia of clinically affected or at trial end 49/50 weeks pc. These samples were snap-frozen in liquid nitrogen and then stored at -80C. The disease status for animals in both studies was scaled on clinical outcome, histopathology, culture and serology (Tables 1 and 2). The frozen JJLNs from the 3 least affected and 3 most affected at 3 time points (Weeks 4, 12, 50) in the 2008 study, and from Week 4 in the 2009 study, were processed and the RNA extracted and subjected to next generation Life Technologies SOLiD SAGE sequencing for gene expression.

Table 1: 2008 study results of JJLN culture and histopathology. Table 2: 2009 study results of JJLN culture and histopathology.

Week 4 12 50									Week 4 13 Euth or 49								
Tag	Sire	LSS	JJLN dtp	LSS	JJLN dtp	LSS	JJLN dtp	Affect	Tag	Sire	LSS	JJLN dtp	LSS	JJLN dtp	LSS	JJLN dtp	Affect
506	A	2	36	6	21	3	34	mild	92	R	0	29	6	22	0	>66	nil
511	A	0	36	4	21	4	34	mild	84	R	0	29	11	22	5	32	mild
509	B	0	36	6	15	5	26	mild	88	R	0	29	6	22	5	39	mild
Mean		0.7	36	5	19	4	31	mild	Mean		0	29	8	22	3	46	mild
510	B	2	36	11	12	9	18	mod	82	R	0	29	11	16	13	8	severe
508	A	0	32	11	15	9	18	mod	85	S	0	39	13	9	13	8	severe
507	A	0	36	11	12	13	18	severe	93	S	0	29	11	10	13	8	severe
Mean		0.7	35	11	13	10	18	mod	Mean		0	32	12	12	13	8	severe

Week: weeks post challenge with MAP; JJLN: jejunal lymph node; dtp: days to positive for Bactec culture of MAP; Euth: euthanasia of clinical cases

LSS: 0=nil, 1-3 nonspecific, 4-7 mild, 8-10 moderate, 11-13 severe.

Results

In 2008 no offspring were clinically affected and there was no sire effect on outcome. In 2009 there was a significant sire effect, with the three animals clinically affected and the offspring of the S sire were significantly worse affected than R offspring (Tables 1 and 2; also see Poster P045 "Heritable resistance / susceptibility in red deer to experimental MAP challenge").

Sequencing generated a total of 373 million "tags" with lengths between 26 and 28 bases. Genes were mapped against three datasets; the deer and elk transcriptome, the cattle transcriptome and the bovine genome. 31,500 genes were uniquely identified, and of these so far 18,000 genes have been annotated

and 17,500 were recognized by the Ingenuity Pathway Analysis (IPA) programme. The number of genes significantly ($P < 0.05$) upregulated in either the 3 S animals or 3 R animals changed over time (Table 3).

Table 3: Number and fold range of significantly upregulated genes in R and S offspring at time points in two studies

	S offspring genes upregulated		R offspring genes upregulated	
	Number	Fold range (mean)	Number	Fold range (mean)
2008 Week 4	161	2.5 – 33.5 (5.0)	38	2.48 – 22.8 (6.5)
2008 Week 12	405	2.4 – 141.9 (6.9)	188	2.4 – 17 (5.0)
2008 Week 50	408	1.7 – 25.4 (3.1)	204	1.7 – 32.9 (3.4)
2009 Week 4	142	3.8 – 178 (16.2)	222	2.6 – 34.3 (5.5)

In the 2008 study the overall number of significantly upregulated genes in both R and S animals increased between Weeks 4 and 50, and the greatest fold changes were in the S animals at Week 12. The 2009 Week 4 R animals had significantly more upregulated genes than the 2008 Week 4 R animals (222 vs 38). While the number of S upregulated genes in 2009 remained similar, the range and mean of upregulation was higher, and the highest fold change in the study was recorded in S animals at 2009 study Week 4 (TCN2 - transcobalamin II; 178 fold). 2008 Week 12 and 2009 Week 4 animals had the greatest number of genes upregulated >10 fold (Table 4).

Table 4: Number and fold range of genes upregulated >10 fold in R and S offspring at time points in two studies

	S offspring genes upregulated		R offspring genes upregulated	
	Number	Fold range (mean)	Number	Fold range (mean)
2008 Week 4	12	10.8 – 33.5 (14.4)	6	10.5 – 22.8 (15.3)
2008 Week 12	59	10.5 – 141.9 (21.8)	13	10.1 – 17 (12.5)
2008 Week 50	7	10.5 – 25.4 (13.8)	10	10.2 – 32.9 (16.8)
2009 Week 4	70	10.2 – 178 (26.4)	24	10.2 – 34.3 (15.3)

A number of genes showed high levels of upregulation at different time points. At least 105 inflammation related genes, including chemokines (C-C and C-X-C), interferon family, tumour necrosis factor family, S100 calcium binding, FAU, NOS and ADIPOQ genes, were upregulated at these 4 time points, including 13 genes upregulated on two occasions and 9 genes on 3 occasions. These genes were especially upregulated in S animals at Weeks 4 and 12 in the 2008 study. ADIPOQ was upregulated on all 4 occasions and is an important adipokine involved in the control of fat metabolism and insulin sensitivity, with systemic anti-inflammatory activities associated with antagonizing TNF-alpha and inhibiting endothelial NF-kappa-B signalling. By contrast, apoptosis and autophagy genes tended to be upregulated more in R animals, especially at Week 4 in the 2009 study. For example genes upregulated in R animals 2009 Week 4 include MAP3K11 (mitogen-activated protein kinase kinase kinase 11) 33.1 fold, RASGRP4 (RAS guanyl releasing protein 4) 8.6 fold, THAP3 (THAP domain containing, apoptosis associated protein 3) 8.2 fold, RNF41 (ring finger protein 41) 8.2, CEBPE (CCAAT/enhancer binding protein (C/EBP), epsilon) 7.9, AMBRA1 (autophagy/beclin-1 regulator 1) 6.6 fold, RASL11A (RAS-like, family 11, member A) 3.8 fold, S100A9 (S100 calcium binding protein A9) 3.7 fold, NFKBID (NFKB nuclear factor of kappa light polypeptide gene enhancer in B-cells) 3.1 fold, TNK1 (TRAF2 and NCK interacting kinase) 2.9 fold. At Week 4 in both 2008 and 2009 studies, 8 genes were significantly upregulated in S animals. One gene (ORMDL3) was significantly upregulated in R animals at 8.1, 3.4

and 32.9 fold for 2008 Weeks 4, 12 and 50, respectively, and 3.3 for 2009 Week 4. ORMDL3 is present in the cytoplasm in most tissues, it may indirectly regulate endoplasmic reticulum-mediated Ca(2+) signalling, and its expression is associated with chronic inflammation. It is a member of a gene family that encodes transmembrane proteins anchored in the endoplasmic reticulum and genetic variants regulating ORMDL3 expression appear to be determinants of susceptibility to childhood asthma. The above are a few examples of relative gene expression and further results will be revealed in due course.

Discussion

Access to biopsy samples of JJLN from 19 animals at these 3 time points in the 2008 study has given a powerful insight into the parallel changes in histopathology, immunology, culture and gene expression over the 12 month period that, in red deer, typically determines the outcome of paratuberculosis in terms of the animal recovering, becoming latently infected or succumbing to clinical disease. Unfortunately only the Week 4 samples from the 2009 study could be sequenced due to lack of resources, although this has enabled interesting comparison with the 2008 Week 4 samples. These 2009 animals showed much greater differences between R and S than the 2008 animals, with R animals minimally affected and S animals developing clinical disease. Work is currently underway to sequence this full set.

Innate immunity did not appear to have any significant influence on disease state in the first 4 weeks of either study, with similar numbers of MAP present in JJLN and no histopathological lesions or measurable antibody. Nevertheless, there appear to be significant differences in gene expression at Week 4, which is likely to reflect differences in the pathway the animals' immune systems were taking at that time and subsequently diverged at Weeks 12 and 50. There was quite a marked difference between R and S groups between Week 4 and 12, with S animals showing a more marked increase in number of MAP and severity of lesions. This is accompanied by a much greater number and degree of genes upregulated in the 3 S animals, compared with R animals, especially genes associated with inflammation. Unfortunately it is not possible to be sure whether these differences in gene expression are due to cause or effect. The task of analysing these results is ongoing because of the number of genes involved, the complexity of the immune response and the fact that our knowledge of many of the genes is nil or incomplete.

Conclusion

Gene expression in JJLN of deer R or S to MAP challenge is at a very informative phase. The results to date appear to be meaningful and will contribute to understanding resistance to MAP in ruminants.

IN VITRO EXPRESSION OF SLC11A1 GENE IN GOAT MONOCYTE-DERIVED MACROPHAGES CHALLENGED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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Introduction

Paratuberculosis (Johne's disease) is an economically significant, chronic, infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), characterized by progressive weight loss and a nonresponsive, persistent or intermittent diarrhea (Chiodini et al., 1984). Genetic factors have been associated with variations in host susceptibility to bovine paratuberculosis indicating, a range of moderate values for heritability of infection (Koets et al., 2000, Mortensen et al., 2004, Gonda et al., 2006). However research on the association between disease susceptibility and polymorphism of specific genes has not provided yet conclusive evidence (Gonda et al., 2005, 2007; Taylor et al., 2006; Hinger et al., 2007; Mucha et al., 2009; Pinedo et al., 2009).

The *SLC11A1* (solute carrier family 11 member 1) gene plays an important role in innate immunity, preventing bacterial growth in macrophages during the initial stage of infection. The *SLC11A1* protein functions as a transporter of divalent cations such as Fe²⁺, Mg²⁺ and Mn²⁺ through the phagolysosome membrane of macrophage cells (Forbes & Gros, 2003), and has been associated with natural resistance to infections induced by intracellular pathogens such as *Mycobacterium* spp., *Salmonella* spp., and *Leishmania* spp., (Paixao et al., 2007). Specific *SLC11A1* alleles have been associated with susceptibility to Johne's disease in sheep (Reddacliff et al., 2005) and bovines (Juste et al., 2005).

Recently we published the sequence for the caprine *SLC11A1* gene (GeneBank FJ388877) and investigated the potential association of the polymorphisms in its 3' UTR with test-positivity of goats to MAP (Liandris et al. 2009). This was further investigated with the results indicating a statistical significant association between the B7/B7 genotype of the *SLC11A1* 3' UTR region and negativity of goats to MAP by ELISA (Korou et al. 2010). Here we present the results of the work performed to investigate the functional role of the most frequent polymorphisms of the 3' UTR region of the *SLC11A1* gene on its expression in goat macrophages exposed in vitro to MAP.

Materials and Methods

In order to define the complete sequence of the 3' UTR of *SLC11A1* gene we performed Classic RACE (Rapid Amplification of cDNA Ends). This PCR is used to amplify partial cDNA fragments representing the region between a single point in an mRNA transcript and its 3' end.

Fifty four (n=54) whole blood samples were collected from goats belonging to the native goat breed from the farm of the Agricultural University of Athens. This herd is considered paratuberculosis-free with no record or evidence of the disease and with all the animals reacting negative to screening that was performed twice within the one year period before sample collection, and consisted of culture and PCR performed on feces, and serology (Pourquier ELISA paratuberculosis Antibody screening, Pourquier, France). The whole blood samples were used for DNA isolation that was subjected to sequence and structure analysis of the caprine *SLC11A1* gene. Two sets of primers were designed to amplify the polymorphic regions A and B. The PCR product was analyzed by sequencing and SSCP analysis. The animals that were tested were divided into three groups based on their genotype. Blood samples collected from 47 animals within each of these groups were used to establish primary cell cultures of peripheral blood monocyte-derived macrophages. These were incorporated to the investigation of the functional impact of the genetic polymorphisms that were identified in the 3'UTR region of the *SLC11A1* on gene expression.

The mononuclear cells were distributed into 24 well-plates (3X 10⁶ cells/well) and they were incubated for 24 h (37 °C; 5% CO₂) in 1 ml RPMI medium supplemented with 4mM L-glutamine and 15% bovine fetal serum. Non-adherent cells were removed and 1ml of fresh medium was added again to each well. The primary cultures of peripheral blood monocyte-derived macrophages were challenged with 15 x 10⁴ MAP cells/ well. Total RNA was isolated from each of these cell cultures at 1, 3 and 24 h after exposure

to MAP, and was used to assess the mRNA level of the *SLC11A1* gene by Real Time PCR using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001). All reagents were supplied by Invitrogen, USA. The relative quantification of the *SLC11A1* gene expression was performed using as reference the *GAPDH* (glyceraldehydes 3-phosphate dehydrogenase) gene (Taylor et al., 2008). The association between the different alleles of the gene and its expression level was investigated using Independent-Samples T Test.

Results

To the best of our knowledge the sequence of the 3' UTR end of the caprine *SLC11A1* gene was fully determined for the first time within the context of this study (GenBank accession number JF431430). The results confirmed our previous findings (Liandris et al., 2009; Korou et al., 2010) indicating that the 3' UTR region of the targeted gene presents 2 microsatellites consisted of a variable number of guanine-thymine repeats herein referred to as regions A and B. The genotypes A(GTn)15/16 - B(GTn)8/8, A(GTn)15/16 - B(GTn)7/7, and A(GTn)15/16 - B(GTn)7/8 were detected respectively in 27 (50%), 11 (20%), and 8 (15%) of the 54 animals that were tested with 8 of them producing genotype patterns that were defined as miscellaneous. Region A was poorly polymorphic and was therefore excluded from further analysis (Korou et al., 2010).

In all time points the mean *SLC11A1* mRNA levels of the B7/ B7 macrophages was higher compared to those of the B8/ B7 and B8/ B8 genotypes. An increase of the mean *SLC11A1* mRNA level was recorded from the monocyte cell cultures with the B7/ B7 genotype 1hour and 3 hours post-exposure to MAP, followed by decrease at the 24-hour time point. This expression profile was recorded also by the heterozygous genotype (B7/B8). The pattern differed with regard to the homozygous B8/B8 genotype that demonstrated an increase of m-RNA expression levels at all-time points. The homozygous B7/B7 genotype was associated at a statistically significant level ($p < 0.01$) with increased expression of the *SLC11A1* gene in comparison to the homozygous B8 and heterozygous genotypes 1 and 3 hour after exposure to MAP. The m-RNA level of the *SLC11A1* gene recorded by the B7/B7 macrophages was 13, 17, and 4-fold higher at the 1, 3, and 24-hour time points respectively compared to the macrophages with the B8/8 allele. The comparison of the evidences that were recorded with connection to the heterozygous and the homogygous B8/B8 genotypes indicates a different pattern of fluctuation of m-RNA concentration only at the 24-hour time point, though not at a statistically significant level ($p > 0.05$). However the absolute values of the m-RNA concentration were in both cases, (homozygous and heterogygous), almost identical at all time points.

Conclusion

Associations of polymorphisms of the *SLC11A1* gene with susceptibility of humans and bovines to infections induced by *Mycobacterium* spp., (Bellamy et al., 2000) and *Brucella* spp., (Capparelli et al., 2007; Barthel et al., 2001) have been reported by others. The (GT)13 allele of the 3'UTR of *SLC11A1* gene in cattle was shown to confer resistance to *Brucella abortus* infection whereas the (GT)14, (GT)15 and (GT)16 alleles were associated with susceptibility to infection induced by the same pathogen (Adams and Templeton, 1998; Barthel et al., 2000, 2001). Martinez et al. (2008) concluded that bovine macrophages with (GT)12 allele display higher antibacterial capacity to in vitro infection by *B. abortus* compared to macrophages from cattle presenting the (GT)10 allele. Admittedly not all results are in agreement. Paixao et al., (2007) who investigated for an association between *B. abortus* infection of cattle and the expression of the *SLC11A1* gene found no correlation. Our results indicate in-vitro up-regulation of the expression of the caprine *SLC11A1* gene after exposure to MAP with connection specifically to the B7/B7 genotype of its 3' UTR end. This genotype has been correlated in one of our previous studies with ELISA negativity to MAP of individuals from goat herds with a record of paratuberculosis (Korou et al., 2010). The combination of these studies provides the first evidence in support of a potential association between up-regulation of the *SLC11A1* gene and resistance of goats to the specific disease.

GENETIC MARKERS FOR RESISTANCE AND SUSCEPTIBILITY TO JOHNE'S DISEASE: A DEER MODEL

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This study looks at disparate genetic breeds of deer to identify polarised phenotypes for either resistance or susceptibility to Johne's disease following natural exposure to high levels of virulent *M. paratuberculosis*.

Methods

Extensive diagnostic screening of more than 5000 purebred and crossbred deer was carried out using ELISA, faecal culture, faecal qPCR and histopathology on animals persistently exposed to high levels of infection with *M. paratuberculosis*. Correlates were established to determine the diagnostic precision of each individual test. The positive predictive value of ELISA for detecting individual animals shedding significant number of bacteria (qPCR +) was 0.79 while the negative predictive value was 0.95. Excellent correlations were seen between qPCR and culture, giving Spearman Rank correlations of -0.9469 and between qPCR and histopathology as -0.7900.

Results

The results show estimated heritability for seroconversion is 0.3 +/- 0.06. Progeny from breeds with a resistant genotype showed very low levels of seroconversion or disease (<5%) while breeds with a susceptible genotype had reactor rates >60%. Mortality in animals with a resistant genotype was extremely low (<3%) while more than 50% of progeny from sire dam combinations that were susceptible succumbed to Johne's disease.

Conclusion

The genetic homogeneity of deer from geographically isolated populations of wild deer appears to offer a unique opportunity to study heritability of susceptibility or resistance to *M. paratuberculosis* infection and Johne's disease. By contrast, deer from disparate populations appear to express heterogeneous genotypes. The high prevalence of exposure to *M. paratuberculosis* in deer studs allows the identification not only of susceptible breeds but also the study of resistant animals that remain unaffected by infection following persistent exposure. More detailed genetic analysis is currently being carried out on these deer breeds using experimental infection and *in vitro* challenge of mononuclear cells from animals with either a resistant R or susceptible (S) phenotype.

IMMUNE PROFILES AND FAECAL SHEDDING OF *MYCOBACTERIUM AVIUM* PARATUBERCULOSIS INDICATE EVENTUAL SEVERITY OF JOHNE'S DISEASE

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Using a well characterised experimental infection model in sheep this study tracked cellular and humoral responses as well as the quantity of the organism shed in faeces for up to 30 months post challenge. Merino lambs were unexposed (controls, n=30) or challenged orally with MAP (n=58). Blood and faecal samples were collected prior to infection and at multiple time points post inoculation. MAP antigen-specific IFN γ , IL-10 and antibodies were detected by ELISA and cell proliferation was assessed by flow cytometry. Quantification of MAP in faecal samples was by quantitative PCR. Disease outcome was classified by tissue culture and assessment of histological lesion type. The IFN γ response increased early after inoculation regardless of disease outcome. Infected sheep with severe Perez 3b type lesions (multibacillary) could be distinguished from animals that developed less severe lesions. Their IL -10 response remained relatively unchanged throughout the course of disease while IL-10 levels increased with time in animals with less severe disease. Also, from early on (4 months post inoculation), the amount of MAP DNA shed in faeces of animals with 3b type lesions was higher than in other diseased animals. Interestingly, the cellular immune response in animals with 3b type lesions showed a similar pattern to animals with no lesions, though the disease outcomes were vastly different. Inoculated sheep showing no signs of clinical disease had a stronger proliferative response than those with clinical disease and continue to do so at more than 2 years after exposure to MAP. These studies demonstrate the complexity of the immune response to MAP and illustrate the fact that measurement of immunological parameters alone at a single time point is insufficient to determine the outcome of Johne's disease.

SEROREACTIVITY OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTED, VACCINATED AND HEALTHY ANIMALS

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The detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in animals is difficult because of the lack of sensitivity of the currently available tools. The purpose of this study was to compare protein profile reactivity of bovine and ovine strains incubated with sera from infected (vaccinated and non-vaccinated) and healthy (vaccinated and non-vaccinated) animals in order to identify antigenic differences that could be used for diagnosis. Animals were classified as infected or healthy based on feces culture, IS900 PCR and PPA-3 ELISA results on repeated samples.

Protein extracts from bovine, ovine, vaccine (bovine origin) and reference MAP strains were separated by 1D electrophoresis (1D-EF) and 2D electrophoresis (2D-EF). Gels were stained with Coomassie or Syprotangerine staining. Protein transfer to PVDF membranes for immunoblot analysis of both 1D-EF and 2D-EF was performed. Reactivity with sera from infected (vaccinated and non-vaccinated) and healthy (vaccinated and non-vaccinated) animals was assayed, and visualized by ECL.

Significant differences were observed in recognition patterns for ovine and bovine strains. In 1D-EF, all strains except for ovine ones presented a 25-37,5 KDa immunodominant antigen that reacted strongly with sera from infected and vaccinated animals. As expected, 2D-EF immunoblot analysis, the antigens present in 25-37,5 KDa molecular weight proteins with 4-5 pI were recognized by sera of vaccinated and infected animals only in bovine strains and not in ovine ones.

From our results, a classification system was devised that differentiates infected from vaccinated animals by seroreactivity profiles on 2D-EF against four groups of proteins (I, II, III and IV). Protein group I reacts with sera from infected or vaccinated animals. Proteins from groups II and IV react with sera from vaccinated healthy animals and proteins from group III react only in vaccinated and infected animals. Healthy and non-vaccinated animals do not react with any of these protein groups.

THERAPEUTIC VACCINATION WITH RECOMBINANT HSP70 OF CATTLE NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SPP. *PARATUBERCULOSIS*

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The purpose of this study was to evaluate if a candidate paratuberculosis vaccine, based on recombinant MAP Hsp70 with DDA adjuvant, has therapeutic efficacy in cattle naturally infected with MAP. Forty-five adult cows, naturally infected with MAP in the field and fecal culture positive, were assigned to three groups of 15 animals each: control (G1), and two vaccine groups that differed in the vaccination regimen (G2: day 28, 112, 196; G3: day 0, 28, 112, 196). The study design included 6-7 sampling points pre-vaccination and maximum 15 sampling points (three week intervals) post-vaccination. The primary outcomes were MAP fecal culture and survival time of cows. The proportion of negative fecal cultures in the post-vaccination period was higher in both Hsp70/DDA vaccinated groups as compared to controls, and for G2 this effect was statistically significant ($p=0.02$). In addition, the contribution of vaccination to survival was assessed. In total five (33.3%) control animals and two (13.3%) vaccinated animals in each group reached predetermined humane end points due to end stage clinical signs of paratuberculosis. Subgroup analysis revealed that vaccinated animals classified as high Hsp70-specific antibody responder ($N=22$) had significantly reduced fecal shedding ($p=0.01$) and a higher survival rate ($p=0.047$) as compared to controls. In conclusion, Hsp70/DDA vaccination showed some beneficial effects in chronic paratuberculosis infected animals, for which the added value in an eradication program has to be assessed in additional studies. The role for antibodies as therapeutic modality in protection during chronic mycobacterial infections needs further attention and could provide a lead for new paratuberculosis vaccines.

MECHANISMS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) INDUCED APOPTOSIS AND NECROSIS IN BOVINE MACROPHAGES

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Abstract

Bovine monocyte-derived macrophages infected by equal number (MOI=1) of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) were not affected, but *Map* at MOI=10 caused macrophage apoptosis. *Map* at higher bacterial burden (MOI=50 or greater) induced both apoptosis and necrosis in macrophages. Activation of caspase-3 and alterations in membrane potential/membrane permeability transition of mitochondria were observed in *Map*-infected macrophages. *Map* at lower bacterial burden induced caspase-dependent and mitochondrial pathway of apoptosis, while *Map* at higher burden induced caspase-independent and nitric oxide-independent apoptosis and mitochondrial damage-associated necrosis in macrophages. It is concluded that under higher bacterial burden and spatial stress, *Map* induced apoptosis and necrosis of macrophages by complex mechanisms as to find a new niche for survival and replication.

Introduction

Little is known about the host-pathogen interactions that regulate the pathogenesis of paratuberculosis (Johne's disease), particularly host cell death or survival of bacteria within infected cells. The interaction of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) with bovine macrophages was found to be a complex processes involving strategies for survival of bacteria or host cell death depending upon the number of bacteria infected per macrophage (multiplicity of infection, MOI). In the present study, we report that *Map* at equal bacterial burden per cell (MOI=1) was not harmful for macrophages, but at MOI of 10 induced apoptosis. Interestingly, *Map* at higher bacterial burden (MOI=50 or greater) induced both apoptosis and necrosis in macrophages.

Materials and methods

A characterized mycobactin dependent bovine strain (C-123/IVRI) of *Map* isolated from clinically infected cattle was grown in the Middlebrook-7H9 broth (10^9 CFU/ml) for infection of bovine blood monocyte-derived macrophages. Bovine macrophages (2 to 2.5×10^5 /wells) were infected with *Map* at different multiplicity of infection (MOI, ranging from 1 to 100). The infected cells were cultured at 37°C for 4 to 48 h post-infection (*hpi*) depending on the experimental design. Apoptotic changes of macrophages were assessed by nuclear morphology and cellular permeability to fluorescence dyes (DAPI/PI and AO/EB). For detection of necrosis in macrophages, the release of lactate dehydrogenase (LDH) was estimated in cultured cells. TUNEL assay and electron microscopic evaluation of apoptosis were performed to confirm apoptotic and necrotic changes. Pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl2) gene expressions were analyzed by qPCR assay. Caspase-3 activity was measured using CaspACE™ colorimetric assay system. Nitrite was measured using quantitative assay based on Griess reaction. The change in mitochondrial inner membrane potential ($\Delta\Psi_m$) was measured using DiOC₆(3). Data were analyzed in GraphPad Prism for statistical significance.

Results and Discussion

Map at lower bacterial burden (MOI=1) did not induce cell death, but *Map* at MOI=10 induced macrophage apoptosis (Fig A, B, C, D). Strikingly, *Map* at higher bacterial burden (MOI=50 or 100) induced both apoptosis and necrosis in macrophages. Thus *Map* induced apoptosis in macrophages is dose dependent as reported previously for *M. tuberculosis* (Lee et al., 2006). LDH assay suggested that *Map* at low level was non-cytotoxic but was cytotoxic at higher bacterial burden (Allen et al., 2001; Weiss et al., 2004; Lee et al., 2006). On fluorescence microscopy, cells undergoing apoptosis had condensed and fragmented nuclei, whereas necrotic cells showed higher cellular permeability for impermeant dyes. At ultrastructural level, apoptotic cells had chromatin condensation and fragmentation, while necrotic cells showed breaks in cell membrane and loss of cytoplasmic and nuclear contents. Apoptotic and necrotic changes were mostly observed in those cells having intracellular bacteria and occasionally were seen in

bystander cells without intracellular bacteria. Activation of caspases, induction of mitochondrial permeability transition (MPT), and structural changes of mitochondria were observed in apoptotic macrophages. Change in mitochondrial outer membrane permeability during apoptosis has been reported previously (Chipuk et al., 2006). Addition of caspase inhibitors (Z-VAD-fmk or Z-DEVD-fmk) (Fig. E, F) or MPT inhibitor (cyclosporine A) (Fig G, H) significantly reduced in numbers of apoptotic macrophages infected with *Map* indicating that caspases and mitochondria were involved in induction of apoptosis. In contrast, *Map* at higher burden (MOI=50 or 100) induced macrophage apoptosis in caspase-independent pathway since caspase inhibitors or MPT inhibitor did not reduce apoptotic changes in macrophages. Macrophage apoptosis in response to high intracellular burden of *M. tuberculosis* was mediated by a novel caspase-independent pathway (Lee et al., 2006). Similarly, NO-dependent and independent mechanisms have been implicated in apoptosis and necrosis at different MOI. Nitric-oxide mediated apoptosis in murine peritoneal macrophages has been reported by Albina et al. (1993). Thus, it is concluded that under higher bacterial burden and spatial stress, *Map* induced apoptosis and necrosis of macrophages by unknown mechanism as to find a new niche for survival. It was further supported by the observation of more number of cells infected with *Map* at 48 h (55%) when compared to 4 h (41%) post-infection at MOI=50 or 100 (data not shown).

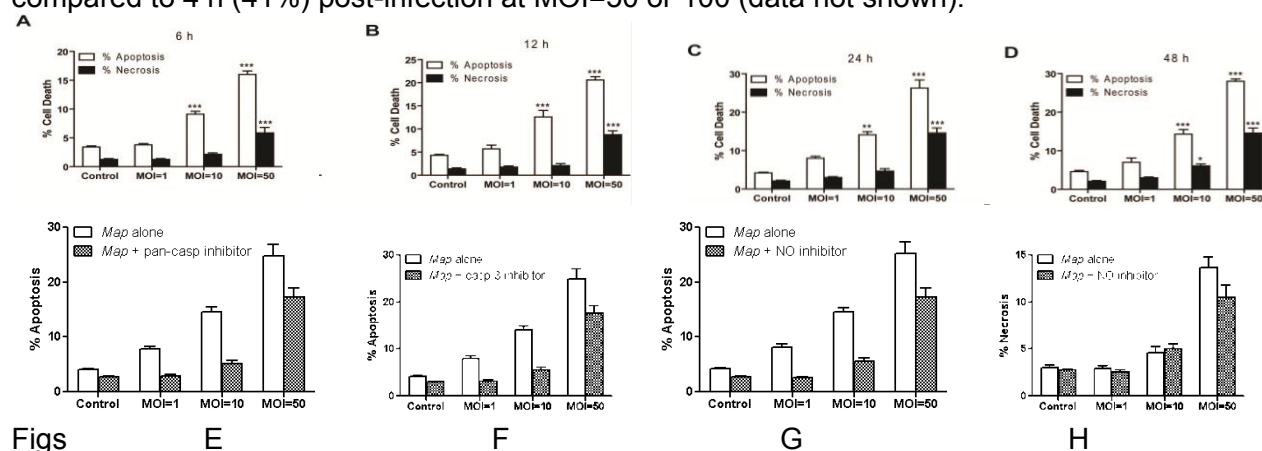


Table : cellular response of bovine macrophages to *in vitro* *Map* infection

	ontrol	OI=1	OI=10	OI=50
ax expression (fold change in mRNA level)	±0	8±0.1	8±0.1	4±0.2
cl2 expression (fold change in mRNA level)	±0	2±0.1	8±0.1	9±0.05
aspase-3 activity (absorbance at 405nm)	06±0.01	08±0.02	4±0.08	25±0.1
itric oxide production (µM)	13±0.06	3±0.06	5±0.1 ^a	3±0.8 ^c
cells showing dissipated ΔΨm	6±0.3	6±0.8	4.3±1.3 ^c	3.3±2.3 ^c

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THE ONSET OF DETECTABLE IMMUNE RESPONSES AND FECAL SHEDDING AFTER EXPERIMENTAL INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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Introduction

Early detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map) infection is hampered by the low sensitivity of the commonly used diagnostics, in part due to the late onset of immune responses and fecal shedding¹.

The immune response in paratuberculosis seems to be driven by strong Th1-type cellular immune responses during the subclinical stages of infection². This Th1 response can be detected by production of IFN- γ by memory T-cells after stimulation with specific antigens. This makes measurement of IFN- γ production after cell stimulation a good candidate for early detection of Map infection. However, reports on the specificity of the IFN- γ assay are conflicting, ranging from 26 to 97.6%³.

Detection of serum antibodies is associated with the level of Map shedding and the age of the animals⁴. Overall, the sensitivities of serum ELISA when compared to tissue culture are very low⁵ and serological detection of subclinical cases is difficult⁶.

Fecal instead of tissue culture is often used as a gold standard for diagnosis of Map for practical reasons⁷. Shedding in the early stages after infection is considered to be low and intermittent, but can potentially lead to infection of pen mates⁸.

The aim of this study was to evaluate the onset of cellular and humoral immune responses and fecal shedding with the available diagnostic tests when animals are infected with Map with different doses and at different ages. Tissue culture and histology were used to determine the infection status of the animals.

Materials & Methods

This infection trial was designed based on the guidelines published by Hines *et al.* (2007)⁹. Thirty Holstein-Friesian steer calves were experimentally infected at 5 different ages (2 weeks, 3, 6, 9 & 12 months). In each age group animals were infected with a high ($5 \cdot 10^9$ CFU) or a low ($5 \cdot 10^7$ CFU) dose of Map, on 2 consecutive days. Samples were collected monthly for measuring the onset of fecal shedding and immune responses using serum ELISA, Map-specific IFN- γ induction assay and fecal culture. All calves were euthanized at 17 months of age to confirm infection status by tissue culture and histology. IFN- γ ELISA was performed in 2 stages. In the first stage, blood samples were incubated overnight with a Map-specific antigen (johnin, provided by Canadian Food Inspection Agency, CFIA) and appropriate controls to stimulate the lymphocytes to produce IFN- γ . In the second stage, IFN- γ in the plasma supernatant of each blood aliquot was determined using the BOVIGAM[®] ELISA (Prionics, USA) according to manufacturer's instructions.

Serum was tested using the *Mycobacterium Paratuberculosis* Antibody Test Kit[®] (IDEXX laboratories, USA).

Fecal and tissue samples were decontaminated and incubated in the TREK ESP[®] Culture System II (TREK diagnostic systems, USA) for 7 weeks. A confirmatory IS900 PCR was performed on all cultures. The following tissues were selected for tissue culture: ileocecal valve, distal ileum and ileocecal lymph node.

The same tissues and one more (ileal lymph node) were embedded and stained with Hematoxylin-Eosin (HE) and Ziehl-Neelsen (ZN). Interpretation of histology was performed by a pathologist.

Results

A Map-specific IFN- γ response was detected in all age groups starting at 2 months after infection. Calves infected with a high dose of Map responded earlier and stronger than the groups infected with a low dose of Map. After the peak response at 3-4 months post infection, response slightly decreases over time.

Analysis of sera using the serum ELISA showed prolonged increased antibody titers in some animals from all age groups, for the high dose animals as soon as 3 months post-infection.

Fecal shedding was low and intermittently present during the trial in all age groups, except the 9 month infection group. There was no consistent onset of shedding in the different groups. However, high dose calves shed more frequently than low dose calves over the duration of the trial.

A combination of tissue culture and histology identified 27 out of the 30 animals as infected.

Discussion

IFN- γ ELISA may offer a powerful tool for early diagnosis of JD. Infected animals respond strongly in the first months after infection. However, a cellular immune response is no proof of established infection, as the cellular immune response is considered most efficient for controlling mycobacterial infections, including paratuberculosis^{10,11}. The observed gradual decrease of the response might potentially lead to negative results when animals are tested long after infection.

Serum ELISA also detected animals in earlier stages than previously observed, although not all infected animals produced a detectable antibody titer during the trial. There is no consistent onset of humoral immune responses, but responses can occur in the first 10 months after infection.

A combination of tissue culture and histology classified most animals as infected. By tissue culture alone, 16 out of 30 animals tested positive. This may be explained by the fact that only 3 tissues were selected from all the samples collected at necropsy. We have reasons to believe that more animals were actually infected than what tissue culture indicates. For example, some animals were repeatedly shedding while tissue culture had a negative result. We plan to analyse the remaining samples collected at necropsy in order to estimate the optimal-but-practical number of necropsy tissues to collect for culture and histopathology to determine the true disease status of challenged calves.

This study provides information about shedding and cellular and humoral immune responses in the first months after infection, up to 17 months after infection for the group infected at 2 weeks of age. Related to the predefined stages of JD¹², this would correspond with the first 2 stages of the disease: the silent infection and carrier stage. This study proves that animals in the silent infection stages can shed the bacteria and can be detected with IFN- γ ELISA and in some cases with serum ELISA. Excretion of Map by young animals may contribute in only a small part to environmental contamination, but it may be a significant risk for transmission of the disease if it occurs when highly susceptible calves are kept in groups⁸.

Frequent testing and using a combination of available tests would be recommended in order to diagnose Map infection in the early stages of the disease with a high sensitivity and specificity.

Acknowledgement

We would like to acknowledge the funders of this project (NSERC, Dairy Farmers of Canada and The Agriculture Funding Consortium). We are very grateful to the many people who have been involved in the trial. We would sincerely like to thank JDIP (USDA-NIFA Award No.2008-55620-18710) and University Research Grants Committee (URGC) for awarding a graduate student travel award, which made it possible to present the results of this project at the 11th ICP.

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NOD2 MEDIATES HOST RESISTANCE TO *MYCOBACTERIUM AVIUM PARATUBERCULOSIS* INFECTION

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The similarities between Paratuberculosis and Crohn's disease have stimulated efforts to investigate whether Crohn's susceptibility genes mediate control of *M. avium paratuberculosis* (MAP) infection. To test this hypothesis in the specific case of NOD2, we have obtained *Nod2*^{+/+} and *Nod2*^{-/-} mice, to assay bacteriologic and immunologic outcomes during *ex vivo* and *in vivo* infection. Following stimulation of peritoneal macrophages with heat-killed MAP, we observed impaired innate recognition of the pathogen with *Nod2* disruption, characterized by decreased TNF- α production. We also observed NOD2-dependent TNF- α responses after infection with live MAP, at a time when the bacterial burden in macrophages was not affected by NOD2 disruption (24 h after infection). When extending the *ex vivo* infection to day 5, we observed a ~ 0.3 log relative increase in bacteria in *Nod2*^{-/-} cells. To test whether these findings translated into differences *in vivo*, we infected mice by the intraperitoneal route; 4 weeks after infection, there was no effect of *Nod2* status on MAP burden in the livers, spleens and mesenteric lymph nodes, indicating that NOD2-independent processes exist to control early MAP infection. However, NOD2 disruption was associated with diminished antigen-specific interferon- γ responses (enumerated by ELISpot). Furthermore, splenocytes from MAP-infected *Nod2*^{+/+} animals when co-cultured with MAP-infected macrophages resulted in a decrease in bacterial numbers; in contrast, splenocytes from uninfected animals or from infected *Nod2*^{-/-} animals provided no anti-mycobacterial activity when placed in co-culture with infected macrophages. In summary, disruption of NOD2 was associated with diminished killing of MAP by macrophages, reduced innate and adaptive immunity in the host and impaired immune responses required for control of intracellular mycobacterial infection.

POSSIBLE MECHANISMS HOW MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS MAY EVADE HOST IMMUNE RESPONSES IN NATURALLY INFECTED COWS

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Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease, is able to dampen or distort immune responses at the mucosal sites and co-exist with a massive infiltration of immune cells in the gastro-intestinal tract. Knowledge of the mechanism how MAP subverts the immune response at the mucosal level in cattle is important for development of improved disease control strategies including new vaccines and diagnostic tests. In this study, 38 cull cows from herds infected with MAP were divided into four groups, based on MAP culture from gut tissues and histopathological lesion score. Cytokine and Toll-like receptor (TLR) gene expression and cytokine secretion from MAP-stimulated peripheral blood mononuclear cell (PBMC) and mesenteric lymph node (MLN) cultures of the animals were compared. Antigen stimulation of MLN cells from the severely-lesioned group resulted in significant up-regulation of the mRNA expression of five cytokines which have a diverse range of functions, IFN- γ , IL-10, IL-13, IL-17A and TNF- α , while there was no significant upregulation of these cytokines for the other groups. There were major differences between the responses of the PBMC and MLN cultures with higher levels of secreted IFN- γ released from the MLN cultures and conversely, higher levels of IL-10 from the PBMC cultures. In the MAP-infected cows there was a significant down regulation of antigen-specific expression of TLR1 gene and a trend for down-regulation of TLR2 gene expression in both MLN and PBMC cultures compared to that for the control group. The up-regulation of a diverse range of cytokines at the site of infection in the severely-lesioned animals and lack of recognition of MAP as foreign by TLR1 and TLR2 suggested possible mechanisms contributing to a failure to clear infection in this group of animals.

DETECTION OF CD4/CD8 RATIO IN MICE IMMUNIZED WITH BICISTRONIC PLASMID CONSTRUCT ENCODING A PPD GENE OF *MYCOBACTERIUM AVIUM PARATUBERCULOSIS* AND A CYTOKINE GENE OF MURINE GAMMA INTERFERON

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In the present study a gene encoding PPE protein of *M.a.paratuberculosis* was cloned with murine IFN- γ in a mammalian bicistronic vector pIRES 6.1 to elucidate the role of gamma interferon on the PPE gene for determination of CD4/CD8 ratio in post immunized mice. Flow cytometric analysis with mononuclear mice splenocytes on 42nd day post immunization revealed significant reduction of the CD4/CD8 ratio in the mice group immunized with pIR PPE/IFN compared to the pIR PPE group. These result suggested that co-expression of murine IFN γ in conjunction with PPE protein significantly enhanced the CMI response.

IMMUNE RESPONSE PROFILES OF LAMBS INFECTED WITH A LYOPHILISED PURE CULTURE SEED-STOCK OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

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Introduction

Johne's disease (JD) or paratuberculosis is a chronic intestinal infection of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Several experimental sheep infection studies have been conducted to study the pathogenesis and immunological aspects of JD and to evaluate protective efficacy of vaccines. These studies are frequently difficult to reproduce because the infectious material is derived from either intestinal mucosal extracts or *ad hoc* laboratory-adapted cultures of *Map* that are not clearly defined. Recently, an experimental model for JD in sheep was developed in Australia (Begg *et al.*, 2010), based on a lyophilised, low passage (level 5), pure culture, seed-stock of *Map* strain, Telford 9.2, isolated from a clinical ovine case. A series of experiments conducted in that study showed authentic experimental reproduction of the natural features of JD. The main objective of the current study was to investigate this model with a defined seed stock strain of *Map* in Merino sheep under New Zealand (NZ) environmental conditions. Interim results pertaining to immune response profiles and faecal shedding of *Map* are presented.

Materials and methods

Experimental animals, *Map* challenge and sampling: Fifty seven October 2010-born purebred NZ Merino wether lambs were procured from a commercial farm during December 2010. They were managed under conventional NZ sheep farming conditions in a specially prepared quarantine paddock. When the lambs were approximately 4 months of age (February/March 2011), they were challenged orally with three doses of *Map* suspension (from cultures seeded with the reconstituted lyophilised stock described in Begg *et al.*, 2010). The intervals between successive doses were 1 and 3 weeks, respectively. The retrospective viable *Map* counts in the three challenge (C) doses were 2.1×10^7 (C1), 9.3×10^6 (C2) and 2.3×10^8 (C3). The lambs were weighed and faecal samples obtained at 7 weeks prior to C1, on the day of C1 and subsequently at monthly intervals. Faecal samples were stored at -80°C, until processed for culturing. Jugular blood samples (for immune responses) were obtained at 7 and 5 weeks prior to C1, as well as at 4, 8, 16, 30, 44 and 49 weeks post-C1. The study protocol was approved by Massey University Animal Ethics Committee.

Immune responses: Interferon- γ responses to purified protein derivative of *Mycobacterium avium* (PPDA; 12.5 μ g) and concanavalin A (20 μ g; positive control) and phosphate buffered saline (PBS; blank) were tested by culturing 0.5 ml whole blood in 0.5 ml RPMI 1640 medium (Invitrogen Life Technologies) containing 5% foetal bovine serum (Invitrogen Life Technologies) and antibiotic and antimycotic solution (Invitrogen Life Technologies), for 72 hours. At the end of 72 hours, 50 μ l of supernatant from the cultures were harvested and IFN- γ assays done using a Bovigam® kit (Prionics AG, Switzerland). IFN- γ responses were expressed as PBS corrected optical density readings at 450 nm (OD₄₅₀). Antibodies in serum against *Map* were detected using a commercial ELISA kit (Paratuberculosis screening kit, Institut Pourquier, France). Serum samples, incubated with *Mycobacterium phlei* extract (in order to remove non-specific antibodies), were added to antigen coated wells and immune-assay carried out as per the kit protocol. Corrected (negative control readings subtracted) antibody responses, corrected OD₄₅₀, were expressed as percentage of corrected positive control readings and individual sheep with % OD₄₅₀ readings of > 70% considered positive for *Map* antibodies.

Faecal cultures: Faecal material collected on the day of C1 and those at 36 and 41 weeks post-C1 were sent to the Infectious Diseases Laboratory, Wallaceville Research Centre, Upper Hutt, for *Map* culture. Results were expressed as days to positive and growth index when first discovered to be positive.

Results and discussion

The mean body weight was 19.65 ± 0.33 kg on the day of C1 and it increased to 41.08 ± 1.06 kg at 49 weeks post-PC1, with an overall growth rate of 61.2 g/day. The stress effect of challenge on growth rate was evident, with pre-challenge growth rate of 72.7 g/day dropping to just 19.2 g/day during the four week period that included administration of the three challenge doses. A few lambs exhibited progressive weight loss, as early as six months post-C1 (samples for histopathology, tissue and faecal culture, have been collected for analysis). The onset of this apparent sign of clinical JD is much earlier (6 months post-C1), compared to around 13 months in the Australian study (Begg *et al.*, 2010). A rapid surge in IFN- γ responses to PPDA was evident (figure 1A) as early as 8 weeks post-C1 and those high levels continued until 44 months post-C1. This is consistent to the findings of the study in Australian Merinos (Begg *et al.*, 2010), where >80% of the animals tested positive for IFN- γ responses up until 13 months post-challenge. In the current study, approximately 36% and 55% of the animals tested positive for antibodies to *Map* by 8 and 16 weeks post-C1, respectively, and reactivity remained high (>40%) until the latest time-point (49 weeks, figure 1B). This early onset antibody response (8 weeks post-challenge) is interesting, considering the reported delayed onset in several sheep *Map* challenge studies. No antibody responses could be detected in Australian Merinos (Begg *et al.*, 2010) by 4 months post-challenge, with only 10% of the sheep testing positive by 8 months. Antibody levels in NZ Merinos (Begg *et al.*, 2005), exposed orally to medium or high doses of tissue homogenates of *Map* remained at background levels until at least six to nine months and peaked at nine months post-challenge (just before clinical disease was evident). In a different study (Stewart *et al.*, 2004) involving Australian Merinos, none of the clinically affected sheep developed antibody responses despite the presence of persistent faecal shedding of *Map*.

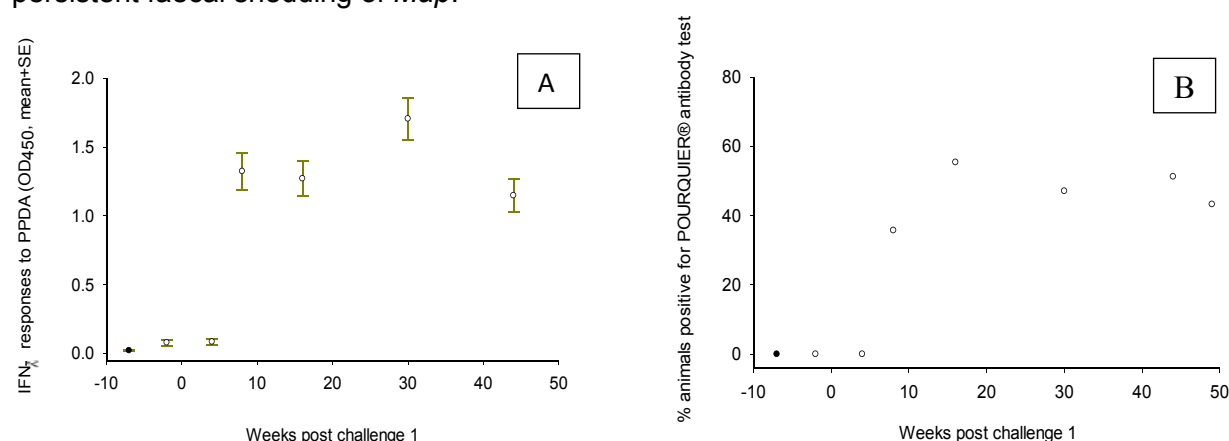


Figure 1: Immune response profiles in sheep challenged with three doses (at weeks 0, 1 and 4) of cultured *Mycobacterium avium* subspecies *paratuberculosis*. A: PBS corrected IFN- γ responses to PPDA B: Per cent sheep positive to Pourquier® antibody assay

Greater than 84% of the animals tested positive for *Map* in the faecal samples obtained at 36 and 41 weeks. This high prevalence of faecal shedding of *Map* is different to that seen in the Australian study (Begg *et al.*, 2010), where just over 20 and 60% sheep were positive at 8 and 13 months post-challenge. None of the pre-challenge faecal samples tested positive. Culturing of monthly samples obtained at other time-points is currently in progress.

In conclusion, results-to-date indicate that the lyophilised, low passage (level 5), pure culture, seed-stock of *Map* strain, Telford 9.2 can be used in an effective JD challenge model with Merino sheep under NZ conditions. The relatively early onset of clinical signs, consistent with JD, may be due to differences in host immunogenetics or environmental conditions, or a combination of the two.

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IMMUNOGENICITY OF EIGHT *MYCOBACTERIUM PARATUBERCULOSIS* SPECIFIC ANTIGENS IN DNA VACCINATED AND MAP INFECTED MICE

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Mycobacterium avium subsp. *paratuberculosis* (Map), the etiological agent of chronic enteritis of the small intestine in domestic and wild ruminants, causes substantial losses to livestock industry. Control of this disease is seriously hampered by the lack of adequate diagnostic tools and vaccines.

Here we report on the immunogenicity of eight *Map* antigens, i.e. MAP1693c, Ag3, MAP2677c, (identified by post-genomic and immunoproteomic analysis of *Map* secretome) (Leroy et al., 2007) and Ag5, Ag6, MAP1637c, MAP0388 and MAP3743 (identified by bioinformatic *in silico* screening of the *Map* genome) (Leroy et al., 2009). Strong, antigen-specific IFN- γ responses were induced in mice vaccinated with plasmid DNA encoding MAP1693c, MAP1637c, MAP0388 and MAP3743. In contrast, T cell responses in *Map* infected mice were directed preferentially against Ag5 and to a lesser extent against MAP3743.

None of the DNA vaccines conferred protection against subsequent challenge with *Map*. We postulate that differences in antigenic repertoire may explain this failure, T cells induced by DNA vaccination being unable to exert a protective role because their cognate epitopes are not presented on the surface of infected cells.

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Work partially supported by grant RT-07/6 PARATUB, Federal Public Service of Health, Safety of the Food Chain and Environment

IMMUNOGENICITY OF A HEAT KILLED *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* VACCINE IN GOATS

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Johne's disease (JD), also called as paratuberculosis is a chronic disease of cattle, sheep and goat caused by *Mycobacterium avium* subsp *paratuberculosis* (MAP). As in case of developed countries, JD is an economically important disease in India as well. Though several vaccines are available against JD, the efficacy of the vaccines are still being debated. India, with a large population of large and small ruminants needs an effective vaccine against JD. In the present study, the immunogenicity of a heat killed JD vaccine was assessed. A clinical local isolate of MAP was heat killed and coupled with chitosan nanoparticles. Immunogenicity of this vaccine was assessed in goats. Chitosan nanoparticle coupled MAP vaccine was found to be stable when injected subcutaneously with no untoward effects. The vaccine elicited good antibody response, six weeks after administration. Significant, antigen specific lymphoproliferation and IFN- γ response was observed in the immunized animals in comparison with un immunized control goats.

INTESTINAL STRICTURES, FIBROUS ADHESIONS AND HIGH LOCAL IL-10 PRODUCTION IN GOTAS WITH SEVERE LESIONS CAUSED BY *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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The purpose of the study was to describe pathological changes in goats naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). We further wanted to investigate how peripheral and local production of interferon gamma (IFN-γ and interleukin -10 (IL-10) was associated with MAP-lesions using IFN-γ testing, immunohistochemistry, immunofluorescence and *in situ* hybridisation. The intestine and associated lymph nodes from 35 goats was sampled and examined histopathologically. A full necropsy was performed on 14 of the goats. Pronounced lesions with a diffuse, transmural enteritis were found in 18 goats. Focal lesions were seen in 9 animals, while only minor lesions were recorded in 8 goats. The cranial and mid jejunum were most severely affected and the pathological changes included jejunal strictures and fibrous, serosal adhesions. Large areas with immunohistochemical labelling for IL-10 were seen in granulomatous changes of goats with diffuse lesions, but not in animals with focal lesions. IFN-γ was detected in peripheral blood and affected organs of goats with focal lesions as well as in goats with diffuse lesions. The distribution of leukocyte markers was analysed by immunohistochemistry. Numerous CD4+ and CD8+ cells, but relatively few gamma delta γδ cells and natural killer (NK) cells were seen associated with granulomatous changes. In conclusion, we found that many of the goats examined had severe, diffuse lesions. The pathological changes included jejunal strictures and adhesions, findings that are not commonly seen in paratuberculosis. IFN-γ appeared unable to prevent disease progress since both peripheral and local production was seen in goats with severe lesions. The high local

HERITABILITY/SUSCEPTIBILITY OF RED DEER TO MAP CHALLENGE

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Introduction: Paratuberculosis (Johne's disease) results in serious losses of farmed red deer (*Cervus elaphus*) in New Zealand. Young deer are particularly susceptible, especially to heavy challenge with the bovine strain of MAP, and clinical disease occurs in deer as young as 8 months of age. Field data suggests that some breed-lines of red deer display heritable resistance to paratuberculosis. Although early immune responses have been studied in cattle and sheep, there is little published information about the progression of disease and immunological responses over the 12 months after challenge with MAP in red deer and no information on how this might relate to genetic resistance. It is believed that early innate and acquired immune responses play important roles in determining the severity of the disease.

Objectives: to assess the heritability of resistance in the offspring of two red stags to experimental oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), to monitor clinical, pathological and immunological changes, and to obtain samples for gene expression studies.

Method: Two red stags from a large deer stud were identified as having offspring that were highly resistant (R) or susceptible (S) to paratuberculosis based on extensive field data, with an estimated heritability of 0.27. Unselected hinds were synchronised and artificially inseminated with semen from these two stags, and 9 offspring of each sire were weaned in March 2009. These 4-month-old offspring were challenged with 4 daily doses of $\sim 10^9$ a virulent bovine strain of MAP harvested as a tissue isolate, obtained directly from the jejunal lymph nodes of clinically affected deer. Samples of jejunal lymph node (JJLN) were surgically biopsied at 4 and 13 weeks post challenge (pc), and also collected at either euthanasia of the 3 clinically affected animals or at the end of the study 49 weeks pc. The disease status for each animal was scaled on clinical outcome, histopathology lesion severity score (LSS), culture and serology.

Results: Three animals (2S, 1R) developed clinical disease and were euthanised, and three (2R, 1S) had nil or non-specific lesions. However, overall the 9 S offspring had significantly more severe lesions than the 9 R offspring (Mantel-Haenszel Chi-square $P=0.017$). The mean LSS of R offspring was 5.9 (mild), and 7/9 had no or very mild lesions. In contrast, the LSS of S offspring averaged 11.7 (severe), and 7/9 had severe lesions. Most of the resistant, but not susceptible animals, showed evidence of resolving lesions and a reduction in the number of MAP between 13 and 49 weeks after challenge. One R offspring appeared to completely cure itself, and progressed from mild culture-positive paratuberculosis lesions at Week 13 to having no signs of disease or infection 36 weeks later. The 3 most S and 3 most R animals showed very different patterns of LSS and JJLN culture (Table 1). There were also key differences in immunological responses; R deer had higher IFN- γ levels, lower antibody titres (Fig 1a, b, c), fewer MAP cultured from JJLN and a less vigorous granulomatous response, while S deer had lower IFN- γ levels, higher antibody, more MAP cultured from JJLN and a more vigorous granulomatous response, indicating their inability to control intracellular MAP multiplication.

Conclusions: This study confirmed that resistance/susceptibility to MAP challenge is highly heritable trait in red deer. Innate immunity appeared unable to prevent infection, while acquired immune responses appeared to control infection and limit disease in the majority of resistant, but not susceptible, offspring.

Acknowledgements: We wish to acknowledge The Johne's Research Consortium for funding and the assistance of staff at AgResearch and the Disease Research Laboratory, Otago University and the Wallaceville Tb Laboratory for field work and laboratory work in carrying out this study.

Fig. a, b and c. Paralisa PPDj titres over the 49 week study for deer bred from an R sire (R suffix; gray dotted lines) or S sire (S suffix; black solid lines). Fig. 2a: The three most susceptible deer that were clinically affected (82R, 85S, 93S). Fig. 2b: Four of the most resistant deer (84R, 88R, 89S, 92R), based on serology, lesion severity and culture; Fig. 2c: The remainder.

Fig 1a.

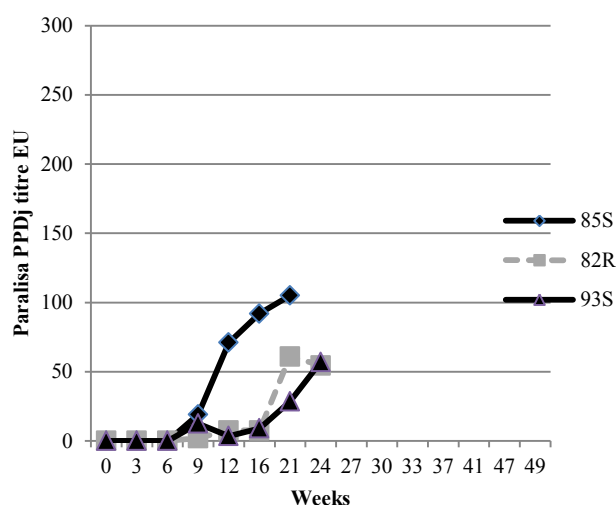


Fig 1b.

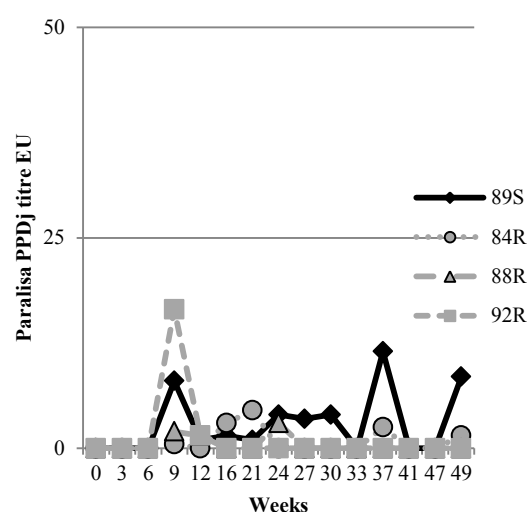


Fig 1c.

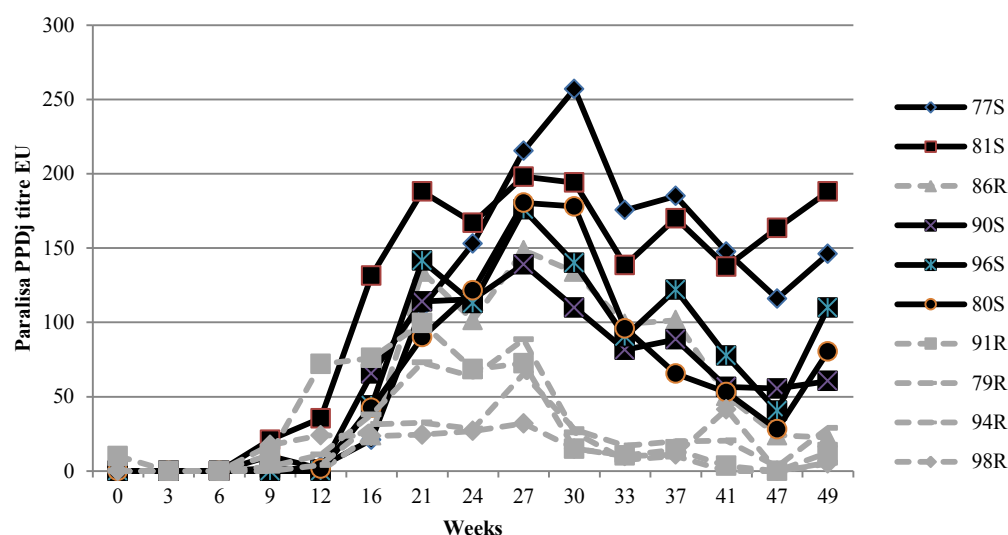


Table 1: Results of JJLN culture , histopathology and overall outcome.

Week		4		13		Euth* or 49		Affect
Tag	Sire	LSS	JJLN dtp	LSS	JJLN dtp	LSS	JJLN dtp	
92	R	0	29	6	22	0	>66	nil
84	R	0	29	11	22	5	32	mild
88	R	0	29	6	22	5	39	mild
Mean		0	29	8	22	3	46	mild
82	R	0	29	11	16	13	8	Severe*
85	S	0	39	13	9	13	8	Severe*
93	S	0	29	11	10	13	8	Severe*
Mean		0	32	12	12	13	8	severe

Week: weeks post challenge with MAP; JJLN: jejunal lymph node; dtp: days to positive for Bactec culture of MAP; Euth*: euthanasia of clinical cases

LSS: 0=nil, 1-3 nonspecific, 4-7 mild, 8-10 moderate, 11-13 severe.

BIOCHEMICAL ANALYSIS ON CATTLE NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

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Summary

The purpose of this study was to measure biochemical parameters (total protein, albumin, calcium, phosphorous, magnesium, total Bilirubin, creatinine) on clinical and subclinical paratuberculosis crossbred cows (Friesian x local Butana eco-type) confirmed by faecal culture. 21 sera were collected (7, 11, 3 from clinical, subclinical and non-infected animals respectively). Total protein, calcium and magnesium decreased significantly with the progress of the disease. Albumin decreased significantly in subclinical and clinical cases. Total Bilirubin increased significantly with progress of the disease. No significant difference was observed in Phosphorous and creatinine in both subclinical and clinical cases compared to control crossbred cows. All above differences were significant at ($p \leq 0.05$) as compared with control (non-infected) crossbred cows.

Introduction

Paratuberculosis (Johne's disease) is a chronic granulomatous enteritis due to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Motiwala et al., 2005). Cattle are most likely to be infected before 6 months of age, but clinical manifestation seldom occurs before the age of 2 years (Chiodini et al., 1984). Clinical signs of the disease in cattle include diarrhoea, emaciation, lethargy, decreased milk yield and Death. Paratuberculosis is now recognised to be of serious economic impact and animal health consequences in domesticated ruminant species (primarily dairy and beef cattle, sheep and goats) throughout the world. Paratuberculosis has the greatest economic impact in dairy cattle, where premature culling, reduced carcass value, decreased weight gain and milk production; estimated to exceed 1.5 billion dollars annually in USA (Stable, 1998; Motiwala et al., 2005). The aim of this study was to measure biochemical parameters (total protein, albumin, calcium (Ca), phosphorous (P), magnesium (Mg), total Bilirubin, creatinine) on clinical and subclinical paratuberculosis crossbred cows (Friesian x local Butana eco-type) confirmed by faecal culture.

Materials and Methods

This study was established in Khartoum State, Sudan. Twenty one blood samples were taken into plain vacutainer tubes from clinical, subclinical paratuberculosis (confirmed by faecal culture) and non-infected crossbred cows (Friesian x local Butana eco-type) and sera were separated and stored at -20 °C prior to examination. Sera were collected (7, 11, 3 from clinical, subclinical and non-infected animals respectively). Sera were analysed for the concentration of total protein and albumin according to the method of Friedman and Young (1997). Calcium was determined by Arsenazo III method described by Smith and Bauer (1979) and phosphorus was measured according to manufacturer's instructions (Randox laboratories, UK). Magnesium was determined according to tietz (1995) and total bilirubin was measured according to Friedman and Young (1997). Creatinine was conducted according to Bowers and Wong (1980).

Statistical analysis

Data were analysed for significance by ANOVA at $P \leq 0.05$ using SPSS computer Software.

Results

Total protein, albumin, Ca, Mg significantly ($P \leq 0.05$) decreased in clinical and subclinical paratuberculosis crossbred cows compared to control crossbred cows. In clinical and

subclinical paratuberculosis crossbred cows total bilirubin increase significantly ($P \leq 0.05$) as compared to control crossbred cows. No significant difference was observed in Phosphorous and creatinine in both subclinical and clinical cases compared to control crossbred cows. (Table 1).

Table 1: Parameters of biochemical changes (Mean $\pm$ SD) in control, subclinical and clinical paratuberculosis Crossbred cows in Khartoum State, Sudan

Group	Total protein	Albumin	Ca	P	Mg	Bilirubin	Creatinine
Control	6.88 $\pm$ 0.24	5.03 $\pm$ 0.1	8.26 $\pm$ 0.46	3.20 $\pm$ 0.1	1.30 $\pm$ 0.1	0.133 $\pm$ 0.05	1.16 $\pm$ 0.05
Subclinical	5.74 $\pm$ 0.8*	4.26 $\pm$ 0.88*	5.40 $\pm$ 1.90*	3.26 $\pm$ 0.52	0.88 $\pm$ 0.13*	0.41 $\pm$ 0.13*	1.50 $\pm$ 0.25
Clinical	4.49 $\pm$ 0.47*	2.97 $\pm$ 0.8*	4.14 $\pm$ 2.01*	3.30 $\pm$ 0.25	0.80 $\pm$ 0.12*	0.66 $\pm$ 0.15*	1.37 $\pm$ 0.26

Values with asterisk in a row differ significantly ($P \leq 0.05$) from

that on control(non-infected) cross-bred cows.

Discussion

A simple serum biochemistry profile of serum proteins and mineral status may provide a useful preliminary diagnosis of Johne's disease in emaciated and unthrifty sheep. Sheep with clinical Johne's disease have decreased serum concentrations of calcium, total serum proteins, and serum albumin compared with controls (Jones and Kay, 1996). In this study, total protein, albumin, Ca, Mg significantly decreased in clinical and subclinical crossbred cows compared to control crossbred cows. This is similar to those described by other investigators (Jones and Kay, 1996; Kopecky, 1972; Patterson et al., 1968; Stewart et al., 1945) but in study of Jones and Kay, 1996 there is no significant change in Mg. The low blood calcium and magnesium concentrations may be due to interference with absorption from the intestine, the loss of cations bound to albumin through a damaged gut or possibly through changes to calcium homeostatic mechanisms induced by the disease. Given that healthy control animals in the same environment possessed normal blood concentrations of calcium, magnesium and phosphorous, and because the severity of disease was positively correlated with the degree of hypocalcaemia, these observations provide no support for the idea that pre-existing nutritional deficiencies of calcium, magnesium or phosphorous could induce disease progression (Lugton, 2004). In the present study total protein and albumin significantly decreased in clinical and subclinical crossbred cows compared to control crossbred cows and this is due to a destructive granulomatous inflammatory response develops that eventually leads to intestinal malabsorption and protein losing enteropathy (Sweeney, 211). Although, the previous studies did not determine significant increase in total bilirubin, in our study total Bilirubin increased significantly with progress of the disease and this may attributed to hepatic dysfunction.

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IMMUNE RESPONSES FROM BLOOD AND MESENTERIC LYMPH NODE CELLS OF CALVES EXPERIMENTALLY CHALLENGED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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Mycobacterium avium subsp. *paratuberculosis* (MAP) infects calves at a very young age, yet the disease does not develop into a clinical state until cattle are at least 2 years of age. Knowledge of early immune response in cattle infected with MAP is crucial for early diagnosis and vaccine development. In this study, 20 calves, 5-8 week old, were challenged orally with a pure culture of MAP and the infection was monitored at 2-3 month intervals by faecal culture and systemic immune responses. Ten non-challenged calves served as controls. Animals were euthanized at 7 months and the remainder at 15 months post-challenge and studies were undertaken on immune cells from blood and mesenteric lymph nodes (MLN). The experimental infection was successful with 19/20 challenged calves shedding MAP in faeces between 2-4 months post-challenge, but thereafter faecal shedding decreased with a proportion of the challenged calves appearing to partially control the infection. The majority of challenged calves produced peripheral blood IFN- γ responses to MAP antigens by 6 months post-challenge and these responses persisted until end of the study. The MLN cells stimulated with MAP induced stronger immune responses than those from peripheral blood mononuclear cells (PBMC). The immune responses from the challenged calves appeared to be dysregulated with induction of both stimulatory and inhibitory cytokines (IFN- γ , IL-10, IL-13, IL-17 and TNF- α) as well as down-regulation of toll-like receptor (TLR-1, 2 and 4) mRNA expression. Different subsets of T lymphocytes were also analyzed using fluorescent activated cell scanning. The results provided information on early immune changes in MAP-infected calves and indicate possible mechanisms how MAP persists in the host.

EFFICACY OF 'INDIGENOUS VACCINE' USING NATIVE 'INDIAN BISON TYPE' GENOTYPE OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS FOR THE CONTROL OF CLINICAL JOHNE'S DISEASE IN AN ORGANIZED GOATHERD IN GUGARAT, INDIA

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Therapeutic efficacy of a new "Indigenous vaccine" prepared from native highly pathogenic 'Indian Bison Type' *Mycobacterium avium* subspecies *paratuberculosis* of goat was evaluated for the control of clinical Johne's disease in naturally infected Mehsana breed of goat in North Gujarat. Fifty Johne's disease (JD) positive goats from Sheep and Goats Research Station, SDAU, Sardarkrushinagar, were randomly divided into 2 groups viz., 'Vaccinated' (N = 35) and 'Control' (N = 15). After vaccination goats were monitored for physical condition, morbidity, mortality, body weights, shedding of MAP in feces, internal condition, gross lesions and humoral immune responses. At the end of the 120 days of the trial there was marked overall improvement in physical condition and body weights of vaccinated goats as compared to 'Control' goats. Vaccinated goats gained significantly ($P < 0.01$) higher body weights, hardly exhibited any lesions characteristic of JD, had significantly higher ($P < .01$) antibody titers and shedding of MAP was significantly ($P < 0.01$) reduced. Few of the vaccinated goats positive for MAP DNA in faecal PCR and blood PCR were negative on 120 DPV. Overall vaccine exhibited effective in restriction of MAP infection and significant improvement in production parameters and reduction in mortality and morbidity due to JD. The trial in the herd is continued.

EFFICACY OF INDIGENOUS VACCINE IN CONTROLLING JOHNE'S DISEASE IN A SHEEP FLOCK USING 'INDIAN BISON TYPE' STRAIN OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* OF GOAT ORIGIN IN FOUR YEARS OF TRIAL

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Efficacy of Indigenous vaccine using 'Indian Bison Type' strain of *Mycobacterium* subsp. *paratuberculosis* (MAP) of goat origin was evaluated in a sheep flock (Mannavanur, Tamil Nadu) endemic for Johne's disease from 2008 to 2011. Body weight performance, reproduction efficiency, survivability, restriction of shedding of MAP and sero-conversion rates between vaccinated and non-vaccinated sheep were compared. Successive progenies of vaccinated sheep were also vaccinated (112 and 53, respectively) in 2009 and 2010, whereas progeny of control sheep (25 and 15, respectively) were kept as control. There was very little difference in body weight gained between sheep of vaccinated and control groups in 2008. However it was distinctly high in 1st and 2nd generation vaccinated lambs. Reproduction performance (tupping rate) and survivability of flock also improved. Morbidity and mortality of flock was reduced. Shedding of MAP was reduced in vaccinated sheep by 3.7, 1.4 and 44.5% in 2008, 2009 and 2010, respectively; whereas it increased in control sheep. Sero-monitoring by indigenous ELISA showed enhanced herd immunity in successive generations. 'Indigenous vaccine', reduced clinical disease and the burden of MAP and raised flock immunity and productivity in the naturally infected sheep flock.

PHENOTYPIC CHARACTERIZATION OF IMMUNOCOMPETANT CELLS AND THE ROLE OF IFN-G AND INOS CYTOKINES IN PAUCI- AND MULTI-BACILLARY CASES OF PARATUBERCULOSIS IN SHEEP

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Various lymphocytic subpopulations and their interactions with IFN-g and iNOS influence the outcome of *M.a.paratuberculosis* (MAP) infection in sheep. In the present study, the density of CD4+, CD8+ and IFN-g secreting cells and quantitative detection of transcripts of IFN-g and iNOS were evaluated in the small intestine and mesenteric lymph node (MLN) tissues of multibacillary (MB), paucibacillary (PB) and uninfected control sheep. Significantly higher density of CD4+ cells was observed in the small intestines and MLN of the PB sheep than the MB and uninfected sheep. In contrast, the MB sheep had significantly higher density of CD8+ cells in the small intestines and MLN in comparison to the uninfected and PB groups. The IFN-g positive cells were found in singles or in groups of 2-3 cells and were found significantly increased in the small intestine of paucibacillary sheep than the multibacillary and uninfected sheep. In MLN, the number of IFN-g cells did not differ significantly between the groups. In the small intestine of the PB sheep, mean relative expression of iNOS (4 folds) and IFN-g mRNA (4 folds) were significantly increased in comparison to uninfected sheep. There were highly significant decreases in the mean relative mRNA expression of iNOS (~ 5 folds) and IFN-g mRNA (~10 folds) in the MB sheep in comparison to the PB sheep. The mean relative expression of IFN-g mRNA was found significantly down regulated (~21.5 folds and 22 folds) in MLN of the MB sheep as compared with uninfected MLN tissues and the same tissues of PB sheep. It was concluded that CD4+and IFN-g cells had definite role to play in the PB sheep by eliciting better cell-mediated immune response along with enhanced expression levels of iNOS and IFN-g cytokines than those in MB cases where CD8+ cells were in more numbers and significantly downregulated expression of these cytokines.

ACTIVATION OF HOST IMMUNE RESPONSES IN NEONATAL CALVES AND INTERFERENCE WITH TB DIAGNOSTICS AFTER IMMUNIZATION WITH A COMMERCIAL HEAT-KILLED VACCINE

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Abstract:

A major drawback of current whole-cell vaccines for *Mycobacterium avium* subsp. *paratuberculosis* is the potential interference with diagnostic tests for bovine tuberculosis and paratuberculosis. The current study was designed to explore cross-reactivity of the current USDA commercial vaccine for MAP with diagnostic tools for bovine TB and to assess host responses to vaccination. Neonatal dairy calves were assigned to treatment groups consisting of: 1) Control – no vaccine (n = 5); and 2) Vaccinate – Mycopar vaccine (n = 5).

Blood and fecal samples were collected prior to the initiation of the study for pre-vaccination measurements.

Calves were vaccinated subcutaneously with a 0.5 ml dose in the dewlap-brisket area as per standard procedure with the wild-type commercial vaccine that consists of a heat-killed whole cell suspension of MAP in oil (Mycopar). Calves were sampled throughout the study on days 7, 14, 28, and at 3, 6, 9, and 12 months. Comparative cervical skin testing was performed at 6 months both as a diagnostic tool and to determine in vivo cell-mediated response to vaccination. For determination of *M. bovis*-specific antibody, the TB Stat-Pak assay and the dual-path platform (DPP) VetT B assay (Chembio Diagnostic Systems) were performed. Peripheral blood mononuclear cells were isolated before and after vaccination and stimulated in vitro for measurement of interferon-(IFN)- γ , interleukin (IL)-4, IL-10, and IL-12, and to assess differences in lymphocyte populations by flow cytometry. Results from this study demonstrated a rapid initiation of MAP-specific IFN- γ in Vaccinate calves by 7 days, with robust responses continuing throughout the study.

Vaccinate calves also had IFN- γ responses to BoPPD, with moderate reactivity to ESAT-6/CFP-10, an *M. bovis* recombinant fusion protein. Interestingly, IL-4 and IL-10 were markedly decreased in Vaccinate calves only on days 7 and 14 of the study and thereafter were similar to Controls. Vaccinate calves began to seroconvert at 4 months with all calves having detectable MAP antibody by 6 months. Only one Vaccinate calf had a positive (suspect) skin test response to *M. bovis* PPD and none of these calves reacted in *M. bovis* serologic tests. These results suggest that vaccination with Mycopar will interfere with diagnostic tools for the detection of paratuberculosis but have low interference with *M. bovis* diagnostics.

Introduction

New serologic tests for the detection of *M. bovis* infection have recently been developed and are demonstrating high levels of sensitivity and specificity in the detection of bovine tuberculosis (Lyashchenko et al., 2008). However, there are no available data to determine if these new serologic test platforms will reduce cross-reactivity with MAP antigens associated with the paratuberculosis vaccine. Further, a more thorough assessment of host immune responses to vaccination will provide us with information about protective correlates associated with reduced clinical disease. The proposed research will explore cross-reactivity of the current USDA commercial vaccine for MAP with new serologic diagnostic tools for bovine TB.

Materials and Methods

Neonatal calves were randomly assigned to treatment groups consisting of: 1) Control – no vaccine; n = 5; and 2) Vaccine group – Mycopar vaccine; n = 5. After pre-vaccination sampling on days -2 and 0, calves were vaccinated intramuscularly (IM) in the brisket area as per standard procedure with the wild-type commercial vaccine (heat-killed whole cell suspension of MAP in oil; Mycopar, Fort Dodge Animal Health, Ft. Dodge, IA). Skin testing was performed at 6 months both as a diagnostic tool and to determine in vivo cell-mediated response to vaccination. To assess the effects of vaccination on standard diagnostic tools for MAP and *M. bovis*, samples were monitored for the presence of antibody and IFN- γ responses. Plasma was harvested after incubation of whole blood with medium only, ConA,

MAP sonicate, JPPD, BoPPD, and Esat-6/CFP-10 fusion protein and assayed for IFN- γ by ELISA (Bovigam, Prionics). Serum was harvested from whole blood and assayed for the presence of MAP antibodies by commercial ELISA (Herdchek) and Western blot. For determination of *M. bovis*-specific antibody, the TB Stat-Pak assay (Chembio Diagnostic Systems, Inc., Medford, NY) and the dual-path platform (DPP) VetTB assay (Chembio Diagnostic Systems). Peripheral blood mononuclear cells were stimulated in vitro with medium control (NS), ConA (10 μ g/ml), MPS (10 μ g/ml), JPPD (10 μ g/ml), BoPPD (10 μ g/ml), and rEC (1 μ g/ml) to evaluate immunologic parameters such as cytokine secretion (IFN- γ , IL-4, IL-10, IL-12), and changes in cell populations.

Results

MAP-specific IFN- γ was highly upregulated in calves within 30 days of vaccination. There were cross-reactive IFN- γ responses to BoPPD stimulation in vitro in Vaccinate calves that paralleled responses to JPPD and MPS (Figure 1). However, responses to rEC were not different between Control and Vaccinate calves.

CCT (skin test) responses were highly specific to AvPPD and did not yield false positive results with BoPPD for vaccinated calves as demonstrated on the scattergram depicting zones of positive and negative results (Figure 2). MAP-specific antibody was demonstrated by 4 months post-vaccination using Western blot analysis (data not shown). Sera from vaccinated calves did not demonstrate any cross-reactivity with the TB Stat-Pak or DPP VetTB assays for *M. bovis* (data not shown).

Vaccination resulted in the increased expression of CD25 and CD26 activation markers on CD4 cells by 6 months.

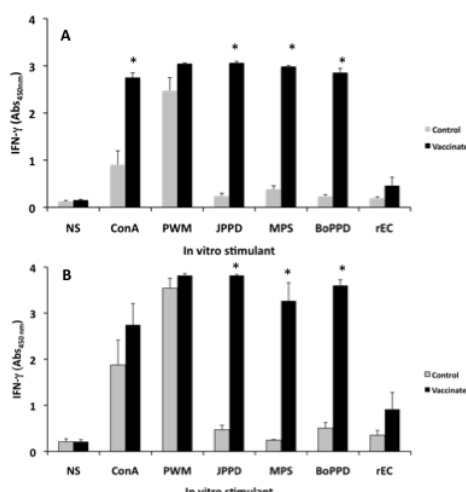


Figure 1. Interferon-gamma (IFN- γ) responses for total PBMCs at 1 month (A) and 12 months (B) after vaccination of calves

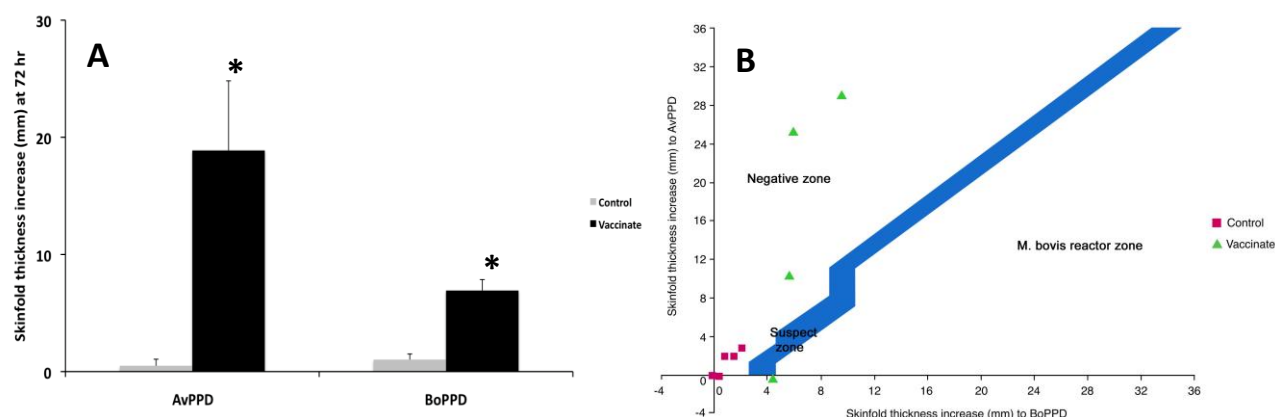


Figure 2. Delayed-type-hypersensitivity responses to *M. avium* PPD and *M. bovis* PPD were determined by the comparative cervical (CCT) test (means $\pm$ standard errors) in Control (n = 5) and Vaccinate calves (n = 5). Responses represent the change in skin thickness relative to pre-skin test (A) with results depicted on scattergram for interpretation of results (B).

Discussion and Conclusions

Vaccination of calves with a commercial heat-killed vaccine invokes an early cell-mediated immune response that is maintained through 12 months and is concurrent with the appearance of MAP antibody. There are several TB test options including the rEC-mediated IFN- γ test, the CCT test, and newly developed *M. bovis* antibody tests that do not result in false positive results in MAP-vaccinated animals and could be used effectively in TB control programs.

THE OTHER WAY AROUND: PROBIOTIC LACTOBACILLUS ACIDOPHILUS NP51 RESTRICT PROGRESSION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) INFECTION IN BALB/C MICE VIA ACTIVATION OF CD8A⁺ IMMUNE CELL-MEDIATED IMMUNITY

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ABSTRACT

The objective of this study was to examine the immune-modulating effects of feeding a novel probiotic *Lactobacillus acidophilus* strain NP51 to specific pathogen-free Balb/c mice challenged with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease (JD) in ruminant animals. We hypothesized that feeding the NP51 would activate the adaptive immunity and impede the development of MAP infection in murine model of JD. Thus, Balb/c mice were randomized to treatment groups in a factorial design including mice that were fed either the viable or heat-killed NP51 (VNP51 or HNP51, respectively) and challenged with either the viable or the heat-killed MAP (VMAP or HMAP, respectively). Mice were fed 1×10^6 CFU of either VNP51 or HNP51 $\cdot$ mouse⁻¹ $\cdot$ day⁻¹ mixed with standard mouse chow throughout the study. On day 45 of the study, mice were challenged with 1×10^8 CFU of VMAP or HMAP injected intraperitoneally. Ten mice from each group were euthanized on days 45, 90, 135, and 180. Spleens were excised and used for *in vitro* splenocyte cell cultures that were either stimulated with sonicated MAP antigen or concanavalin A and examined for frequency of T lymphocyte subpopulations. Also, spleens and livers were cultured on HEYM to evaluate effects of VNP51 and HNP51 on tissue MAP burden. The fecal pellets were collected and examined for MAP shedding. VNP51 and HNP51 differentially stimulated the adaptive immunity and decreased MAP tissue burden and shedding of MAP in fecal pellets. With VMAP as the inoculum, both VNP51 and HNP51 stimulated CD8 α^+ immune cell-mediated immunity and decreased the humoral immunity. When HMAP was used as the inoculum, VNP51 stimulated both the CD8 α^+ T cell-mediated and humoral immunity. In contrast, HNP51 feeding induced CD8 α^+ T cell-mediated immunity only as verified by the differential cytokines and immunoglobulin secretion pattern. These data provide persuasive evidence that NP51 has the potency to prevent JD infection in murine model of JD.

INTRODUCTION

Johne's disease (JD) is a chronic granulomatous enteritis of domestic and wild ruminants. The etiologic agent of JD, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), infects and deactivates the antigen-presenting cell (APC) of the host (Momotani et al., 1988; Sweeney, 1996) leading to suppression of innate and adaptive immunity. Feeding a probiotic to mice programmed dendritic cells (DC) to adopt a tolerogenic or pro-inflammatory phenotype in a strain-dependent manner (Hart et al., 2004), suggesting that probiotics can be used to modify the immune response of mice. It has been shown that *Lactobacillus salivarius* Ls-33 induced IL-10-secreting DC. In contrast, *Lactobacillus acidophilus* NCFM predominantly induced IL-12-secreting DC (Evrard et al., 2011; Gad et al., 2011).

On the basis of previous findings, we evaluated the preventative effects of feeding the probiotic NP51 to Balb/c mice prior to challenge with a virulent strain of MAP isolated from a clinical cow at the National Animal Disease Center in Ames, Iowa. We hypothesized that feeding NP51 to Balb/c mice infected with MAP would induce DC maturation and accelerate the innate and adaptive immunity targeting MAP. To test the hypothesis, we evaluated effects of feeding the probiotic VNP51 or HNP51 to Balb/c mice infected with MAP on their immune responses and resistance to colonization with MAP.

MATERIALS AND METHODS

Three hundred sixty Balb/c mice were randomized to three major treatment groups that included nine subgroups. Group 1 mice were fed the maltodextrin carrier for the probiotic at 3% of their chow diet.

Groups II and III were fed the *Lactobacillus acidophilus* **NP51** at 1×10^6 CFU·mouse⁻¹·day⁻¹ in their chow with Group II fed heat-killed **NP51** (**HNP51**) and Group III fed the viable **NP51** (**VNP51**). Within Groups I, II, and III were subgroups of non-infected controls (n = 40), and mice challenged with heat-killed **MAP** (**HMAP**; n = 40), and viable **MAP** (**VMAP**; n = 40). **HMAP** or **VMAP** were injected intraperitoneally at 1×10^8 CFU·mouse⁻¹ once on day 45 of the study. Ten mice from each subgroup were euthanized on days 45, 90, 135, and 180 of the study. Fecal samples were collected weekly. In addition, the spleen, cecum, ileum, liver, and mesenteric lymph node (MLN) were dissected and cultured to determine MAP burden in tissues. Splenocyte single cell suspensions were stimulated with MAP antigen for examination of T cell differentiation and secretion of cytokines.

RESULTS AND DISCUSSION

Feeding either VNP51 or HNP51 to mice challenged with the VMAP or HMAP significantly ($P < 0.01$) induced proliferation of the antigen-specific CD8 α^+ immune cells (Figure 1A). VMAP burden significantly decreased in spleens ($P < 0.01$; Figure 1B) and livers ($P = 0.01$; Figure 1C) of mice fed VNP51 or the HNP51 compared with that of the VMAP-infected control on days 135 and 180, respectively. In addition, VNP51 and HNP51 feeding markedly ($P = 0.02$) decreased the percentage of mice shedding MAP in feces on day 180 (Figure 1D). These data suggest that feeding the probiotic NP51 to mice infected with MAP induces immune responses that target MAP *in vivo* and curtails progression of MAP infection.

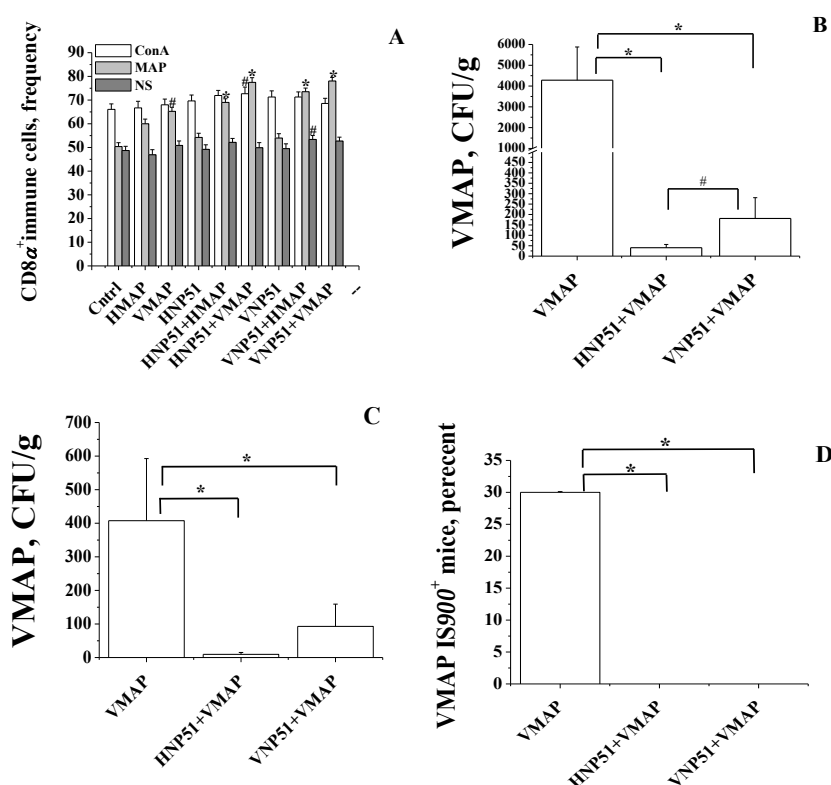


Figure 2. Effect of probiotic VNP51 and HNP51 feeding on CD8 α^+ immune cell proliferation and MAP burden in tissues and feces of Balb/c mice on days 135 and 180. Panel A, splenic CD8 α^+ immune cell proliferation stimulated with ConA (open bars) or with MAP antigen (grey bars) or nonstimulated (dark bars) on day 180. Panel B, Spleen MAP burden on day 180. Panel C, Liver MAP burden on day 180. Panel D, Fecal MAP shedding on day 180. (*) indicates significance difference at $P \leq 0.05$; (#) indicates tendency for a difference at $0.05 < P \leq 0.10$. Data are means $\pm$ SEM.

CONCLUSIONS

The data suggest that **NP51** impedes MAP infection via induction of CD8 α^+ cell-mediated immunity.

ACKNOWLEDGEMENT

We thank the Nutritional Physiology Corporation, Guymon, OK, for the financial support of this study and for donating the NP51.

A MULTI-STAGE PARATUBERCULOSIS VACCINE: EFFECT OF AGE ON IMMUNOLOGICAL RESPONSE IN CALVES

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In this study we evaluated the immune response to novel recombinant multi-antigen subunit vaccines in two separate experiments. The vaccines incorporate proteins expressed during the acute and latent stages of MAP infection and is called a 'multi-stage' vaccine. Experiment 1 was conducted to determine the appropriate age of MAP vaccination in calves to generate strong Th1-type immune response. Five MAP recombinant proteins including ESAT6 family proteins; secreted and latently expressed proteins were formulated with CAF01 adjuvant. 27 male jersey calves were divided into 3 groups with first vaccination at 2, 8 or 16 weeks of age, respectively, followed by booster vaccinations at week 4 and 12 relative to the first vaccination. Results suggest there is a correlation between age and vaccine induced Th1 immune response. We found weakest responses in calves vaccinated at 8 weeks of age which coincides with weaning stress. The results indicate little boosting effect following third vaccination suggesting the possibility of excluding second booster dose at this time point. Three recombinant proteins were found to be strong inducers of Th1-type response. In Experiment 2, two proteins from experiment 1 and three other novel proteins were used as a fusion protein with CAF01. 28 male jersey paraTB-free neonatal calves were divided into 4 groups with 7 calves in each. Groups included early (vax at 2 and 6 weeks) and late (vax at 16 and 20 weeks) vaccine group, Silirium (commercial vaccine at 16 weeks) group and non-vaccine control group. Calves were dosed orally with 1x10⁹ live MAP 10-12 days after birth with a clinical isolate (E2007). Calves will be followed for 10 months for evaluation of vaccine induced immune responses and possible demonstration of protective efficacy. We propose that such a novel 'multi-stage' vaccine should serve both as a preventive and therapeutic vaccine against latent MAP infection.

MACROPHAGE IMMUNE RESPONSES TO MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS (MAP) AND ANTIGENS

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Macrophages, which are components of the innate immune system, play a critical role in mediating the various immune responses to invading pathogens. On activation by microbial products, macrophages respond by the production of various compounds, one of which is nitric oxide. The antimicrobial effect of activated macrophages strongly correlates with the amount of reactive nitrogen intermediates produced. Nitric oxide is produced in response to stimulation of Toll-like receptors (TLR), which trigger host defence mechanisms on binding with microbial ligands. While many questions still remain regarding the bactericidal ability of nitric oxide, it can be used as a marker of macrophage activation. In this study, RAW 264.7 macrophages were stimulated in vitro with live or heat-killed mycobacteria or mycobacterial antigens; MAP specific antigens (316v, Para-LP-01), *M. avium* PPD (PPDA) with or without pre-stimulation with lipopolysaccharide (LPS), or interferon gamma (IFN- γ). Nitric oxide responses were detected by the Griess assay and TLR expression on the cells was assessed by flow cytometry. MAP and its antigens, 316v and Para-LP-01 did not stimulate nitric oxide production. However, this response increased after pre-stimulation of the cells with IFN- γ but not LPS. A similar result was found with primary bovine macrophages pre-stimulated with IFN- γ . Nitric oxide production induced by live MAP was lower in comparison to heat-killed MAP. TLR expression studies showed that while both LPS and IFN- γ pre-stimulation induced TLR2 and TLR4 expression, it was greatest with LPS, therefore this did not explain the effect of IFN- γ pre-stimulation on the ability of macrophages to respond to mycobacterial antigens. The pathway by which IFN- γ overcomes the inhibitory effect on nitric oxide production by MAP and its antigens needs to be established. Further study is needed to understand whether variations in the TLR expression pattern might affect the outcome of the immune response to this infection.

DETECTION OF HUMORAL IMMUNE RESPONSE IN EXPERIMENTALLY AND NATURALLY INFECTED SHEEP WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* AND ITS RELATIONSHIP WITH PROGRESSIVE PATHOLOGICAL CHANGES AND DIAGNOSTIC SIGNIFICANCE

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Abstract

In experimentally infected sheep it was found that the severity of lesions and the duration of infection had positive relationship with humoral immune response and LAM-ELISA was comparatively more sensitive than other ELISAs in detecting early infection and hence may be useful for screening flocks for controlling infection.

Introduction

Paratuberculosis (Johne's disease) is a chronic bacterial disease of the domestic and wild ruminants caused by a slow growing acid-fast bacterium called *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Map infection in ruminants presents bacteriological, immunological and pathological spectrum. Studies on immune responses and their relationship with bacteriological and histopathological findings are not possible in natural disease because of insidious nature of the infection. However it is possible in experimentally infected animals, where we know the time of infection and course of the disease. In the present study, we evaluated the antibody response by different type of ELISAs and AGID at monthly intervals and correlated with faecal shedding and progressive histopathological changes and their diagnostic implication.

Materials & Methods

Twenty four lambs of either sex, aged 8-12 weeks, testing negative to MAP infection were divided into two groups: group I (n= 19) animals were infected by oral administration of Map prepared from a multibacillary sheep and group II animals (n=5) acted as uninfected control (Kurade et al., 2004). Blood and faecal samples were collected at monthly intervals. Animals died or sacrificed were necropsied lesions noted and relevant tissues were processed for preparation of h&E and ZN stained slides. Histological grading of the lesions in each animal was carried out (Kurade et al., 2004). Faecal smear examination and culture were carried out. Sonicated antigen (SA) and lipoarabinomannan (LAM) antigen using Con A-Sepharose 4B chromatography column (Pharmacia) were prepared from *M. a. paratuberculosis* strain 316 F as per the method described previously (Rajukumar et al., 2001; Sugden et al., 1987; Jark et al., 1997).

Enzyme linked immunosorbent assay: Unabsorbed (Unab-) and absorbed (Ab-) ELISA were performed as described previously (Rajukumar et al., 2001). The procedure for LAM-ELISA was similar to unabsorbed ELISA except the antigen LAM (1 µg/well/100 µl) was coated in the citrate coating buffer (pH 6.0) (Jark et al., 1997). The data obtained from the various ELISAs were analysed for their statistical significance using Student's t-test.

Results and Discussion

The Unab- and LAM-ELISAs detected significantly high antibody levels from 60 days post inoculation (dpi), whereas absorbed (Ab-) ELISA detected antibodies after 150 days (Fig. 1-4). The Unab-ELISA and LAM-ELISA detected consistently high level of antibody in grades 3 and 4 lesion sheep. The sheep with grade 3 and 4 lesions exhibited elevated antibody levels from 30 dpi onwards with higher values at different days post inoculation than other grade and control sheep. LAM-ELISA had shown better sensitivity in comparison to Unab- and Ab-ELISA in all grades sheep. Grade-wise performance of various ELISAs was analysed (Table). Taken together, sensitivities were 52.6, 42.1 and 36.6%, for LAM-, Unab- and Ab-ELISAs, respectively. In the AGID test, 3 sheep (2 grade 3 at 270 and 330 dpi, and one grade 4 at 210 dpi) tested positive. Faecal shedding by smear examination was detected intermittently in one grade 2, two grade 3 and all grade 4 sheep. Three infected sheep, two grade 4 (at 150 and 210 dpi)

and one grade 3, (at 330 dpi) were positive in the bacterial culture. In naturally infected sheep, LAM-ELISA detected 15.2% (16/105) sheep.

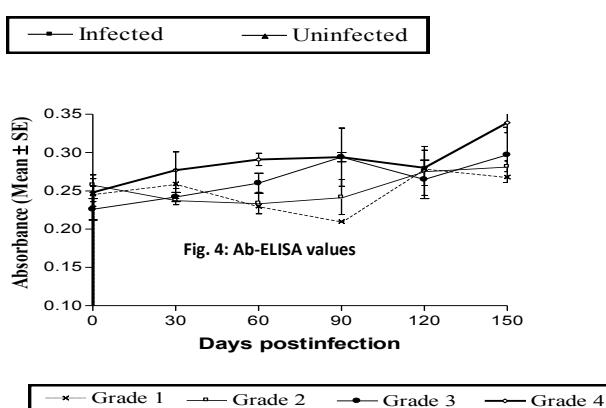
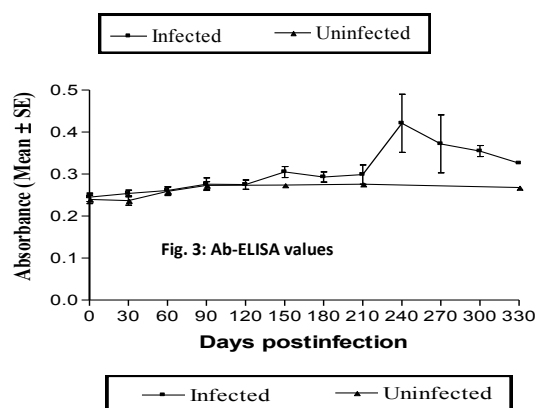
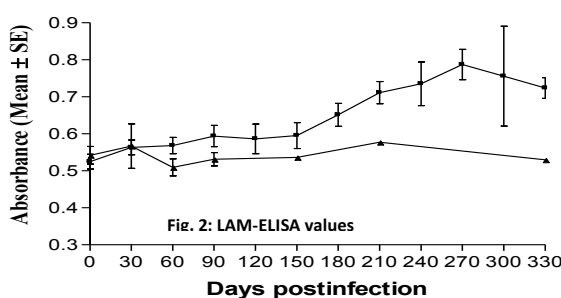
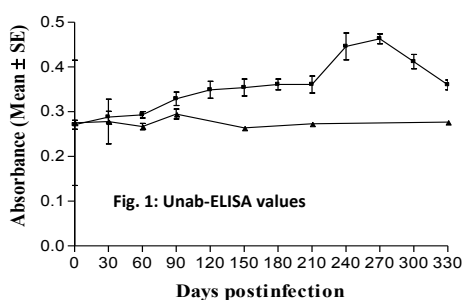
In contrast to earlier held view that MAP specific antibodies develop in the later stages of the infection, most of the experimentally infected sheep in our study showed a rising trend in the level of antibodies as early as from 30 dpi by the unabsorbed and LAM ELISA. This indicated that the antibody response, though minimum, started appearing earlier but significant (diagnostic) levels could reach only late in the course of disease. In a previous experimental study in calf, LAM-ELISA detected antibody 134 dpi and in immunoblot antibodies were detected within two weeks against a ~50 kDa protein of Map (Waters et al., 2003). However in other sheep experiments, antibody was detected after several months of infection (Stewart et al., 2004). This difference could be due to experimental design, dose and strain of Map used for the infection.

Table. Results of different types of ELISAs according to different types of histological lesion categories

	Grade 1	Grade 2	Grade 3	Grade 4	Total
No. of animals	8	4	4	3	19
LAM ELISA	2 (25)	2 (50)	3 (75)	3 (100)	10 (52.6)
Unabsorbed ELISA	1(12.5)	2 (50)	2 (50)	3 (100)	8 (42.1)
Absorbed ELISA	1 (12.5)	1 (25)	2 (50)	3 (100)	7 (36.8)

Figures in parenthesis indicate percentage

Close relationship between pathological findings and serological responses as detected by AGID and absorbed ELISA in the present study has been described earlier (Clarke *et al.*, 1996; Perez *et al.*, 1997). Among the different ELISAs, LAM-ELISA was found to be better and useful in detecting early infection. It was observed that absorption of sera with *M. phlei* powder reduced the sensitivity of the ELISA. Precipitating antibodies with AGID were detected in only three sheep in advanced stages of infection (one at 210 dpi with grade 4 and two at 270 dpi and 330 dpi with grade 3 lesions), which were in accordance with earlier reports (Clarke *et al.*, 1996). Though there has been positive correlation between AGID test results and mycobacterial load in the tissue sections, the positive AGID results in grade 3 sheep in our study suggests that duration of host pathogen interaction is also important for the development of precipitating antibodies in paratuberculosis.



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CHARACTERIZATION OF IMMUNOPATHOLOGICAL FORMS OF BOVINE PARATUBERCULOSIS: IMPLICATIONS FOR INFECTION CONTROL

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INTRODUCTION

The occurrence of delimited granulomatous lesions in the distal small intestine and associated lymph-nodes is frequently observed in subclinical paratuberculosis whereas clinical disease is usually associated with the development of advanced inflammatory lesions. In cattle, the microbiological and humoral diagnosis of subclinical *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections deals with lack of sensitivity and thereby, delimited lesions are mostly unnoticed. The aim of this study was to provide a comparative assessment of histopathological, immunological and microbiological diagnostic methods for natural MAP infections in cattle for control purpose.

MATERIAL AND METHODS

Blood and tissue samples were weakly collected from 1,031 Friesian animals in two local slaughterhouses in the Basque Country, between March 2007 and November 2010. Histological examinations were performed from three tissue sections: ileocecal valve (ICV) and distal ileon (DI), jejunal caudal lymph-node (JC-LN) and ileal lymph-node (I-LN) (1). Humoral and cellular immune responses to the paratuberculosis protoplasmatic antigen 3 (PPA3) and the avian purified protein derivative (PPD) were evaluated with two commercial immunological tests: BOVIGAM® interferon-gamma and Pourquier® ELISA, respectively. Culture and real time PCR assays were applied to a pool of mucosa from ICV-DI and JC-LN (1:1). Tissue cultures in duplicated Herrold's egg yolk and Lowenstein-Jensen media, both supplemented with mycobactine J, were done as previously described elsewhere (2). MAP colonies were confirmed by PCR (3). MAP IS900DNA detection was performed using the combined Adiapure®-Adiavet® extraction and amplification kit. For each pathological form, MAP viability ratio was defined as the ratio between positive cultures and positive rtPCRs.

RESULTS

Paratuberculosis lesions were observed in 46.7% of the studied animals as follows: *focal* forms 38.9%, *multifocal* forms 3.4% and *diffuse* forms 4.3%. Among the *diffuse* forms, *multibacillary* forms (3.3%) were much common than *lymphocytic* forms (0.3%).

Two age-related patterns of paratuberculosis forms could be identified. While *focal* forms appeared to be persistent during adulthood, more extended forms tended to progressively decrease (Figure 1).

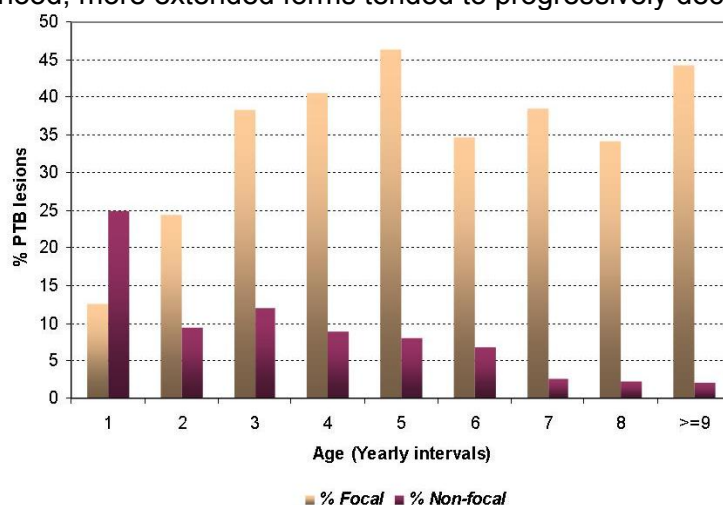


Figure 1. Dynamics of paratuberculosis lesions in cattle and age.

Opposite tendencies for *focal* and *non-focal* forms when yearly intervals of age were considered. Intensity of immune responses as well as MAP isolation and viability rates increased with tissue damage. As seen in Table 1 *focal* forms were associated with low antibody production, cellular responses and slightly higher MAP isolation and viability rates than those for animals without lesions. Conversely, *diffuse* lesions were closely related to the development of specific antibodies (92.9%) and MAP isolation (100.0%).

Histopathology	Number of animals	% ELISA+	% IFN- γ +	%Tissue Culture+	%Tissue PCR +	%MAP viability
No lesions	526 (157)	1.7	23.6	7.0	22.2	31.6
<i>Focal</i>	384 (152)	3.1	36.8	14.3	28.9	49.6
<i>Multifocal</i>	34 (12)	41.2	58.3	64.7	79.4	81.5
<i>D. lymphocytic</i>	3 (1)	100.0	0.0	100.0	100.0	100.0
<i>D. intermediate</i>	6 (4)	83.3	50.0	100.0	100.0	100.0
<i>D. multibacillary</i>	33 (7)	93.9	85.7	100.0	100.0	100.0
Total PTB lesions	460 (176)	14.1	40.3	25.9	39.1	66.1

Table 1. Pathological forms of bovine PTB and their association with the humoral and cell-mediated immunological response as well as MAP isolation and viability rates. The number of animals in which IFN- γ productions were evaluated appears in brackets.

The detection of combined cell-mediated responses varied with the inflammatory grade from 1.3% for *focal* forms to 33.3% for *multifocal* forms and 66.7% for *diffuse* forms. Among animals showing lesions consistent with PTB overall combined antibody and IFN- γ productions accounted for 4.2%.

CONCLUSIONS

Focal paratuberculosis forms are widely present in adult cattle but hardly detected by standard “in vivo” tests. These forms might represent a low epidemiological risk because they also show low MAP isolation and viability rates and thereby, they could be defined as *latent* forms. In turn, *non-focal* forms are related to a higher impact on MAP transmission due to increased isolation and viability rates, especially within the first 4 years. These forms may be considered as *patent* forms.

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ACKNOWLEDGEMENTS

This study was supported by a grant (AGL2006-14315-CO2) from the Ministerio de Ciencia e Innovación (Spain). Patricia Vázquez was in receipt of a FPI doctoral fellowship (BES-2007-17170) from the Ministerio de Ciencia e Innovación (Spain).

DETERMINATION OF SURVIVAL AND ANTI-APOPTOTIC ACTIVITIES OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN EPITHELIAL CELLS

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Mycobacterium avium subsp. *paratuberculosis* (MAP), the etiologic agent of paratuberculosis in cattle, is suspected to be associated with human Crohn's disease (CD), a chronic inflammatory bowel disease, though the zoonotic potential of MAP is controversially discussed. A critical step in the pathogenesis of MAP infections is the invasion of intestinal epithelial cells by MAP.

The aim of our study was to analyse the ability of MAP to invade and survive in intestinal bovine epithelial cells (FKD-R) compared to human cells (Caco-2). Both cell lines were inoculated with MAP strain (ATCC 19689) at a MOI of 100 for 4 h. After incubation, extracellular bacteria were killed by amikacin (200 µg ml⁻¹) treatment, supernatants were replaced by fresh medium and cells were further cultured for 3-24 d. Finally, cells were lysed with Triton X-100 (0.1 %) and serial dilutions were plated for colony counts.

MAP was able to invade both epithelial cell lines and persisted intracellularly. The number of viable intracellular bacteria remained constant over the entire observation period. Infected FKD-R cells formed a closed cell monolayer and showed no signs of increased cell death. By contrast, uninfected cells significantly changed cell morphology within 10 days and succumbed to cell death until day 12.

MAP seems to have the ability to inhibit apoptosis in bovine epithelial cells during infection. Further investigations will aim to elucidate the anti-apoptotic mechanisms involved.

PERSPECTIVES ON: HOST RESPONSE AND IMMUNOLOGY

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Over the years we have seen many descriptive studies that aim to correlate the measurable immune responses with the pathological lesions. As more and more reagents have become available, and novel cytokines enter the field, it has become clear that the classical Th1/Th2 paradigm is a simplification of the immune response in MAP infections. Although some animals seem to elicit this type of immune response other animals have a very variable response. Lately the focus has been more on the balance of the pro-inflammatory immune response that can eliminate the bacterial and the anti-inflammatory response that is necessary to prevent immunopathology. A classical but important question arises in all these descriptive studies, is the measured immune response the cause or the effect? For instance; Does too much IL-10 lead to depressed IFN- γ production and increased bacterial load, or does the very strong IFN- γ response, induced by an uncontrolled infection, lead to IL-10 production in an attempt to avoid immune mediated pathology? Some of the answers may come from the increasing number of studies looking into genes associated with susceptibility or resistance to MAP infection. These studies range from the well defined resistant and susceptible deer lines in New Zealand, huge genome-wide association studies including several hundred thousands of animals, to more targeted studies on possible gene candidates.

Another major focus has been on vaccines against MAP. Today vaccination is not allowed in many countries due to the interference with diagnostic testing for bovine TB. The current vaccines may also interfere with testing for MAP. An ideal vaccine should prevent infection in all animals and not interfere with diagnostic testing. Looking at the long time commitment to develop an alternative to BCG, the first goal might be impossible to reach. Nevertheless a reduction in the number of clinical cases and bacterial shedders might be acceptable providing testing can still be performed. In this regard it is important to remember that different countries may have different aims with their MAP vaccine. In countries where routine screening for paratuberculosis and bovine TB is not done, a vaccine that decreases clinical symptoms and give increased milk production is a good alternative, and such vaccines are already available.

Novel, high through-put technologies have entered the field of paratuberculosis, and I am confident we will see more of these studies that will generate vast amount of data. This will surely give novel information about immune responses to MAP after infection and vaccination. However, experience from the human fields has made it clear that this huge amount of data is not likely to provide any simple answers. What such data can do is to help us generate novel hypothesis that can be tested in functional assays. Hopefully a close collaboration between the “-omic” scientists and the immunologists will bring the field forward.

CONTROL PROGRAMS

KEYNOTE ON: CONTROLLING JOHNE'S DISEASE – LEARNING FROM THE PAST 15 YEARS

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Johne's disease control ...people applying technology

Introduction

The International Association for Paratuberculosis (IAP) is a “scientific organization devoted to the advancement of scientific progress on paratuberculosis and related diseases.” The primary functions are clearly directed at supporting this endeavour by encouraging scientific knowledge, collaboration, scientific freedom and achievement (1). The colloquia and the scientific literature bear testimony to the success of this Association and its members in fulfilling the purpose over the 23 years since it was founded. The advances in the understanding of the biology of *M paratuberculosis* have been astounding and have resulted in many useful technologies, especially diagnostics. We have learned more about how the host and environment interact with the organism and what potential lies in manipulating these (2).

In the end however, the real value of improved understanding and technological developments must be judged against the resulting improvements in prevention and control of paratuberculosis. How have we performed in this area? Manning and Collins posed the following question in 2010: “Given the large scale efforts to control MAP in many countries and the continuing expansion of the epidemic in those same countries, is MAP control possible?”(2). I think that the evidence shows that it is; but we have to be committed, clever and comprehensive in our approach. Presentations in sessions at this of the colloquium outline some novel approaches to the challenges of effective control.

There is not simply one “Johne's disease” and there is no single objective or approach to controlling it. Firstly, in many parts of the world, we are dealing with different types of the organism across several different livestock sectors; for instance, the cattle and sheep types in Australia.

Secondly, control programs may be directed at different priorities with one or more of the following objectives:

- Preventing infection *per se*
- Reducing the impacts of infection on animal welfare and survival
- Reducing subclinical effects on growth and production
- Reducing contamination of farm products and the farm environment
- Assuring markets on the status of animals and products

Thirdly, the size of the arena in which control is attempted also varies from the individual animal and farm level through to regional and national levels, and even the international level. Finally, a major factor is what people want to achieve and what they are prepared to invest to do this.

People's perceptions of what constitutes a risk vary, as does their risk aversion. These differences are especially evident between those whose herds or flocks are infected with paratuberculosis and those who think that they are “free” from infection.

The Association recognises the importance of this human wildcard in the epidemiology of paratuberculosis, listing among its functions “to advance professional and lay education in paratuberculosis... and to increase public understanding and appreciation of the importance and promise of the methods of science in animal health” (1).

The components that should be in place before a major control program is undertaken have been outlined by Thrusfield (3):

- Understanding the aetiology, pathogenesis and epidemiology of the disease
- Veterinary infrastructure and capability
- Diagnostic feasibility

- Adequate surveillance
- Availability and identification of non-diseased replacement stock
- Producers' views and cooperation
- Public opinion
- Public health significance
- Adequate legislation and compensation
- Possible ecological impacts
- Economic costs
- Availability of funds

For various reasons, these are not always in place at the beginning of a program but they do provide a useful framework of technical and socio-economic factors against which current and proposed Johne's disease programs should be assessed.

Australia

Australia's National Johne's Disease Control Program commenced in 1995, under the auspices of the National Farmers' Federation. From modest beginnings, the scope of the program has grown to involve all the affected farming organisations and the national and state government animal health services, under the banner of Animal Health Australia. As both cattle and sheep type infections are unevenly distributed across livestock industries and regions in Australia, the program has a strong interest in prevention of infection at the herd and regional level.

The program has been supported technically by a productive local research program and the results of overseas research and programs, but has largely developed through reviewing and responding to the experience in Australia. Many of these lessons have broader application as more countries attempt prevention and control of paratuberculosis and will be highlighted in the Industry Special Focus Day at this colloquium.

Under the banner of the Australian national program, there is evidence of success in:

- long term prevention and eradication of incursions of Cattle-type infection (bovine Johne's disease, BJD) in Western Australia, the Northern Territory and Queensland
- elimination of infection in many beef herds in south-eastern Australia
- prevention and control of infection in sheep flocks in large parts of NSW and Queensland
- reduction in the flock level incidence and prevalence of OJD in South Australia
- prevention of new infections in the alpaca industry.

Less remarkable has been the ongoing spread of OJD in south-eastern and south-western Australia. Some factors associated with these and lessons drawn are outlined below.

Lessons

As in life generally, equity (or being given "a fair go" in Australia) is a major driver for cooperation; and perceived or real inequity is a major disincentive. The increasing incidence of OJD in New South Wales and its detection in different regions of south-eastern Australia in the mid-1990s, precipitated attempts to control the epidemic. As we found in the early stages however, implementing the available legislative capacity to enforce quarantine and movement restrictions on known infected flocks was ineffective and counterproductive, largely because the program did not include many of the attributes outlined by Thrusfield (3). There were very few technical tools to identify infected flocks and to help affected producers prevent losses or manage infection. Trials into the effectiveness of destocking as a means of eradicating OJD found that apparently disease free replacement sheep reintroduced infection. The national sheep producers' organisations could not reach agreement on providing assistance from national levies to owners of known infected sheep flocks to help them deal with the negative social and economic effects on their businesses. Furthermore, infected flocks that were not known officially to be infected remained free from restrictions. These inequities discouraged other producers from cooperating with surveillance to detect infection and OJD spread between flocks largely unhindered.

In contrast, since 2004, the smarter use of better diagnostic tests, the availability of low risk replacements and the provision of social counselling and financial support to owners of beef cattle herds that are detected to be infected with bovine Johne's disease, have contributed to more successful control in that industry.

In the last decade, the Australian National Johne's Disease Control Program has moved towards emphasising voluntary risk assessment and management in the southern regions and livestock industries where paratuberculosis is endemic. The aim has been to educate producers and advisers to a point where they would change their behaviour to manage the risk that Johne's disease presented to their own business. For instance, the owner of a large beef breeding herd may stand to lose access to markets for bulls and heifers if the herd is infected. The program encourages such producers to manage their herds' biosecurity and only buy replacement cattle that are assigned a high level of assurance that the risk of infection with Johne's disease is low. In turn, other beef producers are encouraged to buy their replacements from herds that can also provide similar assurance, such as through the *Beef Only* declaration, thus educating the market and gradually building demand.

In the absence of obvious market drivers, it has been difficult to encourage producers to voluntarily protect their herds and flocks. Two risk assessment schemes were implemented in Australia in the mid-2000s: the *Assurance Based Credit (ABC) Scheme* for sheep (4) and the *National Dairy BJD Assurance Score* for dairy cattle (5). The former was based on a quantitative risk assessment model and described the risk score as a single number, with each additional point reflecting an approximate four-fold reduction in risk. Designed to simplify risk assessment and communication, it has largely failed as a tool for most farmers as there is little market demand, except where high scores were legislated for movements into and within regions that were trying to protect their flocks.

Within some infected regions, the ABC score was rarely used or used only to meet a low minimum movement standard. Promoting a minimum ABC score for movements not only confused the message of trying to prevent the spread of infection by buying high score replacement sheep but effectively encouraged the selling and buying of sheep that had a high probability of being infected. Abattoir surveillance, conducted under the national program, has demonstrated steadily rising flock prevalence in these regions, and vaccination has recently been increasingly used to reduce mortalities and production losses (Pfizer, personal communication, 2011).

In a heavily infected region of South Australia, however, producers, government and veterinarians have worked together to implement a program, with technical and financial assistance to help producers and neighbours control and prevent OJD. Recent surveillance has found many formerly infected flocks testing negative and fewer new infected flocks.

Business and Trade

Controlling animal diseases can both be driven by trade seeking assurance and be constrained by "business as usual" practices. The potential risk that Johne's disease presents to a business may not be appreciated as the impacts do not occur within the usual business time-horizon. For many farmers and in many countries, sales of livestock are key businesses and income earners. Where control programs are seen to be threatening "business as usual", strong opposition can be expected. Not surprisingly also, in conducting business, the seller will also generally favour his or her own selling potential, so unapparent faults, such as paratuberculosis infection, are unlikely to be declared to potential purchasers unless there are strong incentives, or other compliance pressures, within the trading environment.

Unless the socio-economic environment is also favourable, technical advances will have little opportunity to be effectively utilised in disease control. An illustration of the relative weight given to technology and to trade pressures in decision making has been the OIE's ongoing reluctance to agree on providing guidance to its members on paratuberculosis control in its Terrestrial Animal Health Code. In 2009, the OIE's Scientific Commission for Animal Diseases decided that "considering the lack of reliable diagnostic procedures for paratuberculosis, it would not be advisable to develop such a guidance document. The Commission was also not in favour of the OIE getting involved on the perceived public health issues if there was no clarity on reliable and recommended control measures and diagnostic procedures in

animals”(6). This effectively consigned paratuberculosis to remain a largely hidden ‘fault’ in livestock traded internationally. This may also affect domestic control programs. A trade protocol based on “no known infection” discourages producers from finding out the true disease status of their own herd or flocks; a serious constraint on disease prevention and control.

The Challenge

A real challenge for the IAP is changing people’s understanding, priorities and behaviour so that they want to use the accumulated knowledge and tools to implement sound and effective prevention and control of paratuberculosis at the various levels for which they have responsibility. Getting the biological science and tools right is one aspect of the challenge, but possibly more important for success is to better understand people’s priorities and needs so as to influence them to move away from the status quo and to adopt new business models that include better prevention and control.

This colloquium features some interesting work on human behaviour and the socio-economic aspects of paratuberculosis control. Successful control of Johne’s disease may well depend on people who have complementary expertise in social sciences actively engaging in control programs and sharing their experience and knowledge with the Association at future colloquia.

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CONTROLLING PARATUBERCULOSIS IN UK DAIRY HERDS USING MILK ELISA TESTS AND RISK MANAGEMENT

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Abstract

This paper describes a voluntary Johne's Disease (*Paratuberculosis*) engagement program in the UK, adopted in over 2000 dairy herds from 2008 and 2011, using a standardised web based risk-management program (www.myhealthyherd.com) and milk ELISA testing with the aim of encouraging farmers to determine the risks and disease status for the herd. A Johne's disease module was created which assessed the risk of disease entry & disease spread, predicted prevalence and allowed the development of farm specific strategies for control, biosecurity and biocontainment of the disease. Electronic storage of laboratory test results and a traffic light scoring system for disease risk and status allowed farmers to clearly understand their risks and the likely future prevalence of Johne's disease if these risks were not managed effectively on their farm. Farmers received a consistent education program delivered through milk processors using trained consultant veterinarians. The engagement program has enabled more effective veterinary led control plans to be applied in specific regions of the UK utilising available funding routes for farmer training. The aim is to expand to national, commercially driven control programme with the aim of reducing the economic impact and risk of spread of Johne's disease in the UK dairy herd.

Introduction

The rising incidence of Johne's disease prompted by increases in the risks of transmission between and within herds (herd size, expansion, labour shortages) and the development of practical tools to manage the disease has facilitated a new approach to Johne's disease in the UK.

MATERIALS & METHODS

Health Planning Tools

A specific Johne's disease (*Paratuberculosis*) module was created in MyHealthyHerd.com which assessed the risk of Johne's disease entry & spread. These risks were based on work by Rossiter et al (1998) and Soren Nielsen (personal communication). The module was used to gather information on the biosecurity and biocontainment risks for Johne's disease in each herd. The vet or farmer could enter data onto the program and each answer was then evaluated by applying both a weighting and a score to the answer. The sum total of the risk score was grouped using a traffic light system and given a red, amber or green icon. This allowed a structured prediction to be displayed for the likelihood of disease introduction and spread. This information was then used to help determine the most appropriate biosecurity, surveillance and control plan for the herd.

Seven control strategies were created to suit all types of farmer aspiration, herd risk and prevalence (improved farm management, improved farm management and single test, test and cull, vaccination, Danish 'style' risk-based control, breed to beef (with purchase of low risk replacements) or biosecurity protect and monitor).

The farmers were encouraged to undertake a 30 cow milk ELISA surveillance screen from cows 4-8 years of age who may be of higher risk of paratuberculosis (exhibiting weight loss, scour, high cell count, below average production or lameness).

Results from screening were entered into the program to provide an estimation of the true herd prevalence based on test prevalence. This was an important step in engaging the farmer with the control program.

The Myhealthyherd program used a combination of true herd prevalence and introduction or spread risks to generate a future prevalence prediction. Depending on prevalence and risk of entry and spread, vets were able to create a bespoke control plan for the herd by selecting the most applicable strategy and

then by selecting the tasks required to make a robust plan. This generated a consistent approach by the vets.

Future herd prevalence was illustrated as a graph showing the likely change if the risks remained unchecked. Further improvements allowed each element of the control plan to be scored for robustness. The plan was divided into critical control areas (pen hygiene, manure management, calf separation etc) and if insufficient control points were selected this would score the area and the plan as a red or amber. Further guidance was included to recommend how the plan could be strengthened to generate a plan which is more likely to be effective and helps reduce the risks of creating plans which are destined to fail to block transmission of Johne's disease.

Education

Veterinary practitioners participating in the regional control schemes were required to attend a Johne's training day to update them on Johne's disease and its control, along with training on how to use the Myhealthyherd program. Over 360 vets were trained through this route or through other voluntary training programmes. Farmer education was also a critical aspect of the control programme. The farmer meetings were jointly sponsored by the farmers' milk processor and DairyCo (statutory levy board for the dairy industry). Risk assessments were undertaken at the meeting and entered into myhealthyherd by National Milk Records staff, the vet or the farmer. A total of 80 farmer meetings were held throughout the country through the milk processors and a smaller number at a more local veterinary practice level. The methodology and approach was also integrated into regional control programs within the South and North West of England again using standardised presentations and approaches.

Discussion

The most popular control strategy adopted by UK dairy herds was the Risk Based Control strategy (Nielsen 2007). This involved quarterly testing of all milking cows and dividing the herd into high and low risks groups according to test results. Effective separation of cows at calving allowed for a more practical control option to be applied. This low cost option for management provides options for larger UK herds unable to adopt the traditional improved farm management approaches or to go down the test and cull route.

Conclusion

The success of this industry driven voluntary control program has been due to the adoption of the milk ELISA test as the standard approach for surveillance and in the majority of cases control within infected herds. Creating a structured approach based on best science and incorporating this within a "cloud based" relational database has allowed a methodology based on "ask rather than tell" and avoids the didactic approach often provided by compulsory control schemes. This provides an inclusive framework for managing the disease in increasingly complex and varied farming systems.

Acknowledgements

The support of Dairy UK, Dairy Co, BCVA, NMR & CIS is central to the success of the program having facilitated a consistency of approach to the Johne's programme thus far.

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CERTIFICATION PROGRAMME TO REDUCE RISK OF BETWEEN-HERD TRANSMISSION OF PARATUBERCULOSIS IN THE DANISH DAIRY INDUSTRY

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Introduction

Paratuberculosis has likely been present in Denmark since the 1880'ies (Bang, 1909). However, reliable historical prevalence estimates are not available, partly because of poor diagnostic tests, reporting has been based on clinical disease rather than infection, and farmers have been unwilling to inform the true infection status of herds and animals.

Early control efforts were based on culture-based testing and, to some extent, use of vaccination. Vaccination could only be used if permission had been obtained from the veterinary authorities. To achieve permission, a farmer had to supplement with changes in management to reduce transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Vaccination was banned from 1 January 2008. Culture-based testing was also used to some extent in the 1970'ies and 1980'ies (Flensburg and Munk, 1980). However, because culture-based methods were time-consuming and considered expensive, they were never implemented on a larger scale. Testing based on culture should always be supplemented with changes in management, if the programmes should be subsidised from the cattle health insurance schemes. Due to the major costs associated with testing and lack of success, subsidised programmes were abandoned in 2005.

During the 1990'ies, limited efforts were done to control paratuberculosis. However, research projects from 1999 and onwards led to an increased awareness of infection status in many herds, along with novel ways of testing and management of MAP infections. During this period of time, stigmatisation associated with MAP infections appeared to decrease significantly in the country. Consequently, farmers demanded the initiation of a voluntary programme, which was implemented in 2006 (Nielsen et al., 2007). Participation in this programme was by mid 2011 ~ 29% of Danish dairy herds and 40% of dairy cows. Average herd size in herds participating in the control programme was higher (~170 cows) than the average dairy herd (~150 cows).

Within individual herds, the programme is largely based on separation of cows in the herd into groups with different potential for infectiousness. Cows are divided into risk groups based on quarterly examined milk samples taken from all lactating cows in the herds and examined for antibodies (milk ELISA). Implementation of different management procedures that focus upon reducing risk of transmission from cows with a known high infectiousness to calves and young stock is based on risk assessments in the individual herds.

The programme thus had a major focus on within-herd transmission, whereas the only recommendation on between-herd transmission was to avoid purchase of livestock.

IDENTIFIED FARMERS' NEEDS

A survey carried out in 2009 suggested that reasons for participation varied (Nielsen, 2011). The 1,013 responding farmers specified the following reasons for participation: Control to increase animal health (91%); certify "freedom of MAP-infection" within 4-10 years (87%); control to avoid production losses associated with MAP infections (86%); control to increase consumer safety (64%); certification for sale of livestock (58%); control following production losses (48%). Weighting of responses were not included, so the relative importance of the different reasons could not be assessed. However, the majority of farmers indicated that animal health and potential reduction in production losses were the most frequent reasons, and a number of farmers would be keen to also have a certification programme added to the control scheme. The variation in responses emphasise that many farmers may have different purposes of participation, and these differences needs to be captured by the programme as well as by the herd health advisors.

Furthermore, the results from this survey suggested that many farmers expected that they could participate in a certification programme within the near future. Hence a certification scheme was implemented in August 2011 and has currently approximately 100 herds included. An initial requirement

was that the farms should have been part of the control programme, but from November 2011 all herds can sign up. To obtain a certification status, it is a requirement that minimum 75% of the animals has been tested within the last 12 months. Based on the test-results, the age-distribution in the herd, the specificity and age-specific sensitivities for the milk-ELISA, the following parameters are estimated:

- the apparent prevalence, which is used for most categorisations;
- the true prevalence, which is the apparent prevalence corrected for the herd's age distribution as well as test specificity and age-specific sensitivity;
- the probability that the herd is "free of MAP infection", or more specifically has a lower prevalence than the Danish dairy herd. This estimation follows the principles described in Sergeant et al. (2008), although different parameter estimates for the tests are used due to a change in test;
- the apparent prevalence in all herds, from which livestock has been purchased.

Subsequent to estimation of these parameters, the herd is categorised into one of 10 categories as specified in Figure 1.

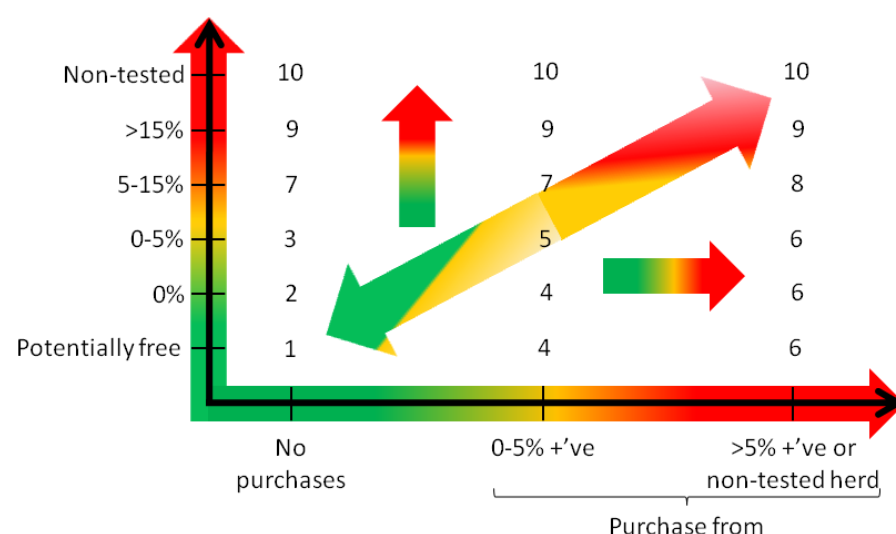


Figure 1. Matrix for categorisation of herds based on annual test results using milk antibody ELISA. Herds are classified based on their own test-prevalence (y-axis) and the prevalence in herds from which they have purchased livestock. To be classified "potentially free", the herd should have no purchased animals in the herd and have a probability of "being free of infection" > 0.95 and an estimated true prevalence of <0.5%.

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COMPARISON OF PARTICIPANTS AND NON-PARTICIPANTS IN A VOLUNTARY JOHNE'S DISEASE CONTROL PROGRAM IN ONTARIO, CANADA

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Introduction

A voluntary risk assessment based Johne's Disease control program for dairy cattle herds was launched in Ontario, Canada in January 2010 (see www.johnes.ca for details). The Ontario Johne's Education and Management Assistance Program (OJEMAP) is funded primarily by dairy producers through a one-time allocation of \$2.1 million by the Dairy Farmers of Ontario. The program has four elements – education of producers, consumers and veterinarians, an Animal Health Risk Assessment and Management Plan (the "RAMP"), optional whole herd testing, and removal of high titre Johne's cows within 90 days of the test. The program offers each producer a financial incentive to test all lactating cows once during the four years of the program. Pending a regulatory change in the Ontario Milk Act, dairy producers will be required to do the RAMP with their trained herd veterinarian each year. The RAMP is a questionnaire that guides the veterinarian and the producer systematically through the calf raising and general sanitation practices on the farm. The goal is to evaluate the farm for risks that could allow *Mycobacterium avium* spp. *paratuberculosis* (MAP) to infect calves on the farm. After completing the questionnaire, the producer and veterinarian decide what can and will be done to decrease transmission risk in the next year. This is the "management plan", and if implemented should not only control Johne's Disease, but will likely reduce new infections caused by other pathogens as well. Testing is optional, but given the number of dairy herds in the province and the limited capacity of the participating laboratories, a testing schedule was produced for the province to allow each producer one opportunity in a 6-week window to complete a whole herd Johne's test. The schedule was created using county divisions and townships within larger counties to form 29 testing windows. The whole herd Johne's testing can be done with the milk ELISA through CanWest Dairy Herd Improvement (DHI) or the serum ELISA or fecal culture/PCR offered by the Animal Health Laboratory (AHL) at the University of Guelph.

To receive the reimbursement of \$8 per cow tested producers in the program must test all lactating animals in the herd on one day during their testing window, complete the RAMP with their herd veterinarian within 90 days of the herd test, and permanently remove all high-titre cows (HTC) [test results of 1.0 or higher on the milk ELISA (Parachek, Prionics®, Zurich, Switzerland) or the serum ELISA (IDEXX MAP Ab Test, IDEXX Laboratories, Maine, USA)], not to another dairy herd or to the food chain, within 90 days of the testing date.

Results from Year 1 (2010) of the Program

Of 1,000 herds eligible to participate in the program in 2010, 700 (70%) elected to complete all three components; the RAMP, herd test and removal of HTC's (*participants*). Demographic, milk quality, milk production, reproductive performance and herd management data collected by the Dairy Farmers of Ontario and CanWest DHI in 2010 were used to compare *participants* to *non-participants* in an attempt to understand the characteristics of *non-participants*, with the hope that this would help in the development of strategies to recruit these herds and thus to increase program participation.

Twenty-three herds tested the lactating herd using the serum ELISA, but most of these herds (97%) chose to complete the whole herd testing using the milk test. In the *participant* herds, of the nearly 50,000 cows tested, 453 (0.9%) of cows tested positive with a milk or serum Johne's ELISA test, and 25% of herds had at least one test positive cow. Just under 0.1% of cows were HTC's.

Of the 1000 eligible herds, 864 were enrolled in milk recording through CanWest DHI and had detailed health and production data available. Milk quality data from bulk tank testing and premise inspections

were available for all 1000 farms. Differences between *participants* and *non-participants* were tested using t-tests for normally distributed continuous outcomes, chi-square tests for dichotomous outcomes and appropriate non-parametric methods where needed, using $p < 0.05$ to determine statistical significance. Herd performance variables found to be significantly different between the two groups are presented in Tables 1 and 2. *Participant* herds were significantly larger (mean of 83 milking and dry cows versus 73), produced more milk per cow (\$5,962 milk value versus \$5,567), produced higher quality milk (lower bulk tank average somatic cell counts in both the cold winter months as represented by the March monthly average and the warm summer months as represented by the August average), kept better health records and were better managed (lower age at first calving, higher pregnancy rate and overall higher herd management score) than *non-participant* herds.

Table 1. Data from 864 DHI Herds Eligible to Participate in the Program in 2010

<u>Statistically Significant Variables ($p < 0.05$)</u>	<u>Participants</u>	<u>Non-Participants</u>
Age at First Calving (Months)	27.5	27.9
Number of Cows (Milking and Dry)	83	73
DHI Herd Management Score in 2010	542	473
Percent that Kept Cow Health Records	58%	43%
Milk produced per cow (\$ Milk Value)	\$5,962	\$5,567
Pregnancy Rate in Cows	13.4%	12.4%

Table 2. Data from ALL Herds Eligible to Participate in the Program in 2010

<u>Statistically Significant Variables ($p < 0.05$)</u>	<u>Participants</u>	<u>Non-Participants</u>
March 2010 Monthly Average BTSCC	218	248
August 2010 Monthly Average BTSCC	308	329

Based on expectations when the program was being developed, the herd and cow prevalence of test positive and high positive animals were lower than projected. When combined with the evidence that the *participant* herds were better than *non-participants* in production and management, there is concern that the *non-participants* include herds more likely to have Johne's Disease. If that is the case, then any estimates of JD prevalence in the province based on program data will be underestimates. There is a need to target these herds to increase the likelihood of their participation in the program.

A survey of *non-participants* was initiated through the CanWest DHI field staff to determine the reasons for not participating in the program. The most common reasons cited, in order of decreasing frequency, were: 1) fear of having to dispose of good cows (if they were found to be HTC's), 2) didn't think they had JD in their herd, 3) didn't want to pay the veterinarian to conduct the RAMP, 4) as farm policy they didn't participate in any formal management programs, 5) didn't have a herd veterinarian who would conduct the RAMP and 6) were planning to sell the herd within the next 2 years. While some of the issues cited by the non-participants are not easily resolved, plans are being developed to address some of these concerns with the hope that participation in the program will remain strong and perhaps increase.

OVINE JOHNE'S DISEASE – HISTOPATHOLOGICAL FINDINGS FOR 13,143 SUSPECT LESION FROM ABATTOIR MONITORING IN NSW AUSTRALIA FROM 1999-2009

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BACKGROUND

Mycobacterium avium subsp. *paratuberculosis* (MAP) “sheep strain” is the cause of ovine Johne's disease (OJD). OJD was first detected in New South Wales (NSW), Australia in 1980 and subsequently became prevalent in higher rainfall sheep-raising areas of NSW (Central & Southern Slopes). Histopathological changes in the lower small intestine and mesenteric lymph-nodes in OJD present in two forms – 1. “multibacillary” – typical granulomatous response with epithelioid cells, giant cells and numerous acid fast organisms (AFOs) or 2. “paucibacillary” – with similar granulomatous response but no AFOs evident on routine examination of Ziehl-Neelsen (ZN) stained sections. Granulomatous enteritis resembling paucibacillary OJD can also occur due to other causes. Abattoir monitoring for OJD was conducted in NSW in 1999-2009 as part of a national program and the occurrence of paucibacillary and multibacillary OJD was determined.

METHODS

The data was stratified based on areas of “High”, “Medium” and “Low” OJD prevalence (HPA, MPA and LPA respectively) implemented on 31st March 2008. Trained inspectors evaluated carcasses of adult sheep (at least 2 years old) for gross evidence of OJD in the target site of lower small intestine. Suspect tissue (generally a single section of terminal ileum) was subjected to histopathological examination and classified as a) OJD positive (“multibacillary” - granulomatous inflammation with AFOs) b) OJD suggestive/ inconclusive (typical granulomatous pathology with no AFOs) or c) OJD negative (absence of granulomatous inflammatory reaction). Lesions with suggestive histopathology were attributed to OJD (paucibacillary form) and included in the analysis if one or more lesions from that consignment were multibacillary or if the flock had a prior history of OJD or was confirmed OJD positive within the subsequent 2 years.

PRINCIPAL FINDINGS: A total of 7.6 million sheep carcasses from 32,032 consignments were examined – 5,109 consignments were confirmed positive for OJD and 26,923 negative. Of the 13,143 lesions submitted for histopathology and included in the analysis, 10,028 samples were attributed to OJD - 8,291 (83%) multibacillary and 1,737 (17%) paucibacillary. There were 3,115 samples negative for OJD on histopathology (2,305 from negative and 810 from positive consignments). In the HPA, the proportion of total samples attributable to OJD (multibacillary plus paucibacillary) was 82% (9,570/11,687), in the MPA 63% (271/428) and in the LPA 18% (87/1,028) – an average of 76% for NSW.

COMMENT

The uncertainty of attribution of typical granulomatous lesions to OJD in the absence of AFOs has major implications in disease control programs. This is particularly so in the LPA where suspicion aroused by an inconclusive result can have major financial implications for the suspect flock. It also creates great uncertainty about the merits of a disease control program in the minds of producers. Examination of serial ZN sections from ileum (and lymph nodes) has been found to increase the sensitivity of detection of AFOs in paucibacillary cases. In a preliminary study 8/9 paucibacillary culture positive animals and 17/30 sections revealed AFOs on detailed examination of 3 sections per lesion (S Hum – unpublished observations). In the current program extra sections of intestine and mesenteric lymph node are generally examined from paucibacillary cases from the LPA, but this is not cost effective for the HPA or MPA. From a comparative pathology perspective, paucibacillary OJD has many parallels with that of Crohn's Disease in man – typical granulomatous pathology in some cases associated with positive culture and/or PCR for MAP.

CONCLUSION

Paucibacillary pathology comprised 17% of lesions attributable to OJD. Inspectors demonstrated high specificity in sampling lesions with 82% of samples attributable to OJD (multibacillary plus paucibacillary) in the HPA. The specificity was lower in the LPA due to intensified sampling of intestines showing any signs of abnormality. Abattoir samples also provide a potentially low cost model for the study of host genetic and immunological factors and bacterial pathogenesis in animal and human mycobacterial infection including Crohn's Disease.

Table 1. Number and Percentage of Intestinal Lesions with Paucibacillary or Multibacillary Histopathology attributable to OJD - 32,032 abattoir consignments - 1999-2009

	HPA	MPA	LPA	Total
Negative	2117	157	841	3115
Paucibacillary OJD	1657 (17%)	56 (21%)	24 (13%)	1737 (17%)
Multibacillary OJD	7913 (83%)	215 (79%)	163 (87%)	8291 (83%)
Total Samples	11687	428	1028	13143

Figures A-F – terminal ileum: A) Paucibacillary - showing granulomatous enteritis (H&E); B) Paucibacillary – no AFOs evident (ZN); C) Paucibacillary – high power, note ground glass appearance of epithelioid cells; D) Multibacillary – small numbers of AFOs (ZN); E & F) Multibacillary – very large numbers of AFOs – (Photographs not shown)

Acknowledgements

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia, and managed through Animal Health Australia and NSW DPI. The project could not have proceeded without the commitment and diligence of the OJD inspectors. The support of abattoir management and AQIS was essential. The contribution from the data entry team at Wagga (Tracey Kingham, James Hamilton & Jenny Fury) and Orange, and pathologists at RVL Orange and EMAI Menangle is gratefully acknowledged.

REDUCTION IN INCIDENCE OF PARATUBERCULOSIS INFECTION IN MINNESOTA JOHNE'S DISEASE DEMONSTRATION DAIRY HERDS

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Certain management practices have been recommended to minimize transmission of paratuberculosis between infected and susceptible cattle. The objective of this study was to evaluate the risk of testing positive and its association with changes in management practices recommended by Johne's disease control program in different birth cohorts. Eight dairy herds were enrolled in the Minnesota Johne's Disease Demonstration Herd Program. Herds were monitored for a period between 5 to 10 years. Annual testing for *Mycobacterium avium* subsp. *paratuberculosis* was performed for all cows (age >24 months) that calved, using bacterial culture and serum ELISA. Risk assessments were performed annually to measure the level of implementation of the recommended management practices. Eight birth cohorts were defined based on the date of cow enrollment in the program. Birth cohorts -2 and -1 corresponded to cows that were born 2 and 1 years before the beginning of the program, respectively, and cohorts 0 to 5 corresponded to cows that were born 0 to 5 years after the beginning to the program. The annual risk assessment score was used to quantify the level of exposure by birth cohort and herd. A time-dependent Cox's regression model was used to model the time to test positive, explained by herd, birth cohort and birth cohort exposure level. Compared to birth cohort -2, there was a reduction of the hazard ratio (95% CI) of bacterial culture positivity of 0.97 (0.70 to 1.35), 0.81 (0.57 to 1.15), 1.11 (0.80 to 1.53), 0.64 (0.42 to 0.97), 0.47 (0.30 to 0.75), 0.44 (0.28 to 0.69), and 0.36 (0.22 to 0.60), for birth cohorts -1, 0, 1, 2, 3, 4, and 5, respectively. Similarly, compared to birth cohort -2, there was a reduction of the hazard ratio of serum ELISA positivity of 0.91 (0.66 to 1.24), 0.71 (0.50 to 1.00), 0.65 (0.45 to 0.93), 0.42 (0.27 to 0.66), 0.24 (0.15 to 0.40), 0.11 (0.06 to 0.20) and 0.04 (0.02 to 0.09) for birth cohorts -1, 0, 1, 2, 3, 4, and 5, respectively (Figure 1). The instantaneous hazard of testing positive for both tests increased with the level of exposure, however, the strength of this association decreased over time. There was a reduction in the incidence of Johne's disease associated with the level of implementation of the recommended management practices.

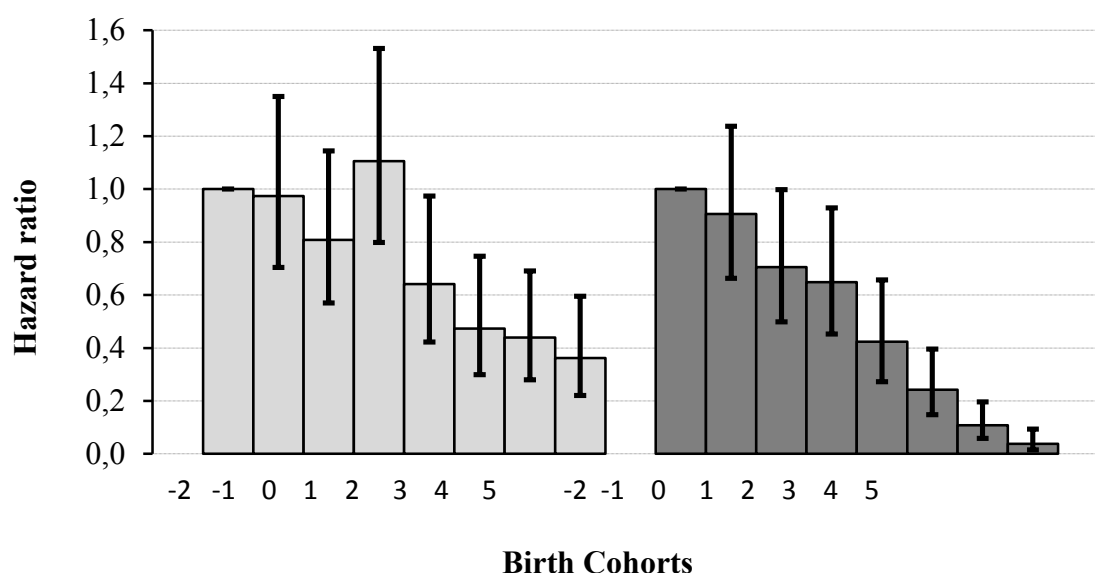


Figure 1. Hazard Ratios and 95% Confidence Intervals of test positive *Mycobacterium avium* subsp. *paratuberculosis* bacterial culture of feces (light grey) and serum ELISA (dark grey) across birth cohorts in 8 dairy farms under a disease control program in Minnesota.

COST-EFFECTIVENESS OF DIAGNOSTIC STRATEGIES TO IDENTIFY *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSI* SUPER-SHEDDER COWS IN A LARGE DAIRY HERD USING ANTIBODY ELISAS, QUANTITATIVE REAL-TIME PCR AND BACTERIAL CULTURE

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Diagnostic strategies to detect *Mycobacterium avium* subsp. *paratuberculosis* (MAP) super-shedder cows in dairy herds have been minimally studied. The objective of this study was to compare the cost-effectiveness of strategies for identification of MAP super-shedders on a California dairy herd of 3577 cows housed in free-stall pens. Eleven strategies that included serum or milk ELISA, quantitative real-time PCR (qPCR) or culture of environmental samples, pooled or individual cow fecal samples, or combinations there-of were compared. Nineteen super-shedders (0.5%) were identified by qPCR and confirmed by culture as cows shedding $\geq 10,000$ CFU/gram feces (median of 30,000 CFU/gram feces). A stratified random sample of the study herd based on qPCR results of fecal pools was the most sensitive (74%) strategy and had the highest cost (\$5,398/ super-shedder). The reference strategy with the lowest cost (\$1230/ super-shedder) and sensitivity (47%) included qPCR testing of fecal samples from ELISA-positive lactating (milk) and non-lactating (serum) cows housed in pens with the highest MAP bioburden. The most cost-effective alternative to the reference was to qPCR test fecal samples of seropositive cows contributing to qPCR positive fecal pools, housed in pens with the highest MAP bioburden. This strategy had a sensitivity of 53% and cost \$1643/ super-shedder. In conclusion, diagnostic strategies varied in their cost-effectiveness depending on the tests, specimen type and labor costs. Initial qPCR testing of environmental samples from freestall pens to target cows in pens with the highest MAP bioburden for further testing can improve the cost-effectiveness of strategies for super-shedder identification.

FIVE YEARS OF MILK QUALITY ASSURANCE FOR PARATUBERCULOSIS IN THE NETHERLANDS

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In 2006, a milk quality assurance program (MQAP) for paratuberculosis in Dutch dairy herds was initiated. The aim of the MQAP is to reduce the concentration of *Mycobacterium avium* subsp. *paratuberculosis* in milk delivered to the milk factories.

Herds participating in the MQAP are assigned a herd status based on the results of herd examinations by individual milk-ELISA. Farmers are entitled to confirm positive ELISA results by faecal culture. Test-negative herds are assigned status 'A'. Test-positive herds are assigned status 'B' (if all test-positive cattle have been removed from the herd) or status 'C' (if any test-positive cattle are retained in the herd). The MQAP promotes preventive management measures and culling of test-positive cattle to reduce the spread of Map. On the herd-level, these measures increase the probability to obtain and maintain status 'A'. On the national level, these measures taken by individual farmers increase the milk quality of the national dairy herd.

Based on the results achieved in the programme, the Dutch dairy industries are requiring all dairy herds delivering milk to their factories to have at least status 'A' or 'B' since January 2011.

The aim of this paper is to present results obtained over a five-year period in the first cohort of herds that entered the MQAP in 2006 - 2007, and to provide a road map to nation-wide participation in paratuberculosis programmes.

FIELD EFFICACY OF SILIRUM™ VACCINE IN TWO AUSTRALIAN DAIRY CATTLE HERDS

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A randomised, controlled, blinded clinical trial commenced in June 2005 to assess the efficacy of Silirum® Vaccine in two seasonal-calving dairy herds endemically infected with *Mycobacterium avium* subsp *paratuberculosis* (MAP), the causative organism of Bovine Johne's Disease (BJD). The herds are located in the Macalister Irrigation District, Gippsland, Victoria. The study is co-sponsored by Pfizer Animal Health and the Victorian Department of Primary Industries. The in-life phase of the study is planned to continue until at least July 2012 (i.e. 7+ years in total). This interim summary covers data generated during the first five years of the study for animals enrolled during 2005-2008.

All classes of female cattle in the two herds (adult cows, 2 year-old heifers, yearling heifers, autumn-born calves and newborn calves) were enrolled in the study at its commencement in 2005, and these animals were randomly allocated to an unvaccinated control group (group NTX) and a Silirum-vaccinated group (group T01). In the four subsequent years (2006-2009) newborn female calves were also enrolled in the study and randomly allocated to one of the two groups. A total of 1351 animals were enrolled in 2005 (675 controls, 676 vaccinates), and a further 1009 newborn calves were enrolled between 2006 and 2009 (502 controls, 507 vaccinates).

The response to vaccination with Silirum Vaccine has been assessed by several measures including humoral and cell-mediated immune responses, faecal shedding of MAP organisms (faecal culture), clinical cases of BJD, histopathology of necropsy specimens, and milk production. Blood and faecal samples have been (and continue to be) collected twice a year from animals >15 months of age. In this interim report, the number of animals removed (died/culled/sold), clinical cases of BJD, and MAP culture results from faecal samples up to the 5-year study time point (July 2010) have been summarised. As the study is still in progress, and most of the data are cumulative in nature, statistical comparisons between the experimental groups have not yet been conducted. Baseline prevalence data (test positive) for the two herds are shown in Table 1:

Table 1. Summary of faecal culture positive and ELISA antibody positive animals at study commencement

Test	Age group	Herd 1	Herd 2	Total
Faecal culture MAP positive	Adult cows (≥ 3 yo)	55/458 (12.0%)	14/269 (5.2%)	69/727 (9.5%)
	2 year-old heifers	14/143 (9.8%)	0/74 (0%)	14/217 (6.5%)
Parachek ELISA antibody positive	Adult cows (≥ 3 yo)	16/458 (3.5%)	5/269 (1.9%)	21/727 (2.9%)
	2 year-old heifers	0/143 (0%)	0/74 (0%)	0/217 (0%)

In all age groups vaccinated in the first year of this study, including newborn calves vaccinated at 3-6 weeks of age, administration of a single dose of Silirum Vaccine induced a cell-mediated immune response, as measured by the mean γ -IFN response to both avian tuberculin PPD and Johnin PPD. Vaccination also induced a humoral (antibody-mediated) immune response in all age groups except newborn calves.

Approximately 50% of animals enrolled between 2005 and 2008 were removed from the study (died/culled/sold) during its first five years. For each age group, the proportion of animals removed was similar between vaccinates and controls. Overall, 42 animals were culled from both herds due to clinical signs of BJD; 26 controls and 16 vaccinates. These clinical cases were from animals enrolled in the study as adult cows, or 1-2 year-old heifers, and therefore were likely to have been infected prior to vaccination. At this stage of the study, no animal enrolled as a newborn calf, either in 2005 or subsequent years, has been culled due to clinical signs of Johne's Disease.

Post-mortem specimens of the gastrointestinal tract from 269 animals were submitted for histopathology and/or tissue culture. A total of 101 animals were classified as positive or equivocal for Johne's Disease. Forty-nine of these animals were definitively positive on histopathology; 27 controls and 22 vaccinates. Of the 49 animals with positive histopathology, seven were enrolled in the study at the age of two years or below; 5 controls and 2 vaccinates.

At the 5-year study time point, there was a lower proportion of animals with MAP-positive faecal cultures in vaccinates compared to controls, across all age groups, as shown in Table 2 (both herds combined).

Table 2. Summary of animals with MAP-positive faecal culture at the 5-year study time point

Age group at enrolment	Negative control group (NTX)		Silirum-vaccinated group (T01)	
	Rate	Percentage (%)	Rate	Percentage (%)
Adult cows (2005)	4/39	10.3	1/30	3.3
1-2 yo heifers (2005)	11/90	12.2	5/88	5.7
Newborn calves (2005-08)	27/365	7.4	5/372	1.3

A comparison of faecal culture data (ante mortem) and histopathology data (post mortem) indicated strong agreement between animals with at least two MAP-positive cultures and a subsequent positive histopathology classification. The proportion of animals with at least two MAP-positive faecal cultures during the first five years of the study was lower in vaccinates compared to controls for all age groups except adult cows (where the proportions were similar), as shown in Table 3 (both herds combined).

Table 3. Summary of animals with two or more MAP-positive faecal cultures during first 5 years of the study

Age group at enrolment	Negative control group (NTX)		Silirum-vaccinated group (T01)	
	Rate	Percentage (%)	Rate	Percentage (%)
Adult cows (2005)	39/313	12.5	41/315	13.0
1-2 yo heifers (2005)	25/182	13.7	6/180	3.3
Newborn calves (2005-08)	10/417	2.4	4/427	0.9

Based on the faecal culture results at the 5-year time point (Table 2), it is expected that the number of calves in the unvaccinated control group with two or more MAP-positive cultures will increase over the next 1-2 years of the study.

A proportion of vaccinated animals developed a firm, subcutaneous nodule at the site of vaccination. Of 60 vaccinates (excluding newborn calves) selected for injection site assessment, 30 (50%) had a visible nodule 6 months post vaccination; of 46 animals still available for assessment at 12 months post vaccination, 13 (28%) had a visible nodule remaining. Newborn calves had a lower rate of visible reactions, which tended to dissipate more quickly, compared to the older animals. Most injection site reactions recorded in newborn calves peaked in size at around six weeks post-vaccination, and the proportion of newborn calves with visible reactions had fallen to 14% by 7 months post vaccination.

Vaccination with Silirum may result in some animals developing a positive response to the caudal fold tuberculin test, one of the recommended screening tests for bovine tuberculosis (TB). Data generated in this study indicate that animals vaccinated with Silirum Vaccine are easily identified as bovine TB-free using the comparative cervical tuberculin test, whereby avian tuberculin produces an invariably stronger skin reaction compared with that produced by bovine tuberculin.

In conclusion, the 5-year interim results of this randomised, controlled clinical trial show very encouraging trends in terms of the immune response to vaccination, a reduction in clinical cases of BJD, and a reduction in the number of animals shedding MAP in faeces. A comprehensive analysis of the data, including statistical comparisons between groups, will be conducted at the conclusion of the study.

Acknowledgement

The authors would like to acknowledge and thank the following colleagues and organisations for their support: E. Abadin and E. Lopez (Pfizer Animal Health); C. Bell (Victorian DPI); J. Malmo and G. Davis (Maffra Veterinary Centre); T. Jubb; Meat and Livestock Australia; Gribbles Veterinary Pathology; CZ Veterinaria; and the herd owners and managers.

USE OF A NATIONAL JOHNES SERUM SURVEY TO HELP DESIGN A LEAST COST SURVEILLANCE PROGRAMME

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A serum prevalence survey was carried out in Ireland in 2009 on 1654 herds picked randomly from all the herds in the country, which were subject to an annual brucellosis test. All 78,123 females and breeding bulls over 24 months-of-age in these herds were tested using a ID vet ELISA test. There were 286 positive herds and 91 herds had two or more positives. The overall prevalence of infected herds, based on the presence of at least one ELISA-positive animal, was 17.41%. The herd prevalence level amongst dairy herds (27.76%) was higher than among beef herds (12.7%). The animal level prevalence for all breeds was 0.60% but some breeds had a higher prevalence than this. Only 5.5% of all herds had more than one ELISA-positive infected animal. The herd corrected seroprevalence excluding herds with only one positive result was 5.50%, dairy herds were 9.7% and beef herds 3.6%.

Animal Health Ireland had been asked for advise by several milk producers setting up control programmes that wanted to screen herds using a sub sample of the total herd and use bulk milk tank testing. Using data from the present survey it was determined that if a sub sample of eight of the oldest animals in the herd were selected 64.58% of the positive herds would have been missed.

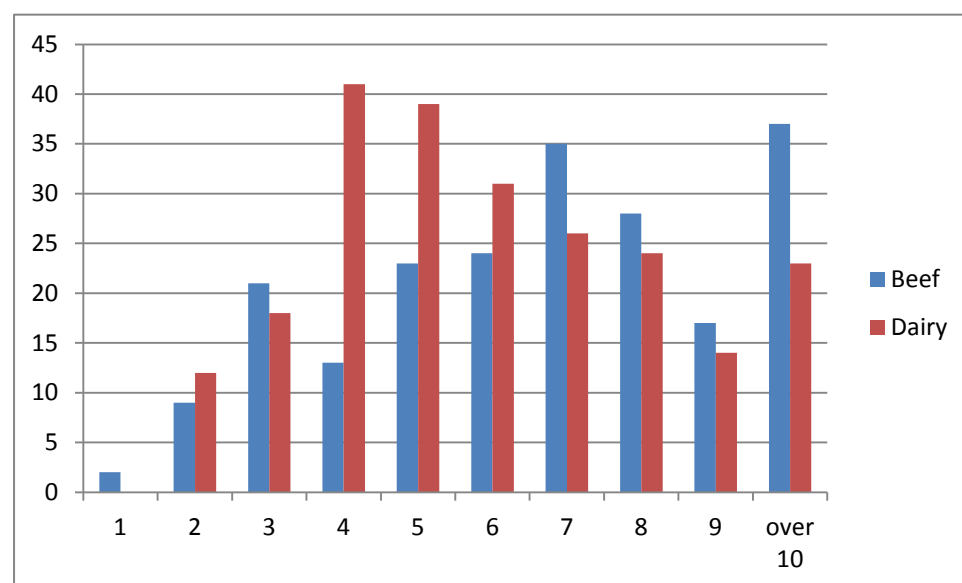


Figure 1: Number of positive animals by age in years of dairy and beef herds.

There were one hundred and twenty seven dairy herds with positive animals. From Figure 1 it can be seen that the age group with most positive animals is three to five years of age. Fifty-five herds would be missed if all animals in this age group were sampled as the positive animals in these herds were older or younger than the selected target group. When the subset sampling was applied to this group rather than the oldest animals in the herd and animals were picked at random, if thirty animals were sampled in the herd seventy-one herds would be missed including the fifty five herds with no positive animals in this age group. There were fifty-seven herds with thirty or less animals in this group so these animals did not need to be picked randomly. If results were only taken from twenty animals in the herd eighty-four herds would be missed including the fifty-five herds with no positive animals in this age group. There were thirty-six herds with twenty or less animals in this group so these animals did not need to be picked randomly. If results were only taken from ten animals in the herd one hundred and four herds would be

missed including the fifty-five herds with no positive animals in this age group. There were five herds with ten or less animals in this group so these animals did not need to be picked randomly.

Following the analysis of the data from the prevalence survey one cooperative, with an existing programme, decided to modify and extend it to whole herd individual testing (over two years of age), risk assessment based on the publication “How to Do Risk Assessments and Develop Management Plans for Johne’s Disease” prepared by the United States Animal Health Association, Johne’s Disease Committee. Programme participants are also given calf rearing and biocontainment advice delivered by the herds’ veterinary practitioner. The programme will be aligned with the national programme as the latter is rolled out. This control programme would be partially subsidised by the cooperative and available to all their suppliers.

OUTCOMES FROM THE VICTORIAN TCP2 (2003-2010)

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Aim

To assess the records of a sample of dairy herds that enrolled in TCP2, the former bovine Johne's disease (BJD) control program administered by the Victorian Department of Primary Industries, and review their retention rates, progress in herd BJD status and seroprevalence at the end of the program.

Method

A random sample of 147 herds was selected from all herds that enrolled in TCP2 in 2003, weighted to reflect the proportion of dairy herds in each of the three major regions (Gippsland, Southwest and Northern).

Veterinary audit reports and laboratory test results for these herds were assessed for their content, completeness and accuracy. Herds were classified into seven categories according to their program outcomes in 2010:

- Tested to RD2* or MAP Standard* – at least 2 consecutive negative herd ELISA tests in 2003-2010
- Tested seronegative – one negative whole herd test in 2010
- Enrolled in TCP3 - tested positive in 2010 and transferred to TCP3
- Left TCP2 in 2010 – tested positive in 2010 but did not join TCP3
- Withdrawn – left the program prior to 2010 without achieving RD2 or tested to MAP standard
- Disbanded – sold or dispersed prior to 2010
- Expelled – removed from TCP2 due to serious non-compliance

*As defined in the National Dairy BJD Assurance scheme

Changes in the BJD herd status were divided into 3 categories (Progressed, No change, Regressed). Herd seroprevalence at the first and last TCP2 whole herd ELISA test was compared using the paired t-test.

Results

1. Review of ADMIS records and veterinary reports

Examination of individual herd records in TCP2 revealed some omissions in the ADMIS database. For example, the herd status in some instances had not been updated to reflect the most recent herd history and herd testing records were not always complete.

Veterinary audit reports in the database also varied in quality, particularly in their format and level of detail. Some were individually tailored to the enterprise, providing the farmer with detailed advice about calf management practices and identifying high risk animals, while others were pro-forma style reports which outlined generic strategies to manage BJD risk.

Reported compliance with the calf rearing practices recommended under the TCP2 program rules was excellent, with few recorded corrective actions for the JDCAP. However some herds continued to produce home-bred seropositive animals years after implementing hygienic calf rearing practices to minimise BJD transmission. Only two properties in the sample were expelled from the TCP2 for major non-compliances.

2. Outcomes for herds enrolled in TCP2 in 2003 Refer to Figure 1.

The most frequent outcome was that herds enrolled in 2003, remained in TCP2 until 2010 and then re-enrolled in its successor, TCP3. Northern herds had a higher rate of attrition from the program than those in the other regions. Southwest herds had the highest rate of enrolment in TCP3. Gippsland herds were overrepresented in testing to RD2 or MAP Std.

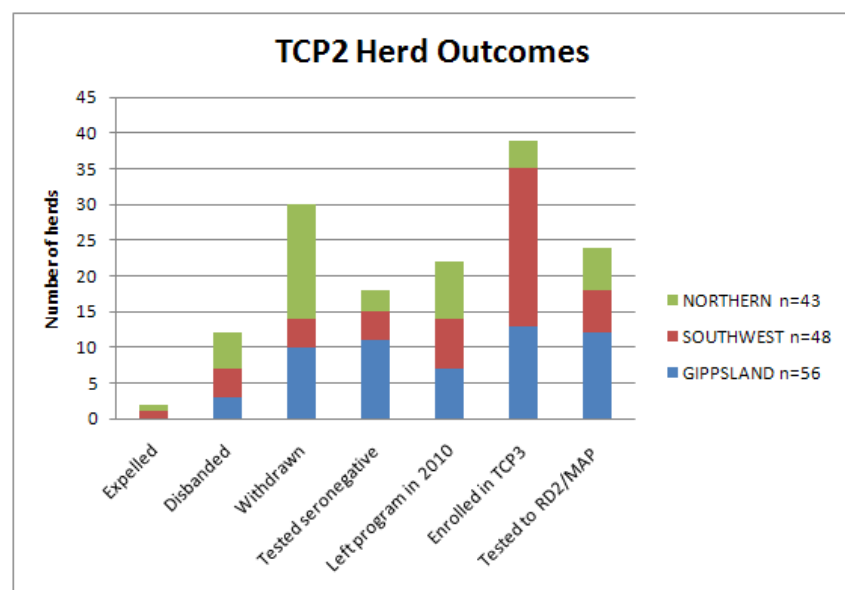


Figure 1. Herd outcomes by region for herds enrolled in TCP2 in 2003 (n=147)

3. Changes in herd BJD status 2003-2010

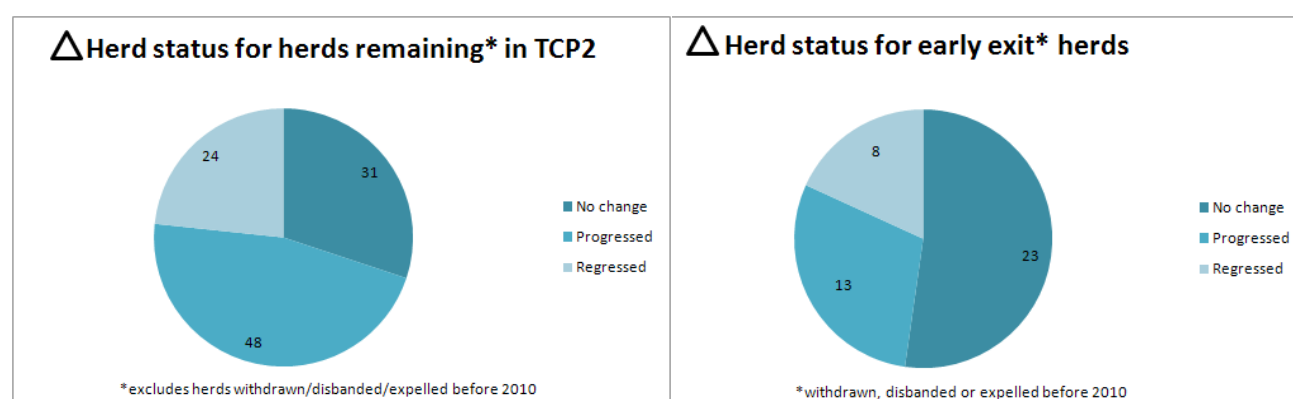


Figure 2. Change in herd status of herds remaining in TCP2 Figure 3. Change in herd status of early exiting herds

4. Effect on herd ELISA seroprevalence

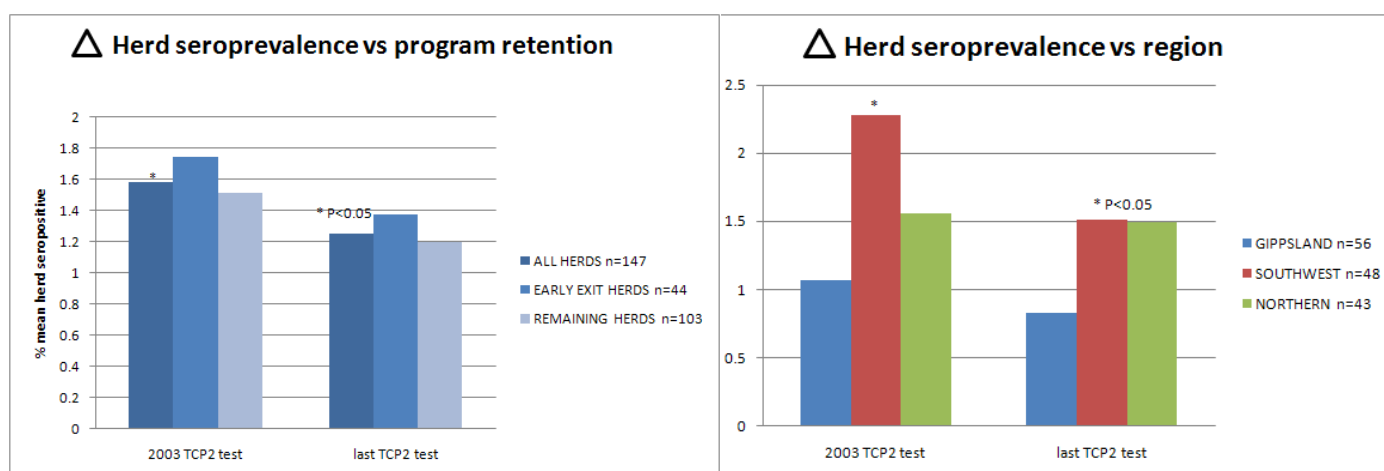


Figure 4. Herd seroprevalence vs program retention Figure 5. Effect of region on change in herd seroprevalence

The mean ELISA seropositive rate within herds enrolled in TCP2 in 2003 fell significantly during this period ($P < 0.05$) (Figure 4). When examined by region, herds in the Southwest region accounted for most of this improvement, which may be related to their higher initial herd seroprevalence rates (Figure 5).

Conclusions

- This review highlights the importance of consistent and accurate recording of data over the life of a BJD control program. Controlling the format and integrity of the information captured is particularly important when it comes to evaluating program outcomes.
- Some herds continued to produce seropositive animals many years after implementing management changes to minimise BJD transmission and having apparently complied with the program rules. Further review of the veterinary advice provided in these situations is warranted.
- It was evident that the standard of veterinary reports varied between practitioners. This is an important finding as BJD control programs often fail because they are not designed to meet the unique needs and capabilities of an individual farm. The recommended practices should be tailored to the individual property and presented to farmers in a way that is prioritised to allow for selective or progressive adoption.
- Attrition of herds from TCP2 was more often associated with lack of progression in herd status than regression in herd status. The lack of progress in these herds could be a reflection of the shorter time these herds spent in the program or may reflect reduced farmer commitment to BJD control when progress is not apparent.
- Herd progression, retention rates and changes in herd seroprevalence varied between regions.

EXPERIENCES AND RESULTS WITH PARATUBERCULOSIS CONTROL IN AUSTRIA

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Introduction

In 2006 paratuberculosis (PTB) in cattle, sheep, goat and farmed deer became a notifiable disease in Austria by government regulation. The district veterinarian has to be notified about animals showing clinical signs of PTB. Blood and faeces respectively lymph nodes and intestines in case of fallen stock or slaughtered animals have to be sent to the national reference laboratory (NRL) for diagnosis. Positive tested animals must be eliminated in combination with implementation of hygienic and management measures on the affected farms under control of the district veterinarian.

Material and Methods

Blood samples are tested by Enzyme Linked Immunosorbent Assay (ELISA) for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) specific antibodies, while faeces and tissue samples are tested by PCR for MAP specific DNA. In the period from 2006 to 2011 samples from 759 cattle (325 farms), 29 sheep (7 farms), 14 goats (7 farms) and 8 farmed deer (5 farms) were sent to the NRL for laboratory confirmation of clinically suspicious animals.

Results

The number of positive tested ruminants and farms are listed in table 1. The predominant breeds among positive tested animals were Limousin (105), Fleckvieh-Simmental (51) and Holstein Friesian cattle (46).

Tab. 1: MAP examinations 2006-2011

	Cattle	Sheep	Goat	Farmed deer
PTB suspicious ruminants	759	29	14	8
PTB suspicious farms	325	7	7	5
PTB positive tested ruminants	254	2	8	1
PTB positive tested farms	125	2	2	1

Discussion

Due to problems with test accuracy and sample selection, the true prevalence of PTB in most European countries is still unknown. The prevalence is thought to be significant and may also be increasing. Therefore, Austria as one of the first countries in the European Union established a control programme declaring PTB a notifiable disease under government regulation. Because of the difficulties in the detection of subclinically infected animals the Austrian control programme is focused on the detection of clinically diseased animals. The removal of high shedders in combination with the implementation of management and hygienic measures is an important first step in the reduction of intra herd transmission. From the occurrence of PTB affected breeds it can be concluded, that PTB seems to be especially a problem in beef herds of Limousin race, which can be explained by suckler cow husbandry and inter herd transmission.

EFFECT OF VACCINATION AGAINST PARATUBERCULOSIS IN THE DIAGNOSIS OF BOVINE TUBERCULOSIS WITH COMPARATIVE CERVICAL SKIN TEST

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INTRODUCTION

Paratuberculosis is one of the diseases that cause the heavy economic losses in cattle industry, especially in dairy herds. Control is based on two main strategies: detection and slaughter of infected animals and vaccination. Vaccination has been widely used in both bovine and ovine with good results when issues such as reduction of clinical cases, excretion rate and severity of lesions are taken into account (1,2). Also the economic balance is quite clear and the cost-benefit analysis shows better ratios for vaccination versus test and culling programs (3,4,5). However, there are two issues that have limited its use. The first is that the goal of those programs until very recently has generally been full and immediate eradication instead of just control to reduce economic losses in affected farms. The second is that vaccination against paratuberculosis can cause a cross-sensitization that would interfere with the diagnosis of tuberculosis. Since most countries have advanced eradication programs against this zoonotic disease in cattle which is also tightly regulated for international trade, Animal Health Authorities tend to be reluctant to allow paratuberculosis vaccination in bovine (6).

Nearly complete eradication of bovine tuberculosis in the Basque Country, as well as the high prevalence of clinical cases of paratuberculosis, led the local Animal Health Authorities to approve a vaccination field trial. The goal of the trial reported here was to investigate the bovine version of the Spanish killed vaccines and to what degree vaccinated cows become false positives in the Comparative Cervical Skin Test (CCST) for bovine tuberculosis.

MATERIAL AND METHODS

Seven bovine herds of the Basque Country with a history of clinical paratuberculosis were selected. They had been officially tuberculosis-free for, at least, the last ten years before joining the trial. Given environmental effects of shedding changes on both vaccinated and non-vaccinated animals, as well as other management difficulties, the trial was designed to compare animals in Vaccinated Herds (VH) versus animals in farms on just Test and Culling (TC). Two herds applied a Test and Culling program, while the other five applied a vaccination one. The trial started in 2006 and data from annual testing were collected until 2011. A total of 4,285 CCST records, 2,033 of them from VH and 2,252 from TC were available for the analysis. At the beginning of the study (M0) and later annually (M12, M24, M36, M48, M60), all animals, both vaccinated and no vaccinated, older than 6 weeks were tested. The CCST was carried out by the Official Veterinary Services according to European legislation (EU Council Directive 64/432/CEE and RD 2611/1996). Vaccination was carried out as described before (1). Briefly, one ml of Silirum® Map vaccine (CZ Veterinaria, S.L.; Porriño, Spain) was administered subcutaneously into the chest area of all the animals at the moment of joining the trial, and then to all new calves older than 1 month. Each dose contained 2.5 mg of heat-killed 316F Map strain and mineral oil as adjuvant. Animals from unvaccinated herds were considered as controls.

RESULTS

When all records were analysed together regardless the time since vaccination and age, 8.9% of the animals from the VH were positive to bovine Skin Test (bST), while only 0.8% scored positive in the TC group. From a comparative perspective, 0.14% of the vaccinated animals were scored as positives, while only 0.04% got that qualification in the TC farms. No lesion of tuberculosis was ever found in slaughtered animals in the post-mortem inspection carried out by the Official Public Health Veterinary Services. In addition, we saw that the percentage of positivity to bST ranged between 20.42% in the M24 and 4.63% detected in the M48 in vaccinated farms. However, the TC herds didn't exceed the 0.74% (M36) in the annual samplings. When the analysis was done in reference to the CCST the main reactivity was found in the M12 with a 0.58% of positives, while in the TC herds only appeared one positive along the study (Figure 1).

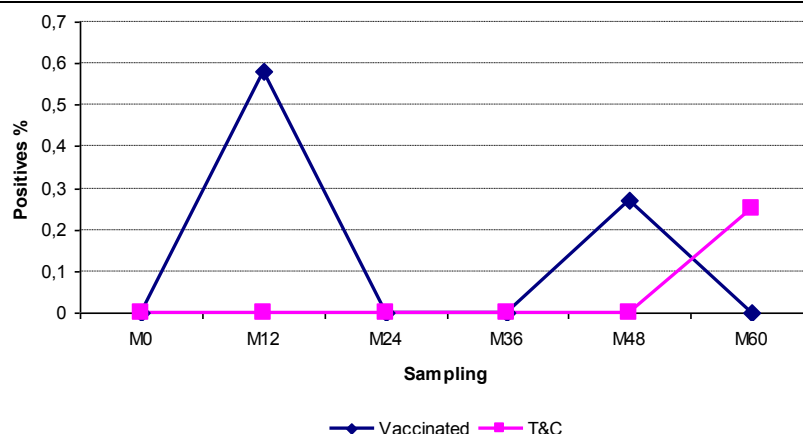


Figure 1. Percentage of positives to CCST in VH and T&C herds in annual samplings

Results according to time elapsed since joining the Program.

Animals joined the program at the time of first testing and vaccination (VH) or just first testing (TC). According to this criterion, significant differences in the percentage of bST positives were found in VH between pre-vaccination (M0), and <6 and 36 months after vaccination; however, such differences were not observed in animals that had been vaccinated more than 36 months ago. On the other hand, no significant difference in the bST or in the CCST regarding time from joining the trial was found among TC strategy animals.

Even though, individually, an increased proportion of reactors might persist as long as three years after vaccination, the overall proportion substantially decreased by the third year of vaccination. This can be explained because the majority of animals were vaccinated as adults at the beginning of the intervention. In the following years, since only the replacers are vaccinated, the overall reactivity in the herd quickly diminishes. If only the CCST results are retained, only three positive results were observed out of 2,033 tests. That can be considered irrelevant because it is a very small fraction of the total number of vaccinated animals. Even though paratuberculosis vaccination was ignored, it would be possible to slaughter this small number of cases to verify that they are not real TB cases. Only one of these animals had the CCST repeated one year later and then it was clearly negative.

Results according to the age at the moment of joining the Program

According to this approach, we found no significant differences between VH or between the TC.

CONCLUSIONS

The CCST is considered a useful tool for the diagnosis of tuberculosis in animals vaccinated against paratuberculosis. The interference caused by vaccination in the diagnosis of tuberculosis is similar to that caused by natural infection in TC herds.

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ACKNOWLEDGEMENTS

This study was supported by the projects INIA RTA2005-00147 and MICINN AGL2008-05820, as well as by the Bizkaia and Gipuzkoa County Councils and the Agricultural Department of the Basque Government. Patricia Vázquez had a FPI doctoral fellowship (BES-2007-17170) from the MICINN. The authors wish to thank the farmers and practitioners involved in this study.

THE ATLANTIC JOHNE'S DISEASE INITIATIVE

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Introduction

Johne's disease has been identified as one of the top health priorities of the Canadian dairy industry (Dairy Farmers of Canada, 2012). Control program standards have been developed on a national basis with delivery of specific initiatives on a provincial or regional basis (Canadian Johne's Disease Initiative, 2012). The Atlantic region consists of 4 small provinces on the east coast (Prince Edward Island, Nova Scotia, New Brunswick and Newfoundland). There are approximately 700 herds in the region with an average herd size of 85 lactating cows. The Atlantic Johne's Disease Initiative (AJDI) is a fully voluntary program of the four Atlantic province's dairy boards and the Atlantic Veterinary College (AVC) at the University of Prince Edward Island. The program provides substantial resources to assist herds that have the disease to decrease spread and overall prevalence on the farm. Additionally, environmental culture (EC) test-negative herds are provided with management plans to maintain their status. Like all other dairy biosecurity program, AJDI discourages the movement of animals between farms. When animal transfers are necessary, the AJDI program promotes informed animal movement by providing an accessible register of EC test-negative herds. The program is initially funded for three years (2011-2014). In addition to a broad-based education program for veterinarians and farmers, it includes three main activities; herd testing, risk assessment and selective cow testing. These procedures are designed to strategically, and in a cost-efficient manner, reduce the impact of Johne's disease on the regional dairy industry by decreasing existing infections and reducing new infections.

Herd testing

Herd testing is being conducted using EC procedures. Six mixed manure samples are collected from prescribed locations by a small number of trained technical staff. Details are available from the authors. Fresh samples are transported to the Maritime Quality Milk laboratory at the Atlantic Veterinary College where they are cultured using the *para*-JEM broth culture system in a ESP or VersaTREK incubator (TREK diagnostics). After incubation, all cultures are acid-fast stained and any samples which are positive for growth through the TREK sensor or visually contain acid-fast organisms are confirmed with PCR (VetAlert™, Tetracore). Herds positive on EC, are eligible to participate in voluntary co-funded animal testing. Herds that are EC test-negative, are eligible to be listed on a web-based register (www.atlanticjohnes.ca). Herds are eligible to be tested annually. For herds with two consecutive negative EC tests, testing eligibility will switch to every second year. The program covers all costs for the herd EC.

Risk Assessment

Risk assessment is the cornerstone of the program. Only veterinarians that have completed a specific advanced education program will be eligible to receive test results for interpretation and conduct risk assessments. In total, 47 veterinarians completed the certification program which included 3 phases: a) reading assignments and an on-line quiz; b) attending a program seminar with specific emphasis on diagnostic test interpretation; and c) an on-farm risk assessment exercise. Herd testing results are delivered by the AJDI certified veterinarian coincident with a farm-specific risk assessment. Risk assessments must be completed for each year that EC is conducted. A risk assessment workbook, designed using the Canadian national standards for risk assessment, is used to evaluate the farm Johne's control strategy (Canadian Johne's Disease Initiative, 2012). A management plan, based on the best management practices identified in the workbook is developed. Copies of these tools are available from the authors. The program will cover up to 1.25 hours of veterinary fees to complete the risk assessment and management plan.

Individual animal testing

Co-funding is available for cow testing in herds that are EC test-positive. All lactating cows in the herd must be tested to receive the program funding. Additionally, cows that are dry at the time of testing will be eligible for co-funded testing. Approved individual cow testing methods include milk and serum ELISA, and fecal PCR and culture. Project co-funding is capped at 60% of the cost of milk ELISA. Individual animal testing is focused on positive EC herds for two reasons. First, because of the test characteristics of MAP ELISA diagnostics, the positive predictive value improves if used in true positive herds. Second, by focusing this activity on herds with a known problem, financial resources can be used to their maximum benefit in the herds with the greatest need. In this manner, infected herds will have access to annual testing to facilitate a sustained control effort.

Environmental culture status register (For EC negative herds)

All samples are collected by project personnel to insure consistency. Environmental culture negative herds may choose to participate in the AJDI status register. To maintain a focus on risk management, there are only 2 levels in the program. Herds are classified as EC Negative Level 1, if their most recent environmental culture is negative and they have not had a positive EC test in the previous 10 months. Level 1 herds must retest within 10-14 months. If the retest is negative, the herd is listed as an EC Negative Level 2. Level 2 herds must retest no earlier than 10 months and no later than 26 months after the previous test to maintain their status. EC negative herds will be eligible to have their status registered on the AJDI website (www.atlanticjohnes.ca). Herds will lose their status level and be removed from the website list if: a) any EC samples are positive on the most recent test; b) more than 14 months has elapsed for EC Negative Level 1 herds and they have not retested; or c) more than 26 months has elapsed for EC Negative Level 2 herds and they have not retested.

Implementation and Research Program

The program was launched in June of 2011 with a communications campaign and training program for the certified veterinarians. The enrolment goals are 50% of herds in year 1 and 60% by the end of year 2. In the first 6 months of the program, approximately 300 herds have enrolled, which is 85% of our first year target. Culture results are available for around half of those herds and the herd prevalence, based on the EC, is approximately 15%.

A portion of funding for the program has been set aside for research activities. Research will focus on 2 key areas including evaluation of program effectiveness and assessment of communication strategies employed in the initiative. Program effectiveness assessment will include uptake of program recommendations through the management plan process, changes in prevalence at cow level among infected herds and economic models of the disease and control strategy. Assessment of communication channels and the effect of these channels and prior beliefs on implementation of program recommendations will also be evaluated.

Acknowledgements

The authors acknowledge the financial support of the Dairy Farmers of Prince Edward Island, Dairy Farmers of Nova Scotia, Dairy Farmers of New Brunswick and Dairy Farmers of Newfoundland. Additional financial support is provided through Agriculture Canada (Canadian Agricultural Adaptation Program) and the AVC, through the Innovation PEI Dairy Industry Research Chair. Oversight of the project is conducted by a Steering Committee from the dairy boards and AVC. Laboratory and technical support is provided by Maritime Quality Milk. This project would not be possible without the support of the private veterinary practitioners, who have enthusiastically promoted the program to their clients.

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CAN JOHNE'S DISEASE BE CONTROLLED BY VACCINATION? DATA ON 12 MILLION OVINE VACCINATIONS AND 7.6 MILLION CARCASS EXAMINATIONS IN NSW AUSTRALIA FROM 1999-2009

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BACKGROUND: *Mycobacterium avium* subspecies *paratuberculosis* "sheep strain" is the cause of ovine Johne's disease (OJD). OJD was first detected in New South Wales (NSW), Australia in 1980 and subsequently became prevalent in higher rainfall sheep-raising areas of NSW (Central and Southern Slopes). Abattoir monitoring for OJD was instituted in NSW in 1999 as part of a national program. Vaccination of lambs and adult sheep with Gudair® killed vaccine (Pfizer Animal Health) was permitted from January 2000.

METHODS: Prevalence of OJD was stratified into "High" "Medium" and "Low" areas (HPA, MPA and LPA) at 31st March 2008. Trained inspectors evaluated carcasses of adult sheep for gross evidence of OJD in the lower small intestine. Suspect tissue was subjected to histopathological examination.

PRINCIPAL FINDINGS: From 2000-2009, 12 million vaccinations were administered (91%; 10.9 million in the HPA). A total of 7.6 million carcasses (76% of sheep killed in 32,032 consignments) were examined (38%; 2.9 million from the HPA). From 2000-2009, 16% of all consignments were positive, of which 94% were from the HPA. The annual percentage of positive consignments from the HPA remained above 23% from 1999-2009 (average 37%, maximum 47% in 2006). In contrast, the proportion of animals positive for OJD from the HPA fell progressively from 2.5% in 1999/2000, prior to widespread vaccination, to 0.83% by 2009 (average 1.65%, <1.0% from 2005 to 2009). There was a marked reduction in the proportion of heavily infected positive consignments (more than 10% lesions): 28% of consignments in 1999-2000, 15% in 2001-2004, decreasing to 2% from 2005-2009. The percentage of animals confirmed positive in the LPA averaged 0.01% while the MPA averaged 0.4% over the decade.

Figure 1: Percentage of Sheep with OJD from the High Prevalence Area 1999-2009 v's Vaccine Sales 2000-2010

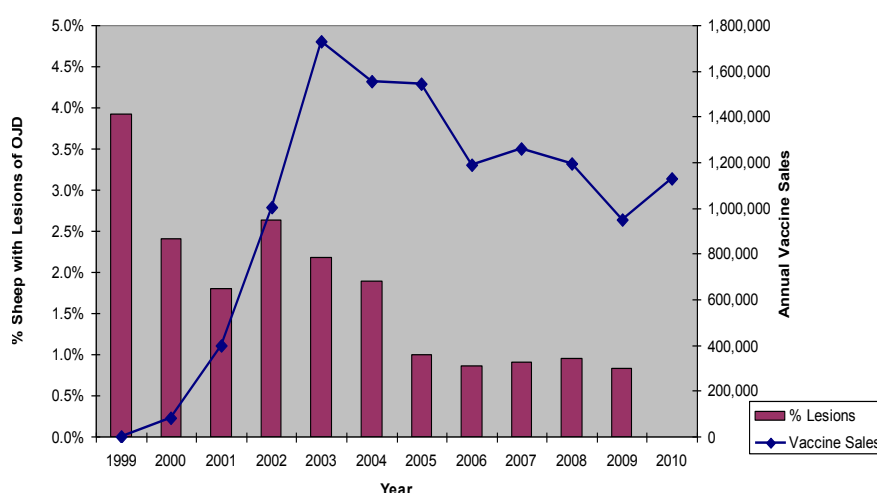


Figure 2: Percentage of Lines Positive for OJD by Prevalence Area (implemented 31/03/2008) - 5109 positive ex 32,032 lines monitored from 1999 to 2009

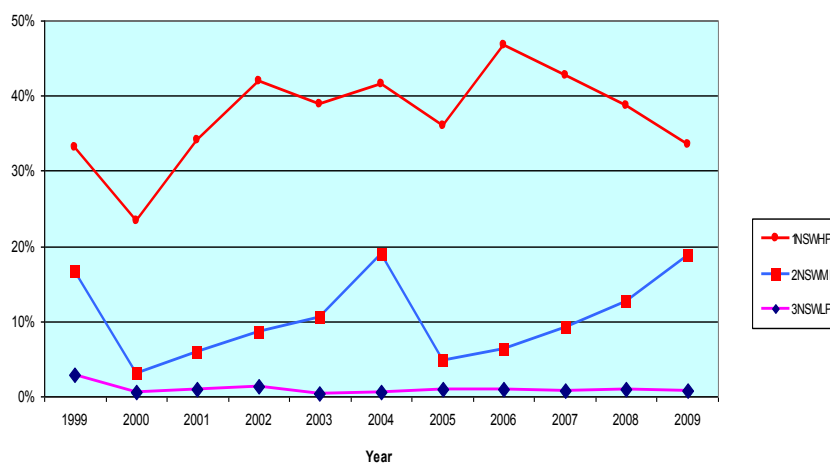
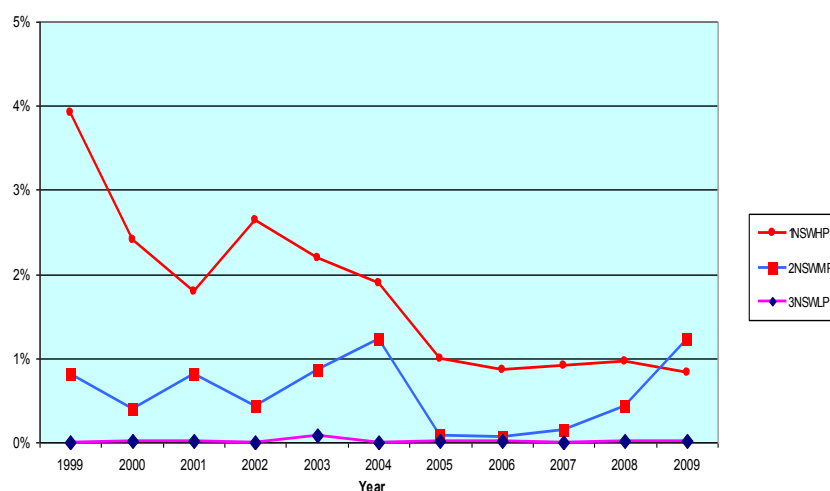


Figure 3: Percentage of Sheep with Lesions Attributed to OJD by Prevalence Area (implemented 31/03/2008) - 7,635,513 sheep in 32032 lines monitored 1999 to 2009



CONCLUSIONS: #1.) Following the administration of 10.9 million vaccinations in the HPA over 10 years, consignments positive for OJD remained constantly high (average 37%). #2.) In contrast, the percentage of animals positive for OJD fell by 67% during this decade. #3.) While these observational data do not prove that vaccination was responsible for the reduction in OJD prevalence, they justify further studies on the efficacy of vaccination to control Johne's disease.

Acknowledgements

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia, and managed through Animal Health Australia and NSW DPI. The project could not have proceeded without the commitment and diligence of the OJD inspectors. The support of abattoir management (Dubbo, Goulburn, Deniliquin & Wallangarra & domestic works at Cowra, Cootamundra, Young, West Wyalong, Junee & Mudgee) and AQIS was essential. District Veterinarians with LHPAs provided ongoing support notifying producers and promoting the uptake of vaccination. Pfizer Animal Health kindly provided vaccine sales data. The contribution from the data entry team at Wagga (Tracey Kingham, Jenny Fury & James Hamilton) and Orange, and pathologists at RVL Orange and Elizabeth Macarthur Agricultural Institute Menangle is gratefully acknowledged.

IS WHOLE OF FLOCK VACCINATION FOR OVINE JOHNE'S DISEASE JUSTIFIED?

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BACKGROUND: Vaccination with Gudair® (Pfizer Animal Health) killed Spanish vaccine was permitted in NSW from early 2000 in flocks with 5% or more annual deaths due to OJD. In April 2002 the vaccine was registered for wider use. This followed vaccine trials demonstrating that it could reduce clinical disease by 90% following vaccination in lambs at less than 16 weeks of age.

A whole of flock vaccination trial in Yass NSW, in a heavily infected Merino flock suffering major mortalities, demonstrated that vaccinating animals incubating infection did not exacerbate or accelerate the onset of clinical disease. This important research finding enabled producers to choose to vaccinate adults if desired. However the benefits from whole of flock vaccination were considered equivocal.

Producers may elect to vaccinate older sheep and, if desired, the whole flock. However this is not an official recommendation as there is no definitive research to demonstrate either a financial or disease control advantage. Based on the evidence that Gudair® vaccination of lambs (prior to significant exposure) provides a 90% protection against clinical disease, producers should feel comfortable in vaccinating nominally unexposed older sheep who may be at risk. The long incubation period of OJD, however, makes the decision to vaccinate older adult sheep difficult for producers in the absence of clinical disease.

Unfortunately there is no clear-cut research evidence that Gudair® vaccine is therapeutic in animals already incubating the disease. In the absence of such evidence, producers with infected flocks are left in-limbo (or worse) pending the 5 years or so that vaccinating only lambs takes to yield a fully vaccinated flock. Unvaccinated infected animals may progress to clinical disease & death or be sold for slaughter with reduced value. In the meantime they excrete increasing numbers of organisms, increasing the challenge to vaccinated lambs as well as any unvaccinated uninfected adults.

The "Therapeutic Effects of Vaccination" have been discussed by GW de Lisle ("Paratuberculosis – organism, disease, control" MA Behr & DM Collins ed., p349 CAB International 2010), particularly vaccination of sheep in Iceland.

With the increasing value of sheep meats and other products, the financial loss to producers and processors due to OJD in mutton sheep at the time of slaughter is substantial. Concurrently, the animal welfare implications of uncontrolled OJD in individual flocks needs to be considered.

With vaccine costing \$2.60 - \$3.00 per dose and a ewe currently valued at an average A\$150, ultimately saving 1 animal (i.e. reducing annual losses by 2%) would justify vaccinating 50 animals. Potential production and animal welfare benefits would provide additional value to whole of flock vaccination. This report identifies a range of scenarios and estimates potential benefits and costs of whole of flock vaccination in different situations.

SCENARIOS: Specific scenarios where whole of flock or adult vaccination should be considered include:

- (1) Flocks suffering substantial losses – anecdotal evidence from producers and District Veterinarians that some flocks experience significant reductions in mortality. Benefits: Potential for reduced death rate and improved animal welfare. Reduced producer anxiety compared with "do nothing" option.
- (2) Flocks where the sub-clinical disease detectable in the abattoir is >10% (usually associated with emerging losses on farm). Benefit: Protect unexposed animals or slow the onset of clinical disease.
- (3) Flocks with low level of sub-clinical infection (1-2%) confirmed by abattoir monitoring but losses not yet evident. Benefit: Protect unexposed animals.

(4) Commercial flocks exposed to ongoing risk from infected neighbours or introductions. Benefit: Protect whole flock of naïve animals.

(5) Uninfected high value trading enterprise in the Low Prevalence Area – e.g. stud or restocker sheep vendor. Benefit: Protect flock and manage major financial risk due to potential loss of trade. Minimise the risk to purchasers and neighbours.

CONCLUSIONS: Whole of flock vaccination can be justified in a range of circumstances based on potential benefit-cost analysis and animal welfare. If vaccination was demonstrated to have a therapeutic effect in adult sheep, the economic benefits to the sheep industry from improved productivity, decreased mortality and decreased wastage at slaughter would be substantial. Further research on the merits of whole-of-flock vaccination are warranted.

THE BENEFITS OF A SIMPLE FINANCIAL & PROJECT MANAGEMENT APPROACH TO THE OVINE JOHNE'S DISEASE PROGRAM IN NEW SOUTH WALES

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BACKGROUND: The National Ovine Johne's Disease Program (NOJDP), with a mix of funding sources, was implemented in NSW in 1999. It involved extensive on farm sampling, laboratory testing, tracing and group extension activities. The complexity of the NOJDP highlighted the need for an innovative financial and project management approach to ensure auditable, transparent and cost efficient delivery of services/outputs. This report describes the approach taken by the NSW Department of Primary Industries (NSW DPI) to manage the state-wide program from 1999 to 2007.

METHODS: Based on nationally agreed rates of payment for a unit of activity (outputs), a range of invoice templates (see attached example) were prepared covering specific activities e.g. on-farm surveillance testing, assurance testing subsidies, farmer group workshops, tracing etc. Each activity (e.g. tracing, field investigation etc.) was allocated a financial group code (WBS – work breakdown structure) and each input a line item code (GL – general ledger line) in the financial system (SAP). Individual properties were identified by their Property Identification Code (PIC or RLPBID) allocated under the National Livestock Identification System (NLIS). The PIC was used as the common link between the on-farm field activities (Disease Monitoring System database), laboratory submissions (LABSYS database) and SAP.

The field activities were coordinated by the Rural Lands Protection Board (RLPB) veterinarians in each region. Activities could be delivered either by RLPB staff or outsourced to private veterinarians. On completion of the activity they completed the relevant form and faxed it to Wagga Wagga for data entry into Microsoft Excel. Key data was uploaded into the SAP financial system. Non-financial data fields (e.g. PIC, no. of samples etc) were concatenated (joined - space delimited) into a single "description" field, prior to upload into the only available field in SAP. The laboratory uploaded their testing charges into SAP and results into the "Labsys" database. Data exported from SAP was analysed in Excel to provide management with financial reports and tax invoices to the various funding bodies. Veterinarians were paid for invoiced activities directly via SAP to their financial institution accounts.

PRINCIPAL FINDINGS: Implementation of a major disease control program with limited resources of skilled personnel in both the veterinary and financial spheres requires an innovative approach. By June 1999 over 98 veterinarians had been enrolled in the program. The reliance on a paper based system of recording with tax compliant invoices faxed to a central location for data entry and upload into the financial system worked very efficiently. It provided a high level of flexibility in utilising the mix of private and government veterinarians & support staff. Timely preparation of tax invoices to funding bodies was facilitated. It streamlined the management procedures dramatically, reducing labour costs. It provided flexibility with allocation of veterinary resources and facilitated timely payment to service providers. The program has also been very effective in reducing the impact of OJD on sheep producers financial situation and minimising any potential threat to domestic or export markets.

CONCLUSIONS: The system provided a transparent mode of payment based on units of output. It was GST tax compliant, auditable and provided real-time financial management. Despite the wide range of activities supported, there was a negligible level of complaint from veterinarians regarding payment for activities. Invoicing the funding bodies was simplified. It provided a permanent record of all activities undertaken and enabled the financial aspects of the >\$17m 8 year program to be finalised only 2 weeks after completion. It demonstrated the collateral financial benefits of implementation of NLIS in Australia. It acts as model for future disease control programs providing effective control of information, a transparent process and protection of privacy via use of PIC.

Acknowledgements

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia and the Commonwealth and managed through Animal Health Australia and NSW DPI. The support of RLPBs in coordinating the activities was essential. District Veterinarians and private veterinary practitioners cooperated to provide a unified enthusiastic & cooperative approach. The Australian Veterinary Association provided ongoing support. The contribution of data entry team at Wagga and financial services in Orange are gratefully acknowledged.

EVALUATION OF 108 ABATTOIR TRACES FOR OVINE JOHNE'S DISEASE TO THE NEW SOUTH WALES LOW PREVALENCE AREA – A STANDARDISED APPROACH

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Background: Ovine Johne's disease (OJD) was first detected in New South Wales (NSW) in 1980. It subsequently spread to infect a large number of flocks in the higher rainfall areas of the Central & Southern Slopes of NSW. Abattoir monitoring for OJD was instituted in NSW in 1999 as part of a national program aimed at controlling OJD with early detection and neutralisation of infected flocks in areas of low prevalence seen as critical to prevent long term establishment of infection.

Methods: OJD positive abattoir consignments were traced to property of origin based on their unique Property Identification Code (PIC). As the Low Prevalence Area (LPA) boundaries have been changed since 1999 only traces reallocated to the LPA implemented on 31st March 2008 were reviewed. A standardised risk assessment process was developed in 2004 (and implemented retrospectively) to determine the initial status of individual flocks with bi-annual re-assessment. Details collated included on-farm testing, status of neighbours, details of introductions and sales, property disease management plans, vaccine use, destocking and, if infection was confirmed on farm, whether it was in homebred or introduced sheep. This data was reviewed in mid-2010 prior to adjustment of prevalence area boundaries in January 2011.

Results: A total of 17,392 consignments were monitored from the LPA in the period November 1999 to December 2009. The results of follow-up risk assessments were analysed for 87 properties representing all 108 positive consignments (one or more animals confirmed with OJD by histopathology on samples of gross intestinal lesions detected in the abattoir). There were 73 properties with a single trace, nine with 2, three with 3 and two with 4 traces. Forty seven flocks were considered to be infected based on a combination of abattoir monitoring (multiple traces and/or multiple animals positive), trace confirmation and risk assessment. Four properties close to the LPA boundary were reallocated to the Medium Prevalence Area (MPA). Based on the risk assessment 21 flocks were considered as highly unlikely to be infected following either failure to confirm the trace to the property or comprehensive negative on-farm testing (usually Pooled Faecal Culture). The initial status of the remaining 19 properties was undetermined.

Table 1: Summary of risk assessments on 87 properties with positive abattoir traces to the NSW Low Prevalence Area – 1999 to 2009

	Yes	No
Infected (initially)	47	21
Confirmed LPA PIC	83	4 (MPA)
>1 animal positive histopathology	38	49
Homebred sheep infected	9	17
PDMP	15 (+ 13 tested neg.)	10
Vaccinating	8	33

Comments: The ongoing risk presented by infected properties has been resolved in many cases by destocking and/ or implementing a property disease management plan (PDMP). The results are summarised in the Table 1. The distribution of the initial traces is shown in the attached Figure.

CONCLUSION: A standardised approach to risk assessment proved invaluable in minimising the risk of establishment of OJD in the NSW LPA and ensuring maintenance of the area status. There were 108 of 17,392 (0.62%) consignments initially identified as positive on abattoir monitoring. Following risk assessment 43 LPA flocks were considered infected, 21 unlikely to be infected and 19 of unknown status.

Acknowledgements

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia, and managed through Animal Health Australia and NSW DPI. The support of Abattoir Management (Dubbo, Goulburn, Deniliquin & Wallangarra & domestic works at Cowra, Cootamundra, Young, West Wyalong, Junee & Mudgee) and AQIS was essential. District Veterinarians with LHPAs for conducting Risk Assessments. The contribution from the OJD Inspectors, the data entry team at Wagga Wagga (Tracey Kingham, Jenny Fury & James Hamilton) and pathologists at RVL Orange and Elizabeth Macarthur Agricultural Institute are gratefully acknowledged.

DEVELOPMENT OF A GROUP APPROACH TO FACILITATE VACCINATION FOR OVINE JOHNES DISEASE (OJD) IN NEW SOUTH WALES

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BACKGROUND: Following the first detection of ovine Johnes's disease (OJD) in New South Wales (NSW) in 1980, it subsequently spread to infect a large number of flocks in the higher rainfall areas of the Central Tablelands & Southern Slopes of NSW. In April 2002 the Gudair® vaccine (CSL Australia Ltd, and subsequently Pfizer Animal Health) was registered for widespread use. Vaccine trials had demonstrated that it could reduce clinical disease by 90%. In May 2003 the NSW Department of Primary Industries, in conjunction with NSW Rural Lands Protection Boards (RLPBs, now Livestock Health & Pest Authorities) & CSL, instigated a series of vaccination workshops for producers in the South West Slopes & Plains. This report outlines the process followed & benefits from this approach.

METHODS: An experienced facilitator liaised with veterinarians & rangers at Rural Lands Protection Boards, livestock officers and CSL to identify local halls & shearing sheds in target localities to conduct "group" meetings.

The meetings followed a standard format:

- 1) Introductory session to identify questions the producers wanted answered;
 - 2) Gudair® vaccine video;
 - 3) Presentation on trial work, claims of the vaccine, preparation & use of the vaccine, precautions to take & benefits of vaccinating;
 - 4) Applying to access the vaccine, tagging requirements of vaccinated animals;
 - 5) Trading options for vaccinated sheep;
 - 6) Autopsy of an infected animal if available or video of autopsy plus display of affected tissues from the abattoir & explanation of abattoir surveillance program;
 - 7) Demonstration of correct vaccination technique – emphasising avoidance of needle-stick injury;
 - 8) Producer presentation on "Why they elected to vaccinate" and display of vaccination site lesions.
- Attendees at group workshops were eligible for a subsidy for each property represented (based on Property Identification Code).

PRINCIPAL FINDINGS: A total of 14 workshops were held between July and August 2003 with 454 producers in attendance. There was a marked upsurge in use of vaccine associated with these workshops and the risk of accidental self-inoculation or unacceptable abscesses in the neck muscle of the sheep was minimised.

CONCLUSIONS: A group approach to extension by trained facilitators assisted by technical advice from experienced veterinarians, livestock officers, vaccine distributor and experienced producers proved a very effective means of communication. It greatly assisted in "taking the heat out of" the OJD program. It provided a means of producer empowerment by encouraging vaccination.

Acknowledgements

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia, DAFF and RLPBs, and managed through Animal Health Australia and NSW DPI. The support of RLPB District Veterinarians, Rangers, directors and support staff, NSW DPI Livestock Officers and Veterinary Officers and CSL was essential to the successful delivery of this project. Producer support for this training program was outstanding, in particular the willingness of producers with heavily infected flocks to share their experiences.

EFFICACY OF KILLED VACCINE WITH DIFFERENT ADJUVANTS FOR THE CONTROL OF JOHNE'S DISEASE

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Abstract

Heat inactivated Johne's disease (JD) vaccine was developed using prevalent isolate of *Mycobacterium avium* subsp. *paratuberculosis* (Bison type) with four different adjuvants for the control of ovine paratuberculosis in farms experiencing 1.19 – 8.2% mortality in Tamilnadu, India. Farm I (674 animals) was vaccinated with montanide 71VG JD vaccine, Farm II (225 animals) with montanide 201VG JD vaccine, Farm III (62 animals) with montanide 763A JD vaccine and Farm IV (1206 animals) with mineral oil JD vaccine respectively. The young stocks and replacement stocks were also vaccinated with the same vaccine following lambing. Periodic blood and faecal sampling was carried out on 0, 2nd, 3rd, 6th, 12th and 24th month post vaccination to assess the impact of vaccination on mortality rate, faecal shedding and cell mediated immune (CMI) response. A remarkable reduction in mortality ranging between 70 – 100% and faecal shedding (75 – 93%) was observed in all the vaccinated farms. The CMI response in farm I and II showed an initial increase by 30th dpv followed by reduction by 6th month whereas, farm III and IV showed an initial increase by 3rd month and the response sustained even at 2 years of the trial. In farm I and II, 50% of the animals showed lameness and were anorectic after vaccination for a period of 15 days post vaccination. Lymphangitis was noticed in 2% of the vaccinated animals which required further treatment. Nodule formation was noticed in 45 – 53% of the animals vaccinated in all the farms which persisted even after 2 years. The farm trial concluded that mineral oil adjuvanted JD vaccine was safe and potent enough to reduce mortality and shedding with sustained CMI response to control JD in infected farms during the trial period.

Introduction

Johne's disease (JD) was first diagnosed in India in 1913 at Hissar followed by reports of JD throughout the country with incidence ranging from 1.78 to 1.9%. Latest reports reveal an increased seroprevalence of JD ranging from 15% to 78%. Control of JD by altered managemental practices was found to be very difficult. Larson et al, (1978) showed that controlled clinical vaccination trial 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). At present, no commercial JD vaccine is available in India and is not cost effective especially to small farmers who have minimum holdings for their livelihood. The commercial vaccines available in other countries have incorporated cattle strain whereas bison type is most prevalent in this region. Hence, an attempt was made to develop a vaccine using the prevalent isolate and assess its efficacy in farms with JD infection.

Methodology

Development of Vaccine

Mycobacterium avium subsp. *paratuberculosis* was isolated and characterized by IS900 PCR (Collins et al., 1989) and IS1311 PCR restriction enzyme analysis (Marsh et al., 1999). The isolate was adapted in Reids synthetic medium and a heat inactivated vaccines were developed as per Sigurdsson (1960) with different adjuvants mineral oil, montanide 71VG, montanide 201VG and montanide 763A (Seppic, France) as per the manufacturers recommendation. Safety and potency tests were carried out in guineapigs as per OIE (2005).

Field Trial

The vaccines were evaluated in four sheep farms with previous history of JD. Farm I, II, III and IV were vaccinated with montanide 71VG (674 animals), montanide 201VG (225 animals), montanide 763A (62 animals) and Mineral oil (1206 animals) respectively. Whole farm vaccination was carried out with the respective vaccines followed by annual vaccination of the new born at 1 month of age and replacement stocks. Periodic blood and faecal samples were collected for interferon gamma (IFNG) assay using BOVIGAM (Prionics AG) and culturing respectively on 0, 30, 180, 360, 720 days post vaccination. The faecal samples were initially screened by Acid fast staining followed by pooled faecal sample culturing (25 animal per group). Positive groups will be subjected for individual culturing and confirmation by

IS900 PCR. Mortality was recorded from the inventories available at each farm. No significant changes in the management strategy were adopted for the control of JD.

Results

The prevalent *Map* isolate was characterized as Bison type and four different heat inactivated vaccines were developed and adjuvanted as per manufacturers recommendation. The safety and potency tests in guinea pigs were found to be safe and satisfactory. An initial pilot study was carried out with the vaccine in sheep with control (Data not included) was found to be satisfactory.

Field trial with different vaccines in the farms showed an increase in IFNG values in vaccinated flock and the OD values are tabulated in table-1. No significant difference in IFNG OD values were noticed between Farm I and II and Farm III and IV respectively whereas significant difference in OD values were noticed on 30 and 180dpv.

Post Vaccination Interferon Gamma response to vaccines

DPV/Vaccine	Mineral oil	Montanide 71VG	Montanide 201VG	Montanide 763A
0 dpv	0.04 $\pm$ 0.01 ^a	0.08 $\pm$ 0.04 ^a	0.02 $\pm$ 0.03 ^a	0.02 $\pm$ 0.03 ^a
30dpv	0.24 $\pm$ 0.03 ^{ac}	0.36 $\pm$ 0.02 ^b	0.28 $\pm$ 0.02 ^a	0.2 $\pm$ 0.05 ^c
180 dpv	0.9 $\pm$ 0.06 ^a	0.19 $\pm$ 0.02 ^b	0.18 $\pm$ 0.03 ^b	0.5 $\pm$ 0.06 ^c
360 dpv	0.43 $\pm$ 0.03 ^a	0.1 $\pm$ 0.01 ^b	0.11 $\pm$ 0.01 ^b	0.22 $\pm$ 0.03 ^c
720 dpv	0.14 $\pm$ 0.01 ^a	0.09 $\pm$ 0.02 ^b	0.09 $\pm$ 0.01 ^b	0.12 $\pm$ 0.01 ^c

Different superscript differ significantly ($P \leq 0.05$)

All the vaccinated farms showed a significant reduction in faecal shedding. Farm IV showed 93% reduction followed by 91% in farm III. 83% in farm II and 75% in farm I. It was observed that the young stock vaccinated during the trial did not excrete *Map* till the end of the trial.

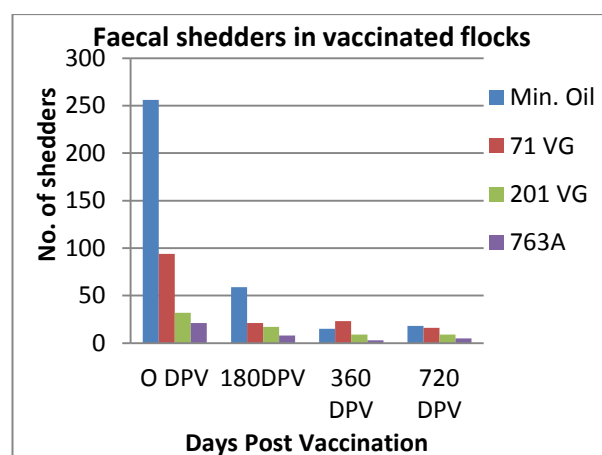


Fig. 1

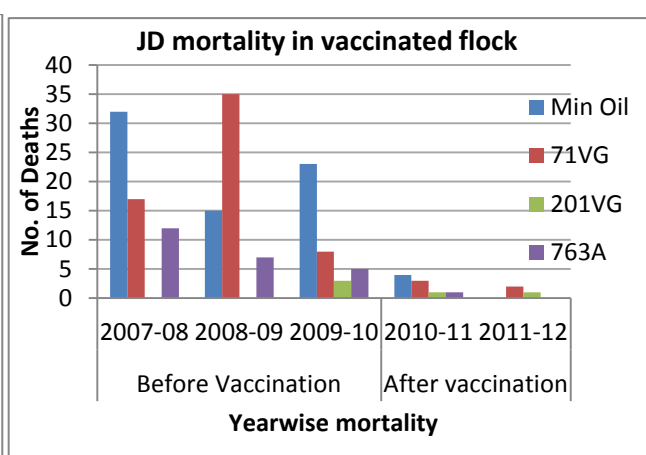


Fig. 2

Figure 2 shows highly significant reduction in mortality due to JD in all the vaccinated farms upto 720 dpv. Mortality was reduced upto 100% in farm III and IV whereas mortality due to JD reduction in farm I and II was 91% and 70% respectively.

The adverse effect of the vaccines included lameness(50%) with anorexia for 2-3 weeks following vaccination with montanide 71VG. This was followed by Lymphangitis in 2% of the sheep which required treatment. In Farm II, lameness and anorexia was noticed in 50% of the animals with lymphangitis in 0.09% animals. Vaccine granuloma was noticed in farm I, II, III and IV at 53%, 50%, 51% and 45% respectively. The size of the vaccine lesion increased to 5-10 cms at 30 dpv and regressed to 2-3 cms and persisted till the end of the trial in few animals.

Discussion

This study was initiated to control JD with the developed vaccine using the prevalent isolate since no commercial vaccine is available in India. In India sheep and goat farming were the predominant livelihood for small farmers and management changes were difficult to implement. Results of an extensive vaccination trial were reported almost 40 years ago for sheep in Iceland by (Sigurdsson, 1960) concluded that the most cost-effective strategy was to vaccinate replacement ewe lambs. Hence *Map* was isolated and identified as bison type was used for vaccine development with mineral oil and different montanide as per the manufacturer's recommendation. It is generally agreed that protection against mycobacterial disease is mainly due to TH1 cells that produce IFNG to activate macrophages which then kill mycobacteria during phagocytosis. Hence the heat inactivated *Map* was blended with adjuvants like montanide 71VG (a water-in-oil emulsion), montanide 201VG (water-in-oil-in-water emulsion), montanide 763A (a water-in-oil emulsion) adjuvant and mineral oil which can induce strong and long lasting CMI response. Whole farm vaccination was carried out since herd vaccination has previously been shown to be an effective tool in the control of paratuberculosis (van Schaik et al. 1996) although infection rates within the herd may remain high. Doyle (1964) pointed out that mixing control and vaccinated animals in the same herd may be contraindicated because, as the immunity in vaccinated animals declines, control animals may become infectious and transmit the disease to vaccinated animals. The two year trial period revealed that all the vaccines stimulated cellular immune response significantly by 30dpv and 180dpv while farm I and II showed a decline in CMI response after 180dpv.. This reduced CMI response could be a reason for only 75% and 80% reduction in JD mortality respectively. Similarly farm I and II has increased faecal shedders. The IFNG response in farm III and IV showed prolonged immune response with complete reduction in mortality and 90 and 93% reduction in faecal shedding. Farm I and II showed lameness with anorexia in 50% of the animals following vaccination which could be due to pain at the vaccination site. In Farm I and II, 2% and 0.09% of the animals developed lymphangitis which required further treatment. This could be due to excessive CMI response. All the animals which died after vaccination were the adult animals which were found to be shedding *Map* at the time of vaccination. Since vaccinated animals showing clinical symptoms will shed more *Map* than animals in subclinical stage it is recommended to cull all the vaccinated animals which were found to be shedders. This trial showed that there was remarkable reduction in mortality and shedding in farm III and IV in controlling JD. But considering the cost of the adjuvant which needs to be seriously considered in Indian scenario the study concluded that mineral adjuvanted JD vaccine with bison type *Map* strain was able to provide better control against JD in the trial period.

Conclusion

The study revealed that mineral oil adjuvanted JD vaccine and montanide 763A were able to provide better control of JD compared to the other vaccines during the trial period. Further extended studies are required to ascertain the vaccine effect on growth parameters and to quantify the faecal shedding.

Acknowledgement

The author acknowledges the support rendered by the staff of all the farms enabling to conduct this trial.

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EFFICACY OF A KILLED VACCINE FOR THE CONTROL OF PARATUBERCULOSIS IN FRENCH GOAT HERDS

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Introduction

Vaccination has been considered a successful measure for controlling paratuberculosis (Corpa et al., 2000). A commercial vaccine (heat-killed vaccine) is currently registered in Spain for sheep and goats, but in France, the use of paratuberculosis vaccines is restricted to goats under one month old. As there were no scientific reports demonstrating its efficacy in these conditions, a field trial was undertaken from 2006 to 2010 to determine the efficacy of a killed *Mycobacterium avium subsp. paratuberculosis* (*Map*) vaccine, GUDAIRTM (CZ Veterinaria, Spain) for the control of paratuberculosis in French dairy goats. The main objectives were to determine the effect of vaccination on the excretion of *Map* in faeces (shedding), on the culling rate and on gross lesions associated with paratuberculosis in culled goats. Secondary objectives were to investigate the cellular and humoral immune responses following the GUDAIRTM vaccination and to determine the vaccine's safety by assessing local reactions.

Materials and methods

Two goat herds with clinical paratuberculosis were selected. In each herd, kids under one month of age were randomly allocated to treatment groups (vaccinated, $n = 115$ and unvaccinated controls, $n = 124$). Kids in the vaccinated group were injected once with GUDAIRTM (1 ml, subcutaneously), but not separated from the other kids.

Samples of blood/sera and faeces were collected approximately every seven months.

Faecal shedding of *Map* was both detected by culture, as previously described (Mercier et al., 2009) and by PCR (TaqVet *M. paratuberculosis*, Laboratoire Service International, France).

Cell-mediated immune responses were measured using a gamma interferon (IFN- γ) assay (BioSource bovine IFN γ EASIA® », Invitrogen, France) after stimulation with avian PPD and PBS (negative control). A sample was considered positive as described by Storset et al., 2005. Humoral immune responses were measured using a commercial ELISA test (ELISA Paratuberculose anticorps bicupule®, Institut Pourquier, France). A sample was considered positive if Sample to Positive ratio (S/P) was greater than or equal to 0.6.

Local reactions to vaccination were assessed in one herd ($n = 75$) three times after vaccination (40, 111 and 168 days post-vaccination). The presence of lesions, their diameter and any abscess were recorded.

Culled goats ($n = 67$) were euthanised and post-mortem examinations performed. The enlargement of mesenteric lymph nodes and thickening of the small intestinal mucosa were recorded as gross lesions associated with paratuberculosis.

The χ^2 -test on 2 x 2 contingency tables, or Fisher's exact test (if an expected cell value in the χ^2 -test was less than 5) was used to test the significance ($p < 0.05$) of association of each of the following parameters comparing vaccinated and control goats: *Map* shedding (proportion of goats shedding *Map* in their faeces), culling, presence of gross lesions and immunological responses (proportion of goats with positive responses).

Results

1 – Efficacy

1 – 1 – Faecal shedding

Vaccination was associated with a reduction in faecal shedding. The percentages of shedding goats detected by faecal culture (Figure 1) dropped significantly to 3% at 23 months post-vaccination and 8% at 30.5 months post-vaccination in vaccinates, versus 16% and 20% respectively in controls. Whenever faecal shedding was detected by PCR, the differences between vaccinates and controls were significant at 15.5 months and 23 months (data not shown). At every sampling time, the percentages of shedding goats detected by PCR were higher than those detected by faecal culture.

1- 2 – Culling

Culling was calculated for each age class across both herds throughout the trial. Vaccination was associated with a reduction in culling. The reduction in total culling due to vaccination was 37% (41 controls and 26 vaccinates) but the difference between vaccinates and controls was not significant.

1 – 3 - Gross lesions in culled goats

Across both herds, there was a 20 % reduction in the proportion of vaccinates with gross lesions compared to controls (nine out of 26 vaccinates had gross lesions, compared to 18 out of 41 controls). This difference was not significant.

2 – Immunological responses

The stimulation of both the cell-mediated and humoral immune systems by vaccination was evident from the elevated proportion of positive reactors for both tests in vaccinates compared to controls, and from the rapid increase in these proportions among vaccinates after vaccination (Figures 2 and 3).

There were significantly more frequent positive responses to an IFN- assay in vaccinates at 8.5 months, 15.5 months and 23 months. There were significantly more frequent positive responses to an ELISA in vaccinates at 8.5 months and 15.5 months. The percentages of positive responses were higher with the IFN-assay than with the ELISA test

3 – Local reactions to vaccination

Local reactions were identified in 79%, 78% and 61% of vaccinates at 40, 111 and 168 days post-vaccination, respectively. Only a few vaccinated goats (7%) developed abscesses. Most local reactions were less than 5 mm in diameter (92%, 97% and 97% respectively). Local reactions were persistent but mild.

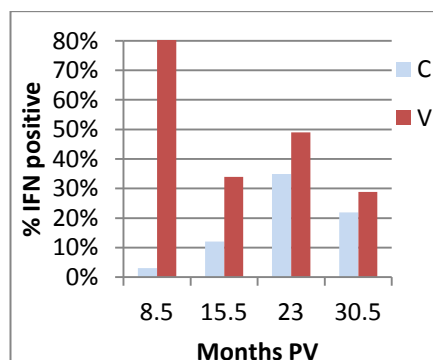


Figure 1: Proportion of goats excreting *Map* in faeces (detected by culture)

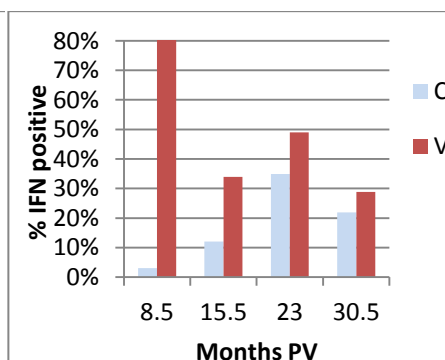


Figure 2: Proportion of goats showing positive gamma interferon responses (stimulation with avian PPD)

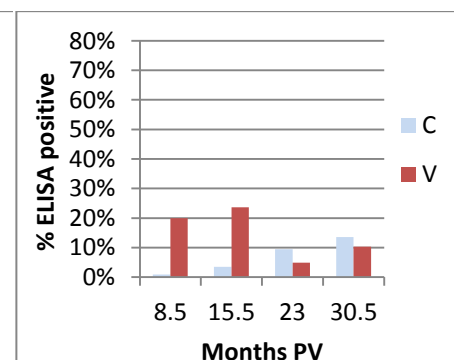


Figure 3: Proportion of goats showing positive ELISA responses

Discussion

Conducted on two commercial goat herds, this trial demonstrated a reduction in faecal shedding of *Map*, stimulation of immune responses following vaccination, and no adverse effect of vaccination.

Furthermore, there was a non-significant reduction in culling and in the proportion of culled goats with gross lesions of paratuberculosis. Similar results were observed with sheep under Australian conditions (Reddacliff et al., 2006). The trial design reduced the efficacy of vaccination. Firstly, the whole cohort herd was not vaccinated because we wished to assess the effect of vaccination against controls. Secondly, we examined only a single cohort of goats over a four- to five-year period. Any effect of vaccination on successive generations of kids over time is not therefore reflected in the results of the current study, which was a “worst-case scenario”.

To conclude, this trial demonstrated the efficacy of a killed vaccine in controlling paratuberculosis in French goat herds.

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Acknowledgements

This study was carried out with financial support from Région Poitou-Charentes (France). The authors wish to thank participating goat breeders.

USE OF ANIMAL IDENTIFICATION AND MOVEMENT SYSTEM (AIM) DATA TO ASSIST RISK ASSESSORS IN A JOHNE'S CONTROL PROGRAMME

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As part of a Johne's Control Programme it is very important that a risk assessment is carried out on participating farms. Cattle movements are the main risk of introduction of Johne's Disease in to a herd. The Irish Department of Agriculture has maintained a database of all cattle movements in the country for the last ten years. A system has been set up so that this movement data is available to Johne's Control Programme risk assessors. At the end of each year all movement data is downloaded to a separate database, which can be queried on line by the risk assessor. This will allow the assessor to determine the risk level

The United States Animal Health Association, Johne's Disease Committee revised their risk assessment manual, "How to Do Risk Assessments and Develop Management Plans for Johne's Disease" in 2011.

Available at

(<http://www.johnesdisease.org/Risk%20Assessment%20&%20Management%20Plans%20for%20Johne's.pdf>) The table on how to rank risk on purchased animals is reproduced below. Until there is a certified control programme in place in Ireland, all purchased animals would fall in to category 3 or 4.

Additions and replacements	Number of Animals				
	1-5	6-12	13-20	21-50	>50
1. Get additions or replacements from level 2-4 status herd	0	2	4	6	8
2. From low-risk herds, level 1 or pre-tested herds	10	11	12	13	14
3. From single-source non-tested or non-programme herds	20	22	23	26	28
4. From multiple sources, non-tested or non-programme herds or markets	30	34	36	38	40

Fig. 1. Risk factors from additions and replacements.

Since the mid-1950s, the Department of Agriculture, Fisheries and the Marine (DAFM) has registered each cattle herd, with a unique identification herd number. The Animal Identification and Movement (AIM) system contains details of all bovine births from 1996 onwards. Data relating to imports, movements, slaughterings, exports and deaths is complete from the year 2000 onwards. At the end of December 2009, the bovine population in Ireland was distributed between 123,500 herds. In total, there were 6.5m animals on the database: including 3.4m females and 300,000 bulls aged 12-months-of-age or over (NBAS Division, 2009).

All movements in to the fifteen herds with ≥ 4 ELISA positive animals, in the five years preceding the study 2004 – 2008 were accessed from the AIM database.

Herd	County	Enterprise	No. cattle	No. +ve	2004	2005	2006	2007	2008	Total	% bought in
A	Louth	Dairy	702	13	113	17	5	44	4	183	26.0%
B	Meath	Dairy	279	10	27	66	0	0	268	361	129.4%
C	Meath	Dairy	179	10	3	0	0	7	1	11	6.2%
D	Meath	Dairy	335	7	0	45	61	0	6	112	33.4%
E	Kildare	Suckler	47	6	1	3	1	0	0	5	10.6%
F	Meath	Suckler	49	5	7	6	1	2	1	17	34.7%
G	Cavan	Suckler	36	5	9	22	53	41	13	138	383.3%
H	Westmeath	Suckler	57	5	1	4	0	1	2	8	14.0%
I	Limerick	Suckler	251	4	124	31	86	39	48	328	130.7%
J	Cork	Dairy	71	4	1	3	0	0	0	4	5.6%
K	Cork	Dairy	207	4	18	20	9	6	2	55	26.6%
L	Clare	Suckler	45	4	63	55	24	22	3	167	371.1%
M	Longford	Dairy	54	4	17	19	8	9	4	57	105.6%
N	Cork	Dairy	78	4	1	0	1	1	0	3	3.9%
O	Kerry	Suckler	233	4	29	13	105	13	6	166	71.2%

Fig

2. Number cattle moved in to each herd 2004 – 2008.

HERD	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Friesian Males under 1	2	3	0	1	0	0	1	0	1	0	0	0	0	0	0
Non Friesian Males under 1	0	0	0	0	1	0	20	2	33	0	1	34	3	0	0
Friesian Females under 1	15	9	0	0	0	0	0	0	0	0	1	0	0	0	0
Non Friesian Females under 1	0	0	0	0	0	4	10	0	9	0	3	9	1	0	0
Friesian Males 1 to 3	0	2	3	1	0	0	2	0	2	0	1	0	0	0	96
Non Friesian Males 1 to 3	0	1	1	0	3	3	58	2	133	0	6	83	15	3	49
Friesian Females 1 to 3	78	98	1	81	0	0	1	0	0	0	32	0	13	0	13
Non Friesian Females 1 to 3	0	1	0	29	1	10	37	4	106	3	8	31	1	0	7
Friesian Males over 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Non Friesian Males over 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Friesian Females over 3	88	245	6	0	0	0	0	0	3	0	3	0	24	0	0
Non Friesian Females over 3	0	2	0	0	0	0	9	0	41	1	0	10	0	0	0
Total Bought In	183	361	11	112	5	17	138	8	328	4	55	167	57	3	166

Fig 3. Ages and types of bought in animals.

This study clearly shows the high levels of movements into multiple-positive herds. We plan to look at whether the level is as high in herds that don't have ELISA positive animals and try and convince dairy farmers to have as few animals entering their herds as possible.

USE OF A LEARNING MANAGEMENT SYSTEM (MOODLE) TO PROVIDE ONLINE TRAINING FOR RISK ASSESSORS IN A JOHNE'S CONTROL PROGRAMME

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As part of a Johne's Control Programme it is very important that a risk assessment is carried out on participating farms. Risk assessors must have an adequate knowledge of JD in order to carry out the assessment. A series of thirteen online lectures were provided through an Open Access Learning Management System (Moodle). A day long seminar was held at which the lectures were delivered by international speakers. The audio was recorded and synchronised with the PowerPoint slides, which were then uploaded to the Moodle server. In addition to the lectures facilities available in Moodle that were utilised included forums, glossaries, quizzes, workshops and surveys. On completion of the lectures participants had to get a passing score in a multiple choice questionnaire and also produce a PowerPoint synopsis of three recently published articles on Johne's Disease before continuing to the next module. The second module consisted of four lectures on risk assessment. Once they had obtained a passing score on this module they were assigned to a herd to carry out a risk assessment under the direction of a monitor. If this was successfully completed they were certified as a Johne's Disease Risk Assessor.

Moodle is available on free websites at : <http://www.e-socrates.org>; <http://www.ninehub.com>

As part of a website maintained by one of the authors Moodle hosting was available for an additional €120 p.a. Moodle has also been installed on the Department of Agriculture's Intranet.

Links were provided to several documents on the Internet including:

- USDA Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program
- A guide for Johne's disease risk assessments and management plans for beef herds
- A guide for Johne's disease risk assessments and management plans for dairy herds
- How to Do Risk Assessments and Management Plans for Johne's Disease

They were then asked to pick two articles from a reading list and develop a ten-minute PowerPoint to be shown to their farmer clients based on the two articles.

Reading lists were developed both for Department of Agriculture employees who were subscribed to several journals through a library subscription to Science Direct and also for those looking for articles on the Internet that did not require a subscription. Articles were freely available through journals such as Journal of Dairy Science, Journal of Clinical Microbiology, Journal of Veterinary Diagnostic Investigation, Veterinary Pathology Online, Veterinary Microbiology, Applied and Environmental Microbiology, Journal of Clinical Microbiology, Clinical Diagnostic Laboratory Immunology, Journal of the American Veterinary Medical Association, Journal of Animal Science.

Participants in the course took 14 different modules

1. Introduction to Course:
2. Irish Johne's Disease Prevalence Studies
3. Johne's Disease Diagnostic Tests
4. Isolation and detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from cattle in Ireland using both traditional culture and molecular based methods

5. Risk factors for the introduction and within-herd transmission of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection on 59 Irish dairy herds
6. Risk factors associated with Johne's Disease test status in dairy herds in Ireland
7. Training veterinary practitioners to carry out risk assessments as part of a Johne's control programme
8. Herd Health Pilot Project
9. Control of Johne's: The perspective of a dairy co-op
10. Control of Johne's: The perspective of the vet and the dairy farmer
11. Animal Health Ireland's plans for Johne's control
12. *Mycobacterium avium paratuberculosis* (MAP) and Public Health
13. Control of Johne's Disease: The French perspective
14. How to carry out Risk Assessments in Johne's Control Programmes

At the end of each module they took a ten question multiple-choice test and at the end of the full course an exam based on these fourteen questionnaires.

On successful completion of the end of course exam and production of the PowerPoint participants were able to print off a course attendance certificate and the Veterinary Council was notified of their success. The Veterinary Council of Ireland has approved e-learning for Continuing Professional Development and it is expected that this course will be assigned 6 CPD points.

SUCCESSSES AND FAILURES OF A GOVERNMENT SUBSIDISED PILOT JOHNE'S CONTROL PROGRAMME

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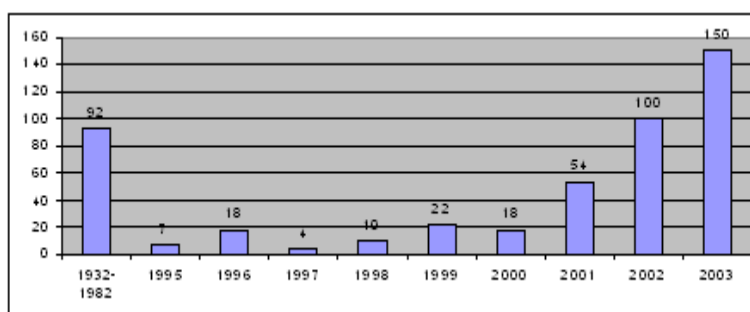
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A pilot herd health programme was established in 2004, which was subsidised by the Department of Agriculture who paid a fee to practitioners for a risk assessment on three to four herds each. Laboratory testing was free to farmer participants. Thirty-five vets attended an introductory course and twenty-two enrolled clients. Sixty-eight risk analyses were carried out on herds. Sixty-three of these herds decided to participate in a Johne's Disease control programme, nineteen in BVD and six in IBR. Following the initial farm visit and risk assessment, a sampling strategy and disease control plan was put in place.

Johne's Disease (JD) was included in the pilot herd health programme because of its increasing incidence worldwide and the possibility that it might be a zoonosis. This was of major concern to the dairy industry in Ireland. JD had been increasing in incidence in Ireland in recent years. There had been ninety-two positive faecal samples submitted between 1932 and 1982, but there were one hundred and fifty in 2003. Much of this increase was due to the large number of cattle that were imported in the intervening years.



Faecal positive samples in Ireland (1932 – 2003)

VET ASSESSMENT OF PILOT PROJECT

An evaluation of the pilot project was carried out by submitting questionnaires to the thirty-one vets initially participating in the scheme and their herdowners. Vets were also asked to repeat the risk analysis for each herd. Sixteen replies were received from the vets. The following questions were asked.

- 2) Five took blood samples and ten took both blood and faeces.
- 3) If you did not take faecal samples, why did you decide not to?
 - a) On the three farms a low number of Johne's ELISA +ve animals were uncovered. The farmers undertook to cull these at the time regardless of how the faecal samples would have come back.
 - b) Bad experience from previous sampling. Concentrated resources on heifer rearing and management.
 - c) Results not very accurate and too slow.
 - d) Too time consuming. Not practical would not get farmer cooperation
 - e) Took mostly bloods for two reasons
 - i) faecal sampling is a very messy process.
 - ii) Farmer unwilling to be "culture positive" vs. antibody positive with legislative issues.

4) What did you do if you got a positive on a blood sample?

Almost all vets recommended culling, some reviewed previous titres before deciding and others confirmed with a faecal sample. One vet said he recommended management change at calving time on positive animals and culled as early as possible.

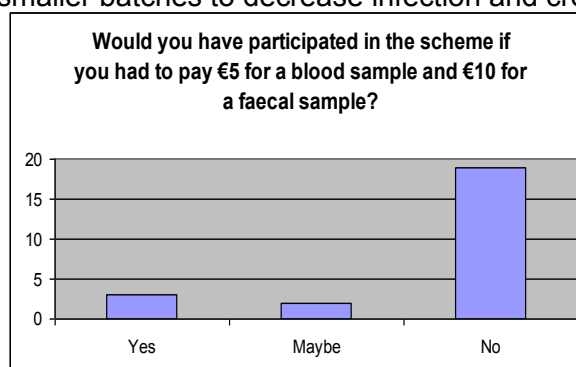
5) What did you do if you got a positive on a faecal sample?

Again most vets recommended immediate culling but one monitored animals and culled some, another recommended culling at an economically viable time.

- 6) If you didn't enrol clients for all three diseases, why not?
 - a) At the time I felt Johne's disease control was a priority.
 - b) Little clinical evidence. Lack of farmer demand.
 - c) Did not know the level and severity of IBR and BVD at the time.
 - d) JD was the primary concern in all three herds.
 - e) Farmers/vet "awareness" of IBR & BVD has increased over last year. Bulk milk and yearling blood sentinels offer "neat" introduction to BVD and IBR health programmes.

FARMER ASSESSMENT OF PILOT PROJECT

- 1) Almost all said they culled if they got a positive on a blood sample either immediately or at end of lactation. One herd owner isolated cow at calving but did not get rid of her. Five herdowners took faecal samples and culled depending on the result. One herd owner stated that "eventually positive cows got sick and I culled them as soon as possible."
- 2) Two herdowners said they culled immediately if they got a positive on a faecal sample. Others were not as proactive, if cows were in calf they allowed them to calve, if any have symptoms they were culled immediately. One monitored and considered when choosing culls at the end of the year. One herd owner said cow was dead by the time we got result. Another said all positive faecal animals were slaughtered and all progeny of all those cows slaughtered also.
- 3) What changes have you made in management of calves since joining scheme?
 - a) Bulls and heifers are separated. Heifers removed from dams immediately. Fed dam's colostrum for 3 days then fed milk replacer for 8 – 10 weeks. No pooling of heifers milk.
 - b) Cows removed from calves within 24 hours of calving.
 - c) Friesian replacements kept separate from birth and fed milk replacer throughout.
 - d) No more pooled colostrum to calves
 - e) Rearing calves in hutches and moving them to a clean out farm. Milk replacer and only two colostrum feeds given.
 - f) Changed from rearing Friesian replacement calves to beef calves.
 - g) Friesian heifers reared on milk replacer only, calf taken from the cow straight away, also Friesian Heifers reared in separate shed.
 - h) Insistence on individual calving. No JD positive colostrum fed to individual calves. Milk replacer fed to all calves from 24 hours. No calf grazing on home farm.
 - i) No longer pool colostrum.
 - j) No fresh milk fed to heifer calves. Heifer calves removed from cow after calving.
 - k) Try to keep bedding area clean water etc. Power hose out sheds.
 - l) No mixing of sucklers with dairy herd.
 - m) Once calves have received colostrum from their own mother they are removed. Improved hygiene in calving area. Calves only fed milk from mother.
 - n) Greater hygiene care was taken. As we have a suckler herd it was more difficult to isolate calves from cows. Stock were kept in smaller batches to decrease infection and cross infection.



HEALTHIER GOTAS, A PROJECT FOR ERADICATING CONTAGIOUS DISEASES IN NORWEGIAN GOATS

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“Healthier goats” is a project with the purpose of eradicating paratuberculosis and two other diseases (caprine arthritis and encephalitis, caseous lymphadenitis) in the Norwegian goat population. The project started in 2001, and to date 369 farmers have joined the project. For eradication of the different pathogens, the technique of “snatching” kids is used. The kids are taken away from the mother and the “infected” barn as quickly as possible after birth. The kids are housed in a clean environment, given cow colostrum and raised separated from older animals. The old goats are kept until the lactating period is finished and are then slaughtered. Thereafter, the barn and the near surroundings are cleaned and disinfected. The sanitized flocks are monitored for infection with MAP by the IFN- γ test which has a high specificity ($Sp = 0.986$) and a higher sensitivity than an antibody assays in the early stages of infection. All animals older than 1.5 years are tested at least once a year for three years. Some herds with confirmed paratuberculosis before entering the programme were tested twice a year with the IFN- γ test and an antibody ELISA. After three years of negative IFN- γ testing, herds will be monitored by a bulk-milk ELISA. Animals with a strongly positive IFN- γ test and animals with a repetitive weak response are culled and subjected to pathological examination and cultivation. A re-infection has to date been confirmed in two herds. In both cases, breaches in the sanitation protocol were identified providing an explanation for the relapses. In addition, several individuals with a positive IFN- γ response from other herds have been culled. Minimal lesions compatible with paratuberculosis were seen in some animals but the infection was not confirmed. Whether these animals were truly infected and the removal prevented a relapse of disease in the herd is thus unknown. We conclude that “Healthier Goats” seems successful in sanitizing herds for paratuberculosis. The herds also show a significant increase in milk yield and animal welfare is improved. So far infection is only seen in two of the participating herds. However the success of the project must still be interpreted with some caution due to the chronic nature of paratuberculosis.

A WEB BASED SYSTEM FOR MANAGEMENT OF JOHNE'S DISEASE

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Abstract

This paper describes how a web based management program (www.myhealthyherd.com) was developed to manage the health of cattle herds and with particular emphasis on infectious diseases and Johne's disease control. The program is a complex relational database with a simple user friendly system interface based on traffic light scoring of risks and status. Simple online questionnaires allow the user to establish the risk of introduction and spread of disease within herds. Each question has a weighting and score and the resultant number creates a red, amber or green icon to indicate the risks. The risks are listed in priority order, graphically displayed and then they are combined with a surveillance result to generate a Prevalence report. The Prevalence report converts the test prevalence into approximate true herd prevalence and then based on the risks indicates the most suitable control strategy for the disease. Six possible control strategies are listed and selection of the most appropriate strategy provides the user with a wide selection of tasks thus providing a simple but effective control plan. The robustness of the control plan is further evaluated by the program and given a "robustness" traffic light indicator for the key control areas required for effective disease reduction.. Control plans which are likely to fail to control disease are readily identified and the user directed towards more appropriate tasks. Biosecurity and surveillance plans can be created based on the aspirations and resources of the farmer. This structured approach allows for all farm types to be included in a Johne's engagement program and is used in over 2500 UK herds

The program has multiple service levels with varying permissions allowing farmers, vets, monitoring organisations and labs to have different levels of access appropriate to their roles. Tracking of progress through the control program allows Johne's disease engagement and control to be monitored at farm, veterinary practice, regional or national level via a secure web portal.

Myhealthyherd Architecture and Principles

Myhealthyherd was created using the core principles of the BCVA Health Planner. The original BCVA Health Program was PC based and proved difficult to maintain and develop and was didactic in its approach and excluded the farmer from interaction with the health planning process.

Myhealthyherd was developed as a "cloud based" system with the main database stored on the National Milk Records servers in Chippenham. The database is a complex relational database with a simple user friendly system interface based on traffic light scoring of risks and status. Multiple distinct service levels were created all with specific levels of access to the database ranging from farmers, vets, consultants, students, labs and account managers which can operate with four different organisations (farms, vet practices, monitoring organisations or laboratories). The key modules within the program allow for complete management of infectious disease, creation of a shared health plan for farm assurance purposes and comprehensive economic evaluation of health outcomes.

The infectious disease module

The infectious disease module has been developed following the principles of the "four pillars" of disease management developed by Dick Sibley. The disease status of the herd for any disease is maintained by the relative strength of the four pillars (biosecurity, surveillance, biocontainment or control and the resilience of immunity of the herd)

This allowed the creation of a mathematical model to quantify relative risks of disease and also to allow the user to alter the strength of each pillar dependent on the farmer's resources and aspirations. This is vital to enable bespoke plans to be created. The key pillar of control are typically managed in the following order.

1. Biosecurity

The assessment of risks is the first pillar to be completed by the farmer. Simple online questionnaires allow the user to establish the risk of introduction of disease within herds. Each question has a weighting and score and the resultant number creates a red, amber or green icon to indicate the risks. The risks are listed in priority order, graphically displayed. This facilitates discussion on the most appropriate approach to the remaining three pillars of infectious disease management.

2. Biocontainment

The assessment of risks of within herd spread are completed following published literature estimates of importance of risks. These are again quantified and graphed. Within the Johne's module the risks for biosecurity and biocontainment are used to generate a Prevalence Report which converts the test prevalence into approximate true herd prevalence and then based on the risks indicates the most suitable control strategy for the disease.

3. Surveillance

The completion of the risks modules allows a logical creation of a surveillance strategy which matches the farmer aspirations and resources for management of the disease. The strategy selected can range from the basic to comprehensive. Surveillance results can also be uploaded into the program by the lab/ vet or farmer and copies of the lab results easily viewed. Reminders for surveillance tasks can be viewed and the icons change colour according to the due date.

4. Vaccination and Immunity

For many diseases vaccination is a core pillar for control. Options for Johne's vaccination management are available with storage of vaccination supplied to the farmer. Four vaccination strategies are readily displayed (ranging from comprehensive vaccination of target animals, partial vaccination, no vaccination despite risks or no vaccine required) complete with icon colour changes for overdue vaccination.

Disease protection and Control Plans

Seven possible control strategies are listed and selection of the most appropriate strategy provides the user with a wide selection of tasks thus providing a simple but effective control plan. The robustness of the control plan is further evaluated by the program and given a "robustness" traffic light indicator for the key control areas required for effective disease reduction. Control plans which are likely to fail to control Johne's disease are readily identified and the user directed towards more appropriate tasks. Biosecurity and surveillance plans can be created based on the aspirations and resources of the farmer. This structured approach allows for all farm types to be included in a Johne's engagement program and is used in over 2500 UK herds

Conclusions

Myhealthyherd has been created with specific aim to "make herds healthier" using an independent robust framework via the internet. For optimal use the farmer and vet work together to create an effective health plan for the herd. The "ask rather than tell" approach has helped improve the uptake and the use of a variety of control options for disease has broadened farmer engagement beyond what would be possible with the more traditional central didactic approach.

Acknowledgements

Myhealthyherd would not have been possible without the support from National Milk Records and the continued unflagging efforts of the Myhealthyherd team and programmer, Rob Dawkins.

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FERTILITY, UDDER HEALTH AND MILK PRODUCTION IN COWS THAT HAVE HIGH MILK ANTIBODIES TO PARATUBERCULOSIS

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Key words: Paratuberculosis, productivity, udder health, fertility, MAP antibodies

Introduction

This paper describes an observational study carried out on a 500 cow dairy herd undertaking a Johne's control programme.

The dairy herd comprising 509 adult Holstein Friesian cows was identified as being of high risk of Johne's disease using a predictive model incorporated within a herd health management system (myhealthyherd.com). Although the herd had high levels of biosecurity, there were high risks of spread of Johne's disease. These were due mainly to the seasonal calving pattern, with many cows sharing communal calving accommodation with a high risk of environmental contamination, had *Mycobacterium avium* subsp. *paratuberculosis* (MAP) shedders been present. A historic biosecurity risk was identified using the biosecurity assessment tool within the myhealthyherd software programme; a stock bull had been introduced some 10 years previously but had been culled due to weight loss and poor performance.

No clinical signs of Johne's disease had ever been diagnosed in the herd, but the risks were such that a screening test was performed using a targeted screen of 30 high risk cows. Two cows showed the presence of high levels of antibody against MAP using a milk ELISA test (IDEXX) which prompted a whole herd screen and the implementation of a control programme of regular testing and management of high risk cows identified by the regular screening.

Method

At the milk recording of May 2011, 48 cows in the herd were consistently testing positive (>20) for antibody against MAP using ELISA tests on their milk recording samples. These cows were identified using an automated testing programme (Herdwise, National Milk Records (NMR)) as having at least three positive tests in the previous six months. None showed clinical signs of Johne's disease. Milk yields, milk quality, somatic cell counts, and all fertility events were recorded by the farmer and the data was downloaded into a cow management programme for analysis (Interherd, NMR). Yields, somatic cell counts, and fertility events of the 48 MAP positive cows were compared with the rest of the herd and with the performance of the 48 MAP positive cows in their previous lactation when they were testing negative for antibodies against MAP. Yields were compared around the date of May 2011 to attempt to avoid the confounding factors that the herd is increasing in yield over time through better nutrition and management.

Results

Of the 48 cows testing positive for antibodies against MAP, 12.5% were 1st lactation, 16.7% were 2nd lactation and 29.2% were 3rd lactation cows. This compared to 27.5%, 24.1% and 20.4% of these lactation animals in the herd as a whole. This demonstrates that there is a high prevalence in 3rd lactation animals, as may be expected by the normal incubation period of the disease and the production of detectable antibodies later in the infection cycle.

The average 305 day yield of the herd as a whole was 10,130kg for completed lactations in the year prior to May 2011, and predicted (using standard lactation curves within the Interherd programme) as 10,203kg for cows in their current lactations in May 2011. The 305 day completed lactations for the 48 test positive cows (completed before they tested antibody positive) was 10,146kg, and the predicted lactations for these cows was 9,424kg. Because first lactation test positive animals had no previous lactation at the time that they tested positive, they were excluded from this analysis.

Third lactation animals had the biggest difference between yields, with the mean predicted production for the 3rd lactation being 10,059kg and 8,562kg for 3rd lactation cows testing positive. The most noticeable effect at farm level was that the test positive cows (easily identified by the presence of a red ear tag to enable their specific management to prevent spread) averaged 26kg of milk per day compared to the test negative cows mean production of 31kg per day over the period from calving to the recording date of 16th May 2011. There was considerable variation between individuals, with some cows producing more than their predicted production.

Somatic cell counts are monitored monthly for all cows in the herd. The lactation mean of the herd at the milk recording of May 2011 was 155,000 cells/ml. The mean somatic cell count in the previous lactation of all cows that tested positive was 270,000 cells/ml and the mean somatic cell count of test positive cows in their current lactation was 238,000 cells/ml. The calculated mean milk somatic cell count at the milk recording of May 2011 was 155,000 for the herd, while the 48 test positive cows had a calculated mean cell count of 334,000.

The management system used to prevent the spread of Johne's disease within the herd as part of the Johne's control programme includes the culling of cows that consistently test positive at the end of their lactations if their milk yield drops or they show signs of mastitis or infertility. Thus, some test positive cows are not submitted for service if they have already tested positive prior to their voluntary waiting period (normally 42 days), or are not pregnant by the time the third test result shows positive. Of the 48 test positive cows, 29 were submitted for service and conceived. The median calving to conception interval in these cows was 136 days, compared to 106 days for the herd as a whole, and 103 days for the cows in the herd that calved up to May 2011. The fertility effect was not seen in the lactation previous to testing positive, where the calving to conception interval was 106 days for the 48 test positive cows.

	Mean 305 day yield (Kg)	Mean somatic cell count (,000 cells ml)	Calving to conception Interval (Days)
Whole herd in current lactation	10,203	155	106
Whole herd in previous lactation	10,130	n/a	102
Test positive cows in current lactation	9,424	238	136
Test positive cows in previous lactation	10,146	270	106

Discussion

This commercial dairy herd is run efficiently and effectively by expert stockmen with considerable veterinary input to maintain the health and productivity of the cows. The high risk of Johne's disease was identified at an early stage by the risk analysis used in the health management programme provided as part of the veterinary service to the farm. Herd screening of targeted high risk cows detected a significant prevalence of infection as determined by the presence of antibodies against MAP in milk. A strategy of regular routine testing of all milking cows and the management of high risk animals to prevent the spread of disease was introduced to control the prevalence of Johne's in the herd. This required a significant financial investment for testing costs, veterinary advice, and the husbandry changes required to manage high risk cows. In this high yielding herd with expert husbandry, classical clinical cases of Johne's disease had not been observed or recognised, although it is likely that many had been culled prematurely due to associated health issues.

The financial investment and resources required for Johne's control can be justified by the association of subclinical disease with the presence of antibodies against MAP, demonstrating that subclinical Johne's disease has a significant effect on productivity, fertility and udder health. An economic cost can be applied to these effects, which justifies to the farmer that Johne's control is worthwhile, despite the prolonged period of investment that is required to reduce the prevalence to insignificance.

PARATUBERCULOSIS CONTROL IN THE CZECH REPUBLIC: 25 YEARS OF EXPERIENCE

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By 1990, paratuberculosis, almost unknown disease, and only in some breeds of cattle and sheep have been known. After cattle imports mainly from the then EU Member States in the mid nineties, the disease situation in the Czech Republic has changed dramatically. When controlling 150 herds of cattle, sheep and wild ruminants for paratuberculosis first serological tests and from 1992 the cultivation of faeces were used. Between 1992 and 1997, more than 65,000 serological tests and 33,861 faecal cultures were performed. Between 1998 and 2001, 50,073 faecal samples were examined by culture. The biggest problem in paratuberculosis control was the strict observance of separate rearing of calves intended for breeding and the removal of offspring of infected mothers. At that time, in 30% of the investigated herds (two negative culture tests performed in the faeces within 6-8 months in animals over 18 months) paratuberculosis was successfully controlled. From 2002 to 2006, 84,539 of faecal samples were examined by culture. Increasingly, however, the control of infected herds manifested in the overwhelming disincentives of cattle breeders. Therefore, between 2007 and 2008 the number of examined faeces samples decreased to 25,763. Breeders started to prefer a serological investigation by ELISA to control paratuberculosis. Since 2007, in selected breeds of cattle and wild ruminants qPCR started to be performed (8,500 were examined faeces samples). Disadvantage in terms of breeding are particularly high financial costs without compensation by the government, preventing wider implementation of this method into routine practice.

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic "AdmireVet" (CZ 1.05/2.1.00/01.0006; ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716202 and QH81065).

HEAT TREATMENT AND GAMMA-IRRADIATION OF BOVINE COLOSTRUM: IMPACT ON SURVIVAL OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) AND OTHER PATHOGENS AND ON IMMUNOGLOBULIN CONTENT

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Correct and profitable management of calves demands the use of high-quality colostrums microbiologically safe and immunoglobulin-rich.

Colostrum can be contaminated directly from infected udders or during its collection, storage and administration by *Mycobacterium avium* subsp. *paratuberculosis* (Map) and other pathogens. Different treatments of colostrums to reduce the risk of disease transmission without significantly affecting the level of immunoglobulins are under investigation (i.e. micro-filtration, pasteurization and gamma-irradiation).

In this study we evaluated the impact of pasteurization (60 °C for 60 min) and gamma-irradiation (5 and 10 Kilograys) on the survival of some pathogens potentially vehiculated by colostrum (Map 106 cfu/ml, *Mycoplasma bovis* 2x10⁵ cfu/ml, *E. coli* 1.6x10⁶ cfu/ml, *Salmonella spp.* 106 cfu/ml, *S. aureus* 106 cfu/ml, *S. agalactiae* 104 cfu/ml). Moreover, we also evaluated the effects of both treatments on immunoglobulins content.

Although both treatments dramatically decreased the level of pathogens (no colonies were found by culture methods) without reducing the immunoglobulin content, they gave rise to some practical problems. In particular, irradiated colostrum was less palatable to calves and required dedicated facilities.

On the other hand, for the pasteurisation, a rigorous control of the parameters was essential to avoid colostrum clotting.

Further studies are needed to assess the efficacy of both treatment on a larger collection of samples.

MEASURING THE IMPACTS OF A PARTICIPATORY APPROACH TO ACCELERATING THE ADOPTION OF PRACTICES AND PROCEDURES TO CONTROL JOHNE'S DISEASE (JD) ON ONTARIO DAIRY FARMS

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The purpose of this study is to effectively design, implement and evaluate a novel knowledge extension program for Ontario dairy producers, focusing on educating producers about innovative and effective management practices for the prevention and control of Johne's Disease (JD). A global search for improved extension methods identified a participatory practice based process called the Focus Farms Process, which has been implemented with great success in important dairy sectors in Australia and parts of the EU. Dairy producers across Ontario volunteered to participate in this process, which includes four separate full-day sessions, where small groups of producers are prompted to discuss and view JD from a variety of lenses (e.g. producer, veterinarian, consumer), using a wide variety of extension techniques (e.g. poster/oral presentations, expert speakers, farm tours, diagnostic tests). The evaluation of this process involves a pre/post survey, with a 'knowledge assessment' component, that is used to evaluate the changes in producer knowledge of JD. Also, pre/post risk assessments are completed by veterinarians to assess changes in herd risk as a result of adopting new management practices. Several focus groups will also be used to qualitatively assess the effectiveness of this process. This information will be compared to data from a group of producers that have not participated in this process, to understand the overall impact of this process. While this project is still ongoing, preliminary results suggest that the Focus Farm process is an effective method for disseminating information to dairy producers, increasing dairy producer knowledge about JD, as well as facilitating the implementation of management practices for the prevention and control of JD. Overall, the process to this point has been successful in addressing many 'knowledge gaps' within the dairy community and we anticipate that this process will become a preferred method for knowledge extension in Ontario.

COMPARATIVE EFFICACY OF 'INDIGENOUS VACCINE' AGAINST 'CAPRINE JOHNE'S DISEASE' VIS A VIS 'A COMMERCIAL VACCINE' IN A CHALLENGE MODEL

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Present study re-evaluated the protective efficacy of 'indigenous vaccine' in protecting the MAP infection in goats after 5 years of passage on solid Herrold's egg yolk medium.

Forty young goats was divided into three groups. Group I comprised of 10 'sham-immunized' goats, Group II comprised of 15 goats injected with 'Indigenous vaccine' and Group III comprised of 15 goats administered 'Gudair vaccine' (CZ Veterinaria, Spain) and were twice challenged with 3 X 10⁹ and 5 X 10⁹ bacilli of 'Indian Bison Type' strain of MAP (S 5) on 50 and on 270 days post vaccination, respectively. Two vaccines elicited similar cell mediated immune response and impact on the proliferation of PBMCs on vaccination and experimentally challenge by MAP S5 strain. Control goats (group I) at 200 DPV showed emaciation and depletion of body fat and mild to moderate lesions of focal / diffuse thickening of small intestine. Remaining goats sacrificed at 450 DPV showed thickening of small intestine in 5 cases each in group II and III with chronic catarrhal enteritis and shortening, thin, atrophied and ballooned villi with infiltration of mononuclear cells and epithelioid cells, some epithelioid cells fused to form giant cells. Mesenteric lymph nodes collected at 200 DPV sacrificed goat (Group I) revealed presence of oedematous fluid and focal infiltration of mononuclear cells with scattered presence of epithelioid cells and few giant cells and on 450 DPV showed infiltration of large number of MNC and epithelioid cells forming sheet like arrangements with presence of multinucleated giant cell. Body score at 200 and 450 DPV on the parameters of body conformation, carcass components and fat measurements revealed better condition (fat measurements) in vaccinated goats (groups II & III) than control (group I). Thus from this study it is conformed that Indigenous vaccine is effective in controlling the Johne's Disease.

CONTROL OF 'BOVINE JOHNE'S DISEASE' IN INDIAN CATTLE BY 'INDIGENOUS VACCINE' DEVELOPED USING 'INDIAN BISON TYPE' STRAIN OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* OF GOAT ORIGIN

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Mycobacterium avium subsp. *paratuberculosis* infection is endemic in low or non productive cattle population of country. Due to ban on cow slaughter cow shelters (goshalas) are home for some of low or unproductive cows. Efficacy of 'Indigenous vaccine' developed using 'Indian Bison type' strain of MAP of goat origin, was evaluated in 4 Goshals consisting of 19, 159, 680 and 37 cows in Herd A, B, C and D, respectively suffering with clinical Johne's disease. Prevalence of MAP in the herds was variable (20 - 85%) and management was below optimum levels (except herd D). Of 895 cattle in 4 herds, 704 were vaccinated and 191 were controls. Efficacy of vaccine was monitored for 6-12 months post vaccination by microscopy, ELISA, blood PCR, mortality and morbidity and histo-pathology. Physical health of cattle of herds improved after of 3-4 M vaccination. MAP shedding was restricted by 42.9, 29.6, 10.8 and 71.4% in herd A, B, C, and D, respectively. Presence of MAP in blood was also reduced by 31.4, 14.8, 6.7 and 57.1% in herd A, B, C, and D, respectively. High vaccine mediated sero-conversion was observed in vaccinated cattle as compared to control. Mortality was lower in vaccinated cattle. Morbidity also reduced in the 4 herds reduced noticeably within 3-6 MPV. Necropsy of 2 vaccinated and 3 control cows of herd B exhibited characteristic advanced lesions of JD. Study showed efficacy of indigenous vaccine in controlling JD in clinical cattle herds despite poor management. Herd D showed better recovery where management was optimum. Indigenous vaccine has potential to effectively control disease in low or non productive cow herds suffering with clinical BJD.

UPTAKE OF THE NATIONAL SHEEP HEALTH STATEMENT BY AUSTRALIAN SHEEP PRODUCERS: KNOWLEDGE, ATTITUDES, AND PRACTICES

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Background

This study was conducted to investigate current attitudes and practices of sheep producers towards sheep health management with a focus on the National Sheep Health Statement (SHS); an on-farm biosecurity tool for managing the risk of ovine Johne's disease (OJD) introduction, and other sheep diseases and parasites. The SHS includes detailed consideration of OJD risk through calculation of an Assurance Based Credit (ABC) score. Credits can be obtained towards the ABC score through vaccinating sheep against OJD, testing sheep for OJD, and the prevalence of OJD in the area (Prevalence Area) from where the sheep originate. SHSs are provided to potential purchasers when sheep are offered for sale.

The aims of this study were to explore current attitudes and practices towards sheep health management with a focus on producer biosecurity practices and use of the Sheep Health Statement (SHS) as the principal tool available nationally to support and protect producers from disease risks during the sales process.

The objectives of the research were:

- to determine the uptake and use of the National Sheep Health Statement as an on-farm biosecurity tool for managing the risk of disease and pest introduction.
- to identify any regional variation in responses and reasons for the variation to the responses, and
- to identify any possible drivers to assist the use of the Sheep Health Statement, and similarly, to identify any social or practical barriers to its uptake that may be amenable to change or influence.

Methodology

A questionnaire was developed by the research team, with the assistance of an expert panel. Survey interviews were conducted using a computer-assisted telephone interviewing (CATI) methodology in which the interviewer follows a script using a software application that is able to customise the interview based on the answers provided.

The questionnaire comprised 53 questions, and covered a range of issues, including details of buying/selling in the last two years (for purposes other than to slaughter), use of the SHS and perceived drivers and barriers to its use. A sampling frame was developed in consultation with industry experts to capture data from a nationally representative sample of sheep producers with 100+ sheep. A total of 870 interviews were conducted with producers identified as the main person responsible for animal health management decisions, from 1-18 March 2011. The survey response rate was 33%.

Results

Descriptive analysis was conducted, for the whole sample and for the sample cross-tabulated by State and by Ovine Johne's Disease (OJD) prevalence area. In addition multivariate logistic regression analysis was conducted to investigate factors associated with uptake of the SHS.

Some key findings from the survey are listed below

- More than half of producers operated closed flocks, in which no sheep were introduced, or partially-closed flocks, in which only rams were introduced.
- Two-thirds of producers buy from a single trusted vendor or small group of trusted vendors.
- The SHS, National Vendor Declaration (NVD), and agents were identified as the main ways to assure others of the health of sheep.

- Over half of producers used agents for ALL their purchasing, and around a quarter used agents for NONE of their purchasing.
- Three quarters of producers used agents for ALL their selling, less than 4% used agents for NONE of their selling.
- Around a quarter of producers had never heard of the SHS and 17% had heard of it but were not aware of any of its content, i.e. around 40% were ignorant of it.
- Use of the SHS was typically 'all or none' with, very roughly, half of producers using it all the time and half using it none of the time.
- The SHS was regarded favourably with 70% of producers, who were aware of it, reporting that it was an effective tool for disease management.
- Agents were regarded as influential in the use of the SHS, with most producers reporting they were willing to supply or request a SHS if their agent told them to.
- Stronger enforcement and education/awareness programs were identified most frequently as ways to encourage uptake of the SHS.
- Current on-farm management practices appeared to be high, especially for inspection, disease monitoring, and movement recording. Some hygiene/cleanliness practices were less widely employed.
- Correct knowledge of current OJD prevalence area was poor with accuracy ranging from 17% to 63%, although producers were highly confident that they were correct. Producers generally tended to underestimate their OJD prevalence area level.

Analysis of the data indicated many differences between producers from different States and OJD prevalence areas. Logistic regression analyses identified the main factors associated with uptake of the SHS. These factors related to operating climate, demographic, and attitudinal factors; specifically whether use of the SHS was mandatory or not, whether agents requested the SHS, producer gender, producer confidence in accurate completion of the SHS, reliance on the OJD Assurance Based Credit (ABC) points system for guiding purchases, and perceived ability to decide on health status by direct inspection.

Recommendations

The main barriers to SHS uptake were around its implementation within State, i.e. whether it was mandatory, or not, and poor levels of awareness and knowledge of it. Those who used the SHS more tend to have more positive attitudes to it and identify more benefits in its use. If uptake of the SHS is to be improved the approach to its implementation needs to be harmonised to simplify the communication with the sheep industry; currently sheep producers receive mixed messages and this is likely to weaken its uptake. In addition there is a clear and ongoing need to raise awareness of the SHS and its potential benefits to producers and the industry more widely.

In terms of the current effectiveness of the SHS as an OJD risk management tool, the low levels of accuracy of producer self-reported current OJD prevalence area raise concern over the validity of information being provided for the ABC score calculation on the SHS. Changes were made to some prevalence areas on 01 January 2011, two months before the survey was conducted, and were thought to explain some of the confusion here, however further investigation did not support this as a major contributor. Given that accurate OJD prevalence area knowledge appears patchy and that the ABC score forms the basis for the national approach to management of OJD an audit of SHS data is recommended to confirm the extent of the problem.

SEROPREVALENCE OF PARATUBERCULOSIS AT AN ORGANIZED FARM IN SERIM-ARID REGION OF RAJASTHAN IN INDIA

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In India, Johne's disease is considered a serious problem more in the ruminants of the organised farms than those reared in villages and semintensive system. The disease has been reported in all domestic ruminants including sheep for more than seven decades. The infection has now widely spread to most sheep and goat farms and is now accepted as a intractable disease to control. In the present study, an organized farm endemic for *M.a.paratuberculosis* infection for the last few years was screened by an in-house absorbed ELISA. This farm was screened two years ago by a commercial ELISA and the overall incidence was found to be 10.3%. Faecal, serum and blood samples were collected from all the sheep (n=546) maintained at this farm. 16.5% of 546 serum samples tested were found to be positive for MAP antibody. The incidence of MAP was higher in hogget females (45.2 %), followed by adult males (20.8 %), adult females (13.9 %) and lower in hogget male (5.6 %). Out of 250 faecal samples tested by microscopic smear examination, 58 (23.2%) sheep were positive for characteristic bacilli of *M.a.paratuberculosis* (MAP). The results suggested that, testing and culling of ELISA positive animals of the farm two years ago, did not lead to reduction in the incidence of the MAP infection and repeated testing is required to reduce the infection at organised farms.

EVIDENCE FOR IMPORTANCE OF MANAGEMENT INTERVENTION IN ADDITION TO PROLONGED VACCINATION FOR CONTROL OF OVINE PARATUBERCULOSIS

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Gudair™ vaccine was registered in Australia in 2002 for the control of ovine Johne's disease (OJD), based on research that showed that the numbers of vaccinated sheep that died of OJD or shed mycobacteria declined by 90% in the first generation of vaccinates (Reddacliff et al, 2006). Vaccination and a risk based trading scheme are now the main management tools used in most states of Australia, but how well does the vaccine perform in the field in decreasing risk of disease and transmission? Experience has shown that vaccination against OJD quickly eliminates the significant mortalities in infected high prevalence flocks. However a series of applied research projects that sought to obtain information on the level of shedding of mycobacteria from vaccinates as determined by pooled faecal culture of 350 sheep using 7 pools of 50 (PFC350), has identified that shedding persists for many years after commencement of vaccination. In a study of 40 flocks in NSW and Victoria that had been vaccinating for at least 6 years, at least one positive pool was present from 82.5% of flocks. Further, in a longitudinal study of 12 NSW flocks, it was found that in several flocks shedding rates increased over time. Studies of risk factors in these flocks indicated that management factors such as straying sheep, leaving wethers unvaccinated (Eppleston et al, 2011) and introduction of unvaccinated sheep were high risk practices. Biosecurity with attention to straying sheep in addition to persisting with vaccination of all sheep in a flock are suggested as important management interventions to optimize the protection offered by Gudair™ in preventing recrudescence of mortalities and transmission of OJD by sale of sheep from infected properties.

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BOTTLENECKS IN BEST MANAGEMENT PRACTICES IDENTIFIED IN THE ALBERTA JOHNE'S DISEASE INITIATIVE (AJDI)

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Introduction

Efforts to control Johne's disease (JD) using test and cull based methods have not lead to successful control of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) transmission on dairy farms (Groenendaal et al., 2002). Transmission models and previous research have shown that implementation of specific management practices that focus on limitation of contact of susceptible animals to manure of infectious animals can decrease the within herd prevalence of MAP infection (Ansari-Lari et al., 2009; Nielsen and Toft, 2011). Therefore, recently implemented JD control programs are based on identification of weaknesses in management and subsequent implementation of best management practices to control the disease. The aim of this study was to identify common management practices on Alberta dairy farms, and to assess whether there are differences in management between MAP-positive and negative farms.

Materials and Methods

Data for this cross-sectional study was collected in the Alberta Johne's Disease Initiative (AJDI), a voluntary control program launched by Alberta Milk and coordinated by the University of Calgary (McDonald, 2009). To participate in the program the herd veterinarian takes six environmental samples at different locations on farm and completes a 34-question risk assessment in which specific farm management practices are assessed. Additionally, up to three changes in management practices can be suggested which are recorded on the management plan. Samples, risk assessment and management plan are shipped to the University of Calgary where samples are processed using the TREK ESP[®] culture system II (TREK diagnostic systems, Cleveland, Ohio, USA) and IS900 PCR as a confirmatory test. Laboratory results and copies of risk assessment and management plan are returned to the veterinarian who discusses the results with the farmer. AJDI procedures are repeated in yearly intervals. Received risk assessments, management plans and environmental sample results were statistically analysed using STATA 11 (STATA Corp, College Station, Texas). Descriptive statistics were used to identify the most common management practices. Logistic regression using the environmental sample result as the outcome and the risk assessment score as the predictor was used to assess the relationship between herd MAP infection status and the use of specific management practices.

Results

As of January 2012, 201 dairy farms participated in the AJDI and 182 farms completed their risk assessment. Analysing answers to the questions in the risk assessment, several management practices that are known to be associated with the odds of MAP infection were identified in over 50% of the received questionnaires: 63% of the farms had no restriction of any visitor access to any animal, 77% of the farms purchased animals within the last 5 years without consideration of the MAP infection status of the seller herd, and 55% of the producers fed calves with bulk tank milk or with pooled milk from several cows.

159 farms had risk assessments and environmental sample results available. Using logistic regression, the following management practices were related to the infection status of the herd:

	% negative herds	% positive herds	P-value
Clinical JD has been observed on the farm	27	52	0.02
Manure is spread on pastures in which heifers graze in the same year	5	12	0.05
At least trace amounts of manure are visible at water troughs and feed bunks of lactating cows	58	76	0.06

Discussion

Most of Alberta dairy farmers did not consider the MAP infection status of the seller herd when they bought animals. In addition, more than half of the farms did not have any restriction of any visitor access onto their farm. This attitude contains a big hazard for introduction of MAP and other infections on a dairy farm (Franklyn, 2011). Feeding of pooled milk from several cows to calves was identified as common practice but did not occur in significantly different frequencies at infected compared to uninfected herds. However, a Danish study reported that use of waste milk for calve feeding influences the JD within herd prevalence (Nielsen and Toft, 2011).

Worldwide, several research projects have found associations between the MAP herd infection status and specific management practices (Chi et al., 2002; Diéguez et al., 2008; Hirst et al., 2004; Johnson-Ifeorlundu and Kaneene, 1998). Results of this study suggest that the manure management and hygiene practices on farm have an impact on the spread of MAP. With an increasing number of participating Alberta dairy herds, the power of this study will also increase. It is very possible that associations between other management practices and MAP infection status will appear when the majority of Alberta dairy herds are included in the AJDI. This will help to adjust the focus of control of MAP infection on dairy farms.

Acknowledgements

The authors would like to thank lab- and field technicians for sample processing and administrative work. Funding for this project was provided by Alberta Milk and Alberta Livestock and Meat Agency (ALMA). This presentation received the Johnes's Disease Integrated Program (JDIP) USDA-NIFA Award (No. 2008-55620-18710) and an award of the University of Calgary (University Research Grant Committee). A big thank you goes to the veterinarians and producers for their participation in the project.

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PERSPECTIVES AND PROGRESS ON: CONTROL PROGRAMS

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In considering the output of the Control Programs theme and providing a perspective on recent progress it is worth considering this theme in context of the rest of the Colloquium. This Colloquium showcases the vast array of international research and activity taking place in relation to *Mycobacterium avium* subsp. *paratuberculosis* (Mptb). It is a sobering thought to realise that most of the program (perhaps 80%) is dedicated to a wide range of research activities in fields as diverse as epidemiology, diagnostics, host response and immunology, pathogenomics, genotyping, public health and environmental biology, for the primary purpose of providing tools and methods for the control of this agent! The presentations and posters presented in this theme build on previous work in these underpinning disciplines and provide a demonstrable outcome for the research funds expended.

At previous colloquia we have seen concepts, ideas and developing regional and national programs, as well as application of a variety of approaches for on-farm control. This Colloquium continues that trend but extends into the realm of evaluation or review of more mature programs. As a snapshot of where “control” is up to, I have broadly classified the 41 abstracts submitted for this theme as: 15 on program evaluation & review; 5 on program planning & implementation; 5 on education and training; 4 on on-farm control and 12 on vaccination.

Obviously there is some blurring across categories, and particularly the vaccination category could also be classified under some of the other headings, as several of the abstracts relate to the role of vaccine in on-farm control or in national and regional programs. So what does this tell us?

Firstly, it is apparent that there are now a number of national or regional programs that are undergoing evaluation and review. An important aspect of program evaluation is that of looking at what aspects have worked well and what has been less successful, so that programs can be modified and improved going forwards. As well as evaluation of existing programs, it is also evident that the need for paratuberculosis control in livestock continues to be recognised, with several papers describing planning and implementation of new or ongoing programs.

Another category which I feel is growing in importance and is now well represented in the program is that of education and training, either for farmers or practitioners (or both). The best program in the world will fail if key participants don't understand or support it. Education/training has been well recognised for many years as essential for the success of any disease control program and it is an indicator of progress to see active research and implementation in this area for paratuberculosis control programs. This is particularly important for a disease where research for many years has been dominated by “hard” science being undertaken in laboratories and then translated into rules-based programs for implementation without adequate communication and education of the target group.

Finally, the number of abstracts relating to vaccination obviously warrants some discussion. Although there were 12 abstracts relating to vaccination in total, this is somewhat misleading, as several research groups submitted multiple abstracts in this area. Nevertheless, this is an important area of research, as vaccination provides one of the few potential tools for effective on-farm control of the clinical and production effects of this disease. This is an important area of research and one which is being watched with interest.

So, where to from here? What are the challenges on the horizon for control programs? How might we pre-empt or overcome those challenges? Firstly, let us briefly consider the principles of disease control. From a scientific perspective, for effective control we need to be able to:

- **Detect the disease or infection:** Current tests still have quite poor sensitivity for early infections. Research is ongoing looking for new diagnostic methods, particularly for early detection before onset of shedding or clinical signs and for tests able to differentiate animals that will succumb to clinical disease from those that remain latent or eliminate the infection.
- **Prevent new cases:** Currently we rely on segregation of new-borne animals at birth and/or vaccination to prevent new cases. Segregation is effective in theory but implementation can be problematic and is an important area of research and education to try and improve uptake and effectiveness. Vaccination is an important but imperfect tool. While it appears to be successful at reducing clinical disease, it does not

prevent infection and ongoing transmission from vaccinated animals. An important area for research is to produce a more effective vaccine, ideally one that prevents rather than suppresses infection.

• **Prevent spread of infection between farms:** In general, paratuberculosis spreads mainly through movement of infected animals. Therefore, in the absence of effective tests for early diagnosis and effective measures to prevent on-farm transmission, the only reliable tool for preventing farm-to-farm spread is to limit animal movements off infected farms to either slaughter or to farms with a similar risk profile. This causes disruption to trade and significant losses for some producers and is therefore a source of angst and resistance to program implementation.

From a scientific/research perspective, the keys to effective control are therefore a better test and a better vaccine. This is not new and is the underlying basis of the many millions of dollars currently being spent internationally on paratuberculosis research each year.

If there are no immediate solutions for these technical issues forthcoming, what other challenges are there and what might be required to address them?

Other factors that should be considered in any disease control program include:

• **Economic cost and funding sources:** Governments in many countries are increasingly reluctant to fund disease control programs, particularly where much of the benefit is seen to flow directly to the affected industry. This is resulting in an increasing reliance on government-industry partnerships or even direct industry-managed programs. The challenge here is to find an appropriate model (and funding) for the specific circumstances in individual countries and industries.

• **Adequate veterinary infrastructure:** Any technical program requires trained professionals to implement the program. Again, with increasing government constraints and the move to industry-based programs, the support of government veterinary services is no longer assured. Alternative approaches using private veterinarians for program management and implementation are increasingly common, but again face the challenge of finding suitable funding and management models, as well as ensuring commitment, technical knowledge and understanding of the program in veterinarians already under significant other workload.

• **Producer and community support:** For a program to succeed it is essential that it has support from the affected industry(s) and from the community in general. Without widespread support the program is likely to struggle to gain momentum and can easily fail to meet its objectives. This is a significant challenge for paratuberculosis control, particularly where programs may be complex and to be effective must disadvantage some producers, often through no fault of their own. The challenge here is to maintain communication with producers and the public; to educate them on key aspects of the disease and the program and to engage them in constructive discussions to help assure program success. This is already starting to happen, as evidenced by the presentations to this Colloquium.

In conclusion, control programs in many countries are maturing, while others are still in the implementation phase. These programs depend on the ongoing support of the research effort and also on the continued communication and education strategies and development of new models for program funding, management and support.

**PATHOGENOMICS AND *MYCOBACTERIUM AVIUM* SUBSP.
PARATUBERCULOSIS BIOLOGY**

KEYNOTE ON: 'PARATUBERCULOMICS' TO DISCOVER BIOMARKERS OF INFECTION AND DISEASE PROGRESSION

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The term 'Biomics' groups all comprehensive studies of entire complexes of the main classes of biological molecules such as proteins, DNA, RNA, small metabolites, lipids or carbohydrates. In recent years, the suffix – omics has been increasingly used to define subareas or combinations of these fields of research. It is exactly that integration and merging of different '–omics' which gives the power to resolve complex questions about host pathogen interactions in Map infections.

'Paratuberculomics' is not only instrumental in teasing apart these interactions, but it also helps in the discovery of biomarkers for Map infection. From a purely diagnostic perspective such biomarkers are very useful to detect infected animals before they become infectious. However, biomarkers of disease progression and biomarkers of protection are also required as they would be very conducive to vaccine development. Biomarkers predictive of protection against infection could significantly accelerate vaccine candidate screening in ruminant infection models of Johne's disease (JD).

In this paper, some important 'biomics' will be discussed in relation to current Map research. First of all, **genomics** is used to study the full genome of both the host and the pathogen. While the entire of genome of Map was first published in 2005 by Li et al., the full bovine genome was only described in 2009, simultaneously by the Bovine Genome Sequencing and Analysis Consortium and by Zimin et al. Both studies have contributed immensely to the study of JD. On the bovine side, this has led to oligo microarrays and SNP chips and consequently to the discovery of alleles for genetic susceptibility of JD. On the pathogen side, this has also enabled countless studies and led to the development of Map specific research tools. However, only recently have more Map isolates been completely sequenced. A bovine, an ovine isolate, a human isolate and a collection of European isolates have now been sequenced. Many more isolates from different regions and host species will have to be sequenced to capture the full genomic diversity of this pathogen. We initiated a project to determine the diversity of Map isolates covering all 10 Canadian provinces by multilocus short sequence repeat (MLSSR) genotyping followed by complete genome sequencing. This project has as objective to determine the contribution of genetic diversity of Canadian isolates to the transmission of the disease. Phenotypic and pathogenic characterizations are part of this pan-genomic approach. For this purpose, selected strains are compared by **kinomic** analysis in collaboration with Drs Napper and Griebel at the Vaccine and Infectious Disease Organization (VIDO). A relevant measure of phenotypic diversity within Map strains can be found in their interaction with the host. Responses of bovine monocytes to infection with different Map genotypes are used as an indicator of bacterial phenotypic variability. Dynamic patterns of protein phosphorylation in those monocytes provide reliable indicators of cellular responses and a variety of approaches are emerging to quantify cellular kinase (kinome) activity. In particular, the highly conserved chemistry of enzymatic phosphorylation permits rapid characterization of kinase activity when an appropriate substrate is available. A validated methodology for creating peptide arrays, based on genomic information, for kinome analysis of bovine species was previously created at VIDO in conjunction with a pipeline for data processing that adds significant power to this technology and its applicability in a high-throughput fashion. Collectively, the kinome analysis allows us to describe the detailed cellular responses necessary to provide specific insight into the precise, and potentially unique, molecular mechanisms by which different Map genotypes interact with the host.

Most of our other biomic studies are based on a calf infection model in which calves are orally infected with different doses, a high dose of 2 times 5x10⁹ or a low dose of 2 times 5 x 10⁷, of a K10-like (by IS900 RFLP) field strain of Map (Cow69). Calves were infected at different ages (2w, 3m, 6m, 9m, 12m) and individually housed till 17 months of age. Non-infected controls were housed under the same conditions. The **transcriptome** of blood cells of these calves was studied by oligo microarray (Affymetrix Gene Chip) at 3 month intervals to discover biomarkers of Map infection. Analysis of differentially expressed genes 9 months after infection between non-infected animals and animals infected at 2 weeks using Genespring software and Ingenuity Systems Pathways identified the following shortlist of

significantly affected pathways: cell trafficking, cell signalling and cell death. The differentially expressed genes had functions relevant to infectious disease, gastrointestinal disease and inflammatory response. Especially the leukocyte extravasation, T cell activation and ERK/MAPK signalling pathways were significantly upregulated. Longitudinal analyses of expression levels of individual genes by microarray and qPCR were compared to the onset of shedding, humoral and cell mediated immune response in the experimentally infected animals. These integrated analyses of gene expression and routine JD diagnostics allow determining the potential and value of newly discovered biomarkers of infection and disease progression. Currently the corresponding individual gene products are being evaluated as biomarkers as they could be more easily developed into tests. The gene expression in ileum and associated lymph nodes from the animals will be investigated by RNA sequencing.

CHARACTERIZATION OF AN EXPERIMENTAL GOAT MODEL OF PARATUBERCULOSIS

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Introduction: An experimental goat model of paratuberculosis was established which will be employed for the identification of novel diagnostic biomarkers and the clarification of pathogenetic pathways of MAP-infection in ruminants.

Methods: Baby goats from a paratuberculosis non-suspect flock were allocated to two control groups (n = 6 each) and four groups (n = 6-7) orally inoculated with a cattle derived field strain of MAP at two time periods (3-24 vs. 42-63 days p. n.) and with two different doses (10 times 10 or 20 mg of bacterial wet weight per dose), respectively. Clinical signs, body weight, MAP shedding, specific interferon-γ reaction to Johnin, and antibody formation were recorded regularly for about 50 weeks after the end of the inoculation period (pi). After necropsy, gross and histological lesions, distribution of mycobacteria in tissue sections as well as cultural recovery of MAP from gastrointestinal tract and other organs were examined.

Results: Body weight development did not differ between control and MAP-inoculated groups and only one inoculated goat developed cachexia indicative for paratuberculosis. MAP transition could be detected at least once per animal during the inoculation period seizing one month pi. The pattern of shedding after inoculation varied markedly among the animals from at least two times (n = 2) up to the whole course of the experiment (n = 5) with no clear group differences. The interferon-γ reaction to Johnin started at 12 wpi and peaked between 28 and 32 wpi in all inoculated groups with large individual variation. Antibody formation started at 16 wpi. Three animals from different inoculation groups did not form any antibodies. Granulomas with caseous necrosis were seen in Nll. mesenterici and ileocolici of 23 goats; multifocal granulomatous lesions occurred in the intestine of 20, in the Nll. hepatici of 5, in the liver of 2, and in the tonsil of 1 out of the 27 goats inoculated with MAP. Intralesional mycobacteria were detected in sections of Nll. mesenterici and ileocolici of 7 and intestine of 4 goats. MAP was isolated at necropsy from the organs of 24 animals.

Conclusions: Using high MAP doses and young age at inoculation, characteristic lesions of paratuberculosis were seen in 23/27 goats at one year pi and MAP was isolated from 24/27 goats. Infection remained subclinical in most of the infected animals. Course of antibody or interferon-γ response were not predictive for the outcome of infection.

INFLUENCE OF REPEATED CYCLES OF MECHANICAL AND PHYSICAL DISINFECTION PROCEDURES ON VIABILITY OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* MEASURED BY PROPIDIUM MONOAZIDE *F57* QUANTITATIVE REAL TIME PCR

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) was shown to have a high degree of resistance to large scale of chemical and physical procedures that are used for the elimination of bacteria. There is only little information about the efficiency of these procedures on MAP survival. Classical method for the determination of viability is culture. However, classical culture suffers from many inconveniences. In our laboratory, detection technique for the determination of MAP viability by propidium monoazide (PMA) *F57*qPCR was optimized and tested on MAP isolates stressed by different chemical factors. The comparison of data from culture and PMA *F57*qPCR showed that although both methods measure viability on different levels, their results are comparable.

Therefore, the aim of this study was to investigate influence of exposition/ concentration and repeated cycles of ultraviolet (UV) light radiation and different concentrations of chlorine and peracetic acid (PAA) on viability of three different MAP isolates.

Materials and methods

Experimental design

- » The isolates for testing were chosen with regard to the length of their culture *in vitro*: collection strain CAPM 6381, low passage isolate 8819 and high passage isolate 12146.

Tab. 1. Experimental stress conditions.

Stress factor	Treatment				
UV (min) at 44 mWs/cm ²	10	20	30	45	60
Chlorine (ppm) for 30 min	10	25	50	75	100
PAA (%) for 10 min	0.01	0.05	0.1	0.5	1

- » The treatments were repeated three times within three days with the overnight MAP cells recovery (Tab.1).

PMA treatment and *F57*qPCR

- » Biological triplicates of MAP isolates suspensions aliquots treated with the stress factors and controls were twice exposed to PMA with dark incubation for 5 min and light exposure for 2 min (Kralik et al., 2010).
- » After washing and heat lysis at 100°C for 20 min, cells were centrifuged and supernatant was used to *F57*qPCR (Slana et al., 2008).
- » Quantification of absolute numbers of MAP by *F57*qPCR was done according to the plasmid gradient
- » MAP viability was determined as the quotient of absolute numbers of PMA-exposed stress factor-treated cells and the PMA-exposed control (stress factor-untreated) cells (Kralik et al., 2010).

Statistical analysis

- » Null hypothesis assumed that the viability will decrease with the increasing concentration and number of treatments.

- » Exploratory Data Analysis showed that the most suitable statistical model for the analyses of experiments with UV and chlorine that will confirm or rebut the null hypothesis is Multiple Regression Analysis like General Regression Models (GRM).
- » The statistical analysis of PAA influence on MAP viability was performed by Two-way ANOVA with subsequent testing of statistical significance of partial differences by Tukey HSD test.
- » P-values lower than 0.05 were considered statistically significant.

Results

- » Increasing concentration/time of exposition of all stress factors tested and number of treatments reduced the viability of all MAP strains/isolates. Null hypothesis was confirmed.
- » In all MAP isolates was shown that repeated UV or chlorine treatment has much more significant influence of MAP viability than increasing of concentration or time of exposition.
- » Strain/isolate specific resistance to factors studied was observed.
- » Although isolate 12146 was the most resistant to one-time UV treatment, it was shown to be the most sensitive to the repeated cycles of UV treatment.
- » Laboratory strain CAPM 6381 was the most resistant to repeated chlorine treatments and increase of chlorine concentration.
- » PAA was shown to be a potent disinfectant in reduction of viability in all MAP strains/isolates.

Conclusion:

- » UV light exposure has only partial effect on MAP viability reduction. Similar effect was observed in other bacteria exposed to UV light (Nocker et al., 2007).
- » As could be expected, all MAP isolates were relatively resistant to chlorine.
- » PAA has the very profound effect on MAP viability and disinfection agents containing PAA will be effective in MAP removal.

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This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic “AdmireVet” (CZ 1.05/2.1.00/01.0006; ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716202 and QH81065).

MAP3776C CODES FOR A NOVEL IRON UPTAKE PROTEIN SPECIFIC TO MYCOBACTERIUM AVIUM PARATUBERCULOSIS

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During the evolution of *M. avium paratuberculosis* (MAP) from *M. avium hominissuis*, the organism developed altered growth phenotypes that are used to characterize the organism in the lab. While it is oft stated that growth of MAP is mycobactin-dependent, the ability of MAP to survive and replicate in its eukaryotic host suggests that MAP has acquired an alternative, mycobactin-independent means of obtaining iron. To study this possibility, we have isolated a transposon mutant of MAP K10, designated MAP::TnMAP3776c, by PCR screening a library of transposon mutants for interruption of genes within MAP-specific genomic islands. This mutant is expected to affect the function of a MAP-specific operon extending from MAP3776c to MAP3772c predicted to encode a siderophore-based metal import system controlled by a Fur-like transcription factor (MAP3773c). In the presence of mycobactin J, there was no difference in growth rate, colony morphology or microscopic morphology of the mutant. However, on ferric ammonium citrate (FAC) agar, the mutant had severely impaired growth and could not be serially passaged on this medium. qRT-PCR analysis demonstrated that expression of MAP3776c was undetectable in the mutant; expression of the genes MAP3775c-MAP3772c was increased in the mutant, consistent with the presence of a strong promoter at the end of the mariner transposon. These data indicate that at the mRNA level, the transposon only abrogated expression of MAP3776c. Complementation studies have been initiated, to test the hypothesis that restoring expression of MAP3776c will rescue the growth phenotype on FAC agar. Future studies will evaluate the effect of this mutation on growth *in vivo*.

IDENTIFICATION OF CHROMOSOMAL REGIONS ASSOCIATED WITH ANTIBODY RESPONSE TO MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN MILK OF DUTCH HOLSTEIN-FRISEANS USING A GENOME-WIDE ASSOCIATION APPROACH

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Heritability of susceptibility to Johne's disease in cattle has been shown to range from 0.041 to 0.159. Although the presence of genetic variation involved in susceptibility to Johne's disease has been demonstrated, the understanding of genes contributing to the genetic variance is far from complete. The objective of this study was to contribute to further understanding of genetic variation involved in susceptibility to Johne's disease by identifying associated chromosomal regions using a genome-wide association (GWA) approach.

ELISA test results of 684,364 individual cows from 12,077 herds from the Netherlands were analyzed to obtain sire estimated breeding values (EBV) for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) specific antibody response in milk using a sire-maternal grandsire model. For 192 sire EBV with a minimum reliability of 70%, a high density SNP panel (50k SNP chip) was available to perform multiple SNP analysis with a random polygenic effect and a random residual component fitting all SNP simultaneously.

Five SNP associated with MAP specific antibody response in milk were identified distributed over four chromosomal regions (chromosome 4, 15, 18 and 28). Thirteen putative SNP associated with MAP specific antibody response in milk were identified distributed over 10 chromosomes (chromosome 4, 14, 16, 18, 20, 21, 26, 27, 29). Results provide evidence for chromosomal regions involved in MAP specific antibody response in milk. This knowledge contributes to the current understanding of genetic variation involved in Johne's disease susceptibility and facilitates control of Johne's disease and improvement of health status by breeding.

INTESTINAL INFECTION OF CALVES WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* FOLLOWING AEROSOL CHALLENGE

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Abstract

In previous studies we have shown the existence of bioaerosols containing viable *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in dairy farms. To investigate if MAP exposure via the respiratory route leads to intestinal MAP infection 12 Holstein Friesian calves were used in a challenge study. Calves were randomly allocated to 3 groups. Six calves were infected with MAP by aspiration of aerosolized bacteria through the nose; 3 orally infected calves served as a positive and 3 as a no challenge control. The challenge was performed with 1×10^7 as a 9 fold trickle dose. Animals were euthanized 3 months after challenge and extensive tissue sampling for MAP culture was performed including lung and intestinal tissue and respiratory and intestinal tract lymph nodes.

No MAP was detected in tissue samples of negative controls. Positive control animals had 1 or 2 positive tissue samples of the intestinal tract. All nasal inoculated calves had at least one MAP positive sample of the intestinal tract (ileum, jejunum, adjacent lymph node). Additionally, MAP positive retropharyngeal lymph nodes were found in 2 calves whereas a positive tonsil was detected in 1 calf. These findings indicate that repeated nasal MAP challenge can result in intestinal MAP infection in susceptible animals.

Keywords: transmission experiment, *Mycobacterium avium* subspecies *paratuberculosis*, calf, bioaerosols

Introduction

The well-established transmission route of MAP is the oral uptake of the bacteria by susceptible calves (Sweeney, 1996). Results of follow-up studies of control programs confirmed that MAP prevalence could be reduced but not eradicated (Benedictus et al., 2008; Collins et al., 2010). Bioaerosols have been shown to contain viable MAP in dairy farms (Eisenberg et al., 2010). The dust containing viable MAP can easily spread within buildings housing several age groups of cattle including young animals susceptible to MAP.

Therefore, those bioaerosols represent a plausible infection route via oral uptake when the environment is explored by licking and suckling or after inhalation. After inhalation dust is trapped in the nose or can reach the alveoli depending of particle size. Only small amounts of dust and thus viable MAP can be taken up via bioaerosol at once, however, due to continuous exposure it might amount to an infective dose. In sheep, experimental intra-tracheal infection has been shown to be successful (Kluge et al., 1968). In calves inhalation has previously only been hypothesized as a possible route of infection (Corner et al., 2004).

The objective of this study was to investigate whether repeated nasal inoculation of a low dose of MAP leads to intestinal infection in young dairy calves.

Material and Methods

Twenty known MAP low prevalent farms (<5%) close to Calgary, Alberta, Canada were selected. Twelve calves of first and second lactation cows were collected. They were fed gamma-irradiated colostrum (Hamilton McMaster Nuclear Reactor, Ontario, Canada) and housed in individual pens. Calves were randomly allocated to 3 groups. Six calves were infected with MAP by aspiration of aerosolized bacteria through the nose with a nasal spray pump producing droplets of variable size for intranasal application; 3 orally infected calves served as a positive and 3 as a no challenge control.

To mimic a real life situation the dose administered was determined using data about dust concentrations in dairy farms, tidal volume of a calf and estimated MAP concentration of MAP detected in dust samples of dairy farms. This plausible respirable dose is at the same time one of the lowest to be infective when administered orally described in literature. The challenge was performed with 1×10^7 as a 9 fold trickle dose. Fecal samples were collected 3 days following inoculation and every 3 weeks throughout the remaining study period. Animals were euthanized 3 months after challenge and extensive

tissue sampling was performed including lung and intestinal tissue and respiratory and intestinal tract lymph nodes. Fecal and tissue samples were cultured in a liquid culture system and presence of MAP was confirmed by IS900 real-time PCR.

Results

Fecal samples of all groups were culture negative so no MAP shedding after inoculation or throughout the study was detected. Tissue samples of negative controls were negative. Positive control calves had 1 or 2 positive tissue samples of the intestinal tract. MAP was detected in tissue samples of all calves of the nasal inoculated group with at least one positive sample of the intestinal tract (ileum, jejunum, adjacent lymph node). The total number of positive samples of the intestinal tract varied between 1 and 6. Additionally, 2 calves of the nasal inoculated group had MAP positive retropharyngeal lymph nodes and 1 had a positive tonsil.

Discussion

These findings indicate that repeated nasal MAP challenge can result in intestinal MAP infection in susceptible animals.

Probably, the low dose administered per treatment probably led to undetectable passive shedding after inoculation in all inoculated calves. The short duration of the experiment made detection of MAP shedding due to infection very unlikely. The absence of MAP in tissue samples of negative control calves supports that measures taken during calf collection, husbandry and management were sufficient to prevent unintended infection of calves. Both, oral and nasal, inoculation procedures led successful to an intestinal MAP infection in all inoculated calves. Bioaerosols produced by the nasal spray pump are mainly trapped in the mucociliary system, presented to local lymphoid tissue and then swallowed so the oral infection route is re-established. The additional detection of positive retropharyngeal lymph nodes and positive tonsils after nasal inoculation indicate that at least part of the inoculated bacteria were able to pass the mucosal epithelial barrier of the nose and enter the reticuloendothelial system to migrate through the body. Although numbers of calves were too small for statistical analysis it seemed that the respiratory route was a more effective challenge compared to the oral infection route in this pilot study. A combination of MAP presentation to NALT and GALT may be responsible for this effect.

Although, these results cannot differentiate if MAP uptake occurred directly via the nose or through swallowing after mucociliary clearance these results indicate clearly that nasal inoculation leads to intestinal infection. Therefore, future control programs should consider taking into account dust bio-aerosols as a potential route of transmission between shedding dairy cattle and susceptible calves

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POLYMORPHISMS OF NOD2/CARD15, SLC11A1, CD209, SP110, TLR2 AND TLR4 GENES AND THEIR ASSOCIATION WITH BOVINE PARATUBERCULOSIS

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INTRODUCTION

Susceptibility to infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been associated with host immunity related genetic factors (1-4), as earlier proposed for human Crohn's disease and other mycobacterial species and intracellular pathogens infecting animals. The aim of this study was to identify polymorphisms in six innate immunity-related genes: *NOD2/CARD15*, *SLC11A1*, *CD209*, *SP110*, *TLR2* and *TLR4*, potentially associated with the genetic susceptibility to the infection in cattle.

MATERIAL AND METHODS

Novel and previously reported polymorphisms were genotyped from blood samples of slaughtered Holstein-Friesian cows using Taqman® OpenArray® methodology. The infection status was established by serological, microbiological and histopathological diagnostic methods using a commercial ELISA, culture and real time PCR from ileocecal valve (ICV)-distal ileum (DI) and jejunal caudal lymph-node (JC LN) and histological examination of three tissue sections (ICV-DI, JC LN and ileal LN). In total 1,031 individuals were investigated. The associations between the frequencies of genotypes and alleles for each gene and the paratuberculosis (PTB) status, according to serial and parallel combinations of the four diagnostic methods, were evaluated using Fisher's exact test at a significance level of $p < 0.05$. Odds ratios (OR) were estimated for significant phenotype-genotype associations.

RESULTS

Genotypes and alleles of *NOD2* (c.2886-14A>G: pAAvsAG=0.0394, pAvsG=0.0273; c.3020A>T: pAAvsTT=0.0393, pAvsT=0.0312; c.*1908C>T: pCCvsTC=0.0297, pCvsT=0.0398) and *SLC11A1* (c.1067C>G: pCCvsGC=0.0423, pCvsG=0.0335) genes, as well genotypes of *TLR2* gene (c.*457T>C: pCCvsCT=0.0266) were associated with PTB status according to parallel estimations while alleles of *CD209* (c.762T>C: pCvsT=0.0274) and *SP110* genes (c.575-88T>G: pGvsT=0.0396; c.587A>G: pAvsG=0.0175) were associated with PTB status according to serial estimations. The genotype CC of *TLR2* (c.*457T>C) gene resulted as the highest risk (OR=5.1576) since the other OR estimations were below 2.0000.

CONCLUSION

In conclusion, our study uncovers the implication of both previously proposed and novel candidate polymorphisms in the immunopathogenesis of bovine PTB by using a large sample and an improved characterization of PTB, due to the performance of post-mortem analysis of tissues.

ACKNOWLEDGEMENTS

This study was supported by a grant (AGL2006-14315-CO2) from the Ministerio de Ciencia e Innovación (Spain) and the Basque Government (Saiotek-10 program). Technical and human support provided by SGIker (UPV/EHU, MICINN, GV/EJ and ESF) is gratefully acknowledged. Patricia Vázquez was in receipt of a FPI doctoral fellowship (BES-2007-17170) from the Ministerio de Ciencia e Innovación (Spain).

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REGENERATING ISLET-DERIVED 3 GAMMA GENE IS STRONGLY EXPRESSED IN THE DISTAL ILEUM OF GUINEA PIGS AND CATTLE INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (Map) causes profound chronic enteritis, but not with mycobacterial species other than Map. Characterization of the differences in host-pathogen interactions between Map and other mycobacterial infections may help us to understand the pathogenesis of Map infection. In the present study, we have used DNA microarray and quantitative real-time RT-PCR (qRT-PCR) analyses to identify differentially-expressed genes in the intestine of guinea pigs (GP) inoculated with Map and other mycobacterial species, and analyzed the gene expression of bovine homolog in the intestine of cattle infected with Map.

MATERIALS AND METHODS

Experimental inoculation of mycobacterial species in GP

Twenty-one 4-week-old female GP were divided into 7 groups, and 9 GP of each group were intraperitoneally injected respectively with 6 strains of live mycobacterium species (Map ATCC19698, Map Kag-1, *M. avium* subsp. *avium*, '*M. avium* subsp. *hominissuis*', *M. intracellulare*, *M. bovis* BCG). At 2 weeks, 1 month and 2 months post-inoculation, the intestinal tract samples were collected from duodenum, jejunum, ileum, ileocecum, colon and rectum. We conducted pathology examination, isolation of Map from 10% intestinal tissues and detection of Map DNA by quantitative PCR. And then gene expression analyses was performed by DNA microarray and qRT-PCR.

Comparison of gene expression in the intestine of GP

In order to analyze the differences in the gene expression in duodenum of GP, we have compared them between Map-inoculated and control GP using the GP DNA microarray. We have also prepared specific primers of identified genes for further analysis and confirmation of differentially expressed genes with qRT-PCR. Total RNA was obtained from the duodenum of GP inoculated with 6 different species or subspecies of live mycobacterium and control GP. The level of gene expression was normalized with the amount of the internal positive control, β -actin in the same RNA sample, and the data were expressed as relative expression quantities of the targets compared to those of control GP. The data was analyzed quantitatively using the $2^{-\Delta\Delta CT}$ method.

Blast analysis

We have identified the differentially-expressed genes by comparing with a GP genome in the "Ensembl genome browser" using Blast analysis. We have also searched the homologous gene in bovine genome in the "NCBI".

Bovine Reg3G gene expression in the intestine of cattle

Five male Holstein calves were orally inoculated with the ileocaecal emulsion of a clinically affected cow. Following 3 to 4 years post-inoculation, the total RNA samples were collected from duodenum, ileocecal lymph node, 4 ileal portions at 10, 30, 50 cm and 1 m from ileocecal region, jejunum, jejunal lymph node, cecum and colon. We have compared relative gene expression between Map-infected and uninfected cattle using qRT-PCR.

Recombinant bovine Reg3G

The recombinant bovine Reg3G (rReg3G) protein was produced and purified using *E. coli* system. Rabbit was immunized with purified rReg3G for the preparation of polyclonal antibody. Furthermore, we have analyzed carbohydrate binding activity of rReg3G which is characterized by C-type lectins. The rReg3G was examined for binding activity to immobilized mono-or polysaccharide (mannose or mannan) in the presence or absence of calcium. Additionally a Dot-blot immunoassay was performed for the detection of the rReg3G binding activity to Map. The suspension of live Map or other *Mycobacterium avium* complex was spotted onto nitrocellulose membrane filters, these spots were incubated with rReg3G or buffer alone. Then after incubation with the polyclonal antibody against eReg3G, peroxidase labeled anti-rabbit IgG was added to the membrane filters. HRP was used as a substrate for visualization of the reaction.

RESULTS AND DISCUSSION

Differential gene expression in GP duodenum

Only the small intestine, particularly in the duodenum, from Map-inoculated GP was noticeably thickened by the infiltration of macrophages and plasma cells, and multinucleate giant cells were observed in the lamina propria mucosae of intestinal villi. A small amount of Map DNA was detected from the duodenum, but not for live Map organisms. More than 30 genes were identified from 15,679 genes by the DNA microarray analysis of differentially expressed genes in the duodenum inoculated with Map and control GP. Following qRT-PCR confirmed that the most up-regulated gene in GP inoculated with Map was the regenerating islet-derived 3 gamma (Reg3G) gene in comparison to those of other mycobacterium species and subspecies ($P > 0.01$). These results appear to suggest that increased expression of Reg3G gene may associated with pathological changes at duodenum of GP inoculated with Map.

Relative expression of bovine Reg3G in the distal ileum of cattle

Even though bovine Reg3G was expressed in the intestine of normal healthy cattle, especially in the jejunum and ileum, Reg3G gene expression was strongly increased in the distal ileum of cattle infected with Map (Fig 1), where the mycobacterial granuloma and Map organism were typically found. The gene expression was not-detected in mesenteric lymph nodes and the large intestine of cattle infected with Map.

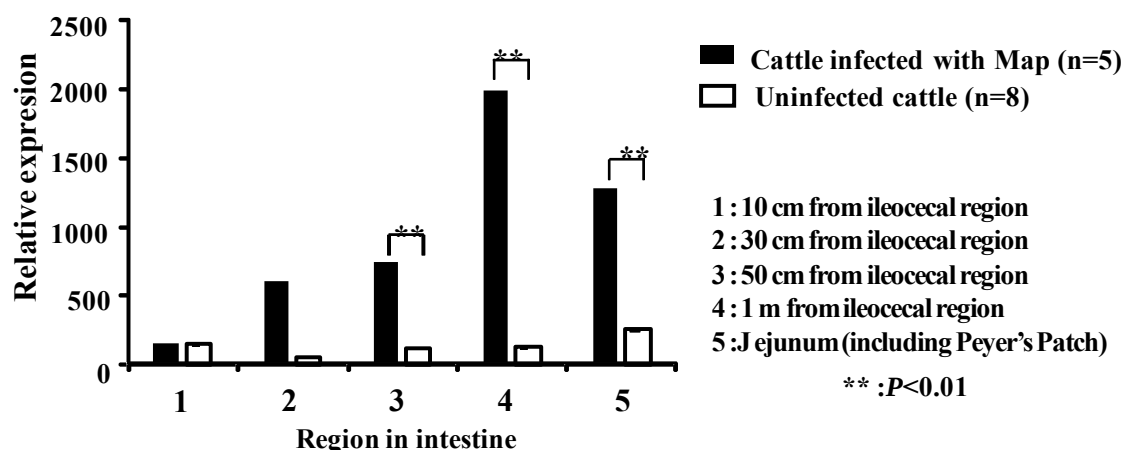


Fig 1. The bovine Reg3G gene expression analysis in the distal ileum of cattle

Binding activity of rReg3G to carbohydrates and Map

The rReg3G bound to mannan but not monomeric mannose, likewise mouse and human Reg3G (Cash et al., 2006). The ligand binding of C-type lectin is calcium-dependent, however, bovine rReg3G binding to mannan was not influenced by calcium. Dot-blot immunoassay demonstrated that rReg3G attached to mycobacterium surface, and binding ligand seems to be lipoarabinomannans, because recent studies have identified the mannosylated lipoarabinomannans as a potential ligand of a C-type lectin of *M. tuberculosis* (Ragas et al., 2007). Interestingly, the interaction of rReg3G with Map seems to be stronger than those of other mycobacterium species compared. The characteristic binding activity of bovine Reg3G against Map may suggest the presence of different ligands.

CONCLUSION

The bovine Reg3G, C-type lectin, is characteristically and strongly expressed in the duodenum of GP and the distal ileum of cattle infected with Map. And the bovine recombinant Reg3G binds strongly to Map surface in comparison to other mycobacterial species. These observations may offer new insights to understanding of the host-pathogen relationship in Map infection.

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TRANSCRIPTOMIC ANALYSIS OF SUB-CLINICAL MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTION IN CATTLE

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Paratuberculosis is characterised by a lengthy sub-clinical phase that may persist for years prior to the development of clinical disease. The mechanisms leading to progression from subclinical to clinical disease are poorly understood although the early sub-clinical phase is reportedly characterised by an increasing cell-mediated pro-inflammatory immune response largely driven by Th1 cytokines. The objectives of this study were to evaluate early gene expression changes in calves exposed to MAP. Microarray chip technology is a tool for probing the expression of thousands of genes in a single experiment and works by exploiting the ability of messenger RNA to bind specifically to the DNA template from which it originated. A comparison of hybridisation between samples derived from control and exposed animals provides an overview of gene expression patterns in response to infection. Blood samples were drawn over three early time points, from a Johne's disease field trial consisting of a mixed population of Holstein and Holstein Red cattle. Four MAP exposed and four unexposed control cattle were sampled at each time point and the 24 resulting samples were processed to Affymetrix GeneChip Bovine Genome Array. Ontological analysis revealed a number of differences in gene expression between the MAP exposed and unexposed control animals at the individual time points. In addition a subset of genes associated with the MHC were found to be consistently altered across time, suggesting significant changes to the antigen processing and presentation pathways in MAP-exposed animals in comparison to the unexposed control cohort. The differential regulation of genes evident in response to MAP is a significant finding in the understanding of the mechanisms of pathogenesis and may lead to a means to determine susceptibility.

EXPLORING *M. PARATUBERCULOSIS* PATHOGENESIS USING AN IN VITRO CELL CULTURE PASSAGE MODEL

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Mycobacterium avium ssp. *paratuberculosis* (MAP) is the etiological agent of Johne's disease, a chronic intestinal inflammatory disease that affects ruminants worldwide. Cattle remain in the subclinical stage of infection for years before the disease progresses to clinical symptoms and this transition has been described as a shift from a TH1 type response to an antibody-dominated type response; however, the cause for the shift remains to be determined. We describe an *in vitro* cell culture passage model in an attempt to gain a further understanding of the changes of the host immune response and the bacterial changes that occur during the progression of the disease. By passing MAP through MDBK epithelial cells, RAW 264.7 macrophages, and MDBK epithelial cells sequentially, and utilizing real-time PCR to determine transcript levels of immune signals, we have observed that cytokine and chemokine levels change in epithelial cell populations after being infected with MAP. After serial passage, epithelial cells express higher levels of immune signal transcripts of pro-inflammatory signals and decreased levels of anti-inflammatory signals compared to the initial infection. These data, as well as previous findings that demonstrate an increase of an invasive phenotype of MAP after intracellular growth in macrophages, suggest that the serial passage of MAP between host cell populations may select for a population of bacteria that optimizes infection and more readily stimulates a pro-inflammatory host response. To fully understand the mechanisms behind these observations, MAP phenotypes have been obtained from both the initial and later stages of *in vitro* infection and microarray analysis and proteomic analysis will be conducted. This hypothesis could potentially explain why the subclinical phase of the disease persists for so many years and our work begins to decipher the dynamics of antigen expression and host response that occur during the progression of Johne's disease.

HUMORAL RESPONSES AGAINST MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS STRESS-ASSOCIATED RECOMBINANT PROTEINS IN CATTLE

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It has been suggested that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) proteins that were regulated in *in vitro* models of physiological stress were also expressed *in vivo* in MAP-infected sheep at an early stage of infection. In this study, 11 MAP stress-associated genes were cloned and expressed in *Escherichia coli* as His-tagged proteins and purified using affinity chromatography. Humoral immune responses in experimentally infected cattle to the recombinant proteins were assessed longitudinally by ELISA. Blood and faecal samples were collected from orally infected male Holstein cattle (n=3) prior to infection and every 2 to 4 weeks post-inoculation and tested by recombinant protein ELISAs, commercial antibody ELISA, IFN γ -ELISA, IL-10 ELISA, direct faecal QPCR and faecal culture. Animals were necropsied at three and half years post-inoculation and tissue samples were processed for histopathological examination, direct tissue QPCR and culture. The commercial antibody ELISA test turned positive only in animal no. 1 after three years post-inoculation. Also, only animal no. 1 showed clinical signs of Johne's disease (JD). The results of histopathological examination suggested that animal no.1 was at a moderate to advanced stage of JD, no. 2 was at an early stage and no. 3 had recovered or was at a very early stage of infection. The antibody response to some recombinant MAP proteins increased early after infection. Although the number of animals in this experiment was low, development of humoral responses to some recombinant proteins was correlated with faecal shedding detected by the QPCR assay. Further study using a wider range of serum samples will be undertaken to evaluate whether these proteins are consistently expressed in MAP-infected cattle and therefore whether they are useful antigenic targets for early diagnosis of JD.

THE EFFECT OF SHORT- AND LONG-TERM ANTIBIOTICS EXPOSITION ON VIABILITY OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* MEASURED BY PROPIDIUM MONOAZIDE F57 REAL TIME QUANTITATIVE PCR AND CULTURE

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) possesses a cell wall rich in lipids. This enables mycobacteria to survive various stresses and persist in the environment. This resistance of MAP is exploited for its culture diagnosis whereby decontamination agents and antibiotics (ATB) are used to suppress the presence of contaminating microflora. However, such treatments can also negatively affect MAP.

The aim of this study was to determine the combined effect of the ATB **vancomycin (VCM)**, **amphotericin B (AMP-B)** and **nalidixic acid (NAL)** on the viability of MAP using propidium monoazide F57real time quantitative PCR (PMA F57qPCR) and culture after short- (3 days) and long-term (5 weeks) treatment.

Materials and methods

Experimental design

- » MAP field isolates 283/08 and 12146 and a laboratory reference strain CAPM 6381 ($OD_{600} = 0.3 - 0.4$) were resuspended in M7H9 with Mycobactin J.
- » A combination of all three ATBs was present in each short- or long-term experimental samples. For each sample, the concentration of one ATB was changed while the concentrations of other two remained the same (Tab. 1).
- » Short-term treatment with ATB – 3 days at 37°C.
- » Long-term treatment with ATB – 5 weeks at 37°C.
- »

Tab. 1. The concentrations of VCM, AMP-B and NAL in MAP cultures (standard concentrations are shaded)

Changing concentration of one of the ATB	Final concentrations of ATB (µg/ml)		
	VCM	AMP-B	NAL
VCM 25	25	50	100
VCM 50	50	50	100
VCM 100	100	50	100
VCM 200	200	50	100
VCM 400	400	50	100
NAL 25	100	50	25
NAL 50	100	50	50
NAL 100	100	50	100
NAL 200	100	50	200
NAL 400	100	50	400
AMP-B 12.5	100	12.5	100
AMP-B 25	100	25	100
AMP-B 50	100	50	100
AMP-B 100	100	100	100
AMP-B 200	100	200	100

PMA treatment and F57qPCR

- » Biological triplicates of MAP isolates suspensions aliquots treated with ATB and controls were twice exposed to PMA with dark incubation for 5 min and light exposure for 2 min (Kralik et al., 2010).
- » After washing and heat lysis at 100°C for 20 min, cells were centrifuged and supernatant was used to *F57qPCR* (Slana et al., 2008).
- » Quantification of absolute numbers of MAP by *F57qPCR* was done according to the plasmid gradient
- » MAP viability was determined as the quotient of absolute numbers of PMA-exposed ATB-treated cells and the PMA-exposed control (ATB-untreated) cells (Kralik et al., 2010).

Culture on solid media

- » Biological triplicates of short- and long-term treated samples were cultivated on Herrold's egg yolk media (HEYM) with Mycobactin J and without ATB at 37 °C for three months.
- » The viability of ATB-treated cells was determined as the quotient of MAP CFU in the ATB-treated sample and the control, ATB-untreated sample (100%) for each MAP isolate. This approach was chosen so as to allow direct comparison of the results of culture and PMA *F57qPCR*.

Results

Viability of MAP isolates after short-term ATB treatment

- » Neither MAP isolates nor changing concentrations of ATB were significant sources of variability using culture.
- » Using PMA *F57qPCR*, isolates were shown to be a statistically significant source of variability, which was manifested by a higher viability (resistance to VAN) of MAP isolate 283/08 compared to the 6381 and 12146 isolates. In experiments with increasing concentrations of VCM and AMP-B, the number of surviving cells was lower in a statistically highly significant manner in the 12146 and 6381 isolates compared to 283/08 ($P < 0.01$).

Viability of MAP isolates after long-term ATB treatment

- » The laboratory strain 6381 responded most sensitively while the 12146 field isolate seemed to be the most resistant to long-term exposure. Five-week stress had a negative impact also on the viability of 283/08, which was the most resistant isolate in response to short-term treatments.
- » According to both culture and PMA *F57qPCR*, MAP isolates and the concentration of ATB were a statistically significant source of variability ($P < 0.05$).
- » As measured by PMA *F57qPCR*, 283/08 noticeably withstood only those combinations of ATB when VCM or NAL were at their lowest concentrations; a similar trend was also observed in the culture experiments.

Conclusion

- » The trend of the responses of MAP isolates to different concentrations of ATBs in short- and long-term treatments was comparable between culture and PMA *F57qPCR*. Therefore, PMA *F57qPCR* can replace MAP culture in experiments aimed at studying the effects of different treatments on MAP viability.
- » Long-term treatment (five weeks) with ATBs had a negative impact on the viability of MAP cells. This effect was very weak in the case of short-term treatment (three days).
- » Data suggest that 50 µg/mL of VNC, 50 µg/mL of NAL and 200 µg/mL of AMP-B are threshold concentrations for decreasing MAP viability. Using culture, the threshold concentrations were determined to be 100 µg/mL of VNC, 50 to 100 µg/mL of NAL and 100 µg/mL of AMP-B.
- » PMA *F57qPCR* can reveal differences in viability between cells treated and untreated with NAL, an ATB, which does not primarily affect synthesis of the cell wall.
- » The 6381 MAP laboratory strain showed lower resistance to ATBs, especially in the case of long-term treatment.

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This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic “AdmireVet” (CZ 1.05/2.1.00/01.0006; ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716202 and QH81065).

INFLUENCE OF REPEATED CYCLES OF MECHANICAL AND PHYSICAL DISINFECTION PROCEDURES ON VIABILITY OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* MEASURED BY PROPIDIUM MONOAZIED *F57* QUANTITATIVE REAL TIME PCR

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) was shown to have a high degree of resistance to large scale of chemical and physical procedures that are used for the elimination of bacteria. There is only little information about the efficiency of these procedures on MAP survival. Classical method for the determination of viability is culture. However, classical culture suffers from many inconveniences. In our laboratory, detection technique for the determination of MAP viability by propidium monoazide (PMA) *F57*qPCR was optimized and tested on MAP isolates stressed by different chemical factors. The comparison of data from culture and PMA *F57*qPCR showed that although both methods measure viability on different levels, their results are comparable.

Therefore, the aim of this study was to investigate influence of exposition/ concentration and repeated cycles of ultraviolet (UV) light radiation and different concentrations of chlorine and peracetic acid (PAA) on viability of three different MAP isolates.

Materials and methods

Experimental design

- » The isolates for testing were chosen with regard to the length of their culture *in vitro*: collection strain CAPM 6381, low passage isolate 8819 and high passage isolate 12146.

Tab. 1. Experimental stress conditions.

Stress factor	Treatment				
UV (min) at 44 mWs/cm ²	10	20	30	45	60
Chlorine (ppm) for 30 min	10	25	50	75	100
PAA (%) for 10 min	0.01	0.05	0.1	0.5	1

- » The treatments were repeated three times within three days with the overnight MAP cells recovery (Tab.1).

PMA treatment and *F57*qPCR

- » Biological triplicates of MAP isolates suspensions aliquots treated with the stress factors and controls were twice exposed to PMA with dark incubation for 5 min and light exposure for 2 min (Kralik et al., 2010).
- » After washing and heat lysis at 100°C for 20 min, cells were centrifuged and supernatant was used to *F57*qPCR (Slana et al., 2008).
- » Quantification of absolute numbers of MAP by *F57*qPCR was done according to the plasmid gradient
- » MAP viability was determined as the quotient of absolute numbers of PMA-exposed stress factor-treated cells and the PMA-exposed control (stress factor-untreated) cells (Kralik et al., 2010).

Statistical analysis

- » Null hypothesis assumed that the viability will decrease with the increasing concentration and number of treatments.
- » Exploratory Data Analysis showed that the most suitable statistical model for the analyses of experiments with UV and chlorine that will confirm or rebut the null hypothesis is Multiple Regression Analysis like General Regression Models (GRM).
- » The statistical analysis of PAA influence on MAP viability was performed by Two-way ANOVA with subsequent testing of statistical significance of partial differences by Tukey HSD test.
- » P-values lower than 0.05 were considered statistically significant.

Results

- » Increasing concentration/time of exposition of all stress factors tested and number of treatments reduced the viability of all MAP strains/isolates. Null hypothesis was confirmed.
- » In all MAP isolates was shown that repeated UV or chlorine treatment has much more significant influence of MAP viability than increasing of concentration or time of exposition.
- » Strain/isolate specific resistance to factors studied was observed.
- » Although isolate 12146 was the most resistant to one-time UV treatment, it was shown to be the most sensitive to the repeated cycles of UV treatment.
- » Laboratory strain CAPM 6381 was the most resistant to repeated chlorine treatments and increase of chlorine concentration.
- » PAA was shown to be a potent disinfectant in reduction of viability in all MAP strains/isolates.

Conclusion:

- » UV light exposure has only partial effect on MAP viability reduction. Similar effect was observed in other bacteria exposed to UV light (Nocker et al., 2007).
- » As could be expected, all MAP isolates were relatively resistant to chlorine.
- » PAA has the very profound effect on MAP viability and disinfection agents containing PAA will be effective in MAP removal.

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This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic “AdmireVet” (CZ 1.05/2.1.00/01.0006; ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716202 and QH81065).

INVESTIGATION ON GENETIC SUSCEPTIBILITY TO PARATUBERCULOSIS IN SARDA SHEEP BREED

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INTRODUCTION

Paratuberculosis is not easily amenable to classical control methods such as treatment and vaccination. Experimental animal models suggest that there could be genetic factors responsible for susceptibility or resistance to infection with the causative agent, *Mycobacterium avium* subsp. *paratuberculosis*. This organism is also of public health concern due to an unconfirmed link to Crohn's disease. In livestock, a number of candidate genes have been studied, selected on their association to susceptibility in other mycobacterial diseases, their known role in disease pathogenesis or links to susceptibility of humans to Crohn's disease. In a previous study we tried to assess whether different genotypes at NOD2 gene may lead to different susceptibility in developing paratuberculosis in sheep, as reported for human Crohn's disease. We identified polymorphisms within NOD2 exon 4 and intron 5-6, however no significant associations between SNPs and the disease were found. A limitation of that study was the narrow number of samples available. We therefore performed a wider sampling and, by analyzing about 95 sheep samples from a previous investigated flock of the Viterbo province, to identify a wider number of affected and controls suitable for genotyping. These samples were then screened for 13 polymorphisms in 8 genes, to verify if an association between phenotype (resistant- affected) and genotype could be observed.

MATERIALS AND METHODS

Serological data

95 adult sheep from a single flock were analysed. We performed a first ELISA screening on serum. A second ELISA verification was performed on serum of positive individuals to confirm results. A commercial kit (Institut Pourquier) was used to diagnose paratuberculosis with the ELISA test. Sera were treated according to the manufacturer's protocol

PCR

PCR on the specific insertion sequence IS900 was carried out on faeces from positive sheep. DNA extraction from tissues and faeces was conducted by means of QIAmp DNA minikit (Qiagen) according to the enclosed protocol. A qualitative IS900 specific PCR was carried out on the total extracted DNA to detect *M. avium paratuberculosis*. A commercial Adiavet paratube (Adiagene) kit was used on purpose.

Genetic analysis

DNA was extracted from blood samples of a total of 95 individuals (44 infected and 57 controls) using NucleoSpin blood kit (Macherey&Nagel) according to manufacturer's instruction. DNA was checked for quality on agarose gel and quantified using a DTX microplate reader (Beckman Coulter) after staining with Picogreen (Invitrogen). 13 Single Nucleotide Polymorphisms (SNPs) in 8 genes (NOD2, NRAMP1, IL2, IL4, TLR2, TLR4, TLR6 and TNF) were selected and genotyped on the 95 samples; SNP genotyping of the total sampling was outsourced to Kbiosciences (www.kbiosciences.co.uk).

RESULTS AND CONCLUSIONS

Elisa test was performed twice on blood samples. Screening and verification resulted in 51 positive and 44 negative samples. Faeces were analysed by PCR, but only 8 samples turned out positive. This is in agreement with the low power of PCR test on faeces in sheep compared, for example, to cattle.

13 Single Nucleotide Polymorphisms (SNPs) in 8 genes (NOD2, NRAMP1, IL2, IL4, TLR2, TLR4, TLR6 and TNF) were selected and genotyped on the 95 samples, for a total of 1235 genotypes assessed. Seven SNPs resulted monomorphic in the analysed samples. We then filtered samples according to serological data, keeping for the statistical analysis only those showing in both ELISA's test a value of 90 S/P or over for a total of 71 sheep analysed.

SNP statistics (allelic frequencies, gene diversity, observed heterozygosity and PIC index) were calculated using Powermarker software (Liu and Muse 2005, Bioinformatics 21:2128-9) (table 1). Fisher test was applied to verify if any correlation between allelic frequencies at the six polymorphic SNPs (three SNPs in NOD2, and one each in IL2, TLR2 and TNF) and affected / control condition exists. None of the SNPs resulted significantly associated with the disease ($p \leq 0.05$) (table 2). It is interesting that two borderline values ($p \leq 0.056$ and $p \leq 0.083$) were obtained for two SNPs in NOD2 gene. This could suggest that another SNPs within the same gene could be associated with paratuberculosis in sheep.

Table 1

Marker	Major Allele Frequency	Gene Diversity	Heterozygosity	PIC
NOD2_ex4	0.8000	0.3200	0.3429	0.2688
NOD2_ex5_6	0.6385	0.4617	0.5385	0.3551
NOD2_int5_6	0.6667	0.4444	0.4928	0.3457
IL2_1	0.7538	0.3711	0.4000	0.3023
TLR2_5	0.5217	0.4991	0.0000	0.3745
TNF_2	0.7537	0.3712	0.3433	0.3023

Table 2

SNP	p-value:
NOD2_ex4	0.1595
NOD2_ex5_6	0.0560
NOD2_int5_6	0.0839
IL2_1	0.1636
TLR2_5	0.1987
TNF_2	0.1357

MHC CLASS II DRB GENE POLYMORPHISM AND GENETIC RESISTANCE/SUSCEPTIBILITY TO JOHNE'S DISEASE IN AN ENDANGERED JAMUNAPARI BREED OF GOATS

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Pathogenesis of *Mycobacterium avium* subsp. *Paratuberculosis* infection in animals is complex and not yet completely understood. Polymorphism in the exon-2 of MHC Class II DRB region and its association with resistance / susceptibility to JD was analyzed in a highly susceptible and endangered breed of Jamunapari goats. Based on clinical signs, microscopy, fecal culture, ELISA and blood PCR, 60 and 143 goats were classified as resistant and susceptible, respectively. PCR-RFLP with two enzymes, *Pst* I and *Taq* I, was used to assess variations in the DRB gene (s) of 203 goats. Two di-allelic SNPs (referred as 'P' and 'T') were identified and in each of the allele there were three genotypes. Minimum allelic frequencies for P and T SNPs were 0.233 and 0.486, respectively. Statistically significant associations were found between alleles, individual genotypes and composed genotypes of both SNPs. Frequency of p and t alleles, of individual *pp* and *tt* and of composed *pptt* genotypes were significantly higher ($p_{corr} < 0.001$) in the 'resistant' group as compared to 'susceptible' group. Whereas P and T alleles, were associated with susceptibility ($p_{corr} < 0.001$). In heterozygous genotypes, susceptibility was dominant over resistance. Effects of both SNP on resistance and susceptibility were comparable and composed heterozygous genotypes showed intermediate levels of susceptibility in terms of odds ratio and p values calculated. Though, associations observed seemed to be biologically plausible, further population and functional studies are needed to confirm these relationships and to clarify the underlying mechanisms before these candidate markers can be considered for marker-assisted selection for the control Johne's disease.

IDENTIFICATION AND CHARACTERIZATION OF NOVEL ADHESINS IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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The pathogenic potential of *Mycobacterium avium* subsp. *paratuberculosis* (MPTB) and the molecular basis for its intestinal tropism are still unclear. Binding and ingestion of MPTB by host cells is fibronectin (FN) dependent and there is a family of proteins present in several species of mycobacteria that allows their attachment and internalization by epithelial cells via interaction with FN. Secott *et al.* (2002) demonstrated that the interaction between FN and FAP-P facilitates attachment and internalization of MPTB by epithelial cells. Dheenadhayalan *et al.* (2002) characterized MPTB Ag85complex, consisting of Ag85A, Ag85B and Ag85C, and observed *in silico* that the three conserved regions involved in FN binding in other mycobacteria were also present in N-terminal regions of 85A, B and C of MPTB. Additionally, Kuo *et al.* (2012) recently showed experimentally that the members of this family possess the ability to specifically interact with FN.

Thus, it is important the identification of adhesion molecules to understand the pathogenesis of MPTB. This could be useful to propose new preventive strategies against paratuberculosis.

The aim of this study was to identify and characterize FN binding-cell wall proteins from MPTB. To accomplish this, a cell wall protein fraction was obtained and resolved by 2D-electrophoresis. The identity of the spots was obtained by MALDI-TOF MS and a homology searching was performed to find if the proteins identified had FN-binding orthologues in other organisms. The proteins selected as candidates and also the organisms where they were previously evaluated and reported in the literature as FN-binding proteins are listed in the following table:

Protein	Organism	Identity (%)	Reference
EF-Tu	<i>Mycoplasma pneumonia</i>	65	Balasubramanian <i>et al.</i> , 2008.
GlnA1	<i>Mycobacterium tuberculosis</i>	90	Xolalpa <i>et al.</i> , 2007.
GAPDH	<i>Candida albicans</i>	52	Gozalbo <i>et al.</i> , 1998

MPTB-EFTu, MPTB-GAPDH and MPTB-GlnA1 were obtained in recombinant form. The complete open-reading frames were amplified. The products of 1190bp, 1437bp and 1020-bp for EFTu, GlnA1 and GAPDH respectively were separated by agarose gel electrophoresis, visualized by ethidium bromide staining and then cloned into pGEM-T vector (Promega). The insert was excised using the appropriate enzymes and ligated to pRSET-A (Invitrogen), which generates an N-terminal fusion with a polyhistidine tag. The resulting plasmid was sequenced to confirm the identity and frame and was further introduced into *E. coli* BL21 (λDE3, pLys). Protein expression was induced by addition of 1 mM IPTG to the cultures at the mid-logarithmic phase. Cells were harvested and lysed in denaturing buffer. Recombinant protein was purified using a Ni-NTA Agarose resin column (Qiagen) and eluted with increasing Imidazole buffer. Eluted protein fractions were dialyzed at 4 °C for 48 h against PBS.

The purified recombinant proteins were electrophoresed in 15% SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare). We demonstrated the FN-binding capability of proteins through a Ligand Blot Assay (LBA), incubating the blot with 20µg/mL FN (Sigma), followed by mouse hyperimmune anti-FN serum incubation and finally, AP-conjugated anti-mouse IgG (Sigma) incubation. The membranes were washed and colorimetric detection of the bound bait protein was performed using NBT/BCIP Substrates (Promega). We observed positive signal indicating the FN-binding capability of EF-Tu and GlnA1. Ag85 recombinant protein was also assayed as positive control and AhpC as negative control.

We also confirmed the interaction with FN through an ELISA assay in duplicate. First, 96-well plates were coated overnight in carbonate buffer at 4 °C with 1 µg of GlnA1, EF-Tu and hsp65 negative control. Plates were then blocked and increasing concentrations of FN (0, 1, 10, 20, 50 and 100 µg/mL) were added. The assessment of bound proteins was performed by incubation for 1 h at 37 °C with hyperimmune anti-FN serum produced in BALB/c mice at the dilution of 1:100 followed by incubation with HRP-conjugated anti-mouse IgG (Sigma) (1:500). The wells were washed three times, and ABTS in citrate phosphate buffer plus H₂O₂ was added. The absorbance at 492 nm was determined in a microplate reader Multiskan Spectrum (Thermo Scientific). We observed a dose dependent interaction in the concentrations assayed confirming the binding of GlnA1 and EF-Tu with FN.

Protein-protein interactions were assessed also by Surface Plasmon Resonance with a BIAcoreT100 system (GE). FN was covalently immobilized on the sensorchip (carboxymethylated dextran matrix) and protein solutions of GlnA1 at different concentrations were injected over immobilized FN. The results indicated that the preliminar GlnA1 dissociation constant (KD) was 35µM. The assay with EF-Tu is in progress.

This results support the hypothesis that these proteins could be involved in the interaction of MPTB with epithelial cells through FN binding. *In vitro* cell culture assays are needed to test this.

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PERSPECTIVES ON: PATHOGENOMICS

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The Pathogenomics and MAP biology stream has a dual focus. On the one hand it is home to the technologies which enable unbiased observational studies of both host and pathogen using genomics, transcriptomics, proteomics and lipidomics.

With the increasing amounts of data provided by sequencing and mass spectrometry the challenge shifts more and more from the actual technical platforms to bioinformatics to turn this data into information and knowledge.

On the other hand the stream also encompasses studies on the biology of MAP. Despite all the progress in the field many questions on basic topics such as behavior in various hosts, growth requirements, dormancy and the nature of the ability of MAP to survive in cells as well as the environment for prolonged periods of time are still actual.

Advances made using these technologies and new insights obtained in the last two and a half years since ICP10 and those presented here will be highlighted during this presentation as well as areas for future studies.

MYCOBACTERIAL DISEASES OF WILDLIFE

MYCOBACTERIUM AVIUM PARATUBERCULOSIS CULTURED FROM THE FAECES OF A BLACK RHINOCEROS (*DICEROS BICORNIS MINOR*) AND OBSERVATIONS SUBSEQUENT TO ANTI-TUBERCULOUS THERAPY

Bryant B

Taronga Western Plains Zoo

Mycobacterium avium paratuberculosis (*Map*) was cultured from the faeces of a wildcaught, female, adult southern black rhinoceros. The animal, which presented with a 4 month history of diarrhoea and weight loss, was prescribed a course of anti-mycobacterial drugs. The clinical signs resolved and the faeces were repeatedly culture negative thereafter. Although the rhinocerotidae are likely to be resistant to Johnes disease, this case raises the possibility that they can become transiently infected with the causative organism.

DIAGNOSIS AND MANAGEMENT OF TUBERCULOSIS (*MYCOBACTERIUM TUBERCULOSIS*) IN AN ASIAN ELEPHANT IN AUSTRALIA

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In 2006 five Asian Elephants were imported to Taronga Zoo, Australia from Thailand. Pre-import tuberculosis screening and initial post-arrival screening was by trunk wash (TW). In April 2009 the ElephantTB Stat-Pak® (SP, Chembio) was used to screen the elephants. A 15.5 year old pregnant cow was reactive. TW frequency for this cow was increased to every three months. TW culture and PCR remained negative on all elephants. In February 2010, this cow reacted on the Dual Path Platform Vet®TB test™ (DPP, Chembio). All other elephants were non-reactive. With concern about the effect of antituberculous drugs on her foetus and ongoing negative TW's, screening continued every three months and treatment was not initiated.

The cow gave birth on 2 November 2010. A routine TW on 24 November 2010 was culture positive for *M. tuberculosis*. Although previous shedding could not be ruled out, reactivation of latent infection due to parturition was suspected. Enhanced infection control, repeat staff screening and staff education sessions were implemented.

Treatment with isoniazid, pyrazinamide, rifampicin and ethambutol commenced. The isolate was susceptible to these drugs and genotyped as a Beijing strain. Retrospective serum from Thailand (2004) and Australia (2006) were positive on SP and DPP.

TW, SP and DPP screening frequency increased to monthly for the positive cow. Monthly serum chemistry indicated drug induced hepatitis. Drug pharmacokinetics was conducted to ensure therapeutic levels were achieved. TW and blood collection training of the infant calf was initiated. For all other elephants, TW and SP screening increased to every three months.

Acknowledgements

We thank the Taronga elephant team, hospital staff, Michele Miller, Susan Mikota, Konstantin Lyashchenko and the many others who were involved and provided assistance.

NON-MAMMALIAN MYCOBACTERIOSIS IN A ZOO SETTING – PATHOLOGICAL AND EPIDEMIOLOGICAL FINDINGS

Sangster C

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Mycobacterial infections are found in all classes of vertebrates, often resulting in severe disease, and are therefore of great importance in the zoo industry. A review of the pathology of mycobacterial infections in fish, amphibians, reptiles and birds will be given. This will be followed by a closer look at the epidemiology of atypical mycobacteriosis in the Taronga Zoo bird collection, which became of significant interest following the discovery of *M. genavense* in 3 figbirds transacted to Melbourne Zoo.

The overall incidence of atypical mycobacteriosis in the avian collection is <1%, with up to 6.6% incidence in birds submitted for necropsy. Cases are present in several orders with most cases in psittacines, passeriformes and columbiformes. Retrospective analysis of avian mycobacterial cases reveals only one other confirmed case of *M. genavense* in the collection, however due to difficulty in culturing this slow growing species, the actual incidence is expected to be higher.

Future plans include development of fecal PCRs for *M. avium-intracellulare* complex and *M. genavense* to further define incidence of atypical avian mycobacteriosis, especially from *M. genavense*. In addition, soil testing will identify atypical mycobacterial burdens in enclosures and aid in monitoring the efficacy of control measures.

**GENOTYPING AND *MYCOBACTERIUM AVIUM* SUBSP.
PARATUBERCULOSIS DIVERSITY**

KEYNOTE ON: MOLECULAR DIVERSITY ANALYSIS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* AS AN AID IN TRANSMISSION STUDIES, DEFINING POPULATION GENETIC FRAMEWORKS AND TRAIT-ALLELE ASSOCIATIONS

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The primary objective of this presentation is to provide a comprehensive and critical overview of the population genetic structure and natural history of the intracellular pathogen, *Mycobacterium avium* subspecies *paratuberculosis* (MAP).

Until relatively recently a major application of molecular diversity studies of MAP have been to describe extent of diversity within the subspecies and some attempts to trace the pathways of transmission within populations. While phenotypic differences among clinical isolates have long been recognized, it was not previously possible to determine whether they were stably associated with specific lineages of MAP circulating in the population. Most of the isolates identified had been associated with large clusters that were widely dispersed both geographically and temporally, raising the possibility that they were either more transmissible or more likely to cause disease, once they are transmitted, than other isolates. The advent of molecular typing of MAP, such as IS900 RFLP, PFGE, AFLP, ML-SSR, VNTRs, SNPs, and more recently comparative genomics (in terms of both the discriminatory index and in vitro stability) have allowed researchers to better describe the pathways of transmission of Johne's disease in well-designed longitudinal studies and strain-specific variations in clinical phenotypes, such as virulence, growth characteristics, immunogenicity, and transmissibility.

Another common theme that has emerged from molecular and population genetic analysis of pathogenic mycobacteria is that biomedically relevant traits, such as host range and virulence are nonrandomly distributed among phylogenetic lineages. Trait-lineage relationships exist, in part, because pathogenic bacterial species often have a level of genetic diversity far in excess of the presence of variation in higher eukaryotic organisms such as animals and humans. In the postgenomic era, SNPs as well as short and large sequence polymorphisms, provide a simple and fast way to compare entire genomes in many bacterial strains. I will compare and contrast anonymous biallelic interrogations such as IS900 RFLP, PFGE, and AFLP against applications of unambiguous single nucleotide polymorphisms, large sequence polymorphisms, short sequence repeats, and direct comparative genomics and their contribution to the development of population genetic frameworks and trait-allele associations of MAP.

Global studies on MAP genotyping using high resolution molecular markers derived from the first sequenced MAP K-10 genome, of isolates of MAP derived from a variety of disease types, geographic localities, and hosts suggests that MAP are indeed a diverse group of organisms and that there exists an association between allele types and host species. Our analyses also show that MAP strains derived from sheep JD are distinct compared to those isolated from cattle, goats or other wildlife species. The strains derived from human Crohn's disease (CD) patients show limited diversity, which may be indicative of either restricted strain sharing between humans and animals or the ability of only a few extant animal genotypes to be associated with the pathobiology of CD. In collaborations with Drs. Kapur and Bannantine, our analysis of genome-wide multilocus- short sequence repeats and single nucleotide polymorphism (SNP) analyses of targets derived by genome mining of the MAP K-10, have provided a highly discriminative methodology to study strain specific variation in the pathobiology of disease.

Furthermore recent studies with microarray based comparative genomics or direct sequence comparisons of multiple draft genomes of MAP reveal genome signatures that can be used to describe disease phenotypes induced by specific strains. While the available literature captures and describes genetic diversity among MAP isolates by multiple approaches, there is little understanding of the functional genomic basis for phenotypic variability – a major gap in the current understanding of underlying molecular basis for variations in pathogenesis in Johne's disease. It is also not clear if the subclinical infection caused by MAP in any animal species represents a subset of strains that may be deemed the most successful and/or more virulent in causing JD. Understanding the functional genomic characteristics of strains that fall into specific ML-SSR, VNTR, SNP, LSP, PFGE, or AFLP phylotypes will be critical in providing a basic understanding of JD pathogenesis. To this end, MAP SSR and SNP genotypes and their associations with macrophage invasion or mouse infection studies as well as those

addressing basic metabolic processes such as iron acquisition have revealed clear molecular mechanisms underlying trait variations. Such functional studies are expected to lay strong scientific foundations for understanding this complex intracellular pathogen.

A LARGE-SCALE STUDY OF DIFFERENTIAL VIRULENCE OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* STRAINS WITH DISTINCT GENOTYPES AND ISOLATED FROM DIVERSE HOSTS IN BOVINE MACROPHAGES

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INTRODUCTION

The purpose of this study was to compare the virulence of 10 clinical strains of *Mycobacterium avium* subsp. *paratuberculosis* (Map) isolated from domestic or wild-life species. The strains were previously isolated and typed in distinct IS1311 PCR-REA profiles (C, S or B).

MATERIALS AND METHODS

Uptake and intracellular growth of the selected Map strains in a bovine macrophage cell line (BOMAC) and in bovine monocyte-derived macrophages was evaluated by quantification of the number of viable bacterial cells at 2 h and 7 days post-infection (p.i.), respectively. In addition, a quantitative qRT-PCR array was developed to test the expression of several cytokines, and genes involved in apoptosis and tissue destruction after the infection of bovine macrophages with the selected strains of Map.

RESULTS

Our results demonstrated that Map strains isolated from diverse hosts have different abilities to grow in bovine macrophages, which grouped largely according to the specific host from which they were initially isolated. In fact, the goat and sheep strains with C or S genotypes had lower infective titers at 7 days p.i. than the cattle strains in the two *in vitro* bovine models. In contrast, the type-C strains isolated from wild-life animal species reached similar infective titers than the field cattle strain at 7 days p.i and were all clustered together. Infection of BOMAC cells with the bovine strain resulted in upregulation of the anti-inflammatory cytokines (IL-6, TGF β 1, IL10) and downregulation of the pro-inflammatory cytokines (IFN γ and IL-1 α), relative to uninfected cells. Cells stimulated with the cattle strain also had high levels of the apoptotic inhibitor BCL2-1 and low levels of matrix metalloprotease-3 (MMP3-1). In contrast, macrophages infected with the ovine strain showed a downregulation pattern of IL10 and a significant upregulation of the pro-inflammatory cytokine IL1 α , which induces macrophage killing mechanisms.

CONCLUSION

Survival efficiency of Map strains within bovine macrophages seem not be associated to the strain genotype but more likely to the specific host environment from which the strains were initially isolated.

EPIDEMIOLOGICAL INFERENCES FROM TYPING *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) ISOLATES FROM MIXED SHEEP, DEER AND CATTLE FARMS

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This study describes the diversity and epidemiological associations of strain types (ST) of MAP. A total of 188 MAP isolates were sourced from 99 farms. Species accumulation curves (SAC), proportion similarity index (PSI) and analysis of molecular variance (AMOVA) were used to describe ST diversity and associations between species, location, species composition of farms, and with farmer observed disease incidence.

It was found that (i) more STs (n=14) were found in the South Island (SI) than in the North Island (NI; n=6); (ii) only 4 STs were found in both islands whereas 12 STs were only found in either one of the islands (iii) SAC suggested that ST diversity tended to be greater in sheep than in deer; (iv) 12 ovine and 4 bovine type strains were found in beef cattle; (v) strains from beef cattle and sheep were more similar than expected by chance (PSI=0.80); (vi) STs from deer and dairy tended to be more similar than expected by chance (PSI=0.50); (vi) the similarity of STs from beef cattle and deer was 3-fold greater when direct contact between these species was considered than when contact was ignored; (vii) there was a trend that the reported incidence of clinical disease in deer was lower on farms where deer were infected with Type S strains (vs. Type C), but evidence was weak due to sparse data.

It was concluded that MAP differed between islands. Beef cattle are likely infected by sheep and are susceptible to infection with sheep strains. MAP is likely transmitted from beef cattle to deer when grazing the same pasture. In view of low observed clinical disease in beef cattle, Type S strains may have less serious pathological consequences in beef cattle than Type C strains.

DNA TYPING OF RECENT NEW ZEALAND ISOLATED OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS FROM CATTLE, SHEEP AND DEER BY VNTR AND SSR

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Abstract

DNA typing and sub-typing of *M. avium* subsp. *paratuberculosis* (*MAP*) isolates is an important tool for any country's paratuberculosis control scheme because of its ability to answer crucial epidemiological questions, and its usefulness in infection, vaccination and pathogenicity studies. All isolates used in this study were categorised as Type C or Type S based on a specific multiplex PCR assay. A system of sub-typing based on a short sequence repeat (SSR) and variable number tandem repeats (VNTRs) that were reported to be useful in other countries was developed using 123 archival New Zealand *MAP* isolates. These isolates came from cattle, sheep and deer and included 65 Type C isolates and 58 Type S isolates. The sub-typing system was then applied to 211 recent *MAP* isolates from dairy cattle supplied mainly by the Livestock Improvement Corporation and 154 recent *MAP* isolates supplied by Massey University that came from properties where two or more ruminant species were farmed. The sub-typing system gave better discrimination of Type C isolates than Type S isolates, indicating a narrow genetic range of Type S isolates in New Zealand. There were two major sub-types of Type C, one found in 70% of dairy cattle and a few animals in mixed farming operations, and the other found in 76% of deer and 2% of dairy cattle. Unexpectedly, the majority of the 19 isolates from cattle in mixed farming operations were Type S strains. These results show that the current host distribution of Type C and Type S strains in New Zealand is substantially different from the situation that prevailed late last century indicating that these changes have occurred recently. These results also raise issues about the pathogenicity of different types and sub-types of *MAP* for different ruminant species.

Introduction

The genotyping of different strains of *MAP* provides a basis for answering important epidemiological questions about sources of paratuberculosis infection and spread of disease and enables potential variation in pathogenicity of different strains to be more easily investigated. Genotyping of New Zealand *MAP* isolates in the 1980s by restriction fragment length polymorphism analysis based on the insertion sequence IS900 (1) showed that cattle and most deer were infected with Type C (later called Type II) strains while Type S (later called Type I) strains primarily infected sheep. The development of newer methods for genotyping *MAP* strains based on VNTR and SSR sequences now provides a faster and potentially more discriminating system that we have applied to recent New Zealand *MAP* isolates.

Methods and Results

All the *MAP* strains studied were determined to be Type S or Type C strains by using a specific PCR assay (2). The Type of strains found in different hosts at two time periods is given in Table 1. The most notable change over time was the recent frequent infection of cattle with Type S strains.

Table 1. Type of *MAP* strains found in different hosts over two time periods

Host	Type	1985 – 1993	2008 - 2010
Cattle	C	33	203
	S	0	27
Sheep	C	3	9
	S	16	61
Deer	C	22	62
	S	4	3

Eight VNTRs and two SSRs, reported to be particularly useful for subtyping, were applied to archived New Zealand isolates of *MAP* from cattle, deer and sheep. VNTR and SSR sequences were amplified by PCR and detected by agarose gel electrophoresis and DNA sequencing respectively. Three of the VNTRs (10, 32 and 47) that gave little or no variation and one of the SSRs (SSR1) that was too

hypervariable to give consistent results for some samples were not further used. The remaining five VNTRs (292, 25, X3, 7 and 3) and SSR8 were used to subtype 365 recent faecal isolates of MAP. These were available as the result of separate studies by the Livestock Improvement Corporation and Massey University and came either from dairy farms or from farms on which two or three different animal species (beef cattle, deer, or sheep) had been grazed. Compared to studies elsewhere, a relatively large number of the samples contained multiple subtypes (Table 2). While this was unsurprising for the mixed-farm samples that came from pools of 10-20 animals, it was not expected for the samples from dairy cattle which came from single animals.

Table 2. MAP samples containing more than one subtype

	Total isolates	Samples with multiple subtypes	Samples with a VNTR giving 2 PCR products	Samples with more than 2 PCR products
Dairy	211	30 (14%)	14	16
Mixed farms	154	10 (6%)	8	2

The results of subtyping are given in Table 3. Two subtypes of Type C predominated in dairy cattle and one of these was not found in beef cattle, deer or sheep. A different subtype of Type C predominated in deer and was also found in small numbers of other animals. While six different subtypes of Type S were found in sheep, one subtype predominated and this was also the predominant Type S subtype in other animals. Surprisingly, beef cattle were more often infected with Type S strains than with Type C strains.

Table 3. Subtyping of recent New Zealand isolates of MAP by five VNTRs and SSR8

Type	Subtype	292-25-X3-7-3-SSR8	Dairy	Beef	Deer	Sheep
Type C		2-3-2-2-2-5	1			
		3-2-2-2-2-5	25			
		3-2-2-3-2-5	2			
		3-3-2-0.5-2-5	6		3	
		3-3-2-2-2-3	1			
		3-3-2-2-2-4	4		1	2
		3-3-2-2-2-5	147	2	8	2
		3-3-2-3-2-5	1			
		4-3-2-2-2-4	5	4	51	6
		4-3-2-2-2-5	1		1	
		5-2-2-2-2-5	1			
Type S		3-3-1-1-1-3	3	1	1	5
		3-3-1-2-1-3				1
		4-3-1-1-1-3	11	12	2	50
		4-3-1-2-1-3				5
		5-3-1-1-1-3	1			2
		7-3-1-1-1-3				1

Conclusions

- While historically, NZ cattle were infected only with Type C strains, recent typing shows that Type S strains are now frequent in NZ cattle and are more common in beef cattle than are Type C strains.
- Subtyping profiles for Type C isolates were always different from those for Type S isolates and VNTR3 by itself distinguished all Type S (1 allele) from all Type C (2 alleles) isolates.
- Some subtypes of Type C are present only in dairy cattle and one subtype of Type C predominates in these animals. In contrast, a different subtype of Type C predominates in deer
- There were fewer subtypes of Type S than of Type C and a single subtype of Type S predominates in all animals. This may reflect a very narrow genetic variation of Type S strains in New Zealand

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USE OF SSDNA-MALDI-TOF TO DETERMINE THE DIVERSITY OF MAP ISOLATES ORIGINATING FROM CANADIAN DAIRY HERDS

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Background

Johne's disease (JD) is primarily controlled through prevention; however this requires a thorough understanding of the transmission patterns and sources of *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Strain typing of *Map* isolates can provide valuable insight into the epidemiology of JD that can ultimately be used to design and evaluate regional control programs. A number of strain typing techniques have been used to discriminate *Map* isolates, though many have proven to be too impractical to encourage large scale studies. Short sequence repeats (SSR), such as the mononucleotide repeats (G1, G2) and trinucleotide repeats (GGT) found in the *Map* genome, were previously reported to allow genotyping of *Map* isolates with sufficiently high discriminatory power (Amonsin et al., 2004). These targets were determined to be the most discriminatory compared with other techniques to investigate the strain variability between and within herds (El-Sayed et al., 2009). Unfortunately, accurate sequence analysis of mono- and dinucleotide repeats is still moderately difficult, expensive, and laborious. This technique also suffers from a lack of reproducibility due to a high frequency of sequencing errors (Ross and Belgrader, 1997) and ambiguous results as illustrated in Fig. 1. This leads to the recommendation for duplicate sample testing, bidirectional sequencing and consensus between two independent readers of the sequencing results (Motiwala et al., 2005), strongly increasing costs and decreasing practicality of the SSR technique as previously performed. This limitation has led to the convention of characterizing repeat lengths greater than 11 as >11 (Thibault et al., 2008). In this study we used single strand DNA (ssDNA) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as an alternative to Sanger sequencing to type *Map* isolates using multiple SSR loci.

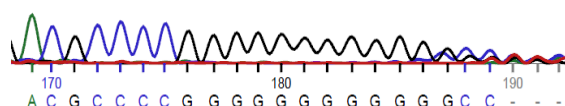


Figure 1: Ambiguous number of guanines in a chromatogram of the G1 SSR locus

Materials and Methods

PCR primers were designed to amplify a short target (< 80bp) surrounding the three most discriminatory loci (G1, G2, GGT) as reported by Amonsin et al. (2004). Synthetic DNA matching the exact size of the PCR amplicons was synthesized to demonstrate the ability of MALDI-TOF MS to accurately determine the mass of the oligo and sufficiently resolve repeat lengths between sequences differing by a single nucleotide. Repeat lengths of 7, 8, 9, 10, 11, and 19 of the G1 locus, 8, 9, and 10 of the G2 locus, and 3, 4, 5, and 6 of the GGT locus were evaluated. These repeat lengths have been previously observed in *Map* isolates, with the exception of 8 and 19 in the G1 locus. Oligos were reconstituted in HPLC-grade water and a 20 picomolar solution was mixed in equal volumes with a 3-HPA (Hydroxypicolinic acid) matrix. 0.5 µl of the sample/matrix mixture was spotted on the MALDI plate and introduced into the instrument. Mass spectrometry was performed on a Sciex MALDI TOF/TOF 5800 (Applied Biosystems) operating in linear, positive ion mode.

DNA was extracted from cultured environmental and fecal samples that were collected on dairy farms throughout Canada. For PCR amplification of SSRs, PCR products were mixed with equal volumes of washed Streptavidin Dynabeads. A magnet was used to immobilize the beads and the supernatant was removed. Beads were washed with HPLC grade water three times before final resuspension in 6 µl of water, followed by elution by heat to capture the non-biotinylated strand for MALDI processing.

Results

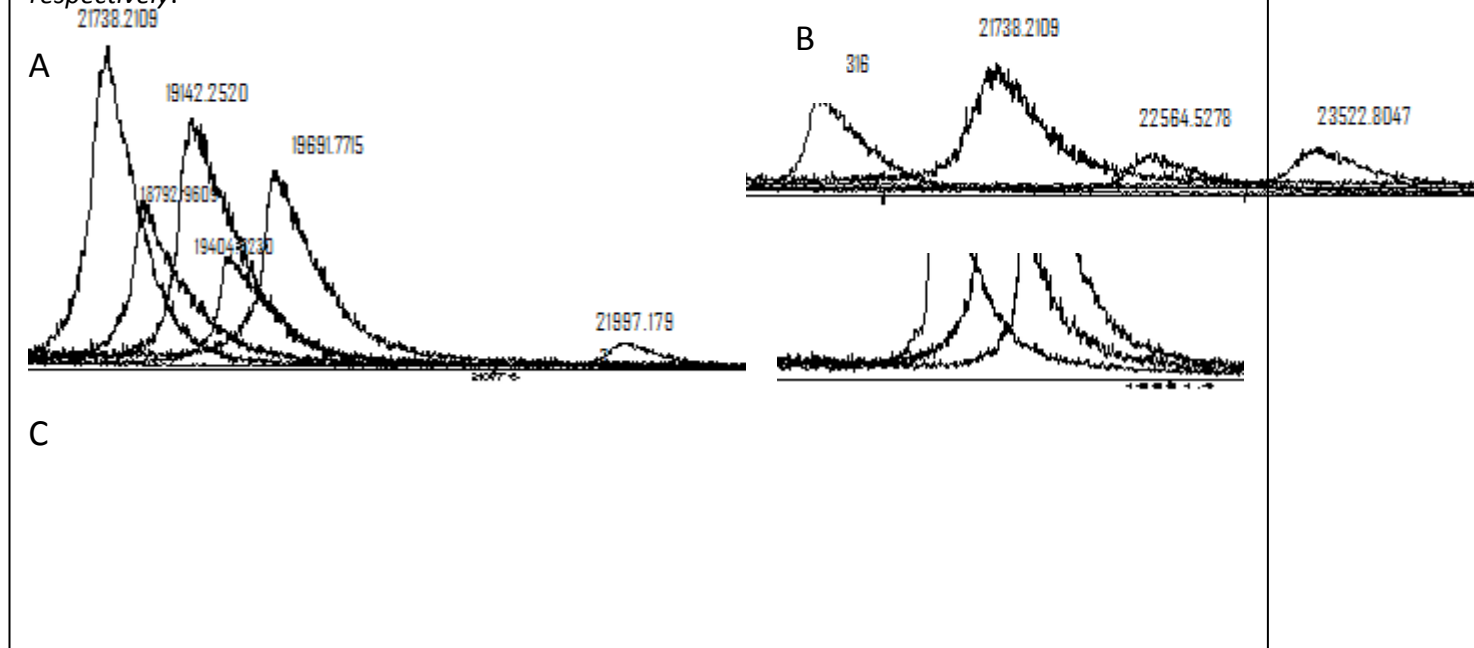
Spectra were obtained for both synthetic DNA and captured PCR products. Observed peaks corresponded to expected masses calculated for each allele. Within each locus, spectra were overlaid as

shown in Figure 2. The resolution of the peaks provided sufficient power to discriminate the mass difference of a single cytosine nucleotide (280 Da) in the G1 and G2 locus and the trinucleotide ACC (891 Da) in the GGT locus. Three distinct peaks were observed when DNA representing three different alleles of the G2 locus was combined in equal molar ratios before spotting on the MALDI plate.

Discussion

Typing of SSRs provides valuable information to understand the diversity, distribution, and transmission patterns of *Map*. The accuracy in which the repeat lengths are determined is critical in this genotyping method. The use of MALDI-TOF MS offers several advantages including accurate, reproducible and inexpensive characterization of SSRs which has traditionally been a challenge using DNA sequencing. The use of synthetic DNA was essential to accurately develop this assay, as we could test a number of alleles with a known sequence. With the detection of a repeat length of 19 in the G1 locus, we demonstrated the potential to accurately identify the number of G repeats far beyond what is currently possible using other techniques. The ability of MALDI-TOF MS to detect multiple alleles in a single sample offers additional advantages over other genotyping methods, as mixed infections may be identified. This technique enables our current efforts to thoroughly investigate *Map* genotypes isolated from dairy herds across Canada.

Figure 2: MALDI-TOF MS spectra of synthetic oligonucleotides matching PCR amplicons of the G1 (A), G2 (B), GGT (C) loci. The number of repeats are 7, 8, 9, 10, 11, 19 (A); 8, 9, 10 (B); 3, 4, 5, 6 (C) respectively.



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GENOMIC VARIATIONS ASSOCIATED WITH ATTENUATION IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* VACCINE STRAINS

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) vaccine strains were first developed 70 years ago, yet the degree and underlying mechanism of their attenuation is still unsure. Most have been widely distributed and regularly subcultured. This study determines the relative attenuation in mice of three vaccine strains, describes their genomic variability and details the changing genomic compliments from low passage 'old' strains of 316F to the currently predominant high passage variants.

Methods

MAP genome microarray, PCR and sequencing determined genomic complements, rearrangements and large tandem duplications of a panel of 10 vaccine strains with varying passage history. Virulence of three 'Weybridge' strains was also assessed using a 12 week infection mouse model.

Summary of Data

Vaccine strains II, 2e and a 316F Norway variant contained large deletion regions. Vaccine strain II and 316F UK and Netherlands variants contained large tandem duplications. 316F variants showed a high variability in transposable element copy numbers but were most similar to the virulent MAP K10 genome. Vaccine strains II, 2e and 316F UK variant were differentially attenuated in the mouse and significantly less virulent than a cervine strain ($p < 0.001$).

Conclusion

Vaccine strains were significantly attenuated in vivo and many showed unique genomic variations. Some of these may contribute directly to their attenuation; however inconsistency in variability suggested no mechanistic consensus.

INVESTIGATION OF INTER-SPECIES DISTRIBUTION AND GENOTYPE DIVERSITY OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN THE RUMINANT AND NON-RUMINANT LIVESTOCK SPECIES CO-HABITING IN CLOSED ECOSYSTEM IN THE TEMPERATE CLIMATE OF SOUTHERN INDIA

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Present study aimed to investigate genotype diversity and interspecies sharing of *Mycobacterium avium* subspecies *paratuberculosis* in a closed ecosystem of Kodai hills of South India. A total of 291 fecal samples from ruminants (n=201) and non-ruminants (n=77) livestock species were analyzed. Of 201 ruminants fecal samples; 45, 33, 123 and 13 were from cattle, buffaloes, sheep and Indian wild bison, respectively. The 77 samples were from a colony of domesticated rabbits. Samples were screened for the presence of MAP using microscopy and IS900 PCR. IS900 positive samples were subjected to IS1311 PCR-REA for genotyping. Of the 291 fecal samples, 126 (43.2%) and 37 (12.7%) were positive for MAP in microscopy and IS900 PCR, respectively. Prevalence of MAP was higher in ruminant species as compared to non-ruminants (Microscopy-33.7%, IS900 PCR-7.7%). Independently, 31.1, 27.2, 56.0, 61.5, 33.7% and 20.0, 15.1, 9.7, 38.4, 7.7% samples were positive for MAP using microscopy and IS900 from cattle, buffaloes, sheep, wild bison and rabbits, respectively. All IS900 positive DNA samples were genotyped as 'Indian Bison Type' using IS1311 PCR-REA. Presence of identical genotype of MAP ('Indian Bison Type') in domestic, wild and non ruminants species in a close ecosystem confirmed the interspecies transmission of MAP. Study indicated the role of wild life in disease transmission and origin, sharing and potential of one genotype ('Indian Bison Type') infecting multiple livestock species in a closed ecosystem.

IDENTIFICATION AND CHARACTERIZATION OF A SPORE-LIKE MORPHOTYPE IN CHRONICALLY STARVED *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* CULTURES

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Mycobacteria represent a group of highly successful organisms that range from free-living saprophytes to those that have adapted full dependence on a living host. During their life cycle, mycobacterial species may encounter a number of stresses including nutrient deprivation, hypoxia, acidic pH, and even competition with other organisms for limited resources and occupation of a specific niche, such as soil and water [3,4,5,6]. In order to survive in such unfavorable conditions, mycobacteria have developed mechanisms to achieve dormancy, latency and persistence. While several studies have investigated persistence in mycobacteria, the definition remains loosely explained and the mechanisms that lead to and sustain this state of non-replication are poorly understood. A recent study by Ghosh et al. stated the formation of endospores in two month old cultures of *M. marinum* and *M. bovis*, which may serve as an unprecedented method employed by mycobacteria to withstand harsh conditions. The concept of sporulation in mycobacteria continues to spark controversy and challenges our current perceptions of the facets involved in mycobacterial persistence. Follow-up studies conducted by Traag et al. could not reproduce endospore formation in 4 week to ~8.5 month liquid cultures of *M. marinum*, which questioned the purity of cultures used in ultrastructural characterization by Ghosh et al. The current research trend focusing on sporulation in mycobacteria remains to reproduce findings by Ghosh et al. using identical isolation methods; however, we investigated whether the potential for sporulation was limited to *M. marinum* or may encompass another saprophyte and animal pathogen, *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

MAP, the causative agent of Johne's disease (JD) in ruminants, is one of the most prevalent and well-documented infections of dairy cattle worldwide. To date, JD eradication remains implausible and control efforts are hampered due to MAP's persistence within soil and water as well as shedding by subclinical and clinical cattle. Therefore, it is critical to augment our knowledge of the events that precede non-replication as well as the various mechanisms used to attain it. We show that dormant cultures of MAP contain a mix of vegetative cells and a previously unknown morphotype resembling a spore. These spore-like structures can be enriched for using sporulating media. Furthermore, purified MAP spore forms survive exposure to heat, lysozyme and proteinase K. Heat treated spores are positive for MAP 16SrRNA and IS900. MAP spores display enhanced infectivity as well as maintain acid-fast characteristics upon germination in a well-established bovine macrophage model. This is the first study to demonstrate a new MAP morphotype possessing spore-like qualities. We propose that MAP may utilize sporulation as a mechanism to persist in unfavorable conditions such as those encountered in soil and aquatic environments. MAP may also commit itself to a spore-like state to survive the pressures applied by pasteurization and thereby provide one explanation for MAP detection in commercialized dairy products. Although significant strides have been made, especially within the last ten years, in understanding mycobacterial persistence, it continues to be fraught with ambiguities and dissension. The findings by Ghosh et al., which identified spores in *M. marinum*, and those presented in this paper for MAP are difficult to grapple with as they defy key concepts and change our perceptions of persistence, dormancy and transmission for MAP. This new MAP morphotype or spore readily invaded bovine MDMs, germinated and developed into acid-fast bacilli. More importantly, enrichment and isolation of this new morphotype was independently conducted by a second laboratory (NADC) using a separate MAP culture grown at that facility. Concerns are raised due to the similarities of spore formation in MAP and *Bacillus* given widely divergent genera. However, 1) certain species of *Streptomyces*, another genus of the *Actinobacteria*, are capable of endospore formation at suitable conditions and 2) DPA is also present in *Streptomyces* despite the lack of *spoVF* operon. Identification of unique MAP spore coat proteins as well as the cues leading to sporulation may aid in future diagnostics for food and environmental safety. Further studies are needed to examine the role of this newly described MAP morphotype in soil and

aquatic environments as well as post pasteurization in dairy products utilizing the above aspects to assess impact in transmission and persistence.

HIGH RESOLUTION MELTING WITH UNLABELLED PROBE FOR SHORT SEQUENCE REPEAT ANALYSIS IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* SUB-TYPING

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ABSTRACT

Among all the methods used for the genotyping of *Mycobacterium avium* subsp. *paratuberculosis* isolates, Short Sequence Repeat (SSR) loci analysis showed the highest discriminatory power. However, this technique requires direct sequencing of amplicons for the correct assignment of the repeat number. We developed a new method, alternative to sequencing, focusing on SSR8 locus. Our approach is based on asymmetric qPCR followed by High-Resolution Melting (HRM) analysis with Unlabelled Probes. Data obtained with our method showed a perfect concordance with those obtained using direct sequencing. Moreover, it resulted faster, simpler and more cost-effective than sequencing.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (Map), the causative agent of paratuberculosis, is characterised by a very slow growing rate and limited genomic diversity [1]. At the moment, methods based on Micro- and Mini- satellites analysis are the most used techniques for the genotyping of Map. Particularly Short Sequence Repeat loci analysis (SSRs) showed the highest allelic diversity, making these loci very useful for the genotyping [2, 3]. However, the requirement of expansive sequencing systems represents a great disadvantage for their use.

We developed a new approach, alternative to sequencing, for the identification of SSR loci repeat number. We focused on SSR8 locus, which is constituted by GGT triplets ranging from three to six repeats (four alleles described in literature).

Our approach is based on an asymmetric qPCR, followed by High-Resolution Melting (HRM) analysis with unlabelled probes [4]. The efficiency and specificity of asymmetric PCR reaction was improved designing the primers according to the LATE-PCR (Linear-After-The-Exponential PCR) strategy [5].

MATERIAL AND METHODS

Map strains were collected at the Italian National Reference Centre for Paratuberculosis. Basically, the DNA was extracted, after homogenisation in Tissue Lyser (Qiagen), with QIAamp DNA mini kit (Qiagen). Primers and probe were designed, referring to Map K10 genome sequence (GenBank no. AE016958), with Beacon Designer 7.60 (Premier Biosoft International, USA) and then modified according to LATE-PCR strategy.

The concentrations of the primers and probe were: 50 nM for the limiting primer (forward), 500 nM for the exceeding primer (reverse) and 500 nM for the probe. Primers and probe sequences were: forward 5'-CGGGTGC GCGAGCTGGTGC-3', reverse 5'-CGCTCCTCGGGCATCTGC-3' and probe 5'-GAGGCGCGGGTGGTGGTGGTGGTGGTGGCGCA-3' (in bold the sequence locus). The 3' end of the probe was blocked with C6-amino group. Cycle PCR conditions were: initial denaturation at 95 °C for 3 min, then 50 cycles with 15 s denaturation at 96 °C and 30 s annealing/extension at 67 °C. At the end of the qPCR, samples were heated to 95 °C for 15 s, followed by 1 min at 60 °C. Then they were heated from 60 °C to 95 °C. The fluorescence was recorded each 0.3 °C step (approximately 3.3 times per degree). Data were exported and processed according to Palais and Wittwer [6]. In our collection was not present a strains containing the allele with six repeats, so a synthetic single strand DNA amplicon holding six triplets was generated copying one µg of the reverse ss DNA (purchased by Eurofins MWG, Ebersberg, Germany) in presence of forward primer.

The number of triplet repetitions for all strains was confirmed by sequencing with ABI Prism 3100 Avant Sequencer (Applied Biosystems), according to the original paper [3].

RESULTS AND DISCUSSION

Representative results carried out by HRM analysis, are shown in Figure 1 as raw semi-log plot (left) and derivative melting curves after normalisation and exponential background removal (right). Two melting domains for each sample were observed: one relative to the amplicon omoduplex (DNA double strand)

and another one to the heteroduplex single strand DNA/probe. According to LATE-PCR strategy, the omoduplex products were generated during the first cycles of amplification, while the single strand DNA during the late cycles.

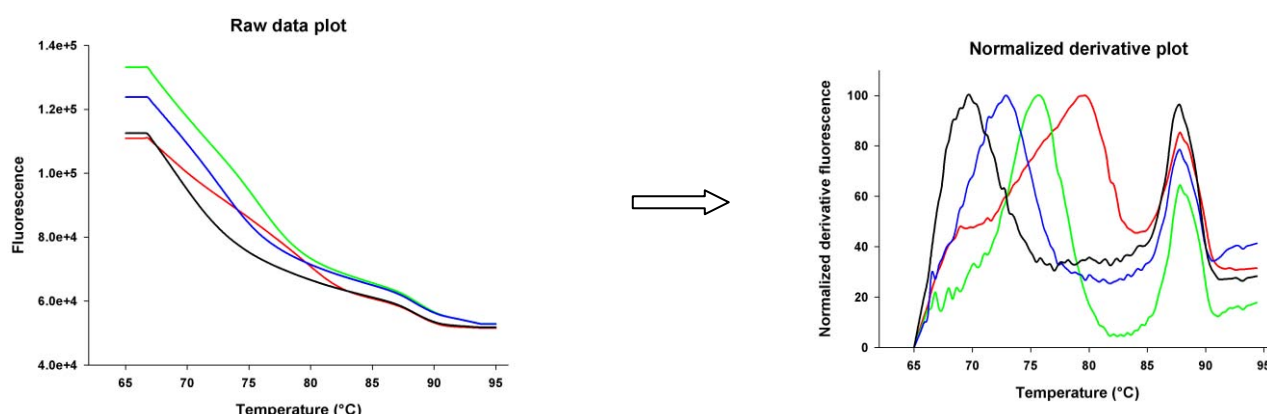


Fig. 1. Raw data and normalized derivative plots. Black: three repeats, blue: four repeats, green: five repeats, red: six repeats (artificial amplicon). The analysis of homoduplex domains did not allow any differentiation among the various alleles, while the analysis of heteroduplex domains allowed a unbiased identification of each allele.

These data were confirmed by the analysis of two strains carrying three repetitions, 20 with four repetitions and 20 with five repetitions [7] while a complete agreement (Cohen's kappa coefficient 1) between our approach and the results of the direct sequencing (data not shown) was found. Our work showed that the use of short unlabelled probe directly in PCR reaction mix enhances the differences between each variant, allowing an unbiased identification of the number of repeats present in the locus.

The method herein proposed is robust, reproducible, cost effective and faster than direct sequencing and could be usefully applied to resolve the locus SSR8. To our best knowledge, this is the first article suggesting the application of HRM analysis with unlabelled probe in short repeat number identification analysis.

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MOLECULAR CHARACTERIZATION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) ISOLATES IN AUSTRIA

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Introduction

In 2006 Austria started a national paratuberculosis (PTB) control programme based on government regulation which affects cattle, sheep, goat and farmed deer. PTB was declared a notifiable disease, so animals showing clinical signs have to be tested for MAP at the national reference laboratory. MAP strains isolated from different herds between 2006 and 2011 and strains from red deer were selected for molecular characterization.

Material and methods

165 MAP field strains from 164 clinically diseased ruminants were analysed by IS900 RFLP with two restriction enzymes (BstEII, PstI) and MIRU-VNTR at 8 genomic loci (MIRU292,X3; VNTR25,47,3,7,10,32) according to Möbius et al. (2008) and Thibault et al. (2007). These field strains included 141 MAP cattle isolates from 73 farms and different breeds (Angus, Aquitaine Blonde, Austrian Brown Mountain, Fleckvieh (Simmental), Tyrolean Grey Mountain, Holstein Friesian, Jersey, Limousin, Murbodner, Piemontese, Red Friesian, Danish Melkrace), 19 isolates from goat and 4 isolates from red deer.

Results

The combination of RFLP and MIRU-VNTR analysis allowed a differentiation of 19 MAP strain types classified AT1 to AT19. Detailed results and frequency in animals and herds are shown in Table 1. The occurrence of MAP strain types in different cattle breeds, goats and red deer is listed in Table 2.

Table1: Molecular characterization of 165 MAP field strains from cattle, goat and red deer (RD)

AT	RFLP patterns	INMV* profiles	MIRU		VNTR						Σ	
			292	X3	25	47	3	7	10	32	animals	herds
1	C1-P1	1	4	2	3	3	2	2	2	8	16	8 1RD
2	C1-P1	2	3	2	3	3	2	2	2	8	58	31 2RD
3	C18-PnewIII	2	3	2	3	3	2	2	2	8	18	6
4	C1-P1	17	3	1	3	3	2	2	2	8	8	6
5	C18-PnewII	2	3	2	3	3	2	2	2	8	4	3
6	C1-PnewV	2	3	2	3	3	2	2	2	8	9	5
7	C1-P7	1	4	2	3	3	2	2	2	8	1	1
8	C1-P1	5									2	2
9	C18-PnewIII	1	4	2	3	3	2	2	2	8	15	9
10	C1-P1	new	2	2	5	2	2	2	2	6	1	1
11	CnewI-PnewIII	2	3	2	3	3	2	2	2	8	1	1
12	C18-P1	17	3	1	3	3	2	2	2	8	1	1
13	C28-PnewI	1	4	2	3	3	2	2	2	8	1	1
14	C18-PI	2	3	2	3	3	2	2	2	8	5	2
15	C1-PnewIV	2	3	2	3	3	2	2	2	8	1	1
16	C1-P1	12	2	2	5	2	2	2	2	8	3	1
17	CnewIII-PnewIV	2	3	2	3	3	2	2	2	8	19	1
18	C14-PnewIII	2	3	2	3	3	2	2	2	8	1	1
19	C1-P1	new	2	4	2	2	1	1	2	8	1	1RD

*INMV: INRA-Nouzilly MIRU-VNTR

Table 2: The occurrence of MAP strain type AT1-AT19 in different cattle breeds, goat and red deer

Breed species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Angus		3																	
Aquitaine Blonde					2	3													
Austrian Brown Mountain	1	3				1													
Fleckvieh	2	10	1	5	1			2											
Austrian Grey Mountain	2																		
Holstein Friesian	1	9			1	3										3			
Jersey		3	3				1								1				
Limousin	5	25	12	3					15		1		1	5				1	
Murbodner			1									1							
Piemontese	4																		
Red Friesian		2	1			1													
Danish Melkrace		1				1				1									
Goat																	19		
Red deer	1	2																	1

Discussion

Despite the fact that 19 different MAP types were detected in a comparatively large and diverse sample selection, 42% of all isolates were classified as MAP type AT2 (RFLP C1-P1; INMV 2). MAP type AT2 was found in 8 of 12 PTB affected cattle breeds and in 2 of 4 isolates from red deer and is therefore the predominating MAP type strain in Austria. 9 new RFLP patterns and 2 new INMV profiles could be determined within the 165 analyse field strains. Some unique occurring strains showed association with certain cattle breeds, goat and red deer (Table 2). This is the first study carried out in Austria providing data about MAP strain types on a national scale. Additional information about routes of transmission between herds and the epidemiology of PTB in Austria could be achieved by using the national cattle data base and the results of MAP typing.

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QUANTIFICATION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* STRAINS WITH DISTINCT GENOTYPES AND ISOLATED FROM DIVERSE HOSTS USING A LIQUID CULTURE SYSTEM

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INTRODUCTION

Standard plate counting methods for slowly growing mycobacteria are laborious, expensive, time-consuming and frequently failed due to either contamination or medium dehydration during the incubation period. In contrast, liquid culture systems have greater sensitivity than solid culture, and shorter time to detection of mycobacteria (1). In this study, the quantitative capabilities of an automatic liquid culture system (ALCS) were evaluated for 11 clinical strains of *Mycobacterium avium* subsp. *paratuberculosis* (Map) isolated from domestic (cattle, sheep, goat), or wild-life species (fallow-deer, deer, wild-boar, bison). The strains were previously genotyped in distinct IS1311 PCR-REA profiles (C, S or B types) (2).

MATERIAL AND METHODS

For each of the Map strains, strain-specific quantification curves were generated by relating the time to detection in the ALCS to the estimated log₁₀ CFU in the inoculum. Universal standard curves and algorithms for the quantification of each cluster strains were generated. In addition, the ALCS system was compared with a *F57* Real-time qPCR assay (Vacunek, Spain) for the quantification of the 11 Map strains.

RESULTS

Our results suggested that Map strains can be classified into two distinct clusters based on their growth curves in the ALCS. The first group included the S-type strain isolated from goat and all the sheep strains (C, S and B genotypes). A second group contained the C or B-type strains isolated from cattle, goat and wild-life animals, with the exception of the fallow-deer strain. Interestingly, the C-type strain isolated from fallow-deer had a standard curve similar to the curves of group I and it was clustered within this group. Correlations between the estimated log₁₀ CFU and Map DNA copy numbers were very high for all the tested strains ($R^2 \geq 0.9$).

CONCLUSIONS

We describe a simple and fast method for quantifying viable Map strains isolated from diverse host, including wild-life animal species. Overall, our results demonstrated that Map strains isolated from goat or from wild-life animals vary in their rate to grow in liquid culture and that strain-specific variation might occur. In contrast, Map strains isolated from cattle or sheep showed similar strain-specific standard curves irrespective of its genotype.

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EXPLOITING MULTIPLE-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS TO EVALUATE STRAIN DIVERSITY OF *M. AVIUM* COMPLEX IN ARGENTINA

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Introduction & Aim. The *Mycobacterium avium* complex (MAC) includes major human and animal pathogens. MAC related diseases have a significant impact on human health, mainly in immunocompromised patients. *M. avium* subsp. *paratuberculosis* (MAP) is the most economically important subspecies due to severe economic losses in infected herds, affecting the sustainability of agriculture even at familiar scale in developing countries. There is still lack of nationwide, reliable epidemiological data. Serological surveys in Buenos Aires province, one of the most important productive regions of the country, reported a prevalence between 0.8%- 19.6% in breeding herds and between 16.7%-39.3% in dairy herds of different locations (INTA-Balcarce). The impact of other subspecies such as *M. avium* subsp. *silvaticum* (MAS), *M. avium* subsp. *hominissuis* (MAS) and *M. avium* subsp. *avium* (MAA) in animal health is largely unknown, and frequently underestimated as a consequence of a poor diagnosis. Molecular typing has contributed with the correct identification and the intra-species differentiation of isolates, helping to understand the epidemiology of these bacteria. The PCR-based methods for molecular typing analysis represent an important technical advance and have simplified the molecular typing of microorganisms. The aim of this study was to determine the genetic diversity of *Mycobacterium avium* complex isolates through multiple-locus variable number tandem repeat analysis.

Methodology. We selected a scheme based on eight MIRU-VNTR *loci* according to Thibault *et al.*, (2007). We analyzed 95 isolates of MAP (bovine, n=76; caprine, n=16 and ovine, n=3) from farms of different regions of Buenos Aires province. The isolates were previously confirmed by IS900-PCR (Collins *et al.*, 1993). Additionally, 25 isolates typed by IS1245-PCR as positive (Guerrero *et al.*, 1995) and characterized by *hsp65*-PRA (Telenti *et al.*, 1993) as *M. avium* type I were also analyzed: Six human samples from patients with non-tuberculous mycobacteriosis (HIV+, Cetrángolo Hospital, Buenos Aires), 2 isolates from cows, 15 isolates from pigs (sampled at the abattoir settled in the same province) and 2 from captive birds. All of the human isolates were identified as MAH since the IS901-PCR (Slana *et al.*, 2010) was negative. All the bird, cattle and pig isolates were IS901 positive. This panel of 120 isolates was subjected to the scheme based on repetitive elements proposed by Thibault and coworkers to type *M. avium* subsp. *paratuberculosis* and further employed to type other subspecies (Radomski *et al.*, 2010). PCR conditions were used as described previously with minor modifications. PCR products corresponding to different *loci* (292-X3-25-47-3-7-10-32) were resolved by 4% agarose gel electrophoresis and tandem repeats were determined using the BioNumerics software (Applied Maths, Belgium). The numerical profiles were classified according of INMV (INRA, Nouzilly, MIRU-VNTR) combinations suggested previously. (Thibault *et al.*, 2007; Radomski *et al.*, 2010).

Results

Profiles & Frequencies. Eight different INMV MIRU-VNTR patterns were identified for the MAP-isolates. The INMV1 pattern grouped 64.2% of the isolates and was the most frequent. The distribution of the other patterns was: INMV2, 10.52%; INMV16, 8.42%; INMV33, 7.3%; INMV13, 3.16%; and INMV11, 2.1%. One cattle isolate presented a pattern that, to our knowledge, has not been previously identified. On the other hand, in three isolates we could not obtain the PCR product for the *locus* 292 and 7. However the alleles in the rest of the *loci* indicated a different pattern to those mentioned above. This

uncomplete pattern represented 3.15% of the isolates. INMV1 and INMV2 patterns were the unique shared by bovine, caprine and ovine strains. Four out of six farms were co-infected with two or more genetically unrelated strains (2 to 6 different patterns). Co-infection at individual level was also demonstrated through the analysis of different isolates from the same animal (n=1). In spite of the low number of MAH isolates studied, we could observe that all the *loci* were polymorphic (5 and 3 *loci* resulted polymorphic in the other subspecies). Five human isolates were grouped in 3 patterns not previously described and one human isolate was identified with a pattern already reported: INMV51. Three patterns were found in pig isolates and cattle isolates shared the same profile. These 4 patterns were not previously reported. In spite of the difficulties to determine the allele corresponding to *locus* 7 in bird isolates (a difference of approximately 6bp was observed), these isolates were classified as INMV100. The presence of polymorphisms in one or more repeats could generate PCR products of different sizes to those expected as previously reported for the same *locus* (Castellanos *et al.*, 2010). We could not establish epidemiological link between different hosts, this was probably due to the reduced number of isolates studied here.

Allelic diversity and Discriminatory index. The allelic diversity and the discriminatory power were estimated as described previously by Castellanos *et al.* (2009). Epidemiologically unrelated strains were selected to this end. The *locus* 292 showed the maximum allelic diversity for MAP strains (0.53) whereas X3 (0.68) and 25 (0.6) were the markers most informative for MAA and MAH respectively. The results obtained for MAP and MAA were coincident with the previous obtained by Radomski *et al.* (2010) and others. We obtained a Hunter and Gaston discriminatory index (HGDI) of 0.86 for all subspecies. The HGDI for MAP was 0.68, lower than the discriminatory index obtained by Thibault *et al.*, (0.75), but similar to that obtained by Stevenson *et al.*, (0.64). A novel panel of MAA and MAH isolates would be suitable to obtain more accurate data in these subspecies.

Conclusions and Perspectives

The most predominant patterns in *M. avium* subsp. *paratuberculosis* strains (INMV1 and INMV2) were also described as the most frequent in other countries from Europe (Thibault *et al.*, 2007, Stevenson *et al.*, 2009). This result could be an indicative of higher transmission rate or infectivity of these clones. This study suggests that co-infection with different strains could be a common feature of paratuberculosis infection in Argentina and may point out trading of animals between herds and highlights the lack of monitoring programs in our country.

Despite the low number of isolates processed in the rest of the members of the MAC, we obtained 9 patterns, most of them not described previously. This highlights the high degree of MIRU-VNTR diversity of *M. avium* subsp. *avium* and *hominissuis*, being congruent with previous studies.

Several authors have used the MIRU-VNTR typing scheme proposed by Thibault and coworkers to type MAC isolates from different countries of the world: France, Germany, Czech Republic, Finland, Greece, The Netherlands, Norway, Scotland, Spain, Japan and more recently Colombia and Argentina among others (Möbius *et al.*, 2008; Stevenson *et al.*, 2009; Inagaki *et al.*, 2009; Castellanos *et al.*, 2010; Radomski *et al.*, 2010; Fernández-Silva *et al.*, 2011, this work). Several studies have included new markers and/or omitted others or have used a different pattern nomenclature. In this way, the results from different studies are very difficult to compare. Hence, the standardization of protocols must be encouraged. Finally, a MAC database could be a suitable tool for understanding *global epidemiology of these bacteria*. Collaborative work should focus to these ends.

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GENOME SEQUENCING TO IDENTIFY PHYLOGENETIC DIVERSITY AND SINGLE NUCLEOTIDE POLYMORPHISMS IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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The aim of this study was to undertake whole genome sequencing of multiple isolates of *Mycobacterium avium* subsp. *paratuberculosis* of different provenance to investigate phylogenetic diversity and identify single nucleotide polymorphisms (SNPs) between different subtypes. One hundred and twenty six isolates from 18 countries and 16 different hosts have been sequenced to date using the Illumina/Solexa approach. The strain panel included pigmented, 'Type III' and 'Bison-Type' isolates, Crohn's isolates and vaccine strains. SNP analysis was performed and a phylogenetic genome tree generated. Two major lineages were observed corresponding to the previously identified 'Type I' or 'Sheep-Type' and 'Type II' or 'Cattle-Type' strains. Two clusters were observed in the 'Type I' lineage that broadly correlated with the pigmented and 'Type III' strains. The 'Bison-Type' was confirmed as a subtype of 'Type II'. We would like to acknowledge all laboratories and collaborators who provided strains for the analysis, too many to list in this abstract.

GENETIC EVALUATION OF IS900 PARTIAL SEQUENCE OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) BRAZILIAN ISOLATES FROM BOVINE MILK

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The heterogeneity of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolates has been exploited for epidemiological purposes and IS900 sequence has been used for identification of MAP. The objective of this study was to verify the genetic conservation of IS900 partial sequences of Brazilian isolates from raw bovine milk samples. DNA extraction and IS900-PCR were performed in 222 milk samples. Six samples were positive and PCR products were cloned and sequenced. The resulting sequences were compared with other MAP sequences from GenBank and it was possible to identify eight polymorphic regions and to form five distinct haplotypes. Using the grouped sequences it was possible to create a network and to determine the number of mutations in each group of haplotypes. Just one mutation was found in haplotypes H3, H4 and H5 when they were compared with H1. In the haplotypes H3 and H4 we found a G instead a A in the positions 598 and 131 respectively. In the haplotype H5, we found a T instead a A in the position 609. Haplotype H2 showed five mutations in relation to H1. We found a A instead a C in the positions 474, 543, 568, 588 and 594. Based on the number of mutations found in the present study, we have verified that IS900 is a very well-conserved sequence even when we compared different isolates from different countries and could continue being used as tool for the molecular detection of this agent and epidemiological purposes. The genetic differences between MAP strains related to geographic factors have a potential use in the epidemiological tracing of the disease, however, there are no studies about the distribution of the MAP genotype or strain types in Brazil. This is the first study that reports genetic differences of MAP in Brazil.

Financial support: CAPES, FAPEMIG and CNPq.

HOW MANY? MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS COMPARISON OF QUANTIFICATION METHODS

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M. avium subsp. *paratuberculosis* (MAP) is a slow growing microorganism with a strong tendency to form clumps. These characteristics have hampered MAP quantification, an important issue when it comes to preparing inoculum or assessing shedding levels in infected animals. In this study, growth characteristics of 8 strains of MAP (K10, bovine reference strain; 316F and 2E, bovine vaccine strains; 81, 764 and 445 bovine field isolates; 22G and Ovicap 49, ovine field isolates) in 7H9 media supplemented with OADC, Tween 80 and mycobactin J have been assessed, and quantification methods (wet weight, turbidometry, agar plating and ParaTB Kuant-VK qPCR) have been compared. Generation times for all strains were dependent on quantification method with lowest value of 30 hours for qPCR and highest value of 15 days for turbidometry. Field isolates showed faster growth and higher production than more laboratory-adapted strains (Table 1).

Strain	Wet Weight	Turbidometry	Viable Count	qPCR
	g (d)	g (d)	g (d)	g (d)
316F	8.99	10.27	1.72	1.54
2E	12.19	12.49	1.38	1.46
K10	8.70	14.00	1.54	1.22
81	10.91	15.28	1.35	1.34
445	8.75	12.34	1.39	1.32
764	10.67	8.65	1.40	1.32
22G	8.41	12.65	1.48	1.49
Ovicap 49	8.34	6.12	1.98	1.54
All				
Mean	9.619	11.475	1.53	1.40
SD	1.437	2.979	0.218	0.119
CV	14.94	25.96	14.28	8.50

g: generation time, d: days

Table 1. Estimated generation times for wet weight, turbidometry, viable count and qPCR.

Correlation analyses between methods showed best coefficients among wet weight, turbidometry and qPCR. Lack of correlation for agar plating with the rest of the methods was partially expected since it is the only method that determines viable and culturable bacteria and because agar plating has repeatedly been reported to underestimate bacterial numbers (1CFU>1 bacterium) (Figure 1).

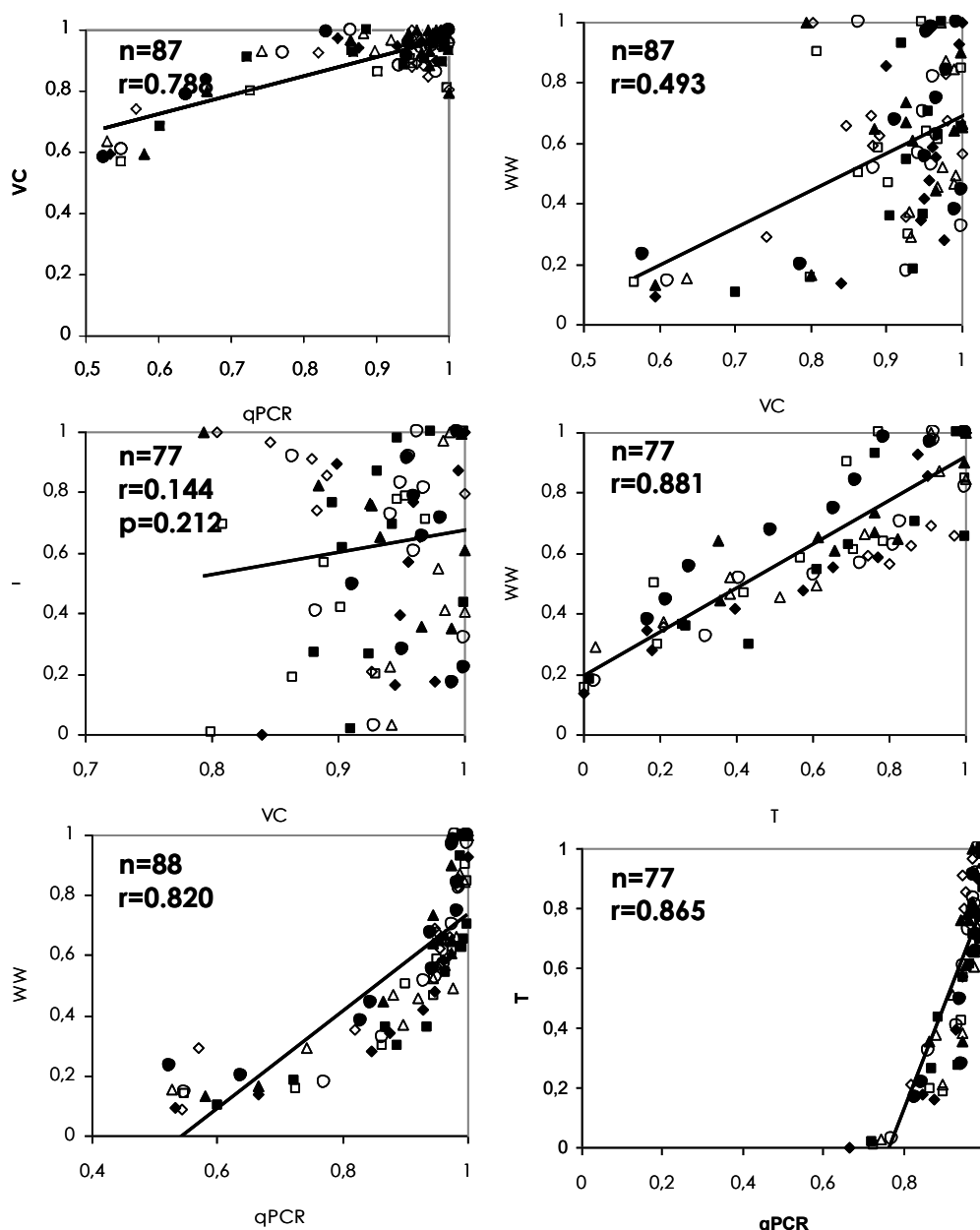


Figure 1. Correlation between quantification methods (WW: wet weight, T: turbidometry, VC: viable count, qPCR: ParaTB-Kuanti qPCR) for all strains (■ 316F, ● 2E, △ K10, ○ 81, ◆ 445, □ 764, ▲ 22G, ◇ Ovicap 49). Trend lines represent least squares estimation. All values have been divided by the maximum value for each strain and quantification method. Pearson correlation coefficients were all significant except for qPCR vs T. It is concluded that wet weight and turbidometry are valid methods only when used during exponential phase or thereafter. Agar plating and qPCR can be used over a wider range of bacterial concentrations. Our results show that ParaTB Kuanti-VK qPCR shows more consistency among repeated samples, and it is fast and accurate. This single copy gene qPCR method provides an opportunity to estimate MAP number in a rapid and reliable way.

MOLECULAR CHARACTERIZATION OF A STRAIN OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS ISOLATED FROM GOAT MILK

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of the chronic, granulomatous, wasting Johne's disease (JD). JD is an economically important disease inflicting huge production losses to both meat and milk industry world over. Apart from the economic importance, another major concern with this pathogen is its suspected involvement in causing Crohn's disease in humans. In the event of confirming the role of MAP in causing Crohn's disease, presence of MAP organisms in milk may raise serious concerns in human health. In recent times, MAP infection has been well established in cattle in India. However, there is paucity of information with regard to JD in small ruminants. India has a goat population of 125.7 million and contributes to 17% of the global goat population. The present study is aimed at screening goat milk for MAP infection by PCR considering the public health and socio-economic factors. A total of 123 milk samples collected from goats were initially tested for the presence of MAP by IS900 polymerase chain reaction. Out of the 123 samples tested two samples were found positive by IS900 PCR. These two samples also gave positive amplification with ISMap02 and IS1311 specific primers. IS900 PCR products were sequenced and phylogenetic analysis of the IS900 gene sequence placed the two goat isolates from in India in two separate clusters.

MLSA-DERIVED PHYLOGENY OF GERMAN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* STRAINS

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Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of paratuberculosis, has a world-wide distribution nowadays. Germany was considered a presumptive point of origin of this disease. To challenge this hypothesis, we aimed to identify MAP genotypes within cattle herds and other hosts in Germany, and to determine their phylogenetic relation to isolates from other regions of the world.

Forty, epidemiologically unrelated MAP isolates were selected for multilocus sequence analysis (MLSA) based on their high genetic heterogeneity as determined by combined IS900 restriction fragment length polymorphism (RFLP), mycobacterial interspersed repetitive unit - variable-number tandem-repeat (MIRU-VNTR), and multilocus short sequence repeat (MLSSR) analysis.

Detected by specific PCR and IS900-RFLP, two of these isolates (sheep isolates) belonged to the MAP-Type-III while the others belonged to the MAP-Type-II group. MAP-Type-II isolates consisted of isolates from cattle, deer, sheep, goat, donkey, and humans and also included two reference strains (DSM 44135 and ATCC 19698). MLSA showed four sequence types (ST): ST 30 and 32 for MAP-Type-II isolates and ST 35 and 36 for MAP-Type-III isolates. These results correspond to four out of seven sequence types published for MAP isolates from United States, Canada, New Zealand, United Kingdom, Australia, Farao Islands and Iceland (1). Within the MAP-Type-II group genotypes detected by IS900-RFLP, MIRU-VNTR, and MLSSR did not correspond to those of MLSA.

MLSA-derived phylogeny shows that German MAP isolates are identical to isolates from above-named regions of the world. The fact that four out of seven known MAP ST can already be detected in a limited number of German strains supports the idea that MAP strains distributed all over the world have the same clonal origin.

Reference

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IMPROVEMENT OF THE ESTIMATION OF G-REPEAT NUMBERS OF MLSSR TYPING USING FRAGMENT ANALYSIS WITH “CHAPERONE MARKER”

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PCR-based typing methods are expected to provide a simpler and faster typing and a better discrimination than before. Multi-locus Short Sequence Repeats (MLSSR) typing appeared at first sight to be a more discriminating typing method than other typing methods. However the sequencing approach for estimating more than 11 numbers of G-repeats were hampered by supposed sequencing troubles. We applied PCR-products of G-repeat loci with the fluorescent-labeled primers to the fragment analysis using AB 3130xl Genetic Analyzer. It appeared that there were sequentially 1bp-different products in our undefined strains including ATCC 700535 and ATCC BAA-968. We prepared 1bp ladder marker from the PCR-products of already-confirmed G-repeat samples with a different fluorescent-labeled primers from the test dye. These homemade ladder markers were named “Chaperone Marker” because the markers moved with approximately half bp difference from the tested-PCR-products in the electropherograms of the fragment analysis. Using Chaperone Markers, it was suggested that precise estimations of the numbers of G-repeat were achieved in the same sample and among the samples. Furthermore, it was indicated that the single nucleotide polymorphism in the adjacent site of one locus of G-repeats was detectable by means of the difference in the precise electrophoretic mobility between the test fragment and the Chaperone Marker. Hence, the fragment analysis with Chaperone Markers would lead MLSSR to come back to the front line of the strain typing of *Mycobacterium avium* subsp. *paratuberculosis*.

SEQUENCE PROFILING OF MULTISPECIES, HIGHLY PATHOGENIC AND IMMUNOGENIC MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS 'INDIAN BISON TYPE' GENOTYPE STRAIN 'S 5' OF GOAT ORIGIN EXPOSES CO-EXISTENCE WITH INTESTINAL BACTERIA POTENTIATING GENE TRANSFER MECHANISMS

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Mycobacterium avium subsp. *paratuberculosis* 'Indian Bison type' genotype (strain 'S 5') is multispecies, highly pathogenic and immunogenic strain inflicting crippling Johne's disease in animals and also recovered from humans. MAP strains implicated to have deviations in genome signifying likelihood lateral gene transfer from non-mycobacterial organisms co-existing in gut environment (Ripoll et al., 2009). Strain 'S 5' is maintained on HEYM with mycobactin J, since isolation in 1999 from advance clinical Johne's disease in a goat and later characterized as 'Bison Type' (2001, Australia and 2004, Spain) and 'Indian Bison Type' (2009, India). Strain 'S 5' though in 35th passage is slow growing, mycobactin dependent and acid fast was subjected to Terminal- RFLP on ABI 3130 automated sequencer. In parallel, clone library constructed for *16s rDNA* using T/A vector was restriction digested and sequenced. Further, multiplexed paired end sequencing of 'S 5' was performed using library prepared with common adapter primers in Genetic analyzer II (Illumina-solexa). High quality reads were *de novo* assembled to contigs and aligned to reference sequence MAP K10. T-RFLP revealed multiple digestion patterns indicating presence of association of more than one species of bacteria. Alignment of *16s rDNA* sequence with NCBI database identified all bacteria besides establishing their homology and phylogeny relationship. Sequencing of strain in Illumina-solexa provided 927877 reads averaging to 72-base pair read length with 200x coverage. Assembly statistics revealed that 31.81% of reads aligned to MAP K10 and 24.9% to *Burkholderia* sp. Remaining showed dispersion of reads randomly distributed over *Salmonella* sp. and other Enterobacteriaceae organisms. T-RFLP and genome sequence profile evidence that 'S 5' tends to co-exist with other intestinal bacteria outside the gut environment. Presumably, such associations have shaped unique characters to high pathogenic 'S 5' strain by lateral gene transfer. This knowledge necessitates skillful diagnostic and prophylactic measures for the control of this raging pathogen.

INDUSTRY SPECIAL FOCUS

KANGAROO ISLAND CONTROL OF OVINE JOHNE'S DISEASE – A SUCCESS STORY?

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Introduction

Kangaroo Island is a small island located off the Southern coast of South Australia at the entrance to Gulf St Vincent. It acquired its name in 1802 after a visit by Matthew Flinders in which he noted the presence of a large number of kangaroos. The island has a length of approximately 150 kilometres and a width of 55 kilometres with a total area of around 4,370 square kilometres. Kangaroo Island has a moderate to high rainfall ranging from 430mm a year to 900mm a year.

Ovine Johne's Disease

Johne's Disease is a significant disease of ruminant animals which is characterised clinically by wasting and lack of responsiveness to remedial measures. Ovine Johne's Disease (OJD) is an enteric condition, caused by infection with the ovine strain of *Mycobacterium avium* subspecies *paratuberculosis*, a member of the *Mycobacterium* family. The bacterium commonly referred to in the abbreviated form as MPtb is an acid-fast bacillus which is closely related to *Mycobacterium avium*. MPtb has a number of phenotypic features that distinguish it from *Mycobacterium avium*; these include its markedly slower growth in culture, its in vitro requirement for the iron transport chemical mycobactin, its rough colony habit on a solid agar culture medium and its ability to infect ruminant mammals rather than birds.

Initial Spread of Ovine Johne's Disease In Australia

Sergeant (2001), in his review of the history of Ovine Johne's Disease in Australia, documents that the disease was first discovered on a property in the central tablelands region of New South Wales in 1980. By the end of 1999 a total of 499 infected flocks had been recognised in New South Wales. In 1997 the first infected flock in South Australia was discovered as result of tracing from a known infected interstate flock. In June 1998 the first property on Kangaroo Island was confirmed with the presence of Ovine Johne's Disease. By the end of 1998 a further 19 properties on Kangaroo Island had been diagnosed with Ovine Johne's Disease.

Progression of OJD on Kangaroo Island

The following table and bar chart illustrates the progression of the discovery of Ovine Johne's Disease on Kangaroo Island since its initial discovery in 1998. Disease detections were made through property tracing (either from other infected properties or from positive abattoir surveillance), by investigation of sheep properties that reported the presence of under-performing animals or the mandatory testing of neighbours to infected properties. Between 2001 and 2003, a program of testing all sheep properties on KI was also carried out.

Year	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
New infections	20	6	9	16	16	11	7	2	3	0	2	0	3	3

Source: Ewers 2011

Diagnosis of OJD

The initial screening tests for OJD were based on serology (AGID and Elisa), with follow-up confirmatory testing using histopathology and/or tissue culture. The introduction of the pooled faecal culture by Whittington et al. (2000) was a significant advance in the diagnosis of OJD on sheep properties. The pooled faecal culture provided a cheaper and more sensitive alternative to the previously utilised serological detection method. The screening test consisted of collecting a single faecal pellet from each sheep and aggregating them in lots of up to 50 pellets during collection. These pooled samples are then homogenised and cultured using the BACTEC system. Confirmation of MPtb is conducted by PCR testing using the IS900 sequence and confirmatory subculture on solid medium. This test has a disadvantage in that it can take up to 5 months to produce a final result, particularly if there is growth on

the BACTEC medium and it is necessary to proceed to subculture on solid medium. This diagnostic technique is still the current procedure.

Control Measures for OJD on KI prior to Advent of Gudair™ Vaccine

Prior to July 2002 and the introduction of Gudair™ vaccine, control of OJD was restricted to the regulatory measures of quarantine and movement controls. In addition, improved biosecurity measures such as upgraded fencing were an important aspect in the control of OJD on Kangaroo Island. Other disease reduction techniques such as total de-stocking, partial de-stocking and judicious grazing and management techniques aided in the OJD control process.

In 1998, after the initial detection of OJD on Kangaroo Island, Primary Industries and Resources South Australia (PIRSA) in consultation with OJD-affected KI sheep producers, established the Kangaroo Island OJD Control Program (KIOJDCP). This program rigorously pursued a program of quarantining any OJD infected or suspect properties. This program advocated the need for properties to improve their fencing and general biosecurity approach to disease management. Throughout the course of the KIOJDCP, 49 sheep properties completed a voluntary de-stocking program. De-stocking consists of the removal from the farm of sheep and goats for a minimum of 15 months, including two summer periods. Short term grazing of these de-stocked properties by terminal sheep during the decontamination period is permitted, although care must be taken to prevent recontamination.

However, the reliance upon de-stocking as a technique for eradicating OJD from sheep properties has resulted in a substantial number of disappointing outcomes, as manifest by frequent recurrence when the property is restocked (Taylor, 2004). The recurrence of OJD on the 49 de-stocked properties occurred in a significant number of them (seven properties) 5 were not vaccinating, 1 purchased it from Vic saleyard and the final property as far as we know had all approved vaccinates and we are currently investigating.

In late 2001 a whole of island Pooled Faecal Testing 350 program was initiated. This meant that every flock on KI (approximately 330) had up to 7 faecal pools of adult sheep collected and tested for OJD. This initiative was completed in mid 2004.

The Use of Gudair™ Vaccine on KI

In May 2002, Gudair™ vaccine became available for use on KI. The initial vaccination strategy was to vaccinate all sheep on infected and suspect properties. In addition any property deemed to be at-risk either by being a direct neighbour or a direct trace to an OJD infected property was included in the Gudair™ vaccination program. Thus, a ring of vaccinated properties occurred around each infected property. Whole flock vaccination was universally adopted in OJD control management on KI.

The South Australian Sheep Advisory Group (SASAG), who advise the relevant parliamentary Minister on ovine matters and administer the Sheep Industry Fund (SIF), orchestrated the provision of subsidised Gudair™ vaccine to OJD infected properties and OJD at-risk properties. This took the form of a 100% subsidy for OJD infected properties for a period of 5 years. At risk properties received 100% subsidy for the first year, followed by 50% subsidy for 4 years. Previously infected properties that had undertaken a de-stocking program were also offered subsidised vaccine along with their neighbours.

In 2006 Dr Evan Sergeant was commissioned to conduct "Bio-economic modelling of future control options for ovine Johne's disease control in South Australia". Using this model the six scenarios were modelled separately for Kangaroo Island and the mainland of South Australia. With such a high initial disease prevalence on Kangaroo Island (~ 30% flock prevalence), the best control measure that would reduce the flock prevalence was a "whole of island vaccination" approach. In February 2007, a five year program began to encourage non-vaccinating flocks on KI to commence vaccination in lambs that would be retained beyond 12 months of age. The Sheep Industry Fund provided a 50% subsidy for this initiative.

Also in 2009, in order to counteract any likelihood of recrudescence of OJD on previously infected properties there has been an extension of the provision of the subsidised vaccine past the initial 5 year period; Gudair™ vaccine is available at a 50% subsidy rate and this decision will be subject to annual review.

Currently all 19 OJD infected properties on KI are engaged in vaccination programs. Of the approximately 200 at-risk properties, 188 of these are vaccinating. In addition, many properties not deemed to be at-risk are using prophylactic Gudair™ vaccine.

Property Disease Management Plans- Vaccination (PDMP-V)

After confirmation of OJD on a sheep property on KI (applicable on all SA sheep properties) a PDMP-V is customised to that particular property. The plan has the following components:

- Whole of flock vaccination in year 1
- Culling of OJD positive mobs and their immediate off-spring
- Non- retention of ewe progeny from positive faecal pool mobs.

The plan also details that a flock is eligible for OJD clearance testing and thus release from their quarantine restrictions when:

- a) The flock is comprised only of approved vaccinate sheep. An approved vaccinate complies with either of the following definitions:
 - (i) The sheep has been vaccinated with Gudair™ as a lamb between 4 and 16 weeks of age
 - (ii) The sheep has been vaccinated with Gudair™ as an adult prior to exposure to MPtb as determined by an accredited Sheep Market Assurance Program (MAP) veterinarian.
- OR
- b) The flock must be a second generation approved vaccinate flock.

In addition to either (a) or (b), at least 2 years must have elapsed since the last known OJD-Infected sheep left the property.

Kangaroo Island Gudair™ Vaccine Efficacy Study

In 2010 a cross-sectional study was conducted by a University of Sydney Honours student on 16 flocks from Kangaroo Island (Masters, 2010). The purpose of this study was to evaluate the efficacy of Gudair™ vaccination and other sheep management strategies in controlling OJD. The primary determinant for inclusion in the study was that the flocks must have been engaged in a vaccination program for at least 5 years. The duration of vaccination for the 16 flocks on KI was: 7 flocks with 8 years of vaccination, 8 flocks with 7 years of vaccination and 1 flock with 6 years of vaccination.

It should be noted that this study also examined 4 flocks from New South Wales and Victoria. No further reference will be made to these 4 flocks.

The aim of the study was to subject the study population flocks to pooled faecal culture in which 12 pools of 50 sheep were sampled to detect an OJD prevalence of 1% with 95% confidence. However, because of limited availability of adult sheep in some of the target flocks, the following sampling regime occurred: 8 pools (or 400 sheep) on 2 properties, 9 pools (or 450 sheep) on 1 property and 12 pools (or 600 sheep) on the remaining 13 properties. The flocks that participated in the study varied with their eligibility for clearance from OJD and thus release from quarantine. A clearance test consists of a PFC 350 test in which the culture process is unable to result in the growth of MPtb. This can be expressed as the inability to produce any culture positive result by a PFC test in which 7 pools of faeces from 50 sheep in excess of two years of age are sampled.

Two of the 16 flocks included in the study utilised the sampling as their clearance testing. A further 8 flocks undertook the sampling whilst the SA OJD Control Program determined their eligibility for clearance testing. The remaining 6 flocks had been previously been cleared of OJD. For the properties undertaking clearance testing it was based upon analysis of pools 1 to 7. The remaining pools 8 to 12 from these properties were de-identified by the University of Sydney researcher. For the flocks that had previously undertaken clearance testing de-identification of the whole 12 pools occurred. The results of de-identified samples were not released to either PIRSA or the producer.

From the 16 flocks assessed in the KI study 14 had zero positive pools, one property had 4 positive pools and the other property had 8 positive pools. The two infected properties had a history that indicated not all sheep on the property at the time of the study were approved vaccinates. Each property had introduced significant numbers of sheep (including 117 and 74 rams) to their respective properties. One of the properties did not carry out whole flock vaccination and, up until 2009, had not been routinely vaccinated replacement stock as lambs or introduced stock upon entry to the property.

All of the 6 properties that had previously undertaken clearance tests returned negative results.

The study was able to determine that the high rate of clearance of OJD from the KI flocks is more likely to be due to the elimination of the causative organism from the flocks than the inability to actually detect persistent infection in these flocks. This finding was previously supported by the conclusions of Whittington and Sergeant (2001) –

“The passage of time and repeated testing are the greatest allies in detecting paratuberculosis because infected animals progress in the disease process and most tests are more effective in the later stages of the disease.”

Discussion on Vaccine Efficacy Study

Whilst the vaccine efficacy study suggests that the approach to OJD on KI is effective, the study also mentions some factors that should be carefully considered in the future management of this disease. The initial whole flock vaccination approach adopted on OJD-infected properties on KI was an undoubted factor that reduced pasture contamination on these properties. On the other hand, it is necessary to consider that a small proportion of vaccinated sub-clinically infected sheep may have multibacillary lesions and be excreting organisms. Whittington et al (2000) indicated that sheep with multibacillary lesions had a total daily excretion rate of 8.36×10^{10} viable MPtb per animal. As noted by Reddacliff et al (2006) this may equate to many hundreds of sheep in the early stages of the disease or with paucibacillary infections. The conclusion from this is, it only takes one sheep with multibacillary lesions and a high excretory output for the disease to persist in a flock.

Another observation from this study is that the continued absence of MPtb shedding in the 6 flocks which had successful clearance tests suggests that ongoing vaccination and the production of second or higher generation vaccinates is a strategy that is likely to prevent the re-emergence of this disease on these properties. Second (or higher) generation vaccinates thus are the most likely sheep to be classified as low OJD risk sheep.

The study also demonstrates that, in some cases, as is apparent with the 2 infected properties, progress with controlling the disease is very slow and it is difficult to define an endpoint for these properties.

Cautious Optimism with OJD Control in the Future

Examination of clearance testing data from Kangaroo Island may shed some light on the future prospects of disease elimination from OJD-Infected properties. The following table looks at the possible association between the initial number of positive pools on Infected properties and the ultimate successful property disease clearance.

Initial number of positive pools	No. of properties with negative clearance test	No. of properties with positive clearance test
1/7 (Low)	22	0
2/7 (Medium)	1	1
>2/7 (High)	3	4
Total	26	5

It would appear that the number of positive pools detected at an initial property testing will provide some guidance to the likelihood of that property being able to ultimately have a successful clearance test. The above table indicates that initial low level (1/7) Infected properties are much more likely to be able to eliminate OJD from their properties than high level (>2/7) properties.

The usual time frame for a property to progress from initial detection of disease until a successful clearance test is in the vicinity of 6 to 7 years. The time frame for a property with high level disease to achieve this goal is unknown. However, it is likely that the process of disease elimination would be to progress from high level infection to low level infection and then ultimately to a successful clearance. Thus, a speculative time frame of between 15 and 20 years could be applicable.

Conclusion

The approach to control of OJD on Kangaroo Island has been moderately successful. The whole flock vaccination in year 1, the accelerated culling of mobs identified as positive by risk profile testing and their immediate progeny and the continuation of vaccination of home-bred replacement sheep or, alternatively through the purchase of approved vaccinate sheep, has resulted in the clearance of OJD from many properties on KI. However, deviations from this approach have resulted in some properties making

slower progress with the disease. The primary recommendation that emerges is that properties should include universal vaccination as part of their normal management procedures.

Acknowledgements

This paper has been only possible with the support of PIRSA and the funding body “Sheep Industry Fund”. A number of individuals have also been major contributors to this dissertation. Firstly, Amy Masters’ Honours study was of invaluable assistance in this paper. The study provided some conclusions that had not been previously characterised. Secondly, Dr Debra Lehmann’s assistance in the Kangaroo Island OJD control program must be mentioned. Dr Lehmann does not randomly select sheep for Pooled Faecal Culture sampling, she actively selects animals that are visually more likely to have OJD. This type of approach along with her due diligence to the correct sampling of sheep and sound advice to producers have been a major contributor to the successful control of OJD on Kangaroo Island. Finally, PIRSA’s Animal Health Officer Andrew Ewers has provided the backbone of the campaign. Andrew’s attention to detail and application to assisting sheep producers affected with OJD, serves as example for future participants involved with the control of this disease.

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HOW AN ARID ZONE PASTORAL COMMUNITY WORKED TO PREVENT ENTRY OF OJD TO WESTERN DIVISION OF NEW SOUTH WALES FROM 1990S ONWARDS

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Background

Ovine Johne's disease is not a disease a veterinarian or flockowner would expect to see in a hot dry area. Thankfully it has not become common in the arid areas of NSW, not just through the admittedly mixed good fortune of its harsh climate, but through action taken over time by the flockowner communities of this area working in concert.

A feature of this action is that it is not the result of central government (bureaucracy) directing, controlling and policing these communities and their livestock, but by the flockowners themselves deciding to act together, to help one another and their sheep, and maintain their reputation for having and providing good, healthy sheep and product.

A central feature of this work has been the *democratisation of process* that we are now seeing around the globe, including the "Arab Spring" and the continuing spread of democracy.

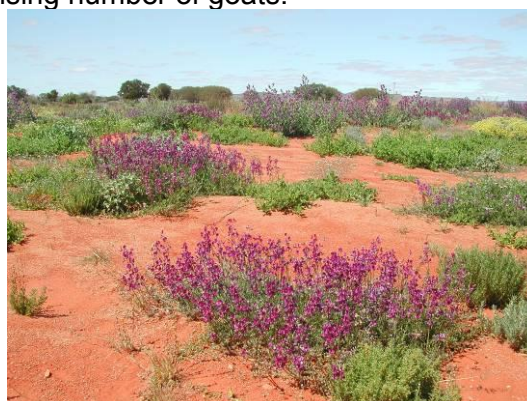
This account of what happened and how it succeeded is offered to encourage similar action elsewhere (where it is possible, or made possible), and to recognise key people and these communities.

I also raise more general questions of how bureaucracy best supports and works through democracy at community and regional levels to control or eradicate animal disease.

The arid zone of New South Wales: the Western Division

The 2012 International Colloquium on Paratuberculosis is being held in Sydney, which is in the high rainfall part of the state of New South Wales. 500 to 1200 km to the west of Sydney is the arid part of New South Wales (NSW), which is called the Western Division. A Google map of the Australian earth (Figure 1) shows Broken Hill (marked A) which is in the west of the Western Division. This area is shown as baked brown and mottled grey-black, with the Darling River running diagonally NE to SW through it; the wetter areas to the east, and the Murray River system's alluvial plains to the south.

The Western Division makes up just under half of the land surface of New South Wales. (See Map 1) In this large area, there are about 1000 flocks of sheep, totally 6 to 10 million, depending on whether we're in a longer or shorter drier or wetter period; how the wool and meat markets are holding; and what flockowners need to do to look after their sheep and their country. There are relatively few cattle in this harsh environment, and a surprising number of goats.



The people of the arid zone of New South Wales

Knowing that there are about 1000 flocks in such a large area points to a number of characteristics of the people of these communities.

They are thinly spread. This does not mean they are "isolated" in the traditional sense. To deal with the distances, they formed a powerful oral tradition, where news and information travelled quickly by word-of-mouth through people moving about the country initially, then property-based radio networks, then telephones and now the internet. Accounts of aboriginal culture show strong similarities, with their

sharing news and information as they travelled and met others. {With aboriginal culture, these networks were formalised into “song lines” and “the Dreaming”, for those who may be interested in antecedents that may have helped the arid zone flockowners establish good community process}.

Figure 1. Google Earth Image of Australia



They live with highly variable rainfall, and hence pasture, and great heat during summer. The long drier or wetter periods can last for 50 years, from the limited records we have, and the shorter wetter periods might be 2 years. This high variability brings high risks, and the environment can punish severely. People and their communities manage risk remarkably well.

They are strong individuals but have strong communities, which is not as contrary as it may seem. Hardship, or the threats about them, means that individuals value their community, their neighbours and their support, and the community gains from the individual. Consequently an arid zone community is effective because it comprises effective individuals.

Not resource-rich, they are resourceful, making the most from what little they have, or can obtain. Although independent, their resourcefulness means they use and value appropriate methods and advice offered, without becoming dependent.

Western Division Rural Lands Protection Boards

One major resource that stockowners pay for, and operate under Act of Parliament was the “*Rural Lands Protection Boards*” (RLPBs). Livestock owners across New South Wales have established this network to deal with important livestock health and pest problems. This network is now the “*Livestock Health and Pest Authority*” (LHPAs). There were 9 RLPBs in the Western Division in 1990s, and now two LHPAs (Darling and Western). See Map1.

RLPBs have staff to deal with livestock diseases and animal and plant pests, and to administer a range of functions. RLPBs were supported by “*Department of Primary Industries*” (DPI) staff. (This government body has had a series of names over time).

Map 1: NSW Rural Lands Protection Boards, showing Western Division



“This is dry country! Why worry about OJD?”

This would have been the response from flockowners, if they had not faced footrot in the 1980s. Footrot had been seen as not an issue for sheep in Western Division; the common view was that it was just too dry. People from the wetter areas once sent their sheep out west to get over footrot, believing that it couldn't survive. A series of much wetter years in the 1980s led to virulent footrot spreading in a number of Western Division areas, as well as across the rest of NSW. Concerted action was taken to control footrot, after a footrot group in the southeast of NSW developed a simple and effective way of eradicating footrot not only in a flock but in a group of flocks, with DPI's Dr Rob Walker (turning sheep, inspecting, paring and treating during hot dry periods).



When flockowners saw the damage done by footrot, they decided to act not only for their sheep's health and well-being, but because sheep from the Western Division were seen as good healthy sheep in the market place. Having footrot damaged the west's reputation.

Not only that, western flockowners generally didn't want to run the risk that people in wetter areas who bought their sheep might get a problem that the western flockowner could do something about. It was clear that once the country dried out, and got back to normal, footrot would not be a clinical problem and that sheep would look normal. Flockowners knew that these sheep would carry subclinical footrot. "A good reputation" is not just a phrase for NSW's arid zone flockowners.

In the Western Division, footrot was usually dealt with by groups of neighbours working together to repeatedly turn, inspect and pare the large numbers of sheep on any one place (sometimes up to 30,000 sheep in a flock), with the help of staff employed by RLPBs and DPI. Contractors were used too. It was expensive in terms of either money or time and effort, but it was successful. That expenditure was largely to retain both market access for their sheep, and the good reputation of their area and the Western Division was a whole.

At about the same time, OJD became a major issue in cooler, wetter areas of NSW. The media's coverage of the disease fuelled and built considerable fear and uncertainty about the disease and its control.

Western Division flockowners asked "*What about OJD?*" after footrot had become their own problem rather than someone else's.

One of the first thoughts was that, after footrot, they had confidence in their own ability to deal with disease at a flock level, and in the technical advice available.



Responding to the threat of OJD (*democratically*)

Broken Hill RLPB lead the way, establishing an OJD committee in 1997, to “*continue trade of sheep and goats based on demonstrated low risk of OJD*”, and to “*assist Broken Hill graziers in protecting their flocks from OJD in other areas of Australia*”. Mr Keith Allison and Mr Max Hams, RLPB Directors and flockowners were both powerful and effective advocates. They requested DPI support and guidance.

I decided to talk to Dr Steve Ottaway, who had deep knowledge of OJD and what was being done institutionally to control it in cooler, wetter areas. Steve recommended talking to flockowners, which was striking for a bureaucrat. He agreed to meet RLPB directors at Ivanhoe in February 1998.

At this meeting, it was agreed that the best approach was to show government and the sheep industry in NSW and the rest of Australia that the Western Division had a very low risk of OJD. The proposal was to achieve this by:

- each RLPB establishing an OJD committee, consisting of invited key sheep industry people (for example, flockowners, stock agents, contractors, transporters)
- voluntary survey of all flockowners asking for details of all sheep introduced onto their land in the last 10 years (purchases, transfers and returns from agistment)
- assessment of survey responses by each OJD committee, to check OJD risk, and call for testing of any high risk sheep introduced
- audit of the survey responses with flockowner permission to test validity against stock agent records of purchases; RLPB records of agistment; and the OJD committee members’ recollection of transfers of sheep not otherwise recorded
- ongoing reporting by flockowners of all sheep introduced, with risk assessment

A series of consultations with each RLPB’s Board of directors (all livestock owners) followed in 1998. Despite the unprecedented nature and scale of the undertaking, directors believed it would be feasible and would be supported. RLPB and DPI staff and directors went to work.

An OJD committee was formed in each RLPB. Each RLPB sent out survey forms to all flockowners, with attached requests to sign permission for auditing purposes.

The response by flockowners was extraordinary by any comparison. In all 9 RLPBs, response rates ranged between 93% and 100%. They provided full 10 year histories of introductions (1988 to 1997). This was not difficult for most, as they were and are breeding operations with most frequent introductions being rams. These tended to come from one or a limited number of studs. Other introductions were remembered because they were related to droughts, when sheep were sent to and returned from agistment. Purchases to help rebuild flocks from drought were less frequent. Flockowners

who traded in sheep were few and well-known to stock agents and other flockowners on OJD Committees, as were their introductions and where they had come from. Purchases of sheep in exceptionally good years were also well remembered.



It had been expected and became clear that there was a very low risk of introduction of OJD into the Western Division because people tended to buy Merino sheep from areas with similar types of country, and with low or very low OJD prevalence relatively close to their areas (other parts of Western Division, South Australia, Western Queensland, northern NSW), to reduce freight costs but more importantly, to have sheep that were likely to adapt well to the harsh environment. This practice had continued throughout the 10 year period, with very few sheep introduced from known or suspected OJD areas. Agistment had related determinants.

Owners of sheep assessed as being “high risk” (based largely on where they had come from) were asked if they would allow testing of these sheep. All agreed, although not all still had these sheep (for example, some were British-bred rams which were used while seasons held and then were sold).

Between 1 and 3 groups of sheep were tested in each RLPB. All were negative (ELISA, GDPT).



Each Board's audit consisted of selecting a sample 5% to 10% of flocks. The selection was not random, and the OJD committee's criteria were often related to perceived OJD risk or not being sure of histories. Stock agents gave full cooperation and access. (The privacy rules of today would make this approach difficult). Audit consisted of comparing survey return with independent records (drought freight rebate, stock agent records of purchase or agistment, stock and land returns). Agreement was graded into 6 categories: (*survey provides more data than records; agreement; substantial agreement; partial agreement; no agreement; or no data from independent record*) with for each of 4 types of record available. Level of agreement differed substantially between records, but overall the OJD committee concluded that the surveys provided more data than the independent record; the independent records were in substantial agreement, or the OJD Committee members were able to correct, confirm and complete the survey returns.

It is important to remember that people on these OJD Committees were not skilled bureaucrats; their work was based on democratic principles and practices. They were prepared to rely on bureaucrats like myself. They made it abundantly clear they wanted a true and complete record and a careful and unbiased assessment of risk, which I found noteworthy. I also recognised they knew their sheep and their management were very low risk, but they did not set out to "*gild the lily*". They didn't want "*a bureaucratic snow job*", as it was once put to me.

All survey returns, audits and assessments were completed in all 9 RLPBs by early 1999.

This work and its outcomes were communicated to NSW government officials, other RLPBs, and other states, together with requests that all 9 Western Division RLPBs be considered "Protected Areas" under the Stock Diseases Act of NSW.

Meanwhile, the work had its own effect on flockowners in the Western Division. All knew that OJD was an issue for virtually all other flockowners in their area, their RLPB, and their region (the Western Division). This meant all established flockowners knew to be careful when bringing in sheep, to be careful with neighbours' sheep when they had doubts, and to bring any problem to RLPB and DPI attention.

OJD eradication and control in NSW's arid zone.

Since 1998, there have been 15 incidents where higher risk sheep were introduced. These were detected from reports of owners or neighbours, from abattoir surveillance, or from risk assessments of introductions.

All cases had investigation and control protocols worked out and agreed with the flockowner. All were documented in Property Disease Management Programs.

Most confirmed infection has been associated with people buying land in the Western Division, and moving their sheep in, only to find they are infected. Several cases resulted from people purchasing sheep after receiving Sheep Health Statement assurance of very low risk or buying supposedly nil risk Western Australian sheep. One infection resulted from a flockowner buying rams from a friend, only to see signs of clinical OJD. The flockowner had a private veterinarian investigate. No case of endemic OJD has been found.

In this environment, it is feasible to aim for eradication, rather than control. In all cases, the infected mob has been destocked. Vaccination is occasionally needed. (The cost of routine vaccination was one of the reasons for wanting to keep the disease out).

Testing of in-contact mobs in the flock and across boundaries has not found evidence of spread, which is believed to reflect the hot, dry climate; the long drier period from 2000 to 2010; and very low stock densities.



There is anecdotal evidence that “infected” sheep do not survive as well as “uninfected” sheep in long dry periods, with low availability of pasture or poor quality pasture.

In all cases, neighbours are informed of the risk, either by the flockowner, or by the RLPB staff at the affected flockowner’s request. Neighbours are encouraged to discuss the risk and its management with the flockowner and RLP and DPI staff.

All suspect cases are subject to confirmation of trace, risk assessment, and testing used Pooled Faecal Culture testing, with more complex cases negotiated and documented under a Property Disease Management Program.

In cases where flockowners were reluctant or unwilling, the RLPB (with DPI support) has contacted the person, and persuaded them of the importance of cooperation in the interests of their neighbours and the area as a whole.

Democracy and bureaucracy

Protected Area status was given to all areas identified as having low risk of OJD in 2002 by the Minister of Agriculture. The requirements of the new Protected Areas were little different to those all Western

Division flockowners were observing to that time. In addition, Broken Hill RLPB introduced flockowner booklets to make it simpler to keep records of all purchases and introductions.

In 2004, after extended media criticism of the whole NSW OJD program, a plebiscite or vote was held across NSW, asking flockowners whether they wanted to retain Protected Area status. This was the first instance of a democratic vote on a major disease control program in Australia, to my knowledge. In western NSW, a series of public meetings was held to discuss the propositions involved the vote. These meetings were set up and run by RLPBs, with all flockowners invited. The meetings were well-attended.

In the far west, the meetings had a formal structure: a flockowner proponent (Mr Keith Allison), a neutral technical advisor and proponent for good disease control (myself), and a neutral facilitator (Mr Lloyd Kingham) who both encouraged and valued all opinions expressed, and helped the meeting formulate its views while retaining diversity of views.

Each RLPB held its own vote, under an independent supervision. In the Western Division, flockowners in all 9 Boards voted solidly for the proposition, despite considerable criticism and at times fierce questioning in the meetings. Each of the 9 RLPBs applied to the Minister for Exclusion Area status. This was granted later in 2004.

In 2009, flockowners of Broken Hill RLPB received the first National Farm Biosecurity Award, in recognition of the work they'd done to prevent OJD. This community would not usually seek such an award, but it wanted recognition of the pivotal role of Mr Keith Allison. Keith died of cancer a few weeks after the award was made.



In 2011, a second vote was required by DPI under Stock Diseases Act, asking flockowners whether they wanted to continue with Exclusion Area arrangements. A larger series of meetings was held across Western Division, to ensure as many people as possible could attend, given higher fuel prices and time constraints. Very few people attended. This poll showed stronger majorities in support for continuing to prevent OJD than in 2004.

In 2012, all very low risk areas will be again asked to prepare cases to justify retaining Protected Area status, under a national approach. At this stage, it is understood that these cases will not involve formal votes.

OJD has affected trade in sheep across Australia to some extent, as is expected when flockowners in one area seek to protect their sheep, and ask that their biosecurity arrangements be respected. In Western Division, there has been very little change to trading patterns since records have been kept from 1988. There have been larger effects on markets: the virtual space where people value sheep. Some areas have been clearly identified as having higher risk; some areas have been shown to have poor disease control and biosecurity; others have higher standards. This change has affected social valuations in what is an industry and profession with certain traditions and standards. Inevitably, this has led to strong political pressure on both bureaucracies and politicians. These political pressures are part of the democratic process. The political pressures lead to some conflict between proponents of good biosecurity and disease control or those wanting to protect their sheep using good biosecurity; and those wanting to return to "normal trade" and "open markets". These conflicts are resolved by those who make both political and disease control decisions. This democratic process will continue to be played out.



A major advance in this process has been the role of Animal Health Australia (AHA), a non-profit organisation made up of both livestock industries and governments. AHA has operated as an honest broker across sectional interests, and a generator and central repository of high quality disease information and intelligence. Two key people in the Johne's disease discussion in Australia have been AHA's Dr Lorna Citer and Dr David Kennedy.

A personal view of requirements for effective democracy in disease control:

In summary:

- Personal action and responsibility
- Group action and responsibility
- Alignment of self-interest and community interests
- Confidence that methods and people will succeed
- Appropriate methods
- Appropriate infrastructure and personnel
- Clear benefits from action and responsibility required and taken
- tangible and *intangible*
- Understanding and respect for the interests of others
- Trust
- Control of process and outcomes by a community through trusted, responsible, committed, altruistic people



A COOPERATIVE APPROACH TO OVINE JOHNES DISEASE CONTROL IN TASMANIA

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Tasmania is an island state of Australia with a sheep population of 2.5 to 3 million sheep. Ovine Johnes Disease (OJD) was first diagnosed on mainland Tasmania in 2001. Abattoir surveillance funded by Animal Health Australia (AHA) showed a rapid increase in the percentage of positive lines over 2010–2011 and disease presence in most sheep-raising areas. AHA and the Department of Primary Industries, Parks, Water and Environment Tasmania (DPIPWE), with support from the vaccine manufacturer (Pfizer), organised meetings in 2010 with rural merchandisers, stock agents, the Tasmanian Institute of Agricultural Research (TIAR) SheepConnect Tasmania¹ extension staff and the Tasmanian Farmers and Graziers Association (TFGA). As a result of these meetings and the recognition that OJD could significantly impact production, the livestock agents decided to make the Sheep Health Statement (SHS) compulsory for store and breeding sheep sales, and Sheep Connect Tasmania staff decided to run a series of workshops. The workshops were well attended and vaccine sales rose sharply. Livestock agents displayed the Assurance Based Credits (ABC) score of the sheep on pen cards at the large autumn store sales and on AuctionsPlus (web-based selling) in 2011 and this increased interest from the sheep producers as well. SheepMAP veterinary practitioners played an important role advising sheep producers on all aspects and show committees have also implemented entry policies based on minimum ABC scores. TAFE lecturers have included OJD in their rural apprenticeship courses. From May 2011, DPIPWE, TIAR, the rural resellers, Proactive Agricultural Safety and Support (PASS), the OJD surveillance abattoir (Tasmanian Quality Meats), and Pfizer ran a number of events focusing on OJD diagnosis, preventing OJD, managing OJD, preventing and managing needle stick injuries, the role of vaccine in disease control, use of the SHS and abattoir monitoring. These included a professional development evening, workshops presented by David Rendell & Associates from Victoria, displays at shows, and workshops in major sheep-producing areas from July to September 2011, attended by more than 250 farm staff. An OJD Task Force initiated by TFGA to consider state-wide policy issues composed of all industry stakeholders has been formed. Staff from other state departments have assisted with advice and Meat and Livestock Australia (MLA) OJD extension publications have been utilised. Interviews have been broadcast by the Australian Broadcasting Corporation (ABC) and several rural newspaper articles have been published. A lot can be achieved when all stakeholders in an industry sector work together on a common goal.

¹ SheepConnect Tasmania (SCT) is the third phase of Australian Wool Innovation's (AWI's) nine-year investment in extension for the Tasmanian sheep and wool industries and is delivered through TIAR. TIAR is a joint venture between the University of Tasmania and the Tasmanian Government.

OJD TEN YEARS ON, OUT OF THE TUNNEL AND TOWARDS IMPROVED PRODUCTIVITY

Reardon J

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Ovine Johne's disease (OJD) first became evident in the Reardon's Merino flock in 1998. The mortality rate at the time was 4.9% and following post-mortem of three young sheep showing signs of ill-thrift, OJD was confirmed as the cause. Subsequently, the property was quarantined and the neighbours notified. Sheep were only allowed to be sold direct to the abattoir or through the slaughter only section of the saleyard. There was no market for sheep that didn't meet the meat buyers' specifications.

The Reardon's property is located in the Southern Tablelands of NSW on the north east shore of Lake George. The land is predominantly flat with a flood plain where water from surrounding higher country flows before entering the Lake. Lambs were regularly weaned onto the flood plain because of the availability of green feed over summer. Prior to their flock's diagnosis, the Reardon's were unaware their neighbours upstream had OJD or that one of the main methods of the spread of the disease was by waterways.

Stock losses increased to 17.1% in 1999 and reached 25% by 2000. One and two year old sheep weaned onto the contaminated flood plain were the worst affected. With assistance from their district veterinarian, a property disease reduction plan was implemented. Key elements were:

- Spell paddocks for one summer by cropping, grazing cattle or haymaking.
- Put lambing ewes in paddocks away from neighbours that were known to be infected.
- Run weaners on paddocks spelled for 12 months, then progress to run with hoggets on pasture previously grazed by cattle.
- Quarantine all sheep showing clinical signs of OJD and euthanize if they do not recover.
- Implement an internal parasite monitoring and control program.

Vaccinating all sheep with Gudair® - if permitted - was recommended to delay the onset of and reduce shedding. At this time, Gudair® vaccine was not registered and use was only allowed in certain areas. The first application to vaccinate was rejected by the NSW Chief Veterinary Officer. In July 2001, changes were made to the rules regulating the use of Gudair® and approval was given to vaccinate lambs born in 2000 and 2001.

The Reardon's also worked closely with their 20 neighbours forming the North Eastern Lake George OJD Local Catchment Management Group. Knowing now, the conditions were ideal for inter-property spread, the most effective way to control the disease was to work together. The Catchment Management Group was successful in changing the regions status so they could access vaccine as well as securing financial assistance.

In the following years, the health of the Reardon's flock improved to the point where no clinical signs of OJD could be seen. Even during the prolonged drought from 2003 to 2009 signs of the disease were not evident. In 2006, 80 sheep were inspected by abattoir monitoring and all were negative. Today, the Reardon's continue to vaccinate and follow the property disease reduction plan and see no clinical signs of OJD.

IT COULDN'T HAPPEN HERE: THE IMPACT OF JOHNE'S DISEASE ON PRODUCTIVITY

Lean G

Graham Lean and Associates

Ovine Johnes disease has spread from initial infection points through to nearly all regions of Australia that run sheep. This presentation outlines that despite OJD being a costly, insidious disease, inadequate biosecurity measures, low vaccination rates with Gudair have resulted in a disease that is now endemic in most sheep regions of Australia, whether the local farming community is ready to accept that it is present or not. "It couldn't happen here" appears to be the most popular method of farmer preparedness for a disease. In the past, mortality rates from OJD were underestimated and conservative economic modelling undertaken has shown an attractive benefit from protecting all the flock via Gudair vaccination. Mortality rates are not only associated with economic loss; they are also an animal welfare issue.

AN INDUSTRY FUNDED MANAGEMENT AND CONTROL PROGRAM FOR BOVINE JOHNE'S DISEASE IN SOUTH AUSTRALIAN DAIRY INDUSTRY USING A DAIRY SCORING SYSTEM

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Introduction

In late 2004 the SA Cattle Advisory Group (SACAG) allocated funding for voluntary herd serological testing and management programs to assist in the control of Bovine Johne's Disease (BJD) on South Australian dairy farms. This was to be implemented by the introduction and development of a "Dairy BJD Assurance Score". The program has been successful in recruiting more than 97% of SA dairy farmers, in the period 2004 to 2011. In 2004 SACAG agreed to fund a new program in SA that promoted awareness of BJD in the dairy industry, provided some simple tools to assess the risk when purchasing cattle and examine the impact on the herd status as a result of these purchases.

SACAG is an industry group comprising members of all cattle related enterprises. This group manages a budget derived from a levy on cattle NLIS ear tags, and provides advice to the SA Minister responsible for cattle industries. The group membership is evenly divided between beef and dairy producers. (see Table 1 and diagram below) The risks to the beef industry of infection with BJD, particularly in the wetter and more highly productive areas of the state, were perceived as significant enough to justify expenditure of the majority of the SACAG budget over a 3 to 5 year period.

In South Australia dairying is widely dispersed, and has occupied a niche that is unfavourable climatically for *Mycobacterium paratuberculosis* survival in some areas. Until recently approximately half of SA dairies were located in areas of 700mm annual rainfall or lower, although dairying is now increasingly moving to the higher rainfall regions of the state.

Table 1. Approximate numbers of cattle in SA, 2011

beef	dairy
1.15 million total	145,000 total
91,000 breeders	(54 infected herds)
543,000 breeders	(2 infected herds)

BJD Surveys in SA

In South Australia dairying is primarily based on irrigated pasture and supplementary feeding of cows. Prior to the implementation of the SACAG initiative in 2005 five BJD surveys had been conducted in SA.

In 1955 Rac29 reported the estimated prevalence of BJD in SA dairy cattle to be <1%. In 1988 Koh et al 31 reported a prevalence of 3.5% of herds in the Murray River area. In 1994 Vandergraff et al 32 reported BJD in 1.5% of herds in the Murray River area, Central Southern drylands and South East. In 1997 Durham & Paine 30 reported that a 1991/1992 serological survey of 617 cattle from 10 beef cattle properties in the northern pastoral area of SA found no evidence of the disease. Another serological study on 4,640 adult slaughter cattle of SA origin also found no evidence of the disease (Van Wijk 1996 – beef herd survey, unpublished data). This 1996 survey included 565 cattle from the northern pastoral area of SA30.

Impetus to investigate BJD in SA dairies

A review of the SA BJD program in 2003/2004, commissioned by SACAG, found that there was a reluctance to report suspicion of BJD infection on SA dairies. The reason appeared to be the punitive measures resulting from an Infected Herd status; the end result was widespread fear and ignorance about the disease.

Some neighbouring states and some regions of SA have a higher prevalence of infected herds and, due to industry restructuring, an increasing number of cattle are being sourced from these areas. This has resulted in an increase in the number of infected herds in SA. This also leads to the increased risk of spill-over of infection into other cattle herds, especially beef herds and, in particular, herds using dairy cross bred mothers for vealer production.

Since the prevalence of BJD in all Australian beef herds is very low¹¹, beef producers are concerned about the risk from contact with dairy and dairy cross cattle. The financial consequences of BJD infection in Australian pasture based beef herds are large, particularly for seed stock producers and producers wishing to access to export markets. The SA beef production sector was faced with the severe consequences of being infected with BJD from a co-existing dairy industry population with unknown, but rising herd prevalence. Therefore, it was important that a new means of managing BJD was sought.

The Plan- called “Dairy ManaJD”

In 2004 there was a National agreement to adopt a “Dairy Assurance Score” for BJD that would apply to all dairy cattle in Australia⁶ (see Appendix 2) and this became the tool to develop the “Dairy ManaJD” program. In SA in the period from late 2004 extending to mid 2005, the “Dairy ManaJD” program was launched. This was achieved by means of a series of regional meetings, along with press releases and extension publications.

PIRSA¹⁰ modelled the SA “Dairy ManaJD” (pronounced “Dairy Managed”) program on the successful National BJD Market Assurance Program ¹² and expanded the categories to include infected herds, as well as the benchmarks to reflect control measures in these herds. Dairy processors⁸ were engaged at the development stage of the project, so that Johnes’s disease management could be monitored as part of regular farm management and auditing. Other industry organisations involved in the development of the Dairy ManaJD program were SADA (South Australian Dairy Association), AHA¹ (Animal Health Australia) and Dr David Kennedy³ (AUSVET) as a technical advisor.

Aims of the program

The “Dairy ManaJD” program aimed to:

- Engage SA dairy farmers in management of Johnes’s disease on farm.
- Engage private veterinarians in BJD management on client dairy farms.
- Provide tools to assess BJD risk and provide assurance when buying or selling cattle and/or property, and assessing agistment land risk.
- Reduce regulatory activities in SA dairy industry, whilst encouraging biosecurity principles, and managing the risk of introduction of BJD to beef producing properties.
- Enrol up to 30% of dairy farmers in the first 2 years

“Dairy ManaJD” has 4 main components:

1. Enrolment

Herds enrol with a private veterinarian who explains the processes and consequences of herd testing, the importance of BJD conscious calf rearing practices and the audit & accreditation processes. A necessary prerequisite is that the private veterinarian must have qualified as an Accreditation Program for Australian Veterinarians (APAV)¹ and be MAP ¹² accredited. APAV veterinarians are those who have completed post graduate training through Animal Health Australia (AHA)¹ specifically with respect to Johnes’s disease.

At enrolment a “Dairy ManaJD” manual² is provided for the dairy farmers. This manual was designed to be compatible with the on-farm QA (Quality Assurance) manual. It was developed in close collaboration with milk processor companies in SA.

There was a fairly rapid uptake of the program and enrolment within the first 12 to 18 months but there was a resolute group that had not enrolled. This group was targeted for specific, individual attention. Dr Geoff Manefield, an experienced veterinarian from the South East dairying area of SA, visited each farmer (approximately 50) to explain the program in detail and encourage enrolment. This strategy was successful in enrolling the majority of SA dairy producers.

Enrolment in the Dairy ManaJD program resulted in access to:

- Subsidies for all laboratory testing costs in the herd.
- A meaningful Dairy BJD Assurance Score based on herd testing results
- Subsidies for veterinarians to perform sampling, provide management advice and issue a certificate detailing the dairy property’s Dairy BJD Assurance Score.
- Compensation for reactors and for high risk offspring of reactors that are slaughtered.

Bovine Johne's disease remains a notifiable disease in all jurisdictions in Australia, and in some states Infected or Suspect⁴ (see official definitions)⁴ properties are quarantined. In SA, dairy properties infected with Johne's disease have been exempt from quarantines on the basis of enrolling in the Dairy ManaJD program. Infected herds not enrolled or infected dairy herds that cease dairying are subject to quarantine.

A flowchart describing the steps and processes of enrolment in the program is shown in Appendix 1.

Table 2 Enrolment of herds in the Dairy ManaJD program, commencing 2004

There has been a small attrition of herds leaving the program, and failing to maintain certification over time and the table below summaries current enrolments.

Table 2. Distribution of dairy herds in South Australia by Dairy Score, December 2011

Dairy Score	Number of herds	Percentage
0	23	8
1	0	0.0
2	0	0.0
3	2	0.6
4	41	14.3
5	11	3.8
6	0	0
7	194	67.8
8	3	1
9	3	1
10	9	3.1
Total herds	286	

2. Testing

Herds tested either all cattle over 2 years (to join the CattleMAP) or all cattle over 4 years (to join Dairy ManaJD) using a commercial absorbed ELISA test. This testing was also extended to known Infected herds. The majority of herds tested cattle 4 year and older cattle to decrease testing costs without a significant loss in test sensitivity. Several studies (Nielsen 21, Jubb 22) have shown that the positive predictive value of ELISA tests increases with age and progression of the disease. Further testing of ELISA reactors was either using serial faecal culture or autopsy with histopathology being performed on a range of gastro-intestinal tissues. The details of the faecal and tissue culture are described in other Test and control programs in Australia.

After the completion of testing dairy herds could then be assigned a "Dairy BJD Assurance Score", based on a Wisconsin scoring system (see appendix 2), that classified the herd according to apparent prevalence (Scores 0-6 for infected herds), or Tested Negative (7 - 10).

Tested Negative herds range from Score 7, (1 negative herd test) to Score 10 (3 negative herd tests over a 4 year period and maintained on the National BJD Market Assurance Program(MAP) at MN3). See an excellent and comprehensive paper by Pamela Lawson⁵ describes the complex relationship between zoning credits, MAP, and Dairy Assurance scores⁶ (see also Citer and Kennedy 13).

Infected herds (herds with a Score 0 to 6) could choose to attempt eradication or use management to minimise BJD on the farm. Eradication is by means of a Test and Cull program (see details in SD & Rs⁴), including removal of high risk cohorts of confirmed infected animals, and improved calf rearing protocol. Typically an eradication program on an endemically infected property takes 6 to 10 years, and is documented in a specifically designed Property Disease Management Plan⁴ that is managed by the herd Approved Veterinarian. If the eradication process is successful the herd Dairy Assurance Score rises accordingly (see below).

Other infected herds (typically larger herds that frequently purchase cattle from high prevalence locations, of unknown or known BJD Infected status) can manage and minimise JD by means of improved calf rearing hygiene and other monitoring and audit processes as described in the Manual.

In 2010 a new Herd environmental culture (HEC)⁴ test was approved in Australia to replace the Check Test. A Check Test (see SD & Rs 4 for complete definition) is an ELISA test of 50 of the older cattle from

a herd. The HEC test involves collecting an aggregate sample of faeces from the dairy milking parlour yard, and culturing the sample. This test has now replaced the Check Test in SA tested negative herds as it is cheaper, quicker and less stressful to collect than previous serological samples. Unpublished data collected by Dairy Australia²⁸ in the process of validating this test in Australian conditions indicates that the test is of equivalent herd level sensitivity to a Check Test.

Milk ELISA tests are not used in Australia to date, and have not been approved for use by Animal Health Committee.

3. Auditing

The audit process in “Dairy ManaJD” herds was designed to focus on management factors likely to impact upon the prevalence of JD in Infected herds and the biosecurity in tested negative herds. A “Check List” (see below Appendix 4) was developed in close cooperation with SA Dairy Authority auditors⁷, processor auditors⁸, and veterinarians.

Farmers were able to see the “Check list” and thus the audit requirements since they were included in the Farmer Manual.

The SA Dairy Authority agreed at the outset of the Dairy ManaJD program in 2005 to provide audit services and reports free of charge. The audits were done in conjunction with the on-farm food safety audits. These audit reports were sent to PIRSA annually. In addition, a copy was forwarded to the approved veterinarian for the herd.

On the basis of processor or SA Dairy Authority reports and his own assessment and audits of the herd, a herd veterinarian recommends the appropriate Dairy Score and forwards an advice form to PIRSA. This results in a unique “Dairy ManaJD Certificate” being produced. A copy is sent to the farmer and the veterinarian annually (see Appendix1).

Farmers receive direct and relevant feedback about their on farm management of BJD and an annual certificate printed on parchment paper. Show societies, buyers and saleyards in SA began demanding declarations of the Dairy Score, (as a legal requirement of sale) and tested negative herds (Score 7 and above) rapidly achieved price premiums in sales, and increased export opportunities relative to some other States of Australia. However, some Dairy Score 7 farmers initially found purchasing options too restrictive and elected to downgrade their scores by introducing lower score animals. Many of these later regretted the decision and the consequent lost trading opportunities.

4. Scrutiny of Calf Rearing Practices

It is well recognised that calf rearing management has an important role in perpetuation of Bovine Johne’s Disease on a dairy property (Refer 27). A number of authors 14, 19 have described strategies to reduce the risk of BJD transmission to calves and the importance of young dairy cattle grazing strategies. These include:

- Removal of the calf from the dam in under 12 hours
- Removal of the calf from the dam in under 24 hours
- Separate rearing facilities that exclude adult cattle
- Various hygiene procedures and bedding arrangements in rearing areas
- Concentrate or hay feeding to young calves in rearing areas.
- Feeding of pasteurised or irradiated colostrum that is derived from low risk sources
- Feeding of pasteurised, irradiated or low risk milk and water
- Culling of calves from high risk dams eg cows with confirmed BJD
- Grazing strategies for calves after weaning and up to 12 months of age
- Vaccination against BJD

A comprehensive paper by Ridge et al¹⁶ describes the impact and success of calf rearing practices in some Infected Victorian dairy herds in Australia, and concludes that adherence to improved calf rearing practices in herds enrolled in a Test and cull program does result in significantly reduced disease transmission in these herds. This is consistent with other experience in the literature, for example Collins et al²⁰ where a testing component is seen as an integral part of a management program on infected properties. Whilst it was not possible to identify the specific aspects of calf management in Ridge’s¹⁶ study that resulted in reduced disease transmission, two major calf rearing programs are documented below.

- (1) The Johne's disease Calf accreditation Program (JDCAP) 17 has mainly been associated with Infected herds on a test and control program and requires:
- The property must be subject to inspection and approval by a DPI (Department of Primary Industries) approved veterinarian prior to the commencement of accreditation.
 - All replacement calves must be separated from their mother within 12 hours of birth.
 - Cows must calve in an area that is free of dairy effluent or large amounts of manure.
 - The calf-rearing area must be free of any dairy effluent or cow manure.
 - Only clean water, preferably tank, and town or bore water, must be supplied for calves for drinking or preparing calf milk-replacer.
 - Only milk from low-risk cattle or calf milk-replacer must be fed to calves.
 - Once calves are weaned they can only graze paddocks that have not been grazed by adult cattle during the previous 12 months
 - The grazing area for weaned calves must be free of any drainage or effluent.

The owner then signs a Herd Owner Agreement form which is endorsed by the approved veterinarian. In the next step the veterinarian provides the herd owner with a JDCAP registered Certificate of Compliance which is valid for 12 months. The calf management program is reviewed and audited annually by the veterinarian.

- (2) The second, more abbreviated version, is referred to as the 3 Step calf rearing plan⁶: The 3-Step Calf Plan is a simple hygienic calf rearing program that encompasses the critical control points for minimising exposure of calves to BJD. The steps are:
- Calves should be taken off the cow within 12 hours of birth.
 - Management of the calf rearing area to ensure no effluent from susceptible species comes into contact with calves.
 - Calves up to 12 months old should not be reared on pastures that have had adult stock, or stock that are known to carry BJD, on them during the past 12 months.

The dairy industry is encouraging all farmers in Australia, regardless of their BJD status, to implement the 3-Step Calf Plan. Dairy processing companies have included the 3-Step Calf Plan as part of their on-farm quality assurance systems.¹⁶

In order to encourage farmers to comply with these recommendations additional Calf Credit Points are available to add to a calf Dairy BJD Assurance Score, if the producer is complying with these requirements.

Calf Rearing Program BJD Assurance Scores Bonus Program

+3	Victorian Johne's Disease Calf Accreditation Program (JDCAP)
+1	3-Step Calf Rearing Plan

Compliance with improved calf rearing and management programs can be difficult to assess and has been reported as being between 20% and 76% (see table below).

Table 3. Compliance with calf rearing requirements

Compliance with calf rearing requirements	Method of assessment	Source
14% to 38%	Postal survey	Wraight et al ¹⁵
Less than 20%	On farm audit JDCAP	Ridge et al., 2010 ¹⁹
40%	On farm audit 3 step plan	Ridge et al., 2005 ¹⁶
76%	Telephone survey 3 step plan	Padula et al., 2009 ¹⁷
60%	Annual on farm audit 3 step plan	J Rogers, 2011 (unpublished)

Future BJD Management for Dairy Herds in SA

Historically there have been few known infected dairy herds in SA (see Table below).

Table 4. Infected herd test prevalence results by year

Year ending June	No. of herds	No. of animals tested	No. of animals test positives (number confirmed positives)	Av. within herd test prevalence (range)
2005	43	6251	57 (38)	1% (0.5 – 2.0)
2006	58	9906	96 (63)	1.2% (0.2 – 9)
2007	59	11143	83 (38)	0.9% (0.2 – 2.8)
2008	57	8158	69 (22)	0.9% (0.1 – 2.0)
2009	58	5835	68 (19)	1.0% (1.0 – 3.2)
2010	58	1346	36 (16)	1.5% (0.4 – 2.7)
2011	54	701	25 (15)	2.4% (0.5 – 10.0)
Totals		43340	434 (211)	

Experience in Victoria with test and control programs (Jubb et al23) suggests that these programs are slow and frustrating and may not be feasible in eradication of JD on commercial farms. Whilst this has been SA experience as well in some cases, a total of 28 dairy herds eradicated BJD from 2004 to 2011. These farms tended to be closed, small to medium sized herds (100-300 cattle) with very low initial herd prevalence.

Infected dairy herds in SA that have a low likelihood of eradication of JD receive funding for investigation of all cases where JD may be a differential diagnosis, compensation for reactor cattle and high risk offspring culled, and the opportunity to commence a vaccination program using Silirum25 vaccine, although no SA herds have taken this option as yet. These are herds that have an open trading policy, some very large herds, and some where there has been historically low compliance with requirements in the past.

As Industry funds for the Dairy ManaJD program have progressively decreased, funding priorities have focussed more on herds that are not infected (to maintain status), and infected herds in which there is likely to be a greater chance of successful eradication.

Tested negative herds (Scores 7 to 10) must maintain this status with biennial serological Check tests or HEC tests as described above, and annual audits. If cattle of a lower Dairy Assurance Score are introduced in to a herd, the whole herd status degrades to the Score of the lowest introduced animal.

Conclusions, lessons learned, and measures of success

By 2011 over 97% of SA dairy producers have enrolled in the Dairy ManaJD program and test results confirmed the low prevalence of BJD infected herds (19% in 2012) in the SA dairy sector. The widespread uptake of the voluntary program has enabled producers to trade cattle using the Dairy Score as a risk assessment tool.

Throughout 2002 to 2004 there was increasing concern in the Australian dairy industry about the risks and consequences of JD infection in the dairy population. These concerns were linked to a perceived link with Crohn's disease in humans and the possible economic costs of JD in dairy herds.

In South Australia there was evidence that the SA dairy population had a low prevalence of infected herds and protecting this population with a program of education rather than regulation was seen to be a worthwhile investment for SACAG. Since there are only 2 known infected beef herds amongst approximately 10,000 herds in SA, SACAG was keen to prevent spillage of BJD from the Dairy industry in to the beef breeder population.

Clinical cases of BJD on SA dairy or beef farms are very rare, and although there have been a number of good international papers on the economic consequences of JD on farms the economic losses due to subclinical disease in SA conditions are perceived to be low.

Economic drivers for control of JD on SA dairy farms appear to be related to market access nationally and internationally of excess heifers, and the loss of cows slaughtered as reactors to ELISA tests.

Enrolment in the Dairy ManaJD program was cautious initially. Farmers were uncertain about the consequences of being "caught" with BJD and the negative effects that this may have on land or herd values. However, as more farmers enrolled, tested, discovered that, in most cases, they were not infected herds and were able to achieve a Dairy Score 7, the program gained momentum.

In addition, the requirement to declare a Dairy Score at sales of cattle in SA, and that this is audited by PIRSA inspectors meant that there is a driver for the dairy farmer to get the herd tested for BJD. Show societies and large sale venues now demand evidence of the Dairy Assurance Score prior to sale.

In contrast to dairy populations of high or moderate infected herd prevalence, certification of tested negative status has been economically feasible²⁷ in SA. The relatively small size of the dairy herd in SA also meant that the program could be managed with a modest budget, and expenditure has been approximately \$2.9M up to June 2011. SACAG have seen the program as a worthwhile investment in the SA cattle industry to assist in protecting the industry from BJD incursion, by achieving the aims of the program to:

- Engage SA dairy farmers in management of Johnes's disease on farm.
- Engage private veterinarians in BJD management on client dairy farms.
- Provide tools to assess BJD risk and provide assurance when buying or selling cattle and/or property, and assessing agistment land risk.
- Reduce regulatory activities in SA dairy industry, whilst encouraging biosecurity principles, and managing the risk of introduction of BJD to beef producing properties.

Some larger infected dairies with an open trading policy have been difficult to attract to the program, so the mandatory requirement to declare their scores has involved them in the Dairy ManaJD program, and most of these herds tested at least once and have become involved in a dialogue about BJD.

In addition, changes to BJD management nationally from July 2012 mean that cattle sales to beef properties in SA from all infected (or herds lower than Score 7) dairy herds will be traced, and buyers advised of the risk status of these cattle, and the consequences to their property. This will underscore further an awareness of Biosecurity, and reduce the sale options from infected properties.

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Q-ALPACA-SURVEILLANCE AIMED AT THE EARLY DETECTION OF JOHNE'S DISEASE WITHIN THE AUSTRALIAN INDUSTRY

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Q-Alpaca is a quality assurance program designed by the Australian Alpaca Association Ltd for voluntary use by its members. The program, which commenced in 2005, was developed to assist in the detection of Johne's disease (JD) if it re-occurs in the Australian alpaca population, as well as to identify emergency animal diseases quickly and monitor endemic diseases effectively. Information collected is used to provide advice to alpaca owners relating to disease management in their herds.

The bovine strain of JD was detected in a number of Australian alpaca herds during the 1990s. Fortunately no cases of Johne's disease have been detected in Australia for a number of years and there are currently no known infected alpaca herds in Australia.

In relation to JD, Q-Alpaca was developed with the aim of monitoring the Australian alpaca herd for Johne's disease as well as enabling owners of alpaca herds to provide assurance to potential alpaca purchasers that their herds are not infected with JD.

During the 2010-11 reporting year, there were 270 alpaca herds enrolled in the Q-Alpaca program, representing 19,516 alpacas. Q-Alpaca participants work with their private veterinary practitioner to monitor for disease in their herd. The herd owner and veterinarian develop a Herd Management Plan which identifies management practices to reduce the risk of introducing JD or other infectious diseases. The veterinarian assesses herd records and management practices relevant to the program every 12 months. All alpacas over 12 months of age that die are necropsied by the participating veterinarian and examined for signs of JD. Any alpacas that are less than 12 months but show signs of wasting or diarrhoea and die or are euthanased are also necropsied and examined for signs of JD.

In addition to examining for signs of JD, the veterinarian also records if there is evidence of conditions such as internal parasitism, gastric ulceration, chronic liver disease or congenital abnormalities. The alpaca owner provides information regarding treatment dates for internal parasites and routine vaccinations.

All information is collated at a national level and an annual report provided to Q-Alpaca participants and the alpaca industry. Epidemiological analysis includes factors such as the age and sex of alpacas in relation to the total number deceased, and the spatial distribution of total deaths and specific causes of death. Emergency, emerging or unusual diseases (including JD) can be identified and appropriate action taken, including the provision of management advice to alpaca owners.

This program provides an alternative to the Alpaca JD Market Assurance Program (AlpacaMAP) which involves regular testing of a representative sample of the herd. Similar to AlpacaMAP, Q Alpaca is endorsed by all State and Federal animal health authorities.

This program provides an excellent example of livestock owners, other relevant industry participants (e.g. private veterinary practitioners) and government agencies working in partnership to both monitor and protect the health status of a targeted livestock industry.

PROTECTING THE AUSTRALIAN BEEF INDUSTRY FROM BJD

Keatinge N

Historically the Australian beef and dairy industries have developed in parallel with only limited crossover of enterprises. Although more widespread in the past, the dairy industry is now confined to higher fertile rainfall regions and irrigated valleys. Beef breeding herds tend to be more extensively grazed and mostly do not run dairy cattle. There is some interface between the industries where dairy cows are used to breed first cross calves but the progeny are usually slaughtered as young cattle.

As a result there is very little BJD in the Australian beef population. The prevalence of known infected herds is only 0.04% and all of these herds are located in the south-east temperate zone. The national industry seeks to protect this favourable status and has already implemented a population based risk assessment system based on the '*Beef Only*' classification which is supported by a National Financial and Non Financial Assistance Package for beef producers whose herds are found to be infected.

A current review of the Australian National BJD Strategic Plan has provided the opportunity for the beef industry to further protect the beef cattle population. Cattle Council of Australia, the national body responsible for the development of national industry policy, has led the establishment of an animal health compartment in the south-eastern temperate zone that recognises the lower disease risk of Beef Only herds in regions where Johne's disease is established in the dairy industry. The peak council has worked with state governments and Animal Health Australia to establish business rules for the compartment, for the movement of stock to regions where the disease is rare or unknown, and for maintaining the robustness of the system through verification and audits.

This presentation will further explain the background to the policy and outline the responsibilities of the various parties involved in developing the business rules.

THE ROLE OF THE BJD COUNSELLOR AS AN INDUSTRY ADVOCATE IN THE DEVELOPMENT OF INDIVIDUAL DISEASE MANAGEMENT PLANS; IN PARTICULAR THE BENEFITS THIS ARRANGEMENT PROVIDES FOR THE PRODUCER, GOVERNMENT AGENCY, PRIVATE PRACTITIONER AND THE WIDER INDUSTRY

Allan D

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Role of the BJD counsellor

The Cattle Council of Australia supports the goals and objectives of the National BJD Strategic Plan (NBJDSP). One sub-program, the Financial and Non Financial Assistance Package (FNF) aims to minimize the trade, economic and social impacts of BJD at herd, regional and national levels. The BJD counsellor's role is to facilitate a process which separates the regulatory role of government from the development and supervision of Enhanced Property Disease Management Plans (EPDMPs)¹ with the goal of reducing economic and emotional stress and social stigma associated with BJD. In carrying out this role the counsellor adopts a client focused approach based on empathy, good communication, a sound understanding of the disease and the associated regulations and an appreciation of the trade, economic and emotional impacts of the disease and regulations at herd, regional and national levels.

Benefits for the producer

The counsellor provides a single point of contact with an independent professional who can articulate unbiased, consistent information regarding the disease and regulations in lay-person language. The counsellor provides the producer with the opportunity to discuss the economic and emotional impacts of BJD with a trusted independent person in their own non-threatening environment. The counsellor develops a unique understanding of the affected producers needs, issues and concerns which, when considered within the regulatory framework, enables the development of EPDMPs which meet the producer's needs whilst complying with the Standard Definitions and Rules (SD&Rs).

<http://www.animalhealthaustralia.com.au/programs/johnes-disease/national-johnes-disease-control-program-njdcp/>

Benefits for the government agency

The role of government is to ensure that animal health legislation is being complied with, provide technical advice where necessary and undertake other regulatory activities such as tracing. These activities are quite separate from the development of EPDMPs. With the counsellor facilitating the development of EPDMPs by approved veterinarians the government agency can focus on its core responsibilities.

Having the counsellor as an independent facilitator and primary point of contact for the affected producer removes much of the angst relating to regulatory requirements that often occurs between affected producers and government agency field staff. This intermediary role of the counsellor has the effect of improving the relationship between the government agency and the affected herd owner. The provision of constructive feedback to the government agency by the counsellor improves overall service delivery.

Benefits for the approved veterinarian

With the counsellor dealing with the emotional and financial issues and communicating with the government agency the approved veterinarian is better able to focus on their core function of developing and supervising the implementation of the EPDMP. The counsellor provides a single point of reference for the veterinarian in relation to FNF program requirements and facilitates the technical approval of the EPDMP and financial assistance approval. The counsellor manages the expedient payment for any approved testing undertaken by the veterinarian and the development of the EPDMP.

Benefits for the wider industry

This facilitated team approach, where each member of the team is able to utilize their unique skill-set to focus on their core responsibilities, is more efficient in delivering the best outcomes for individual affected producers and the wider industry and is a more effective use of industry funding.

The counsellors are accountable to industry and are required to report on a regular basis to both Animal Health Australia and Cattle Council of Australia. The de-identified data provided in these reports is invaluable in terms of monitoring the effectiveness of current programs associated with the NBJDSP and providing further insights into the epidemiology of the disease in an industry sector with very low herd prevalence.

BOVINE JOHNE'S DISEASE CONTROL IN THE DAIRY INDUSTRY – A PRODUCER'S PERSPECTIVE

Westacott T

The Vet Group

Background

Since 1982 Trevor Westacott has been involved in the management of dairy enterprises (both family run and large corporate operations) in Western Victoria, South Australia and Tasmania. He shares his experiences from a producer's perspective in successfully controlling BJD on a large corporate dairy in South Australia, focussing on the challenges of implementing a calf accreditation program (JDCAP). Prior to the introduction of JDCAP, farm management had already developed a good set of standard operating procedures for the farm systems, with good buy in and a high level of staff compliance. The standard operating procedures were tweaked to accommodate the principles of the JDCAP program. The principles of a JDCAP calf rearing program were seen as good for overall calf health, with the benefit of Johne's control. The three main focus areas of the program were:

1. Management to prevent the spread of BJD to calves through contaminated manure
2. Management of colostrum collected for calf consumption
3. Documentation of procedures

Summary of experiences

On farm

1. It is difficult to pick up calves 12 hourly without engaging extra help.
2. Collection of uncontaminated colostrum maybe a challenge for some farmers.
3. Staff must buy in and be well trained in procedures and recording.
4. Intensive dairy farming throws the challenge of how to create a 12 month adult free grazing area
5. Double fencing is expensive and in some cases unsightly.
6. Deer movement is hard to control if the farm adjoins forest precincts.
7. It is less difficult to cull cows that test infected, than to kill off the progeny which the farmer views as his future. Calves and heifers are also perceived as more valuable.
8. Ponding, water runoff and drains pose extra challenges on wet or irrigated farms.
9. Some farmers would rather dispose of clinically infected cattle before detection, to ensure that their herd does not get a BJD status.
10. Loss of farmer commitment to the program due to high workload or setbacks. Even a simple episode like an escaped calf may have the farmer questioning the worth of the effort.

For Industry

12. The perception that a BJD herd, even lightly infected, is viewed as worse than a non-tested herd .
13. Farmers on programs have not been rewarded by the market.
14. Farmers must be convinced of the value of reducing BJD. A proper value proposition has not been sold to farmers.
15. There must be uniform BJD policies across the states.
16. BJD has been used as a barrier to movement of cattle to some states, even low risk cattle.
17. While BJD control is achievable, until there is uniformity in the approach to BJD many farmers will put control measures in the too hard basket or adopt a "head in the sand" attitude to the problem. BJD is not seen as the biggest cost disease on a commercial dairy farm so farmers allocate their resources elsewhere.

THE IRISH JOHNE'S DISEASE CONTROL PROGRAM

Graham DA

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In 2009, Animal Health Ireland (AHI; www.animalhealthireland.ie) was established to provide a partnership approach to national leadership of non-regulatory animal health issues (those not subject to national and/or EU regulation). The objective prioritisation of non-regulatory animal health issues was undertaken through an expert Policy Delphi study and farmer surveys. As a result of this and subsequent work, JD, along with bovine viral diarrhoea and infectious bovine rhinotracheitis, were identified as the prioritized diseases with a biosecurity component. The model by which AHI addresses each of these groups is to convene a technical working group (TWG) comprising experts in each of the diseases. For JD, a group drawing representatives with appropriate experience from academia, the advisory services, government and field veterinarians has been convened.

An initial task of the TWG has been to develop information resources for farmers and veterinary surgeons and to raise awareness and understanding of JD. An information leaflet on JD, accompanied by a more detailed document giving answers to frequently asked questions has been prepared and will form the basis of a series of roadshows for industry (see www.animalhealthireland.ie).

AHI has been actively involved in consultations with the industry at processor level and one outcome of these discussions has been that the TWG has been working on a proposal for a voluntary national JD programme. It is intended that this will involve herd classification. However, optimal testing strategies for initial herd screening and subsequent testing for JD in suckler and dairy herds in Ireland are currently not known. In recognition of the difficulties in categorisation of herds as infected or uninfected, the TWG has sought to use a confidence-based approach to this problem, and has invested considerable effort in developing an epidemiological model to evaluate a range of testing strategies in an Irish context, with a focus on detection probability (given a specified design prevalence) and cost effectiveness. The TWG intends to deliver its recommendations to industry early in 2012. Thereafter, a cross-industry Implementation Group will be convened to take the programme forward.

The overall goals of the programme are as follows:

- To increase awareness within industry and the advisory services, including veterinary surgeons, thereby facilitating informed decision-making
- To reduce herd and within-herd prevalence, thereby minimising on-farm losses and the between-herd spread of infection, and safeguarding the quality of Irish livestock and livestock products
- Safeguard the quality of Irish livestock and livestock products

Initial herd categorization will take into account the type (suckler or dairy) of herd, as each of these have different prior probabilities of infection, its purchase history (bioexclusion) and source of these purchases. For herds with negative test results, the model will use all of this information to assign a probability of infection value, and this in turn will be used to assign the herd to a given risk ranking. For herds with positive results, the principle emphasis will be on bio-containment practices, with ongoing veterinary risk assessment envisaged to identify and prioritize required management changes.

Potentially these biocontainment risk assessments may also be used within test negative herds with poor bioexclusion practices. In these herds, optimisation of bio-containment practices will serve to minimize the spread of infection which is present but thus far undetected.

The Irish dairy industry has clearly signalled its intention to address JD and to seek to build on the current low prevalence of infection. Already, one processor has put in place a programme of whole herd individual testing (over two years of age), risk assessment and biocontainment advice. This is delivered by the herds' veterinary surgeons and will be aligned with the national programme as the latter is rolled out.

Acknowledgements

The contribution of the members of the AHI TWG and Evan Sergeant (Ausvet) to this work is gratefully acknowledged.

**SOCIOLOGY AND *MYCOBACTERIUM AVIUM* SUBSP.
PARATUBERCULOSIS CONTROL**

THE ONTARIO JOHNE'S DISEASE EDUCATION AND MANAGEMENT ASSISTANCE PROGRAM

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The Ontario Johne's Education and Management Assistance Program (OJEMAP) was launched in January 2010 after several years of planning, a pilot and a formal evaluation. The program was initiated by dairy producers and is administered provincially by a committee made up of dairy producers and representatives from the provincial government, the University of Guelph, dairy industry and breed organizations, and veterinarians. The program is supported by \$2.4 million, most of which has come directly from milk producers through the Dairy Farmers of Ontario (DFO). Additional funding for the program is provided by the Ontario Ministry of Agriculture, Food and Rural Affairs; the Holstein, Guernsey and Jersey breed associations; CanWest DHI; the Ontario Cattlemen's Association; Progressive Dairy Operators, and the Ontario Association of Bovine Practitioners.

The program has four pillars and focuses on educating dairy farmers, veterinarians and consumers about Johne's Disease (Paratuberculosis); conducting annual on-farm risk assessments; implementing a milk testing program that reimburses farmers up to \$8 per cow tested; and working with producers to ensure that cattle actively shedding the Johne's bacterium (*Mycobacterium avium* subspecies *paratuberculosis*, or MAP) are removed from the herd and don't end up in food chain.

The education component includes traditional approaches, such as oral presentations and displays at industry meetings and articles in professional and lay industry journals, as well as novel interactive participant-lead learning through the Johne's Focus Farm initiative.

The Animal Health Risk Assessment and Management Plan (RAMP) is a questionnaire that guides the herd veterinarian and the producer through a step by step assessment of calving, calf raising and hygiene practices associated with good calf and cow health, and excellent milk quality. The goal is to identify risk factors that could allow MAP from the manure of an infected cow to infect calves on the farm. After completing the questionnaire (risk assessment), the producer and the veterinarian decide what can and will be done in the next year to mitigate some of the identified risks as part of developing the "management plan". Generally acceptance of recommendations is good when producers realize that steps taken to reduce new MAP infections will also reduce other calf diseases caused by fecal-orally transmitted pathogens.

Funding is available to cover the cost of one whole herd test (all adult animals) for every herd in the province once over the 4 years of the program. Testing is done either by milk ELISA through CanWest DHI or serum ELISA (or fecal culture) through the provincial diagnostic laboratory. To prevent overwhelming the testing capacity of the laboratory system, a province-wide testing schedule was produced that allows each producer one opportunity in a 6-week window to complete their whole herd Johne's test. The schedule was created using county divisions and townships within larger counties to form 29 testing windows.

To receive the reimbursement of \$8 per cow tested, producers in the program must test all lactating animals in the herd during their testing window, complete the RAMP with their herd veterinarian and submit a copy to the program administrator, and remove all cows found with high titre (HT) tests (based on the milk ELISA test currently in use a positive test result is 0.1 or greater, while a High Titre is 1.0 or higher) NOT to another dairy herd or to the food chain, within 90 days of the testing date. Producers who remove these HT cows as required by the program receive \$500 per cow to assist with on-farm changes to prevent MAP spread..

Based on the first 18 months of the program, 1066 of 2429 eligible herds completed all aspects of the program and received payment for testing and high positive cow removal, if applicable. Of the 1253 herds which tested all cows, 1218 used the milk ELISA test and 35 opted for blood testing. Of 84,927 cows tested, 652 (0.7%) had positive ELISA tests. 260 (23%) herds had at least one cow with a positive test result and 85 (7%) herds had at least one cow with a HT test result.

The number of test positive and HT cows identified in the herd tests is quite low, in fact lower than expected. It is encouraging that after the first year and a half, the results indicate that among the eligible herds enrolled, there is generally a low prevalence of herds with a serious Johne's problem, and an overall low prevalence of test-positive and HT cows.

The OJEMAP is a voluntary program and the uptake thus far has been good. However, of concern is the continued lack of participation among herds which we believe are more likely to have Johne's positive animals. In an attempt to shed light on this concern, a study was undertaken to compare some frequently measured herd performance parameters of both Johne's program participating and non-participating herds. While the measurements compared are not directly related to Johne's, they do relate to the overall quality of herd management. Herds that have participated in the Johne's program to date were shown to be better managed, larger herds, with more milk production per cow and lower bulk milk somatic cell counts (these differences were statistically significant at the 5% level). The implications of this difference for future program implementation and endeavours are currently being considered. During the first year of the program there were 659 RAMP's completed by producers with their herd veterinarians. The recommendations from the management plan most commonly cited are as follows:

Frequency Top 10 Recommendations:

- (14%) Don't purchase more cows/ minimize purchases/ buy from low risk herds
 - (11%) Remove heifer calves quickly from maternity area/ pasture to individual pens/ hutches
 - (8%) Feed more colostrum and feed it on time.
 - (6%) Don't feed non-saleable milk of high SCC milk to heifer calves
 - (6%) Separate newborn calf from cow (create mini-pen, calf box/ cart, tub)
 - (3%) Calving pen is NOT to be used as a hospital pen (need separate pen)
 - (3%) Separate heifers (bred/pregnant/breeding) from dry cows
 - (3%) Feed low risk milk
 - (3%) Retest herd in 12- 18 months/ continue testing
 - (3%) One cow in calving pen at a time/minimize cows in calving pen
- Further details about the Ontario Johne's Education and Management Assistance Program can be found at the following web link: www.johnes.ca

A TWO-LEVEL TRAINING AND COMMUNICATION PROGRAM ON BOVINE PARATUBERCULOSIS IN THE VENETO REGION

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Bovine paratuberculosis is highly prevalent (>70%) in the dairy cattle population of the Veneto region (North Eastern Italy), the third largest milk producing area in Italy [1]. To reduce the impact of the disease, an experimental control program was implemented during the years 2004-2007. This program, based on the application of biocontainment measures and on-farm management of the animals testing positive, provided interesting results, but also showed critical points [2]. Over the subsequent three years, the program was extended, on a voluntary basis, to other herds in the region, providing free technical assistance for the development of individual control plans. Disappointingly, a low number of farmers joined and then followed the proposed control schemes. Therefore, a new approach was studied to involve farmers and their veterinarians in the disease control activities, starting from the analysis of the veterinarians' knowledge and perception levels.

A strategy to reduce veterinarians' knowledge gap is the realization of training courses tailored to the target group's real needs and the specificity of their working area. Therefore, the aim of the project was to find sustainable approaches for paratuberculosis control. A participative process was identified as an approach capable of increasing the awareness of veterinarians and farmers.

The process was divided into different phases:

1. Assessment of veterinarians' knowledge and perception of cattle health and management issues with particular focus on paratuberculosis
2. Design of a training path enabling veterinarians to acquire specific knowledge of disease control and communication skills
3. Evaluation of the awareness of a subset of farmers performed by the trained veterinarians
4. Definition of training and communication strategies for cattle farmers

In this framework, the IZSve Verona diagnostic laboratory, in cooperation with the National Reference Laboratory for Paratuberculosis and national experts, produced a set of guidelines on paratuberculosis. These guidelines were developed following the standards of Evidence-Based Medicine to ensure conscientious, explicit and judicious use of current best evidence in making decisions about paratuberculosis control and certification of freedom. The present work describes the procedure followed in the first phase of the project to explore the needs of the target population (veterinarians involved in buiatrics) in terms of training.

Materials and methods

Both quantitative and qualitative methods were adopted to study veterinarians' perceptions and knowledge of disease control, especially with regard to paratuberculosis. The purpose of the project was to develop a shared intervention in which scientific knowledge about the management of paratuberculosis was integrated with the opinions of experts. Therefore social research tools (focus group and questionnaire) were defined in collaboration with a group of stakeholders working on paratuberculosis. The involvement of experts was fundamental to the definition of the information to be investigated with the survey. The questionnaire was tested and validated through a focus group with 10 veterinarians managed by an expert in social research techniques. It was then proposed to the veterinarians working in the Veneto region and involved in animal health (n=250), both large animal practitioners and official veterinarians.

The quantitative survey was designed to assess:

1. veterinarians' perception of the impact of the disease on cattle farms
2. their awareness of the health status of the farms
3. their willingness to participate in a training program.

Data were collected using CAWI (Computer Assisted Web Interviewing) method.

Results and discussion

A total of 96 veterinarians filled in the questionnaire: 47.2% were private vets and the others worked for the local veterinary services. As shown in Table 1, although paratuberculosis is widespread in the area [1], the vets' perception of the prevalence of the disease and its impact on farmers was low. On the other hand the analysis revealed the veterinarians' high interest in paratuberculosis, and especially the application of good management practices in the herds. Moreover vets declared that no more than 10% of the farmers apply paratuberculosis control programs.

Table 1. Ranking of the vets' perception of disease frequency (A) and the economic burden (B) in dairy cattle herds.

A. Frequency of diseases	Position	B. Economic burden of diseases	Position
Mastitis	1	Hypofertility	1
Hypofertility	2	Mastitis	2
Q Fever	3	Q Fever	3
Methabolic diseases	4	Methabolic diseases	4
Lameness	5	Lameness	5
Neonatal diseases	6	Neonatal diseases	6
BVD	7	Paratuberculosis	7
Paratuberculosis	8	BVD	8
Parasites (endo-ecto)	9	Parasites (endo-ecto)	9
Neosporosis	10	Neosporosis	10

As shown in Figure 1, the level of knowledge of the vets involved in the study, calculated by an index based on weighted answers, was quite high, (mean 0.6 – 0= low; 1= high), and higher although more widespread, among private vets than among public ones.

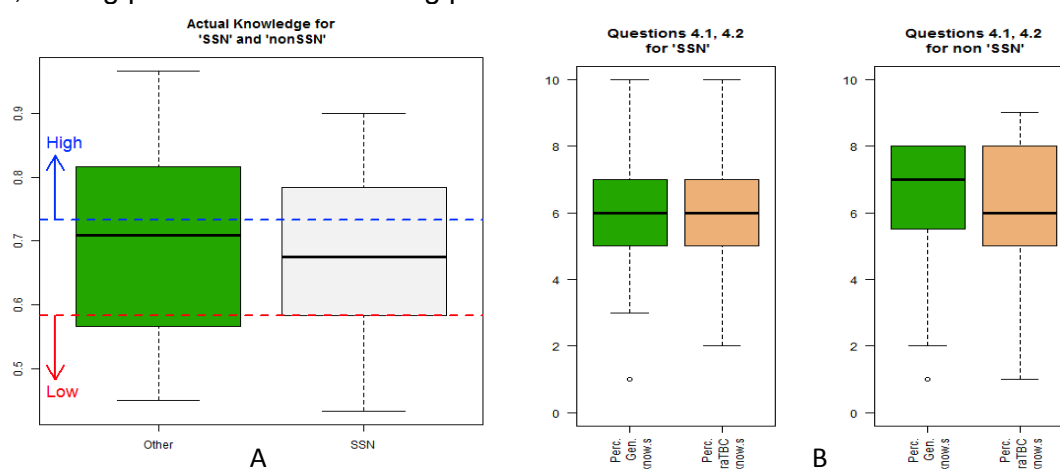


Figure 1. A. Level of knowledge calculated by index 0-1 and distributed by type of veterinarians. B. Vets' self-perception. Question 4.1 measures the vets' self-perception of their ability to evaluate health risks in farms. Question 4.2 measures vets' self-perception of their knowledge of paratuberculosis. Note: 'SSN' refers to Vets working in local veterinary services; 'non SSN' to private Vets

In addition, the self-perception analysis shows that, despite having adequate knowledge of general disease risk assessment in farms (mean 6.08), veterinarians feel less qualified on paratuberculosis (5.91). In particular public vets feel less prepared to manage diseases than private ones.

Moreover data show that both groups of veterinarians express the need to increase their knowledge about paratuberculosis through a training course (92.1% of public vets; 100% of private vets). Respondents requested an in-depth training focused on procedures for managing infected farms (7.98), epidemiology (7.67) and diagnostic test application (7.21).

Conclusions

This project combines the use of scientific knowledge of paratuberculosis and social-research tools which place veterinarians and farmers at the centre of the disease control planning and decision-making processes. The involvement of stakeholders for the planning of disease control activities is an effective strategy to meet the target's information needs. The next step will be the definition of a training path for the vets aimed at providing them with both knowledge of the disease and communication tools to transfer it to the farmers.

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LIVESTOCK AGENTS IN AUSTRALIA AND THEIR INFLUENCE ON SHEEP PRODUCERS' ANIMAL HEALTH MANAGEMENT PRACTICES

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Background

Livestock agents are used widely across the Australian sheep industry to assist producers in the purchase and sale of sheep. The National Sheep Health Statement is an on-farm biosecurity tool used for managing the risk of ovine Johne's disease (OJD) introduction and other sheep diseases and parasites. A SHS is made available to potential purchasers when sheep are offered for sale. In relation to OJD specifically, the SHS includes the calculation of an Assurance Based Credit (ABC) score for the sale sheep; this indicates the likelihood that the sheep may have OJD. In the sheep sales environment the SHS provides different benefits to purchasers and vendors of sheep. For purchasers it provides a level of assurance of the health of the sheep and reduces the chance of introduction of diseases. For vendors, use of the SHS provides an indication of the quality of their livestock and may also increase buyer interest and the price at sale.

Despite the obvious potential benefits of use of the SHS, uptake has been inconsistent across the country and its implementation has been different across jurisdictions; mandated in some, encouraged in others. As a biosecurity tool to protect against OJD and other diseases it is important to ensure that uptake is maximised as widely as possible.

This study was conducted to investigate agents' attitudes and practices regarding use and advocacy of the SHS and perceived barriers to producer uptake of the SHS. It also assessed the degree of perceived influence agents felt they had on sheep producers' use of the SHS and their animal health practices more broadly. This study formed part of a wider study, funded by Meat and Livestock Australia (MLA), in which sheep producers were also surveyed about their uptake of the SHS.

The objectives of the agent research were:

- to determine the uptake and use of the National Sheep Health Statement by agents.
- to identify agents' views on any possible drivers to assist the use of the Sheep Health Statement, and similarly, to identify any social or practical barriers to its uptake that may be amenable to change or influence, and
- to investigate whether agents might be influencers of producer uptake of the SHS and what their attitudes were towards this as part of their role.

Methodology

The questionnaire was developed by the research team in conjunction with an expert panel, and survey interviews were conducted using a computer-assisted telephone interviewing (CATI) methodology, in which the interviewer follows a script using a software application that is able to customise the interview based on the answers provided.

The survey comprised 35 questions, and covered a range of issues, such as details of operating environments, relationship with clients, perceived influence on clients with regard to sheep health management and use of the SHS, and perceived barriers and drivers to use of the SHS. An agent population was obtained from member agency contact details openly available on the Australian Livestock and Property Agents' Association (ALPA) website. A total of 300 interviews were conducted with agents from 1-10 March 2011. The survey response rate was 44%.

As the sample size was large and the response rate for the survey was satisfactory for this type of survey approach, this provides confidence that data are representative of the agent population. The use

of CATI methodology and experienced interviewers provides additional strength; with standardised procedures for data collection and coding and high levels of interview completion. In addition the survey was structured to optimise the methodology and questions were time-bounded and related to *recent* experience and *current* practice to reduce recall bias and improve data accuracy.

Results

Descriptive analysis was conducted, for the whole sample and for the sample cross-tabulated by State and by Ovine Johne's Disease (OJD) prevalence area. In addition multivariate logistic regression analysis was conducted to investigate factors associated with use of the SHS.

Key survey findings are listed below.

- As found in the producer survey, use of the SHS was generally 'all or nothing' with similar proportions of agents (40-50%) using the SHS ALL the time or NONE of the time.
- Agents had strong established and trusted relationships with producers and they believed they had a high degree of influence on producers; 86% felt that producers relied on their advice 'a great deal' or 'a lot'. These findings were mirrored in the producer survey.
- Over half of the agents believed that the majority of their clients (>75%) relied solely on their judgment to purchase disease-free sheep.
- Agents advised producers on a range of issues, but mostly around correct completion of paperwork; the National Vendor Declaration (NVD) and the SHS.
- In relation to selling, agents identified the main drivers for uptake of the SHS as it being mandatory, achieving better prices, and increased buyer interest.
- In relation to purchasing, agents identified the main drivers for uptake of the SHS as providing protection from buying diseased sheep, providing useful information, and assurance.
- Main barriers to use of the SHS were reported as being a lack of mandatory requirement, apathy, ignorance/lack of awareness of the SHS, and lack of perceived benefits of its use.
- Agents were generally positive about the SHS; considering it effective, useful and necessary.
- At least half of agents reported that they encouraged producers to supply a SHS when selling and to request a SHS when purchasing
- Agents acknowledged their influence on producer uptake of the SHS and were able to identify ways to improve its uptake; however, some felt that this should not be their responsibility.

Analysis of livestock agents' uptake of the SHS indicated that this was based on the jurisdiction in which the agent was based and the operating 'norm' in that location. Logistic regression analyses indicated that higher levels of SHS uptake were associated with higher proportions of work based in areas in which the SHS was mandated, use of the SHS irrespective of producer demand for it, and positive attitudes towards the SHS, such as it being useful and effective for managing disease risk.

Agents regarded the main barriers to producer uptake of the SHS as being a lack of mandatory requirement, general apathy, lack of awareness and knowledge of it, and a perceived lack of benefits to its use. Agents themselves were generally positive about the SHS and when asked how *they* could influence producers' uptake of the SHS suggestions were mostly around their promotion and endorsement of it, increasing awareness and improving education of it, and insisting/advocating its use.

Recommendations

Data collected from agents suggested that they were highly trusted and influential in relation to use of the SHS, and this was further supported by data gathered from producers. It is possible that agents are an untapped resource in the industry's animal health and biosecurity system and that this (much smaller) group could be a potential focus for future SHS-related extension and promotion activities. Evidence provided in the current research indicated that agent advocacy of the SHS would have an exponential effect on producer uptake and that uptake of the SHS is significantly associated with more positive attitudes towards it, which should help to sustain uptake once established.

MOTIVATION OF FARMERS TO REDUCE BETWEEN-HERD TRANSMISSION OF PARATUBERCULOSIS THROUGH REPEATED ON-FARM MEETINGS WITH DISCUSSION ON CERTIFICATION

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Motivation of farmers to control paratuberculosis is challenging. This project aimed at motivating farmers to establish measures to reduce within and between-herd transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Eighty farmers from an organic co-operative were divided into 8 groups. These groups met twice a year for 3 consecutive years at different farms. Discussions and knowledge sharing was used as means to motivate farmers to reduce their MAP transmission. Changes in risk scores were monitored in the study period. Herd-level certification was introduced to reduce between-herd classification and was reported to farmers at the meetings. Fifty-four percent reported to have improved their trading patterns as a result of the certification classifications, eighty six percent improved calving procedures and ninety-one percent gained knowledge of how to improve the management of paratuberculosis. The certification system is designed to motivate farmers' behaviour to limit the spread of MAP between herds. The method in which the certification system was implemented appeared to engage the farmers to use it.

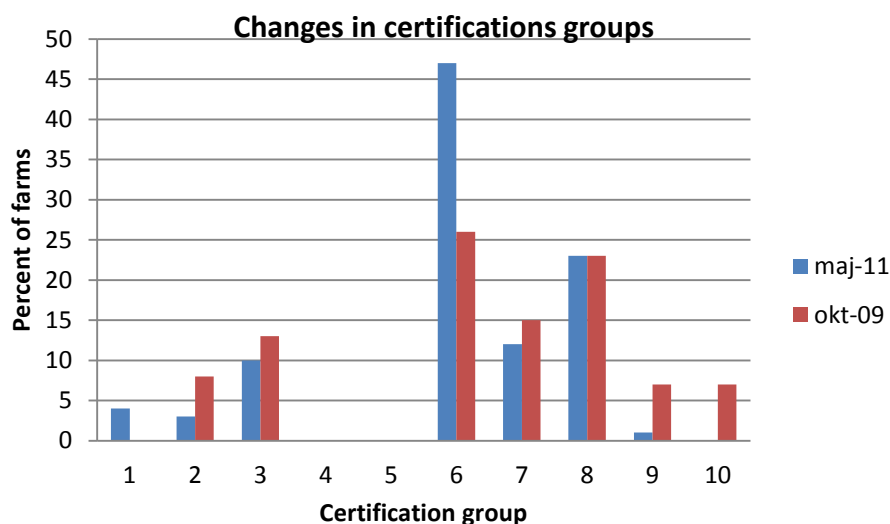


Figure: Distribution of the 80 herds in the 10 certification groups in Oct. 2009 and May 2011. Certification groups are based on the within-herd test-prevalence combined with the within-herd test prevalence in herds from which the farmer has purchased animals (Krogh et al., 2012, 11ICP Proceedings p. 149)

JOHNE'S DISEASE EDUCATION: CREATING TEACHABLE MOMENTS TO ENGAGE LEARNERS

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Online Johne's disease education in the US began with a 6 hour certification program for veterinarians in 2005. Since then we've created modules for special species, producers, and milk sampling field technicians and laboratory technicians. However, we felt that modules that create a "need to know" would make the messages we were trying to teach more relevant and more memorable. The first generation of "simulations" were virtual farms; two dairy operations and two beef operations. These early attempts gave veterinarians practice in gleaned important information from the stories told by the producers and veterinarians, as well as using observation of environmental conditions and management practices to fill out a risk assessment and management plan. Once the forms were submitted the learner could compare their plan to that of an expert.

Using gaming principles and strategies we were able to improve on the earlier virtual farm tours, creating the JD Consult game. The game creates a situation where the learner is an apprentice Johne's consultant where they need to give the producers recommendations, thereby again creating a teachable moment. Learners are given opportunities to learn more about tests and interpretation, different management risks, and best management practices. Feedback is customized to the specific choices they made. Again, expert opinions, sometimes with different viewpoints, are given for comparisons. Most recently, we have created a simulation for producers in which they are led through a risk assessment. Producers are asked pertinent questions about the different risk areas, creating teachable moments. Each area provides opportunities at just the right time for producers to learn about what the risks in that area are, why they are risks, and what management practices could be used to reduce the risk. Based on the responses producers give, specific feedback is given about their business and their management styles. While the primary recommendation is for producers to contact their veterinarian, the simulation creates a personalized experience and the "need to know". A teachable moment is created and the learner is engaged. Success!!

INTERNATIONAL CONSORTIA

JOHNE'S DISEASE RESEARCH CONSORTIUM (JDRC) – NEW ZEALAND INDUSTRY'S PARATUBERCULOSIS RESEARCH INITIATIVE

Larking K

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Johne's disease was first identified in cattle in central New Zealand in the early 1900's and progressively spread to other species and locations in the country in the following years. By 2002 JD was noted as being "endemic in sheep and cattle and on the rise in farmed deer". The prevalence and impact of the disease in New Zealand has not been well understood and efforts to control it have been sporadic and often ineffective.

The Johne's Disease Research Consortium was established in 2008 as a joint venture between the New Zealand livestock industry, research providers and the New Zealand Government to coordinate industry resources and research capability for the study of Johne's disease in New Zealand. The Consortium's primary aim is to develop practical tools that can be utilised by farmers to reduce the impact of Johne's disease on farm in New Zealand. The research programme is focused on four major areas; diagnostics, pathobiology, genetics and epidemiology, and employs and coordinates the expertise of New Zealand's leading paratuberculosis researchers, overseen by an expert international panel.

Early outcomes from the 5 year program include important information describing the profile of Johne's disease in New Zealand. Advances in strain typing methodology have been used to investigate the distribution and spread of JD in New Zealand, revealing previously unknown patterns of infection. There is clear evidence that MAP is transmitted between species and that regional variations are seen in the disease distribution. JDRC has also funded the development of the first comprehensive prevalence data set for New Zealand. Surveys undertaken from 2008-2011 have established that while infection appears to be widespread, very low rates of clinical disease are recorded for the major ruminant species (beef, dairy, deer and sheep), so that only a small proportion of farms have a significant issue with mortality and production losses. Economic data assessing the true impact of the disease in NZ is due for release in 2013.

Alongside the research program the Consortium's is working to provide up to date Johne's disease information and tools for the control and management of the disease for the New Zealand livestock industry. In this area the Consortium works closely with the NZ Deer Industry. The Deer Industry has responded proactively to the emergence of the disease in farmed deer by developing resources and systems to monitor and manage the disease. These resources are now serving as models for the development of farmer guidelines for JD management and control in the wider New Zealand livestock industry. Work is also underway by the Consortium to define national targets for disease control, which in turn will determine ongoing priorities for research in New Zealand.

JDRC has proven an effective vehicle for improving coordination of industry objectives with scientific research in New Zealand, and will have achieved its scientific targets at the conclusion of its first term. It is intended that there will also be a framework in place for ongoing disease monitoring and provision of farmer and veterinary resources for effective disease control and management for New Zealand farmers.

JOHNE'S DISEASE MANAGEMENT AND CONTROL IN AUSTRALIA: A DYNAMIC PARTNERSHIP

Citer L

Animal Health Australia

INTRODUCTION

In 1995, the Australian livestock industries took the first steps towards a nationally coordinated and consistent approach to controlling Johne's disease. The program aimed to manage the spread of bovine and ovine Johne's disease and to provide an objective basis for disease control and the assessment of herd and flock risk.

The catalyst for the development of the NJDCP was the continued spread of Johne's disease within Australia despite the control programs that had been initiated in some states. There were also additional concerns about the capacity for the transfer of disease from cattle and sheep to other livestock industries. Around this time, interest was also increasing in the hypothesis that there could be an association between Crohn's and Johne's disease. Further history about the early control programs in Australia is described by Kennedy and Citer (2010).

SCOPE

The initial focus of the national program was to

- develop national standards for diagnostic procedures, supported by quality control testing for laboratories,
- establish a set of National Standard Definitions and Rules to ensure complementary and mutually respected regulatory control across jurisdictions, and
- implement assurance programs for herd and flock status
- establish liaison with public health authorities.

The principal goals of the National Johne's Disease Control Program are: to provide effective coordination of Johne's disease programs across all jurisdictions and affected industries, to protect the favourable Johne's disease status of the country and to reduce the impact of the disease and control measures on the industries. Since those early days, the focus of the program has expanded to include activities that also aim to protect the economic and trade interests of the various livestock industries through a better understanding of the disease, development of effective control and eradication strategies and establishment of additional assurance schemes and other tools for effective risk management. Although initiated by the cattle industries, other industries soon joined the program as Johne's disease became a broader, cross industry issue, and the benefits of a collaborative partnership approach to managing the disease became apparent. A number of customized sub-programs have been added to address the special needs of, and particular risks faced by, each industry. The over-arching NJDCP provides coordination for these sub-programs and projects.

PARTNERSHIP

Initially the NJDCP used a traditional regulatory animal disease control approach and even though the program was initiated by industry, the bulk of the decision making responsibility rested with governments. However, the establishment of Animal Health Australia in 1996 provided state governments and the livestock industries a mechanism to benefit from national coordination of the program.

The 'partnership model' which underpins a number of national programs coordinated by Animal Health Australia is a proven one that brings together governments, industry representatives and research and development corporations, each with defined roles and responsibilities. This model has enabled the program to benefit from the input of more stakeholders than might be traditionally involved, with livestock research and development corporations, agents and research providers.

Each party within the partnership contributes to the development of national policies, implementation of control strategies and a targeted research and development program, either through direct activity or financial support. This has enabled the development of a robust, flexible and technically sound program to address the challenges arising from the uneven geographic spread and variable prevalence of Johne's disease between industry sectors. It has contributed to the continued relatively low prevalence of Johne's disease in significant geographic regions and industry sectors in Australia. Highly motivated

industry sectors and regions actively work to maintain or develop a 'protected population' status. That the program continues to achieve nationally agreed objectives is testament to the endurance of the partnership model that the livestock industries put in place with government some 15 years ago.

CHANGING CIRCUMSTANCES

The circumstances in which the Johne's disease partnership currently operates are very different from those of 1995. Despite this, with its dynamic partnerships model, the National Johne's Disease Control Program is sufficiently flexible to withstand the challenges of a changing political and business environment. As program coordinator Animal Health Australia has encouraged the livestock industries to adopt an 'outcomes' approach to accommodate the differing approaches of industries in managing disease risk. This is further supported by environmental scanning to identify any emerging issues and novel solutions that could be applied from other business contexts to improve the effectiveness and efficiency of the NJDCP. In recognition of the finite resources available for disease control programs some of these solutions are found through linkages with other animal health activities such as the promotion of biosecurity and the collection of surveillance data.

The experiences in Australia are similar to those in Europe, where changing community attitudes and government policies in relation to agriculture have seen the application of the 'public good/private benefit' test, and general financial constraints for all parties have influenced the program's operation. (More, 2008) General global concern about food security and emerging diseases brought about by climate variability is also having an effect, causing the partnership to further evolve and change. This is particularly so in the areas of disease surveillance, research and development and communications. In the early years of the NJDCP, functions such as R&D and surveillance were undertaken principally by the state departments of primary industries and funding was jointly contributed by the government and industries. Over time the industries have increasingly underwritten this work and have taken a lead in developing new 'partnerships' to ensure the objectives of the program are achieved. Livestock industries have used a formal process to evaluate and prioritise research projects utilising their research and development organisations. This ensures that the applied research program is coordinated and directed at delivering the benefits industry is seeking to further reduce the impact of Johne's disease on their enterprises.

Consequently the livestock industries through their respective research and development corporations, have been willing to invest significantly in applied Johne's disease research. This has delivered improved understanding of the epidemiology of the disease, and effective control tools for Australian conditions. In addition the industries collectively have also contributed to funding basic research on *Mycobacterium avium* subsp. *paratuberculosis* with the objective of better understanding the early infectious process, leading to the development of new tests and vaccine candidates.

Some members (mainly government) have reduced the resources they contribute to the program for a variety of reasons. The potential impact of that shift has largely been minimised by a broader range of contributors from the private sector joining the program e.g. from the food processing and pharmaceutical industries.

The welcome support from these new contributors has been most noticeable in the areas of communication, surveillance and the implementation of new control approaches, which are often based on regional biosecurity models. Surveillance data is now regularly collected from abattoirs as part of a broader production disease surveillance program. This information allows monitoring of the effectiveness of regional control programs as well as generating individual producer animal health status reports. With the recent developments in technology, the communication strategies of 15 years ago are steadily being strengthened. Through Animal Health Australia, the livestock industry councils have worked with pharmaceutical companies and livestock agents to develop new ways to reach producers. This is an area that will become more important in the future.

A partnership approach has been the hallmark of the Australian Johne's disease control program and will continue to be so as circumstances continue to change..

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EPIDEMIIOLOGY

KEYNOTE ON: BAYESIAN TOOLS IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* EPIDEMIOLOGY: WHAT WE SHOULD AND WHAT WE SHOULDN'T DO

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The foundations of Bayesian inference were set by the Reverend Thomas Bayes. The French Mathematician Pierre Simon de Laplace was the first to give statistical applications of Bayes' theorem, while the field of Bayesian statistics was not fully conceptualized until the end of World War I and did not appear in epidemiology until the mid 70's. Computer intensive sampling methods of estimation, like the Gibbs sampler and the Metropolis-Hasting algorithm (Hastings, 1970; Geman and Geman, 1984), have revolutionized the application of Bayesian methods and offer a comprehensive approach to complex model estimation. The advent of user friendly software such as WinBUGS (Spiegelhalter et al., 2003) has further facilitated the use of Bayesian inference and its constantly increasing application in veterinary epidemiology and, in particular, the study of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection.

Persistence of MAP infection in the host for a long latent period is one of the key features to its successful survival, hampering early and accurate diagnosis, quantification of the actual infection level and, eventually, control efforts. Bayesian inference has proved to be a valuable tool in the study of MAP epidemiology due to its inherent ability to incorporate prior information in the estimation process. This is a primary practical advantage that permits the combination of existing knowledge with the data at hand. It, therefore, constitutes an efficient way of filling in information gaps, inevitably arising from the long latency period. To this end, advanced latent class models have been recently developed and employed for the evaluation of MAP diagnostics in the absence of a reference test (Branscum et al., 2005), the overall discriminatory power of tests with continuous results and the subsequent estimation of the actual prevalence of MAP infection (Choi et al., 2006). Importantly, a holistic approach in the diagnostic interpretation of continuous tests has been lately proposed (Toft et al., 2005) that optimizes diagnostic utility: the actual test responses are interpreted rather than been dichotomized. This interpretation is specific to different stages of MAP infection and has been used to formulate groups different risk profiles, which must be subjected to different interventions to effectively control the disease (Nielsen, 2009). Other Bayesian applications in MAP epidemiology involve the development of survival models that adjust for the latent infection period (Kostoulas et al., 2010) and quantification of the heterogeneity of MAP infection for groups of clusters (i.e. herds) that possess a specific risk profile (Kostoulas et al., 2009).

The abovementioned advances are primarily resulting from the ability of Bayesian inference to incorporate priors. Inevitably and undoubtedly, subjectivity is introduced through prior incorporation, which is often mistaken for a subjective treatment of truth and has been a major point of criticism. Yet, Bayesian methods do not mistake beliefs for evidence but rather use evidence to modify existing beliefs. They do so in a formal and very specific way as opposed to what scientists often do but in very implicit and incoherent ways (Rothman et al., 2008). The relative influence of the prior and data on the updated posterior estimates depends on how much weight is given to the prior and the strength of the data. Large datasets have a predominant influence on the updated beliefs, while the same informative prior would have greater influence if the sample size is small. Malpractice of Bayesian inference can indeed transform its major advantage (i.e. prior incorporation) to a tremendous drawback. The mathematical capabilities of Bayesian inference should not overshadow biological reasoning and must not be seen as a substitute for data quality. This is a non-technical overview of the sound application of Bayesian methods in the study of MAP epidemiology, their strengths and their limitations.

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EFFECT OF POSITIVE TEST RESULTS FOR *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* ON WEANING WEIGHTS IN BEEF COW-CALF HERDS

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Johne's disease (JD) is a disease of worldwide economic importance (Johnson-Ifeorunlu et al., 1999; Harris and Barletta, 2001). Efforts to control this disease are hindered by the limitations in diagnosis including lack of gold standard (Wang et al., 2006), low sensitivities of available tests (Kudahl et al., 2007; Kudahl et al., 2008) and potential for impaired test specificity attributed to other mycobacterial exposure (Osterstock et al., 2007; Roussel et al., 2007). Control is further constrained by the lack of economically feasible and practically useful treatments for this disease (Manning and Collins, 2001). With limited information on losses due to JD and associations with relevant production parameters, it is difficult for producers to make sound decisions regarding disease management and disposition of animals after testing (Nielsen and Toft, 2006; Pillars et al., 2009). To address these gaps, this study attempts to estimate the economic losses associated with test status in herd-level measures and individual-animal-level weaning weight measures from beef cattle herds enrolled in a control program. The US National Johne's Disease Demonstration Herd Project (JDDHP) was launched to evaluate management related control measures for cattle and to educate veterinarians and producers about the importance of diagnostic testing, management and control strategies. This study attempts to estimate the difference in weaning weight, and associated monetary losses, of calves from dams with positive serum ELISA or culture of feces for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in beef cattle herds enrolled in JDDHP.

Data from JDDHP comprising of 22 herds from 8 states collected during the period of 1999 to 2009 were obtained to evaluate losses associated with Johne's disease in beef cow-calf herds based on serological and microbiological tests for MAP. The primary economic outcome for the study was calf weaning weight. Data for 205-day adjusted weaning weight (AWW), fecal culture result (n=2,103 cow-calf pairs) and ELISA results (n=3,482) were analyzed. Fecal culture results were classified as positive or negative. ELISA results were classified into four categories based on manufacturer recommendations: negative, suspect, positive, and strong positive.

Multilevel mixed models were developed including random effects to account for repeated tests within cow, and cow nested within herd using a linear mixed-effects model in the *lme4* package of R (Bates, Douglas and Martin Maechler. 2009. Package lme4. lme4.r-forge.r-project.org). Potential confounding of the associations between test status and AWW due to herd and animal-level covariates was evaluated on the basis of change in regression coefficients after inclusion of the covariate in the model. Age of cow, parity of cow, and home raised or from another herd, years since the inception of control program, herd size, breed, and laboratory used for testing samples were used for the analysis.

Univariate analyses identified a significant reduction of 60.2 lbs (95% confidence interval (CI): 7.23 to 93.28) in AWW when the dam was fecal culture positive. Similarly, compared to the AWW of calves from ELISA test negative dams, calves from strong positive cows were estimated to have a 49.5 lbs (95% CI: 28.12 to 70.89 reduction in AWW. The associations of other test categories compared to calves from ELISA negative dams were not significant but models estimated a reduction of 6.2 lbs (95% CI: -5.11 to 17.61) and 7.4 lbs (95% CI: -0.88 to 15.64) for positive and suspect categories respectively.

Final models were selected on basis of reduction in BIC between nested models. For both test types, the selected multivariable models were adjusted for cow age, lactation number and years since the inception of a control program. There was a significant reduction of 73.3 lbs (95% CI: 41.43 to 105.21) in the AWW of a calf when the dam was faecal culture positive (Table 1). Similarly, compared to the AWW of calves from ELISA test negative dams, calves from strong positive cows were estimated to have a significantly lower AWW by 47.4 lbs (95% CI: 25.71 to 69.00). The AWW of calves from positive cows were reduced 6.3 lbs (95% CI: -4.69 to 17.61) and calves from suspect cows were estimated to have 6.1 lbs (95% CI: -1.99 to 17.28) lower AWW.

Table 1: Reduced weaning weights in calves from cows with positive ELISA or fecal culture test results in US beef cow-calf operations

Test status of the cow	Difference in 205-day adjusted weaning weight of calf ¹		
	Lbs (% lost) ¹	95% confidence interval (lbs)	US\$ Value ²
ELISA: Strong positive	47.4 (9.7%)	25.71 to 69.00	\$ 57.59
ELISA: Positive	6.2 (1.3%)	-4.69 to 17.61	\$ 7.53
ELISA: Suspect	6.1 (1.3%)	-1.99 to 17.28	\$ 7.41
Fecal culture positive	73.3 (14.9%)	41.43 to 105.21	\$ 88.57

¹ Loss relative to test negative classification; adjusted for the effects of age and parity of the dam and number of years since inception of herd-level control program

² Based on 5 year average feeder calf value from <http://www.nass.usda.gov>

Results from this study indicate that there is a significantly lower AWW in the offspring of cows positive for MAP in fecal culture or strong positive serum ELISA results. These findings support the contention that Johne's disease is associated with significant economic losses attributed to decreased AWW within infected beef herds.

Positive test results for MAP in dams are associated with reduced calf weaning weights and subsequent economic losses in beef cow-calf herds. The observed impacts on weaning weight can be used by producers and stakeholders in the cow-calf sector to make economically justified decisions regarding Johne's disease control programs

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EFFECT OF DELAYING EXPOSURE TO *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* UNTIL ADULthood ON INCIDENCE OF INFECTION IN ADULT DAIRY COWS

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It is generally considered that susceptibility to infection is highest in youngstock, but that horizontal transmission is insignificant in adults. Also, due to the long incubation period of clinical JD (usually > 2 yrs), it is hypothesized that even if dairy cows could become newly infected as adults, the economic impact of these late infections would be insignificant because most cows will likely be culled or removed from the herd for other reasons prior to the animal experiencing negative effects of subclinical or clinical JD.

Control programs for Johne's disease (JD) in dairy cattle are therefore designed with focus on preventing exposure by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in young replacement cattle, however the role of adult cow infection in JD infected dairy herds is not well understood.

The objective of this study was to compare the incidence of subclinical and clinical MAP infection in cattle raised in an environment free of JD to those in cattle raised in an infected environment. Through a survey of presumed uninfected Minnesota dairy herds (Levels 3 or 4 of the Voluntary Johne's Disease Herd Status Program for Cattle), we identified JD infected herds that previously purchased replacement cattle from uninfected herds. Over a 3 year period, blood and fecal samples were collected in infected herds from 79 purchased replacement cattle that were raised in uninfected herds (unexposed) and homebred cows of similar age and stage of lactation (exposed controls). Serum samples were tested using an ELISA (IDEXX Laboratories) for detection of antibodies to MAP and fecal samples were tested for detection of MAP using bacterial culture on Herrold's egg yolk media.

While results from the first year of testing of cattle across multiple ages indicated that dairy cattle raised in JD low risk herds (Level 3 or 4) and introduced to Johne's infected herds were less likely to test positive for MAP than herdmates raised in infected herds (OR = 0.10, 95% CI = 0.01-0.75) for antibody to MAP and OR = 0.38, 95% CI = 0.14-0.98 for bacterial culture for MAP in feces; Kovich et al, 2006), results over time from survival analyses showed that this difference in risk was reduced later in life. The hazard ratio for testing positive at least once from Cox regression for unexposed compared to exposed cattle was 0.70 (95% CI=0.40,1.23) and 0.64 (95% CI=0.33,1.24) for fecal culture and ELISA, respectively. This longitudinal approach suggests that the reduction in test-positivity based on delayed exposure to MAP is lost through time in the herd. Study results suggest risk of MAP infection in adult dairy cattle should be considered in development of comprehensive JD control programs.

EVALUATION OF THE EFFECTIVENESS OF VACCINATION FOR REDUCING OVINE JOHNE'S DISEASE PREVALENCE IN SHEEP FLOCKS IN AUSTRALIA

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Vaccination with Gudair™ is a principal control measure for ovine Johne's disease (OJD) in Australia. However, despite the effectiveness of vaccination in rapidly reducing flock mortalities, there are concerns that shedding of *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) organisms may continue for many years and be continual source for recrudescence or disease spread. This study was conducted to evaluate the effectiveness of Gudair™ vaccine in reducing the prevalence of *Mptb* shedding in flocks of varying initial prevalence.

Self-replacing Merino flocks (38) that had been vaccinating lambs with Gudair™ for at least five years were enrolled in the study. These flocks had been tested prior to or at commencement of vaccination using pooled faecal culture test and/or agar gel immunodiffusion test. These pre-vaccination test results were used to classify flocks as low, medium or high prevalence flocks. Post-vaccination prevalence was similarly estimated from culture of pooled faecal samples collected from the enrolled flocks approximately five or more years after commencement of vaccination and used to classify flocks into ordered categories. Post-vaccination prevalence was compared with the pre-vaccination prevalence using ordinal generalised linear mixed models, with flocks as a random effect and the study time (pre- or post-vaccination) as a fixed effect.

Of the 38 flocks, 13 (34.2%), 11 (29.0%) and 14 (36.8%) flocks had low, medium and high pre-vaccination prevalence, respectively. Results of the ordinal generalised linear mixed model indicated that the post-vaccination prevalence was about six times lower than the pre-vaccination prevalence.

However, despite 18.4% (7/38) of the flocks apparently not shedding post-vaccination, about half of the flocks (18/38) had a cohort prevalence of >0.1% and a third (13/38) had a prevalence of >1%.

The results suggest that vaccination with Gudair™ is usually effective in reducing prevalence of shedding but the response to vaccination is variable between flocks.

FERTILITY, UDDER HEALTH AND MILK PRODUCTION IN COWS THAT HAVE HIGH MILK ANTIBODIES TO PARATUBERCULOSIS

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Key words

Paratuberculosis, productivity, udder health, fertility, MAP antibodies

Introduction

This paper describes an observational study carried out on a 500 cow dairy herd undertaking a Johne's control programme.

The dairy herd comprising 509 adult Holstein Friesian cows was identified as being of high risk of Johne's disease using a predictive model incorporated within a herd health management system (myhealthyherd.com). Although the herd had high levels of biosecurity, there were high risks of spread of Johne's disease. These were due mainly to the seasonal calving pattern, with many cows sharing communal calving accommodation with a high risk of environmental contamination, had *Mycobacterium avium* subsp. *paratuberculosis* (MAP) shedders been present. A historic biosecurity risk was identified using the biosecurity assessment tool within the myhealthyherd software programme; a stock bull had been introduced some 10 years previously but had been culled due to weight loss and poor performance.

No clinical signs of Johne's disease had ever been diagnosed in the herd, but the risks were such that a screening test was performed using a targeted screen of 30 high risk cows. Two cows showed the presence of high levels of antibody against MAP using a milk ELISA test (IDEXX) which prompted a whole herd screen and the implementation of a control programme of regular testing and management of high risk cows identified by the regular screening.

Method

At the milk recording of May 2011, 48 cows in the herd were consistently testing positive (>20) for antibody against MAP using ELISA tests on their milk recording samples. These cows were identified using an automated testing programme (Herdwise, National Milk Records (NMR)) as having at least three positive tests in the previous six months. None showed clinical signs of Johne's disease. Milk yields, milk quality, somatic cell counts, and all fertility events were recorded by the farmer and the data was downloaded into a cow management programme for analysis (Interherd, NMR). Yields, somatic cell counts, and fertility events of the 48 MAP positive cows were compared with the rest of the herd and with the performance of the 48 MAP positive cows in their previous lactation when they were testing negative for antibodies against MAP. Yields were compared around the date of May 2011 to attempt to avoid the confounding factors that the herd is increasing in yield over time through better nutrition and management.

Results

Of the 48 cows testing positive for antibodies against MAP, 12.5% were 1st lactation, 16.7% were 2nd lactation and 29.2% were 3rd lactation cows. This compared to 27.5%, 24.1% and 20.4% of these lactation animals in the herd as a whole. This demonstrates that there is a high prevalence in 3rd lactation animals, as may be expected by the normal incubation period of the disease and the production of detectable antibodies later in the infection cycle.

The average 305 day yield of the herd as a whole was 10,130kg for completed lactations in the year prior to May 2011, and predicted (using standard lactation curves within the Interherd programme) as 10,203kg for cows in their current lactations in May 2011. The 305 day completed lactations for the 48 test positive cows (completed before they tested antibody positive) was 10,146kg, and the predicted lactations for these cows was 9,424kg. Because first lactation test positive animals had no previous lactation at the time that they tested positive, they were excluded from this analysis.

Third lactation animals had the biggest difference between yields, with the mean predicted production for the 3rd lactation being 10,059kg and 8,562kg for 3rd lactation cows testing positive. The most noticeable effect at farm level was that the test positive cows (easily identified by the presence of a red ear tag to enable their specific management to prevent spread) averaged 26kg of milk per day compared to the test negative cows mean production of 31kg per day over the period from calving to the recording date of 16th May 2011. There was considerable variation between individuals, with some cows producing more than their predicted production.

Somatic cell counts are monitored monthly for all cows in the herd. The lactation mean of the herd at the milk recording of May 2011 was 155,000 cells/ml. The mean somatic cell count in the previous lactation of all cows that tested positive was 270,000 cells/ml and the mean somatic cell count of test positive cows in their current lactation was 238,000 cells/ml. The calculated mean milk somatic cell count at the milk recording of May 2011 was 155,000 for the herd, while the 48 test positive cows had a calculated mean cell count of 334,000.

The management system used to prevent the spread of Johne's disease within the herd as part of the Johne's control programme includes the culling of cows that consistently test positive at the end of their lactations if their milk yield drops or they show signs of mastitis or infertility. Thus, some test positive cows are not submitted for service if they have already tested positive prior to their voluntary waiting period (normally 42 days), or are not pregnant by the time the third test result shows positive. Of the 48 test positive cows, 29 were submitted for service and conceived. The median calving to conception interval in these cows was 136 days, compared to 106 days for the herd as a whole, and 103 days for the cows in the herd that calved up to May 2011. The fertility effect was not seen in the lactation previous to testing positive, where the calving to conception interval was 106 days for the 48 test positive cows.

	Mean 305 day yield (Kg)	Mean somatic cell count (,000 cells ml)	Calving to conception Interval (Days)
Whole herd in current lactation	10,203	155	106
Whole herd in previous lactation	10,130	n/a	102
Test positive cows in current lactation	9,424	238	136
Test positive cows in previous lactation	10,146	270	106

Discussion

This commercial dairy herd is run efficiently and effectively by expert stockmen with considerable veterinary input to maintain the health and productivity of the cows. The high risk of Johne's disease was identified at an early stage by the risk analysis used in the health management programme provided as part of the veterinary service to the farm. Herd screening of targeted high risk cows detected a significant prevalence of infection as determined by the presence of antibodies against MAP in milk. A strategy of regular routine testing of all milking cows and the management of high risk animals to prevent the spread of disease was introduced to control the prevalence of Johne's in the herd. This required a significant financial investment for testing costs, veterinary advice, and the husbandry changes required to manage high risk cows. In this high yielding herd with expert husbandry, classical clinical cases of Johne's disease had not been observed or recognised, although it is likely that many had been culled prematurely due to associated health issues.

The financial investment and resources required for Johne's control can be justified by the association of subclinical disease with the presence of antibodies against MAP, demonstrating that subclinical Johne's disease has a significant effect on productivity, fertility and udder health. An economic cost can be applied to these effects, which justifies to the farmer that Johne's control is worthwhile, despite the prolonged period of investment that is required to reduce the prevalence to insignificance.

BAYESIAN LATENT CLASS PREVALENCE ESTIMATION OF HERD LEVEL INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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The study aimed to estimate the true herd level prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in mixed species pastoral farmed livestock in New Zealand. A stratified-random sample of 238 single- or multi-species farms was selected from a postal survey population of 1,940 farms. The sample included 162 sheep flocks, 116 beef cattle and 99 deer herds from both main islands. Twenty clinically normal animals from each species flock/herd present on farm were randomly selected for blood and pooled faecal culture (PFC), sampling one pool of 20 animals from sheep flocks and two pools of 10 animals each from beef cattle and deer herds. Pooled faeces was tested by Bactec culture with mycobactin-J dependent confirmation. To increase flock/herd sensitivity (HSe), blood serum of all 20 animals from culture negative herds and flocks was tested by ELISA (sheep and cattle) or ParalisaTM (deer). Additionally, blood and faeces from up to five clinical suspect animals was collected, if present at sampling. Thus, the apparent herd status was based on four test protocols made up by the combination of the two tests with optional sampling of suspect animals. Results were adjusted for lack of sensitivity and specificity of tests through latent class Bayesian statistical modelling. True prevalence was corrected for sampling fractions to present prevalence in the total survey population stratified by island. Overall 164/238 farms (68.9%) tested positive. The highest true prevalence was observed for sheep flocks (0.68, PCI 0.60-0.75), followed by deer (0.62, PCI 0.39-0.82) and beef herds (0.31, PCI 0.23-0.39). Farms with two or more species tended to have higher true prevalence. True flock/herd prevalence estimates of MAP infection in sheep and beef cattle were significantly higher in the North Island, whereas for deer it was higher in the South Island. HSe ranged from 0.52 to 0.99 and herd level specificity (HSp) from 0.43 to 1.00, depending on the combination of test, sample type and species sampled. HSe increased by 11-40% when testing PFC-negative herds/flocks by Elisa, and by 8-20% when testing additional clinical suspect animals by PFC. HSp decreased due to using Elisa/ParalisaTM. The HSp decrease was small in cattle herds (1-2%) due to few positive tests, but was large in sheep (18%) and deer (56%). Bayesian latent class analysis of survey data provided biased-adjusted estimates of prevalence and accuracy of the detection of MAP-infected herds.

A DYNAMIC SIMULATION MODEL FOR COST-BENEFIT ANALYSIS OF PARATUBERCULOSIS CONTROL STRATEGIES IN DAIRY FARMS

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Efforts to reduce prevalence and consequences of paratuberculosis can be jeopardised if farmers don't enrol in control programmes. For these decision-makers, the expected efficiency of the programme in the situation of their own farm is paramount. Moreover, methods to assess the balance between costs of the programme and expected benefits should account for the goals and constraints of the farm management. The objective of this study was to evaluate the costs and benefits of control programmes in infected dairy herds, taking into account the production goal and the constraints on replacement resulting from management of mastitis and infertility that can be high when these disorders are frequent. A stochastic dynamic herd model was developed and simulated. Assumptions for transmission of *Mycobacterium avium paratuberculosis* (Map) were based on an epidemiological model where transmission can result from either a contaminated environment of the calf, or in *utero* contamination. Herd dynamics and management were detailed to simulate decisions and all inputs and outputs and to calculate the gross margin. Different scenarios of Map control were simulated (surveillance and culling of clinically affected animals or systematic test-and-cull, improving hygiene of calf rearing) and compared to a do-nothing scenario. A sensitivity analysis was carried out with high or low levels of mastitis and infertility. When culling for infertility or mastitis was high, the Map control programme resulted in an annual herd milk yield much lower than the production goal of the farmer, and in reduced gross margin for all scenarios. When it was low, several scenarios of Map control were profitable after 7 to 13 years. This model shows that the efficiency of Map control can be reduced. It enables to identify the conditions for positive return on investment in a variety of production contexts representative of goals and constraints of dairy farms. Integrating the overall farm management when assessing Map control programmes provides results more likely to be accepted by farmers.

OCCURRENCE OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN MILK AT DAIRY CATTLE FARMS: A SYSTEMATIC REVIEW AND META-ANALYSIS

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Introduction

Presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk for human consumption is a concern due to its possible relationship with Crohn's disease in humans. Pasteurization is effective for 10^4 - 10^5 reduction of the MAP load, but the efficacy depends on the initial MAP concentration, which further depends on the prevalence among contributing herds and individuals (Grant et al., 2005, Rademaker et al., 2007). Considerable variation of MAP in bulk tank milk (BTM) and individual cow's milk (IM) is reported (Eltholth et al., 2009), but factors associated with MAP occurrence in milk at farm level have not been described. This study systematically reviewed published studies aiming at estimating the occurrence of MAP in on-farm BTM and IM using a meta-analysis.

Materials and methods

The review followed a publicly available guideline (Sargeant et al., 2005) and included five steps; literature search, initial screen, quality assessment, mapping of articles to studies and data extraction, followed by the meta-analysis. The inclusion criteria of the review included: description of the milk samples and the test method, reporting of the numbers of samples and the results. The selected articles were summarized to study level, i.e., the combination of milk samples (BTM or IM), animal species (cow, or other ruminants), test used (culture, IS900 PCR, or F57 PCR) and infection status (infected or unknown). The descriptive analysis summarized all studies, while the following meta-analysis excluded some studies to keep equal weight for each set of samples. Apparent prevalences (AP) in BTM and IM, both overall and for stratum specific, were estimated by the meta-analysis using a random-effects model. The estimated APs and corresponding 95% confidence intervals were shown using forest plots. Furthermore, heterogeneity and dispersion in the reported APs were examined using Cochran's Q and Higgins' I^2 statistics (Dohoo et al., 2009; Borenstein, 2009).

Results

A total of 692 articles were identified through electronic databases and initially screened based on title and abstract. The quality of the 61 potentially relevant articles was assessed using full text and 31 articles comprising 18 BTM and 27 IM studies were eventually included in the meta-analysis. The AP of MAP in BTM and IM on farm were summarised in relation to strata defined by the test used to identify MAP and the infection status of the herds/animals. Detection limits of the test used and possible MAP load in the test-positive milk samples were rarely reported. The stratum specific AP and 95% confidence intervals of MAP in BTM based on culture of MAP were summarised to 0.06 (0.00-0.77) for known infected herd and 0.01 (0.00-0.08) for unknown infection status herd. Quantifying the MAP load in test-positive milk samples was not possible.

Table. Summary of the meta-analysis of apparent prevalence of MAP in BTM and IM, stratified by diagnostic test and infection status

Stratification	Apparent prevalence (95% confidence interval)	
	BTM	IM
Culture	0.03 (0.01-0.13)	0.23 (0.07-0.54)
Known infected	0.06 (0.00-0.77)	0.39 (0.24-0.57)
Unknown infection status	0.01 (0.00-0.08)	0.20 (0.05-0.56)
IS900 PCR	0.30 (0.16-0.49)	0.16 (0.11-0.25)
Known infected	0.68 (0.56-0.78)	0.36 (0.17-0.61)
Unknown infection status	0.22 (0.15-0.31)	0.14 (0.09-0.21)
F57 PCR	0.05 (0.03-0.10)	Not available

Discussions

The different APs in BTM and IM by culture and IS900 PCR could be explained by higher sensitivity of PCR than culture and the ability of PCR being able to detect non-viable MAP. Estimated APs in BTM and IM by the infection status could be used to reduce MAP positivity in milk by using the test history. Meta-analysis and accompanied examination of heterogeneities might be useful to explore the variability in the estimated APs that could be explained by the stratum. There was considerable inconsistency in the reporting, resulting in missing information potentially explaining the dispersion in the estimated AP.

The results of this study can be used in exposure assessments and studies modelling occurrence of MAP in milk at cow and herd level.

A full-length version of this article is scheduled for publication in Veterinary Microbiology. For a full version of the research see 'Okura, et al. (2012). Occurrence of Mycobacterium avium subsp. paratuberculosis in milk at dairy cattle farms: A systematic review and meta-analysis'. Veterinary Microbiology (DOI: 10.1016/j.vetmic.2011.12.019)

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PRESENCE OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN ALPACAS INHABITING THE CHILEAN ALTIPLANO

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Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the infectious bacterial cause of paratuberculosis, a disease characterized by granulomatous enteritis, diarrhea, abrupt and severe loss of body weight and death.

Disease subsequent to *Map* infection occurs in ruminants, both domestic and free-ranging. In the last two decades, the organism has also been detected in the tissues of non-ruminant species although pathologic lesions and disease due to the infection are much less infrequently noted than in ruminants (Beard *et al.*, 2001; Corn *et al.*, 2005). These findings have raised the question of whether the natural host range, might be wider than previously thought, thereby suggesting that the epidemiology of this disease is more complex than previously realized. The occurrence of *Map* infected wildlife species found in farming areas suggests at least a potential transmission of *Map* from livestock to wildlife. Since a clinically affected cow may shed over 10⁸ bacteria per gram of feces (Cranwell, 1997; Whittington *et al.*, 2000), and an infective dose is considered to be 10⁶ the likelihood of transmission to free-ranging immature ruminants sharing pasture with infected cattle is high. This mycobacterial infection has been recorded since 1958 in domestic ruminants in Chile (Grinbergs *et al.* 1958, Zamora *et al.*, 1975; Salgado *et al.*, 2007). The pathogen has also been found in Chilean wildlife in close proximity to domestic agriculture ruminants (Salgado *et al.*, 2009 and 2011).

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the etiological agent of paratuberculosis. The organism primarily causes disease in domestic agriculture ruminant species, but it has also been reported to affect free-ranging ruminant wildlife. South American camelids (SACs) have a long shared history with indigenous people in the Andes. Over the last few decades increasing numbers of alpacas were exported to various countries outside South America. No paratuberculosis surveillance has been reported for these source herds. The aim of the present study was to investigate the presence of *Map* in an indigenous animal population in an isolated geographic area: the Chilean altiplano.

In this study, individual fecal and serum samples from 68 adult alpacas were collected from 7 separate herds in the Chilean altiplano (4,000 mt. above sea level). A liquid culture system (BACTEC-MGIT 960) for *Map* presence was used followed by real-time PCR confirmation of acid-fast isolates. DNA extracts from a subset of confirmed *Map* isolates were subjected to MIRU-VNTR typing. For *Map*-antibody detection, a commercial Johnes's disease was adapted for use in camelids by using an anti-llama IgG conjugate instead of the kit's anti-bovine IgG conjugate. Five alpaca were both culture and ELISA test-positive. All isolates tested belonged to the same MIRU-VNTR type showing 4 repeats for TR292 (locus1) in contrast to the 3 repeats typical of the reference strain K10. The number of repeats found in the remaining loci was identical to that of K10 strain. Neither is how nor when *Map* was introduced into the alpaca population in the Chilean altiplano not known. The most plausible hypothesis to explain the presence of *Map* in these indigenous populations is transmission by contact with infected domestic ruminant species that occasionally may share pastures or range with alpacas. These results for such isolated populations suggest that *Map* infection has found its way beyond the boundaries of organized domestic agriculture; evidence of *Map* in free ranging ruminant populations should no longer be considered a surprising finding.

Acknowledgements

This study has been funded by UACH - UST collaboration

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MODELLING TRANSMISSION DYNAMICS OF PARATUBERCULOSIS IN RED DEER UNDER PASTORAL FARMING CONDITIONS

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This study aimed to describe disease dynamics, virulence of strains of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and the effect of management interventions on paratuberculosis (PTB) in farmed red deer (*Cervus elaphus*). A mathematical state-transition model was developed and calibrated to observed data about prevalence on infection and incidence of clinical PTB. To reproduce observed data, the model included a fast and a slow track for progression of infection to disease. MAP on pasture was the source for horizontal transmission, and infected dams for pseudo-/vertical transmission. Transmission was dependent on one of two strains with equal or difference virulence.

The proportion of infected weaned deer that rapidly developed clinical disease had strong, but seasonal variation of MAP survival on pasture had little impact on infection prevalence and annual PTB incidence. A hypothesis of competing MAP strains was more consistent with observed data than the assumption that strains do not interact and their effect on transmission is additive. The model suggested that rotational grazing followed by pasture spelling versus permanent grazing of the same paddock reduced prevalence and clinical PTB by about one half. This was similar to a reduction achieved by test-and-cull using whole herd PCR twice a year, whereas test-and-cull by ELISA had little effects. Early detection of young deer in clinical state was the most effective means of controlling PTB among the tested scenarios. The study identified data-sparse areas with strong impacts on model outcomes, such as the mechanism of interactions between MAP strains and the host in herds with multiple strain infections, the dispersal of MAP in the environment, and determinants for the time to clinical disease. It was concluded that test-and-cull using PCR was more effective than using ELISA for reducing the MAP infection prevalence and clinical PTB incidence.

MAP AT THE WILDLIFE-CATTLE INTERFACE IN SOUTHWESTERN ALBERTA

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Introduction

The role that wildlife may play in the transmission of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in the environment, and the potential consequences of infection in these wildlife populations are being given increasing consideration. Southwestern Alberta has a high density of cow-calf ranches where cows often share the pastures with various wild ruminants. In particular, elk (*Cervus elaphus*), white-tailed deer (*Odocoileus virginianus*) and bighorn sheep (*Ovis canadensis*) often share pastures with beef cattle. Our team has investigated MAP occurrence in both elk and Bighorn sheep and cattle in the same area.

Material and Methods

Bighorn sheep study

In August 2009, a yearling Rocky Mountain bighorn sheep from the Pincher Creek region in the foothills of southwestern Alberta was found moribund with diarrhea, weakness, and emaciation. The animal was euthanized and submitted to the University of Calgary's Faculty of Veterinary Medicine for necropsy. A standard necropsy was performed and sections of the ileum and mesenteric lymph nodes were routinely fixed in 10% neutral-buffered formalin, sectioned and stained with hematoxylin and eosin (HE). Additional sections of the ileum and mesenteric lymph node were stained with the acid-fast Ziehl-Neelsen (ZN) stain. Feces were collected from the rectum during necropsy and kept frozen until processed for culture.

Subsequent fecal herd-level surveys for MAP were done in the Pincher Creek region as well as further north in Sheep River Provincial Park. For each herd fecal samples were collected from specifically identified individuals. Herd size of the Pincher Creek herd was estimated around 150. A minimum sample size of 46 fecals from individually identified sheep needed to be collected for the detection of a minimum of 5% prevalence. Herd size of the Sheep River herd was estimated at 40 and we aimed for 31 fecal samples for the same detection limit.

Cattle and elk study

Fecal samples were collected in the winter of 2009-2010 from 10 wild elk herds both in the foothills of southwestern Alberta as well as in the National Parks of Banff and Jasper, of which five herds with high probability of interaction with cattle and five with low probability of interaction with cattle. Per elk herd 30 fecal samples were selected for culture allowing us to detect a 5-10% herd-level prevalence with 95% confidence (depending on herd size). Blood samples were available from a subsample of elk from the same herds during GPS collaring activities. Additionally, both blood and fecal samples were collected from 30 cows in each of 30 cow-calf ranches in southwestern Alberta.

All fecal samples were processed using TREK ESP culture system followed by IS900 PCR as a confirmation. Fecal culture positive samples were genotyped through sequencing of three discriminatory short sequence repeat (SSR) regions and additional genotyped as is described in detail by Forde et al 2012 (1). The serum samples were tested in our USDA-approved laboratory with the commercial IDEXX MAP Ab Test ®. The elk samples will also be tested using a modified test system with either Protein-G or anti-deer as conjugate instead of anti-bovine.

Results

Bighorn sheep study

MAP was cultured from the ileum, lymph nodes and feces of the moribund yearling ram. Clinical signs of emaciation and diarrhea, as well as histological findings were consistent with previously described cases of Johne's disease in bighorn sheep (2).

In the herd-level survey, three of 44 (7%) fecal samples from individual bighorn sheep from the Pincher Creek region tested positive by culture and polymerase chain reaction (PCR) and were identified as Type II MAP strains by MAP1506 gene sequencing. All three strains and the strain from the clinical case had unique multi-locus SSR profiles. Twenty-five samples collected from the Sheep River herd were all culture- and PCR negative.

Table 1: Strain-typing results of four isolates of MAP from the Pincher Creek bighorn sheep herd in Pincher Creek, southwestern Alberta, Canada.

ID no.	Location	Age	Sex	Map1506	G1	G2	GGT
Clinical case							
UC130	Yarrow Creek	Yearling	Male	Type II	7	12	4
Prevalence study							
BS10-39	Drywood North	2-3 yr	Male	Type II	10	10	5
BS10-58	Yarrow Creek	Adult	Female	Type II	7	11	4
BS10-77	Yarrow Creek	Adult	Female	Type II	7	10	4

Cattle and elk

Of the total of 870 cows over 3 years of age from 30 ranches, only two cows from a single farm were FC+. These strains have not yet been genotyped. All 284 elk fecal samples tested negative by culture and PCR. Eight of 848 (0.9%) cattle serum samples were positive for serological response to Johne's disease. These included the two culture positive individuals. Twenty-seven of the 270 (10%) elk blood samples tested seropositive in the commercial ELISA using anti-bovine conjugate. No significant correlation was found between the sero-prevalence within herds in contact with the other susceptible species.

Table 2: Descriptive statistics and p-values for the test of MAP occurrence with the cattle-elk interaction association.

	Sero positive test result (%)	95% CI	P-value
Cattle	<1%	(0.2;1.4%)	0.884
Elk	Medium titer*: 10.7%	(7.0;14.3%)	0.700
	High titer: 1.4%	(0.03;2.8%)	0.123

*□medium high titres are consistent with positive animals.

Discussion

A better understanding of pathogen transmission at the wildlife-livestock interface is extremely important and an essential tool for increasing insights can be molecular diagnostic techniques. Our work has shown a cattle strain circulating in bighorn sheep without being detected in the cattle population in that area. On top of that four different strains of MAP seem to circulate in a wildlife population suggesting either multiple introductions or strain differentiation in a susceptible population of wildlife species. Although not prominently found in elk, the role of this species cannot be excluded as there home ranges are rather extensive and cattle contact is often observed. Either sampling strategy or sample size might have led to an underestimation of the true prevalence of MAP in elk. A better understanding of bacterial transmission between wildlife and domestic livestock can support management strategies for both herds of interest.

Our future research will focus on more intense screening of the cattle in the Pincher Creek area that are in close contact with the bighorn sheep population in which MAP is circulating. Additional strain typing will help us understand the strain distribution within and between herds.

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INVESTIGATIVE WORKSHOP FOR MATHEMATICAL MODELING OF JOHNE'S DISEASE EPIDEMIOLOGY AND IMMUNOLOGY

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ABSTRACT

Despite long and intensive national-level efforts for Johne's disease (JD) control, we are still far from preventing the significant economic impact of this disease. One of the major reasons for the continuing struggle with JD is that there are many unknown factors in JD epidemiology and immunology. For example, we do not properly understand the host immune responses to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that lead to persistence and sudden exacerbation of the infection. Since the early 1990's, mathematical modeling approaches have been applied for better understanding of JD epidemiology and for estimation of the cost-benefit of alternative JD control strategies. However, there has not previously been an opportunity to gather a multidisciplinary group of scientists to help facilitate mathematical modeling studies in JD. Further, there has been no mathematical modeling approach for studying the immunology of JD. We organized an investigative workshop for mathematical modeling of JD epidemiology and immunology (July 6-8, 2011). The workshop was hosted by the National Institute for Mathematical and Biological Synthesis (NIMBioS) at the University of Tennessee, Knoxville. The objectives of the workshop were to gather diverse groups of scientists for facilitation of interdisciplinary discussions on the mathematical modeling of MAP epidemiology, to establish an initiative in mathematical modeling for immunology of JD and to investigate methods for linking the epidemiology and immunology models. A total of 40 participants including 11 international scholars attended the workshop. In the first two days, 11 presentations were made on epidemiology, immunology and their mathematical models, followed by breakout sessions to discuss challenges, opportunities, and future directions for each objective of the workshop. On the last day, two scientific presentations were made, concluding remarks were presented for each objective, and a whole group discussion was held. In addition, the Directors of the NIMBioS and JD Integrated Program described possible synergies between the two programs. Through the workshop, new mathematical models were proposed, new research opportunities emerged, and future activities/goals identified. Our proposal for forming a working group based on the workshop was accepted by NIMBioS and we will organize three meetings for "Within host modeling of MAP infections" in this and next year.

SUMMARY OF THE WORKSHOP

Host/ Place: National Institute of Mathematical and Biological Synthesis (NIMBioS) at the University of Tennessee, Knoxville, Tennessee

Organizers: Shigetoshi Eda, Ynte H. Schukken, Ian A. Gardner, John P. Bannantine, and Judith R. Stabel

Date: July 6-8, 2011

Participants: Forty participants, including 11 international scholars and 4 Executive Committee members of the JD Integrated Program, attended the workshop. A list of participants can be found at http://www.nimbios.org/workshops/WS_JohnesDisease_participants.

On Day 1 and 2, 5-6 presentations were made on JD immunology, epidemiology and mathematical modeling. After the presentations on each day, participants were divided into three groups - JD immunology, epidemiology and immunology-epidemiology link. Challenges, opportunities and future plans were discussed in each group. On Day 3, whole group sessions were held to summarize the discussions.

I. Immunology (within host level model)

The following key questions were identified during the whole group meeting on Day 3. (1) Can cows 'cure' infection?: cows in infected herd are likely exposed but do not become infected, (2) Why/when are MAP infected cows starting to shed bacteria into intestinal lumen?, and (3) Why/when are cows switching from low to high (super) shedding? Also, stressors (parturition, dystocia, etc.), dam status (e.g. level of infection), and vaccination were listed as factors affecting immunologic parameters. The most important aspect of JD immunological model would be prediction of fecal shedding (timing and level).

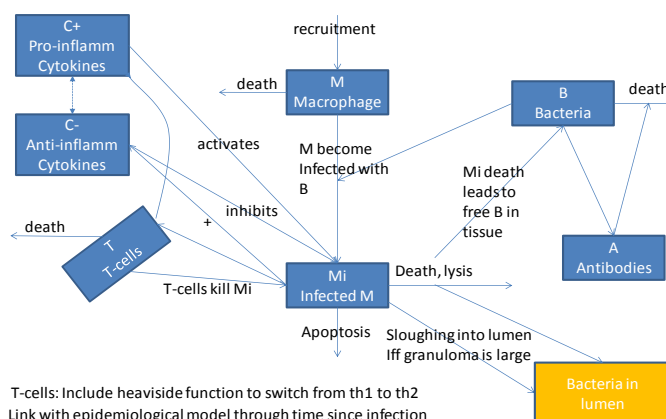


Figure 1. Preliminary within host model. An initial model was created to include key components of JD immunology -- macrophages, T-cells (Th1, Th2, Treg, Th17), pro-/anti-inflammatory cytokines (IFN- γ , TNF- α , IL-4, 6, 10, 12), antibodies, and bacteria.

II. Epidemiology (population level model)

Mathematical models for JD epidemiology are well developed and data from longitudinal studies available; however, some modifications may be required for integration of epidemiology and immunology models. Also, we currently do not have good between-herd model. Possible compartments to be included in future models are environment (MAP contamination), wildlife animals and other pathogens (co-infection). Purpose of the model include identification of effective interventions and better understanding impacts of JD on economy and public health.

III. Immunology-epidemiology integrated model

A major focus of this group was to discuss how to model transition from latent to shedding status. The group discussed factors that impact the transition, such as host characteristics (gestation, calving, stress, genetics), age at MAP exposure, dose and duration of the exposure, and immune control. Suggested approaches for development of epidemiology-immunology model are use of longitudinal data, use of hidden Markov model, and incorporation of pathogen, host and environment.

DELIVERABLES

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3. Eda, S., Lenhart, S., Stabel, J., Bannantine, J., Gardner, I., Schukken, Y., "Investigative Workshop for mathematical modeling of Johne's disease epidemiology and immunology", 2012 meeting of International colloquium of Paratuberculosis, Sydney, Australia

Based on this workshop, we submitted a proposal for establishment of a working group for "Within host modeling of *Mycobacterium avium* subsp. *paratuberculosis* infections". The proposal was approved by NIMBioS and the organizers will start discussing schedule/content of the working group soon. Thirteen scientists will be attending the workshop which will be held at the NIMBioS three times in 2012-2013.

ACKNOWLEDGEMENT

We are truly thankful for generous supports of NIMBioS for the workshop and future working group meetings. NIMBioS leadership team and staffs provided financial/administrative supports for participants' travel, accommodation and meals. The workshop was also partly supported by 2011 UT AgResearch and Extension Innovation Grant.

DETECTION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN CARIBOU

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Abstract

Caribou (*Rangifer tarandus* ssp.) herds from across northern Canada and Greenland were tested for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) using faecal culture and polymerase chain reaction (PCR). Faecal samples were collected by several key collaborators within the CircumArctic Rangifer Monitoring and Assessment (CARMA) Network. Samples from 561 individual caribou from 16 different populations were cultured using the TREK ESP liquid culture system followed by IS900 PCR. A total of 31 faecal samples (5.5%) were positive by PCR, and one of these samples was also culture positive. Sequencing of the MAP1506 locus determined the culture positive isolate to be a Type II strain. The proportion of faecal samples testing positive was highest in the Akia-Maniitsoq herd from Greenland, and the Rivière-aux-Feuilles and Rivière-George herds from northeastern Canada, with 23.4%, 11.5%, and 10.0% of samples testing positive by PCR respectively. Positive samples were detected among all age groups with 3/19 calves (15.8%), 2/23 yearlings (8.7%) and 26/511 adults (5.1%) testing positive. The results of this study suggest that MAP is present in several free-ranging herds of caribou in both Canada and Greenland, and that it can be detected in animals as young as 9-10 months of age. This study also indicates that MAP is able to circulate in an environment that is free of interaction with domestic livestock species, and that circulation within wildlife populations should be considered as an important component in the epidemiology of MAP.

Introduction

Caribou is a keystone species in several northern ecosystems, and is of high cultural importance to northern Aboriginal communities. Although many factors influence fluctuations in caribou populations, infectious diseases can play an important role.¹ It is therefore important to understand and monitor potential pathogens in caribou populations that could have an impact on the health of this species. Johne's disease, caused by MAP, has been previously documented in semi-domestic reindeer (*Rangifer tarandus tarandus*),^{2,3} suggesting that caribou are likely susceptible to this disease. However until now, no systematic survey for MAP has been conducted among wild caribou herds (*Rangifer tarandus caribou/granti/groenlandicus*). The objective of this study was to survey for the presence of MAP among several herds of caribou in northern Canada and Greenland and to gain baseline information on levels of infection within these herds.

Materials and Methods

Faecal samples were collected by several collaborators within the CARMA Network during large-scale health monitoring activities between 2006 and 2009. Animals were not selected randomly since the sampling objectives varied for each herd. Consequently, the age and sex of animals selected for study were variable. Most samples were collected from harvested or culled caribou, although some were collected during live capture-release activities. Faeces were obtained directly from the rectum using a fresh glove for each animal, and these samples were stored at -20°C until processing. Although every effort was made to avoid freeze-thaw cycles, due to challenging field conditions, the time interval between sample collection and freezing was variable, as was the duration of freezing prior to processing. Faeces were cultured using the TREK ESP culture system, and following eight weeks of incubation at 37°C, PCR was performed on the culture supernatant from all samples using the IS900 target.⁴ Culture positive samples were additionally confirmed by subculturing on Middlebrook 7H11 medium and by acid-

fast staining. Strain typing of culture positive samples was performed by sequencing the MAP1506 locus.⁵ Logistic regression was performed to determine whether a relationship existed between a caribou's sex or age and the probability of testing positive for MAP. Herd, season, and year of sampling were included as fixed factors, sex or age class (as a categorical variable) as explanatory variables, and MAP status (positive or negative) as the dependent outcome variable (IBM SPSS Statistics 19).

Results

Samples were collected from 561 caribou (114 males and 446 females). A total of 31 faecal samples (5.5%) from eight different populations were positive by PCR. One sample from a yearling female from the Rivière-aux-Feuilles herd was also culture positive. This isolate was determined to be a Type II (Cattle type) strain. The proportion of faecal samples testing positive was highest in the Akia-Maniitsoq herd from Greenland, and the Rivière-aux-Feuilles and Rivière-George herds from northeastern Canada, with 23.4%, 11.5%, and 10.0% of samples testing positive by PCR respectively. Positive samples were detected among all age groups with 3/19 calves (15.8%), 2/23 yearlings (8.7%) and 26/511 adults (5.1%) testing positive. All but one positive sample were from females, however, in several herds only females were sampled. When corrected for herd, season, and year of sampling, there was no statistically significant association between either sex ($p=0.05$) or age class ($p=0.24$) and the MAP status of an individual.

Discussion and Conclusions

This is the first major survey for MAP in free-ranging caribou across much of their range, and results of this study suggest that MAP is present in several herds in both Canada and Greenland. At this time, conclusions cannot be made about the potential impact this could have on caribou health. MAP was detected in animals of all age categories, including from calves as young as 9-10 months. Shedding in animals this young is surprising, since it is rare for domestic ruminants such as cows and sheep to shed MAP prior to 2-3 years of age.⁶ Further study into the pathogenesis of MAP in *Rangifer* species is merited. This study demonstrates that MAP is able to circulate in an environment that is free of contact with domestic livestock species. None of the populations included in this study have contact with ruminant livestock, with the possible exception of domestic sheep that may graze on caribou range in portions of western Greenland. The possibility for wildlife species to act as reservoirs for MAP independently of interaction with livestock should be given appropriate consideration when studying the epidemiology of MAP.

Acknowledgements

Many people, ranging from community members to wildlife managers, were involved in sample collection through the CARMA Network and without whom this survey would not have been possible. We would like to thank Amanda Reith, Dean Brown and Jesse Invik for processing the faecal samples and Julie Ducrocq and Ryan Brook for database management. Funding for this study was provided by the Canadian Federal International Polar Year Program, the Natural Sciences and Engineering Research Council of Canada, and Alberta Innovates.

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EVALUATION OF COMBINED ENVIRONMENTAL AND POOLED ANIMALS SAMPLING TO DETERMINE DAIRY CATTLE HERD INFECTION STATUS FOR *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* (MAP)

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Abstract

The objective of this study was to evaluate the informative value of combinations of pooled samples, submitted to direct or indirect tests, to determine the infection status for *Mycobacterium avium paratuberculosis* (Map) of dairy cattle herds. Three reference status of 247 dairy herds were determined based on the within-herd seroprevalence of lactating animals (WHP+), and the number of confirmed paratuberculosis clinical cases (MapC) in the past 3 years : A (WHP+ 0% and no MapC since 2008), B: (WHP+ 0% and at least one MapC since 2008 or WHP+ in]0-8%]); C(WHP+ >8%). In each herd, 2 environmental samples were taken. Milk was sampled from all cows, pooled every 5 animals within the same lactation rank and faeces samples from primiparous cows were pooled similarly. All the milk samples were tested using a commercial ELISA, whereas the faeces were tested using *para-JEM liquid culture* system. The sensitivity and the specificity of each combination were calculated. The selected combinations were the ones maximising the Youden index ($Y = Se + Sp - 1$). The best combination (i) to discriminate A from B&C herds was either a PCR applied to two environmental samples, or a PCR applied to walking area and an ELISA on primiparous pooled milk ($Y=0.49$); (ii) to discriminate C from A&B herds was a PCR applied to walking area and a PCR applied to pooled faeces samples from primiparous ($Y=0.44$).

A limited number of sample-tests could be used to determine at lower cost a proxy of the Map status of dairy herds enabling to classify herds in three categories with different priorities of action for Map control.

Introduction

Control program aim at both decreasing prevalence of *Mycobacterium avium subspecies paratuberculosis* infection in infected herds and at limiting incidence at herd level. Sensitivity of direct or indirect test is poor [1] and individual animal testing cannot prevent introduction of asymptomatic Map infected animals in free herds. In addition, certification programs are very expensive and few herds are certified Map-free. Therefore, herd level tools are needed, both to detect herds with the highest prevalence as target for control action and to identify herds with a low risk of harboring infected animals, to promote safe trade of young stock. The review of the literature provides data based on pooled samples to determine herd status [2] but to our knowledge, no data based on combination of direct and indirect tests were published. The objective of this study was to evaluate the informative value of combined and pooled animal sampling to determine a Map infection herd status in dairy cattle.

Material and Methods

A total of 308 dairy herds of Western France were recruited, either enrolled in a Map control program, or involved in a Map-free certification program. Within herd seroprevalence in lactating cows was assessed yearly using ELISA on blood sample. Herds were classified in 3 reference statuses, according to prevalence and number of clinical cases in the last 3 years (table1).

Table 1. Definition of the reference Mapinfection herd statuses

A Status	B Status	C Status
Last known Within herd seroprevalence=0 %	0< Last known Within herd seroprevalence < 8 %	Last known Within herd seroprevalence < 8 %
	Or	
No Map clinical case from 2008 to 2011	Last known Within herd seroprevalence=0 % and at least one clinical case from 2008	> 8 %

To determine the reference herd status, blood samples were collected from all dairy cows. To evaluate new tools, samples were collected 1 to 6 months later: milk samples were collected from all lactating cows and pooled every 5 cows, within same rank of lactation; bulk tank milk (BTM) was collected; 40g of feces from primiparous cows were collected and pooled every five cows; finally, 2 standardized samplings of feces manure in Walking Area and Milking Parlor were collected, both in 4 different locations.

Blood and milk samples were submitted to a commercial ELISA (IDEXX). A sample was considered positive when E/P was $> 30\%$. Feces samples were tested for Map detection using liquid Culture system: (Parajem TREK®) followed by real time PCR (Taqvet *M. paratuberculosis*®, LSI). A sample was considered positive when a typical amplification curve was observed for $Ct < 45$. Herds were classified according to the result for each sample type (table2). Sensitivity (Se), specificity (Sp) and Youden index ($Se + Sp - 1$) of each sample type was calculated. To discriminate (i) A Status from others, Se was defined as a/X while specificity was $(Y+Z-b-c)/(Y+Z)$. To discriminate (ii) C status from others, Se was defined as c'/Z while specificity was $(X+Y-a'-b')/(X+Y)$. Finally, the best combination of samples was systematically searched by maximizing Youden index to discriminate A from (B and C) herds and C from (A and B) herds.

Table 2. Distribution of herds according to the result of each test

Herd status	Number of herds with Negative Result	Number of herds with Positive Result	Total number
A	a	a'	X
B	b	b'	Y
C	c	c'	Z

Results and Discussion

Discrimination of herds status

Complete data were available for 247 herds. The distribution in reference herd status was 21% ($n=52$), 62% ($n=153$), and 17% ($n=42$) for A, B, C, respectively. To discriminate A from B and C herds, the best combination based on two samples was either PCR applied to Walking Area (WA) and Milking Parlor (MP) ($Y=0,49$) or PCR applied to WA and ELISA applied to primiparous pooled milk (table 3).

Table 3. Sensitivity (Se), specificity (Sp) and Youden index (Y) of selected combinations to discriminate herds with a Map infection status

Combination of negative results	Sensitivity	Specificity	Youden index
PCR on WA	0.91	0.53	0.44
PCR on WA and MP	0.82	0.68	0.49
PCR on WA and ELISA on primiparous pooled milk	0.89	0.59	0.48
PCR on WA and ELISA on BTM	0.90	0.55	0.45
PCR on WA and MP and ELISA on primiparous pooled feces	0.49	0.82	0.31

To discriminate C from (B and C) herds, the best combination based on two samples was PCR applied to WA and pooled feces samples from primiparous ($Y = 0.44$). Combinations including ELISA on BTM, such as PCR on WA, Primiparous and BTM are not effective ($Y = 0.12$), because of a lack of sensitivity (table 4).

Table 4. Sensitivity (Se), specificity (Sp) and Youden index (Y) of selected combinations to discriminate herds with a Map infection status C versus (A or B)

Combination of negative results	Sensitivity	Specificity	Youden index
PCR on WA	0.78	0.64	0.41
PCR on WA and MP	0.53	0.90	0.43
PCR on WA and PCR on primiparous pooled feces	0.56	0.89	0.44
PCR on WA and MP and primiparous pooled feces and ELISA on BTM	0.14	0.99	0.12

These results suggest that it should be possible, in a two-step procedure, to distinguish A herds from other herds by two negative environmental samples (WA and MP), using PCR after liquid culture. In a second step, herds with at least one environmental positive sample could be tested using PCR after liquid culture on primiparous pooled feces, aiming at distinguish C herds (positive on primiparous pooled feces) from others. Delay between estimation of within herd seroprevalence and other sampling (1 to 6 months) can have resulted in a few misclassifications into the reference status (farmers could have slaughtered seropositive cows before the second step). In this study, combinations were defined on concomitant positive or negative results of all tests; other combinations of elementary samples, including “and/or” criteria, could be tested. The procedure proposed here is simple and could be applied in a large population of herds, in order to set priorities at a regional level to implement control programs with both detection of herds with the highest prevalence and identification of free herds to promote safe trade.

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SHEDDING PATTERNS OVER TIME OF *MYCOBACTERIUM AVIUM* SUSBSPECIES *PARATUBERCULOSIS* (MAP) IN CATTLE

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Abstract

The objective of this study was to describe the heterogeneity of shedding of *Mycobacterium avium* subspecies *paratuberculosis* (Map) over time in cattle and to investigate the existence of different shedding patterns of interest. In three herds (2 dairy and 1 beef) with a high apparent seroprevalence, 402 cattle were sampled over a 12-18 months period. In each herd, individual feces were collected from every cattle aged >14-18 months every 3 to 6 months. Feces samples were tested using ESP *para-JEM* liquid culture system followed by PCR. Among the 716 fecal samples tested throughout the study period, 297 were positive (41.5%), Ct values ranging from 18 to 44 (Median Ct value: 27). Among 100 animals sampled three time consecutively, 31% were persistently positive and 33% persistently negative. When considering only the 10% of animals with lowest Ct values (assuming they correspond to the highest shedders), 90% of them were also found to be persistent shedders.

Introduction

Within infected herds, early detection and culling of shedder animals who are likely to be persistent heavy shedders is a key factor to control Map transmission [1]. The review of the literature provides very few data based on longitudinal follow-up of shedding by infected cows over a long period [2,3]. The objective of this study was to describe the heterogeneity of shedding of *Mycobacterium avium* subspecies *paratuberculosis* (Map) over time in cattle to investigate the existence of different shedding patterns of interest.

Material and Methods

2 dairy herds (Prim'hosstein) and 1 beef herd (Limousin) were recruited for the study. To maximize the probability to detect Map shedding, they were selected on the basis of at least one clinical cases and a seroprevalence >8%. All adults and heifers (>14-18 mois) were included and sampled. At each sampling time, individual feces were sampled (Table 1). All feces samples were tested for Map detection using liquid Culture system: (Parajem TREK®) followed by real time PCR (Taqvet *M. paratuberculosis*®, LSI). A sample was considered as positive when a typical amplification curve was observed for Ct < 45.

The analysis was conducted in three consecutive steps:

- The distribution of observed Ct value was described at each time
- The proportion of the different observed shedding pattern (persistently positive or negative and all the combination over the 3 sampling times) were quantified
- The Ct values were described according to shedding patterns

Table 1. Study sample characteristics and sampling schedule

Study sample characteristics	Dairy herd 1			Dairy herd 2			Beef herd 1		
Number of tested animals at least once	79			86			224		
Number of animals tested 3 times	29			19			27		
Number of animals tested 2 times	30			28			67		
Number of animals tested once	20			39			130		
Minimal age at sampling (months)	24	18	14	24	18	18	18	18	18
Date of sampling (month/year)	04/10	03/10	12/10	03/09	11/09	03/10	01/10	10/10	01/11
Number of sampled animals	47	62	59	55	67	20	71	74	101

Results and Discussion

Distribution of shedding at a given time (figure 1)

Among the 716 tested feces, 297 were PCR positive feces samples (41.5%) with 43 PCR positive feces samples on heifers < 24 months out of 109 (39%).

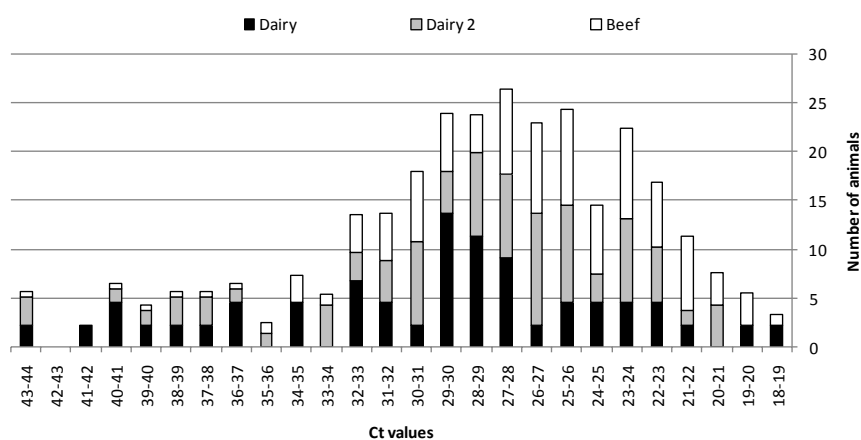


Figure 1. Distribution of Ct values of PCR after liquid culture to detect *Mycobacterium avium subsp. paratuberculosis* in 297 positives samples in cattle from 3 herds over a 8 to 11 months follow-up

Distribution of shedding patterns over time (figure 2)

The distribution of different shedding patterns over the 3 sampling times is displayed in figure 2. Among 311 animals sampled twice consecutively, 32% were found persistently positive, 43% persistently negative and 25% positive only once. Among 100 animals sampled three time consecutively, 31% were persistently positive, 33% persistently negative and 36% shed intermittently. All possible patterns of intermittent shedding were found. Around 50% of shedders were persistent shedder.

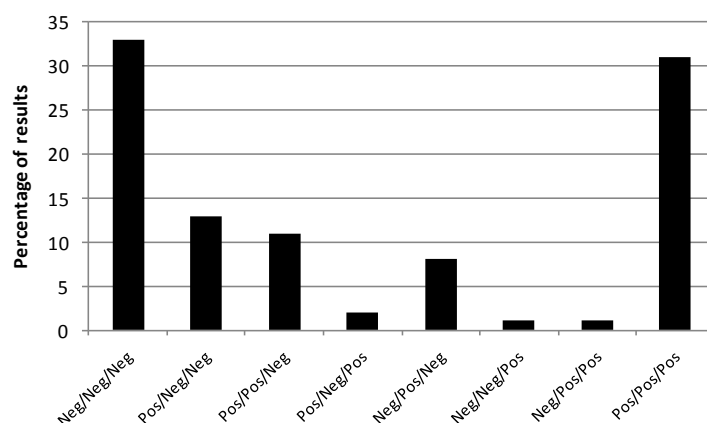


Figure 2. Distribution of different shedding patterns of *Mycobacterium avium subsp. paratuberculosis* at 3 consecutive sampling times (PCR after liquid culture on 100 cattle from 3 herds)

Distribution of shedding level according to shedding patterns (Table 2)

The lowest Ct values were observed for persistent shedding pattern only. Further studies aiming to assess the relationship between Ct value and precise quantification of Map are needed.

Table 2. Distribution of mean Ct values according to the different shedding patterns

	Pos/Neg/Neg	Pos/Pos/Neg	Pos/Neg/Pos	Neg/Pos/Neg	Neg/Neg/pos	Neg/Pos/Pos	Pos/Pos/Pos
Ct time 1	33.7	28.7	36.2	–	–	–	27.7
Ct time 2	–	25.5	–	33.9	–	23.4	26.0
Ct time 3	–	–	40.0	–	26.9	22.6	27.7

Conclusion

The present findings support the existence of different *Map* shedding patterns over time. Among shedder cows, approximately 50% were considered as persistent shedder. Additionally, very low Ct values (<20) were reported only in persistent shedder: Thus, persistent shedder could be a target population for early culling in infected herds. Diagnostic methods predictive of persistent shedding should be looked for to target culling decisions in control plans.

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PREVALENCE ON MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) INFECTION IN DROMEDARY CAMEL (CAMELUS DROMEDARIUS) IN IRAN

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Abstract

Dromedary camel (*Camelus dromedarius*) is one of the highly valuable domestic animals in tropical and subtropical area that can be used for meat, milk and wool production. In addition to the previous traditional uses, modern applications in the dairy industry lead to the development of camel dairy farms that are capable of producing camel milk on the commercial level. Camel milk and meat are considered an important source of proteins for wide range of population (Alhebab and Alluwaimi, 2010). Camels in our country are farmed for their meat and milk. They are subject to many diseases and paratuberculosis, which is the most economically limiting one. Paratuberculosis in *Camelus dromedarius* is poorly documented in the literature. Johne's disease affects camels worldwide causing characteristic clinical illness of severe diarrhea ending in death (Manefield and Tinson, 1997; Wernery and Kaaden, 2002). The course of disease is often more rapid than that in cattle (Higgins 1986). There are no published reports of JD in camels in Iran. It is unlikely to exist within the Ardebil camel herd. Mycobacterium paratuberculosis has been detected in dairy herds throughout Iran (Anzaby et al., 2006; Pourjafar and Badiei, 2005; Kasravi and Nowrouzian, 2004; Khodakaram Tafti and Rashidi, 2000). Fecal culture is considered as the gold standard for the diagnosis of MAP infected animals but requires 12–16 weeks (Stabel and Whitlock 2001); therefore the development of a rapid, sensitive and specific diagnostic method for the detection of Map is essential in the control of Johne's disease in economically important animals (Vansnick et al., 2007). The IS900 element is an insertion sequence considered to be a MAP-specific gene with 15–20 copies per genome and is a target for rapid detection of MAP by PCR (Collins et al., 1989; Ikonopoulou et al., 2004). The aim of this study was to estimate the prevalence of MAP infection in *Camelus dromedarius* in Iran by acid fast staining and PCR. A total of 50 faecal samples from apparently healthy dromedary camel (*Camelus dromedarius*) at different ages from Semnan province, Iran, were taken, and stored at -20 °C until use. For Ziehl-Neelsen staining, fecal smears were stained for 1 hour with TB carbol fuchsin Ziehl-Neelsen acid-fast stain (Quinn et al., 1994). DNA was extracted from all fecal samples as previous protocol (Stabel et al., 2004). IS900 PCR was conducted as described by Corti and Stephan (2002) with the primers P90, 5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3' and P91, 5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3'. Using the Ziehl-Neelsen acid-fast staining technique, *M. paratuberculosis*-infected fecal samples were identified by the red staining of bacteria. Acid fast staining results showed that only 6/50 (12%) samples were suspected to MAP.

PCR analysis revealed expected size corresponding to MAP in 5/50 (10%) samples. Johne's disease (JD) is an infectious disease of cattle and other ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease distribution is worldwide. A recent work in dairy cow herds in Fars province, southern Iran, showed a herd-level prevalence of 11% based on IS900 nested PCR on bulk-tank milk samples (Haghkhah et al. 2008), indicating that the prevalence of infection is low. Traditionally, fecal culture for MAP is considered as the gold standard for diagnosis. However, fecal culture is time-consuming and detects only 38–50% of infected animals (Stabel, 2004). Serologic tests such as ELISA are even less sensitive than fecal culture, particularly in apparently healthy or subclinically infected animals. Use of PCR methods in contrast to culture and serological tests, allowed to detect nonviable as well as viable micro-organisms and would be a more sensitive detection method. Therefore, in comparison with serologic or culture methods, detection of MAP directly from bulk-tank milk or feces by IS900 PCR could be considered a valuable test for the estimation of herd-level prevalence. This is the first investigation on MAP incidence of dromedary camels in Iran. Although the incidence of MAP infection was low, further studies should be conducted to get more information on MAP infection in camel population, especially in areas which camels are close to other ruminants such as dairy cows, sheep and goats.

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CONCORDANCE BETWEEN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* ELISA RESULTS IN PAIRED SERA AND MILK OF DAIRY GOATS

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The objective of this study was to estimate the concordance between *Mycobacterium avium* subsp. *paratuberculosis* (MAP) ELISA results in paired sera and milk samples obtained from Greek dairy goats four consecutive times: at kidding, 2 and 4 months later and at the end of their 7-month-long lactation period (Nielsen et al., 2002). All samples were from the animals (n=225) of a dairy goat flock with a history of clinical paratuberculosis and were tested with a commercial ELISA kit (Pourquier ELISA Paratuberculosis). For each lactation stage, results were grouped in two separate two-by-two tables, one using the recommended cut-offs (S/P ratio of 0.45 for sera and 0.2 for milk) and one using cut-offs at 50% (Kostoulas et al., 2006) of those recommended by the manufacturer and were evaluated for significance by Mc Nemar's χ^2 test for symmetry. Additionally, for each stage of lactation, we estimated the concordance correlation coefficients (rccc) between the S/P ratios of the paired sera and milk samples. Only for the samples obtained in late lactation there was significant difference between the proportion of positive sera and milk at either cut-off (Mc Nemar's $\chi^2=12$, $p=0.0005$ and $\chi^2=7.14$, $p=0.0129$ for manufacturer-recommended and 50% reduced cut-offs, respectively). At late lactation the proportion of positive milk samples was higher. The rCCC's were high in early (rccc=0.887), mid (rccc=0.805) and late (rccc=0.892) lactation but were low-to-moderate at kidding (rccc=0.409). In conclusion, ELISA testing of milk/ colostrum samples may be as accurate as serological testing for the detection of MAP antibodies. Furthermore, at late lactation milk testing may outperform serological testing.

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CASE REPORT OF PARATUBERCULOSIS IN A MINIATURE DONKEY (*EQUUS ASINUS F. ASINUS*)

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) has been isolated from different non-ruminant wildlife species by culture or detected by PCR, but no clinical cases of paratuberculosis have been recorded in these animals to date [1]. Macroscopic lesions have been observed only in naturally MAP-infected rabbits [1]. Otherwise, four cases of natural infections of domestic equids with clinical signs of paratuberculosis have been described worldwide: in a pygmy ass, a Sicilian ass, a miniature donkey [2, 3, 4], and a horse [5]. In addition to the detection of acid-fast organisms in the intestine of these animals, MAP was cultivated only from the pygmy ass [2], and, furthermore, from a healthy horse killed for human consumption [6]. The isolates were not genotyped. Such a rare case of paratuberculosis in a donkey with typical clinical and pathomorphological signs is reported here again. The infection strain was isolated, genotyped and epidemiological data were discussed.

CASE REPORT

Origin of the animal. A male miniature donkey born in France spent the first eleven months of life at a donkey breeding farm with free ranging cattle in the neighbourhood, commingled in common pastures. The farm was localized in Burgundy, west of the Parc Naturel régional Morvan. Together with a female donkey the animal was moved to a private owner in Saxony, Germany. Both animals were kept together with their foal born in Germany, two Cameroon sheep, a cat, and a dog. In between, the male donkey had contact with two other donkeys and two sheep. It appeared healthy.

Course of disease. At the age of two years and eight months the male donkey progressively lost weight, associated with foul-smelling diarrhoea. Treatment by Equest[®] (Pfizer, Germany), later by Equimax[®] (Virbac, Germany) adduced no result. Examinations for parasitic or bacterial diseases were negative. Different blood parameters, including blood cell counts and differential haemogram, and blood chemistry were inconspicuous. Only a low anaemia was revealed. The winter coat remained up to May, later the animal grew a dull coat with areas of alopecia at the hooves and ankles. However, the donkey showed an attentive alert behaviour and appetite but was more frequently found recumbent. Further treatment with different antiparasitics as well as antibiotics and cortisone were not helpful. The emaciation proceeded continuously. Diarrhoea persisted. After eight months a repeated examination of the fluid faeces resulted in the detection of bacterial dysbiosis and intestinal mycosis caused by *Candida albicans* connected with a low pH-value of 5.5, but no parasites. After 9 months - the animal was lying down more often – a decubitus developed. Comprehensive bacteriological and parasitological examinations were again without result. Ten months after the onset of the first clinical symptoms the general condition fell off rapidly and the animal had to be euthanized.

Pathologic-anatomical, histological, and microbiological analysis. Clinical and pathomorphological signs of this equid were similar to manifestations of paratuberculosis described for ruminants. Proliferative enteritis was visible and the intestinal lymph nodes were swollen. In the area of cardia and fundus erosions and ulcerations were observed. The intestinal loops were segmentally thickened, corrugated, and signs of granulomatous lymphadenitis were noticed within the distended mesenteric lymph nodes. The intestinal content was viscous and dirty grey; no parasites or their ova were detectable, but *Candida albicans* was found in high amounts. Yeasts were also detectable within the gastric mucosa. Large quantities of acid-fast bacilli were revealed by Ziehl-Neelsen (ZN) staining in the mucosa and submucosa, mainly in the small intestine and in the intestinal lymph nodes, especially in macrophages and giant cells. In the liver moderate interstitial hepatitis and a low number of acid-fast bacilli within the macrophages were found. In the kidney, profound chronic interstitial nephritis was observed, but acid-fast bacilli were not detectable. Brain, lung, and spleen were without pathological findings. No appreciable morphological signs of immune suppression were seen.

Cultural isolation and molecular biological analyses for diagnosis and genotyping. After four weeks of culture on Herrolds Egg Yolk Medium (HEYM) with Mycobactin J, MAP was successfully isolated from intestinal content, also from the small intestine, liver, kidney, and even muscle tissue. The Mycobactin J dependence of the isolates was tested by comparative cultivation on HEYM with and without Mycobactin J. Other mycobacteria could not be detected. MAP-specific DNA sequences (IS900 pos., F57 pos., IS1245 neg.) were verified by PCR after direct isolation of MAP-DNA from faeces and from cultures of the above-mentioned tissues. The MAP-isolates were characterized by high resolution multi-target genotyping: MIRU-VNTR typing [7], MLSSR analysis [8], and IS900-RFLP using *BstEII*- and *PstI*- digestion [9]. Isolates from different tissues exhibited identical typing results. The MAP isolates belonged to the MAP-type-II group which was revealed by a specific PCR [10]. A unique genotype with the profile 52332228 (MIRU-VNTR loci 292, X3, 25, 47, 3, 7, 10, 32) and 9g-12g-4ggg-5tgc (MLSSR loci 1, 2, 8, and 9) was detected which is different from MAP strains previously studied in Germany [9, 11, 12] and France [13]. In contrast, by IS900-RFLP the strain exhibited the common pattern C1-P1.

Check-up of contact animals. Within the faeces of the female donkey MAP could be detected by nested PCR after direct isolation of DNA. But cultural isolation was negative for about 42 weeks. The other animals of the German owner, the female donkey, the foal, the two Cameroon sheep, the dog, the cat, and four other contact animals (two donkeys, two sheep) from two further animal holders in Germany were also MAP negative using faecal culture for about 42 weeks. Up to now (one year after the death of the male donkey) all these animals have remained healthy. Unfortunately, samples from the donkey breeding farm in France were not available.

DISCUSSION

In the present study a case of paratuberculosis in a miniature donkey (*Equus asinus f. asinus*) was described with typical clinical and pathomorphological signs, inter alia diarrhoea, emaciation, and very high numbers of MAP organisms in intestine, lymph nodes and faeces; parts of this study have recently been published in German [14]. The initial good condition of the animal, the course of disease with examinations by the veterinarian, the pathological, histological and microbiological investigation demonstrated that paratuberculosis was the basic disease of the donkey. *Candida albicans* may have settled in because of the damaged intestine after excessive antibiotic treatment. No evidence for an immune deficiency was traceable by the clinical and pathomorphological examinations carried out by the veterinarians; no specific tests were performed. In future cases it is indicated to use additional methods to find out if a kind of specific immune deficiency of “naturally” infected non ruminant animals exists. However, most probably the animal was infected during infancy in the breeding farm in France by contact with large amounts of MAP from shedders: infected ruminants or related donkeys on the breeding farm. After more than two years there must have been a stress situation which triggered the onset of disease. Equids such as donkeys and horses are often permitted to mingle with cattle; therefore a high danger exists to infect individual infant equids which possibly exhibit a specific unknown susceptibility to MAP. In Germany, paratuberculosis is a notifiable disease in cattle, sheep, goats and other ruminants, but not in equids. Equids are considered resistant to natural infection by MAP. No case of paratuberculosis in a donkey has been noticed in Germany before. Therefore, the veterinarian did not consider paratuberculosis as a cause of chronic diarrhoea. This can be dangerous for farms where different animal species are kept together, including precious species.

CONCLUSION

Donkeys should be considered as paratuberculosis-susceptible animals in exceptional cases and as possible reservoirs or disseminators of infection. Donkey breeding farms should be strictly separated from cattle herds, sheep or goats with an unknown paratuberculosis status.

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The genotyping studies within this report was funded by the German Ministry for Education and Research, Consortium ZooMAP, Grant No. 01KI 0755.

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BETWEEN-FARM MOVEMENT OF LIVESTOCK: EPIDEMIOLOGICAL IMPLICATIONS FOR THE TRANSMISSION AND CONTROL OF PARATUBERCULOSIS IN NEW ZEALAND

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INTRODUCTION

Paratuberculosis (PTB) has a worldwide distribution among farmed ruminants and its control remains a challenge. In New Zealand, PTB is endemic in cattle, sheep and deer on pastoral livestock farms. Movements of livestock between farms are thought to be the primary reason for the introduction of MAP into herds (or flocks) previously free of disease, though sparse evidence exists. In this study, we combined data about movements of livestock with information about the PTB infection status of herd and flocks. The aim of this analysis was to evaluate between-farm transmission of paratuberculosis via livestock movements.

MATERIAL & METHODS

Landcorp farms
non Landcorp farms

All the data for this study were provided by Landcorp Limited, who own more than 100 farms of all production types throughout New Zealand.

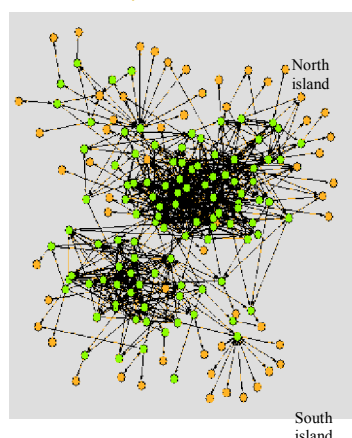


Figure 1: Network of Landcorp Ltd livestock movements in

Paratuberculosis data: Between May and September 2010, 103 Landcorp farms with 162 mobs of sheep, deer, dairy or beef cattle (excluding finishing farms) were tested for Paratuberculosis infection. Farms of Landcorp Ltd. are made up of approximately 50% mixed-species and 50% mono-species properties. For each species within a farm, a random sample of 20 individuals was tested, and additional samples were made in case of the presence of clinical suspects in the mob. Faecal culture pooled at the mob level as well as individual Elisa tests were carried out. A combination of the number of animals Elisa positive in one mob and the Elisa titres, the pooled faecal culture result and the presence or absence of clinical suspects was graded into a score of PTB prevalence for each mob. The scores were 0 (non-infected), 1 (moderate prevalence) and 2 (high prevalence).

Movement data: Landcorp Ltd. keeps systematic records of livestock movements. They include transfers of animals between farms and temporary shipments for grazing management. Between 01 July 2006 and 30 June 2010, details of these movements were retrieved for a total of 117 Landcorp farms, representing 3,532 movements and 1,148,946 animals moved (Figure 1). Only movements of breeding stock were considered as these would generate new infections, whereas no PTB-status was available from finishing stock because these were considered to be minor contributors of new herd/flock infections as they were usually grazed on separate pasture blocks away from the breeding herd/flock. The data generated a list of pairs of mobs from different farms exchanging stock over the past 4 years (2006-2010). A pair described the farm sending animals, the farm receiving animals and the species. We also used network analysis to calculate the annual number of animals received in each farm, including all species. We used movement data of the last year (2009-2010) since these revealed to be more complete and accurate. This resulted in a so called 'indegree' for each destination farm. The indegree was categorised as receiving-cat =1 (low), 2 (moderate), 3 (high), or 4 (very high).

Analysis: Multinomial logistic regression was used to examine associations between the probability of a PTB-score of 0, 1 or 2 ($P(\text{PTB-score})$) for the destination mob, and the category of the annual number of animals received for the destination farm (receiving-cat, 1-4) and infection status of the source mob (PTB-score, 0-2). The analysis was adjusted for possible confounding effects of species, island and herd size. The model was:

$$\text{Mean } P(\text{PTB-score}_{\text{destination}}) = \beta_1 \text{PTB-score}_{\text{source}} + \beta_2 \text{receiving-cat}_{\text{destination}} + \beta_3 \text{species} + \beta_4 \text{island} + \beta_5 \text{herd size}$$

RESULTS AND DISCUSSION

15% (25/162) species mobs had a high PTB- score, 20% (31/162) a moderate and 65% (106/162) were herd/flock-test negative (Figure 2). The PTB score of a mob increased as more animals were received onto the farm, with a threshold effect. Receiving large numbers of animals (categories 3 or 4) increased the odds of a mob being in a high PTB score (2) **2.6 to 2.8 times** ($p < 0.05$), whereas moving smaller animal consignments was not associated with a moderate or high PTB score (Figure 3). Thus, moving large number of animals could potentially increase the infection prevalence of the receiving farm.

The frequency distribution of the annual number of animals received per farm was skewed: few farms received large numbers of animals (Figure 4). These highly connected farms ('hubs') might therefore be an important risk group for controlling the spread of PTB between farms.

The PTB score of the destination mob was not significantly associated with the PTB score of the source mob after adjusting for covariates significantly associated with the score of PTB (species, island, herd size). The analysis of movement data from farms of Landcorp Ltd. was a unique opportunity as Landcorp Ltd. is New Zealand's largest farming enterprise with a network of farms throughout the North and South Island, and because their livestock are mainly moved within the company with little contact with other, non-Landcorp properties.

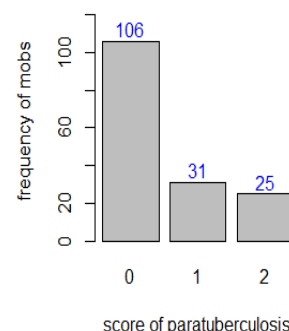


Figure 2: Distribution of the PTB score of receiving mobs

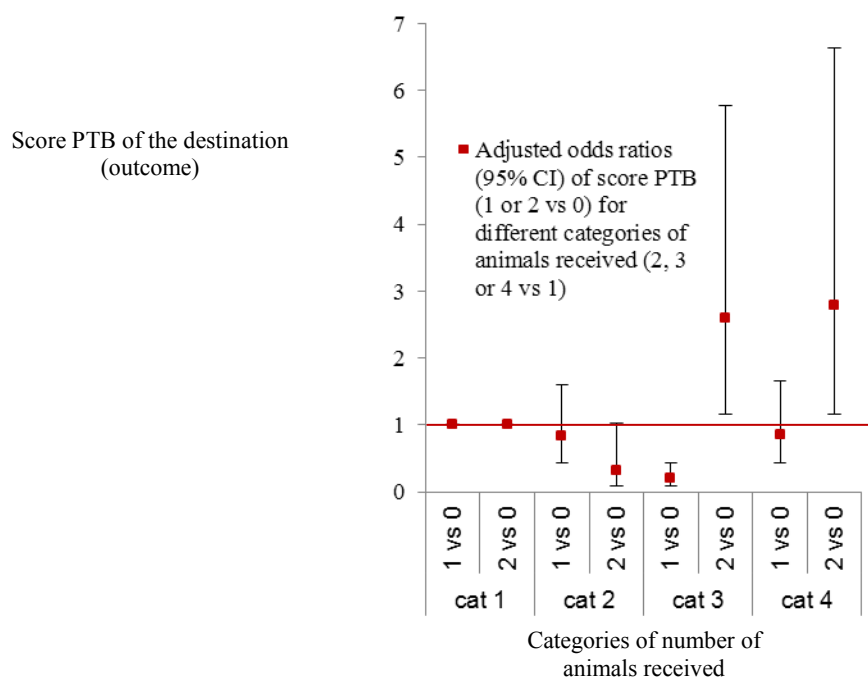


Figure 3: Association between the PTB-score of a receiving farm and the consignment size (cat1-4; cat1 =reference).

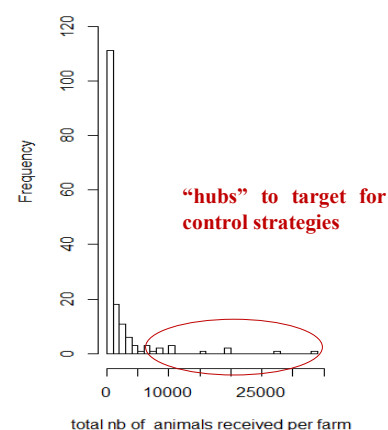


Figure 4: Distribution of the annual number of animals received in year 4

CONCLUSION

By associating movements with data from a recent survey of PTB infection prevalence, this study provides evidence that moving large numbers of animals between farms is a potential risk for increasing the intra-farm prevalence of PTB. The lack of a relationship between the prevalence score of PTB in source and destination mobs may be explained by the already high endemic level of MAP infection in New Zealand pastoral livestock farms. Moreover, it is possible that there was no clear difference between scores '0' (negative) and '1' (low level of infection), due to a lack of herd-sensitivity for detecting low levels of infection.

PREVALENCE OF CATTLE PARATUBERCULOSIS IN FARMER'S FIELD OF TAMILNADU, STATE, INDIA

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Johne's disease or paratuberculosis is an emerging disease, a chronic infection affecting the livestock industry. To assess the prevalence of paratuberculosis in white dairy cattle population random samples were included faecal and whole blood for sera from cattle of various districts of Tamilnadu. The sample size included approximately 20 gms each of 1034 faecal samples in a sterile plastic container and 1034 whole blood samples for sera separation in a clot activator tube. Direct smear examination was performed by acid – fast staining. Shrimpex stool DNA extraction kit (Synergy, India) was used to extract the DNA from faecal samples and IS900 primers were used to screen cattle. The amplified PCR products gave product size 229 bp product size. Acid –fast staining showed 79 animals were positive (7.60 per cent). The humoral immune response was assessed by cattle type absorbed ELISA kit (labor Diagnostik, Leipzig, Germany). Out of 1034 cattle sera samples screened by ELISA 126 animals showed positive (12.19 per cent), 180 animals showed PCR positives (17.41 per cent). To confirm the strain type IS1311 was used and restriction enzyme digestion and it gave product size of 67, 218, and 323 bp indicating bison type. Comparison was made between PCR and ELISA conducted on 1034 cattle faecal and sera samples in chi-square model and found that there was highly significant difference between these two tests (233.18**, $P < 0.01$). From this study it was concluded that the IS900 PCR can effectively used for screening of white cattle paratuberculosis diagnosis.

SLAUGHTER HOUSE PREVALENCE OF OVINE PARATUBERCULOSIS IN CHENNAI CITY, INDIA

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Ovine paratuberculosis is a threat to small animal holders in terms of economic loss such as reduced growth performance and early culling. In order to study the Slaughter house prevalence of ovine paratuberculosis the slaughter house sheep samples were collected from 296 male sheep aged between 16 and 18 months slaughtered at local abattoir. The slaughter weight and body condition score were also measured. DNA was extracted from intestinal tissue and mesenteric lymph nodes using Qiagen Dnase (Synergy, Germany) tissue extraction kit. The humoral immune response was assessed by cattle type absorbed ELISA kit (labor Diagnostik, Leipzig, Germany) and showed 20 animals were positive with the percentage positivity of 6.75 (20/296). The ZN staining technique was also used to examine 296 slaughter house sheep comprising of mesenteric lymph node (MLN) and intestine. A positive percentage of 5.06 (15/296) was observed in the case of ZN staining of intestinal tissue and MLN. In IS900 PCR out of 296 examined, 27 were positive with the percentage positivity of 9.12. In general the mean body weight of paratuberculosis affected animal was 22.3 kgs where as unaffected animals had 28 kgs body weight at the same age of slaughter. The average economic loss per sheep / farmer is around Rs 1200/ (US\$ 32) animal in paratuberculosis affected sheep. From the study it was observed that there was highly significant difference between the two diagnostic tests used and it was also observed that the paratuberculosis affected animals had significantly lower body weight, moreover the increase in the incidence of slaughter house paratuberculosis may be due to early culling of paratuberculosis affected animals because of poor growth rate and other inter current diseases.

HERD LEVEL PREVALENCE OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN ALBERTA DAIRY HERDS USING ENVIRONMENTAL SAMPLES

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Introduction

Many control programs have been implemented worldwide to control John's disease (JD) in cattle and other susceptible ruminants. Not all programs have been equally successful as many have relied heavily on testing of individual animals, and insufficient attention has been paid to preventive management practices. Additionally, many JD control programs have been designed based on the assumption that the between and within herd prevalence is relatively low without being based on a valid prevalence studies. The Alberta John's Disease Initiative (AJDI) is a producer driven program, built on the foundation of the national standards created as part of the Canadian John's Disease Initiative. The AJDI is designed to meet the needs of Alberta's dairy industry, and is coordinated by the Faculty of Veterinary Medicine at the University of Calgary (UCVM). The overall objective of the program is to increase awareness of JD within the Alberta dairy industry and encourage the implementation of best management practices to reduce the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The AJDI aims having a participation of 80% (480 farms) by December 31, 2012.

The goal of the research component of this control program is to obtain an indication of the prevalence of MAP-positive dairy herds in Alberta, and secondly to determine which locations best predict the true herd status through environmental sampling.

Materials and Methods

The AJDI was launched in November 2010. The program evaluates the status of a herd with six environmental samples collected at different sites in and around the dairy barn selected based on previous research results (1).

The first site consists of manure storage areas of adult cows (2 samples). This can either be lagoons, manure piles, or pits (10 cm beneath the surface). The second site consists of manure concentration areas of adult cows in the barn (2 samples), which are identified as any area where there will be a good mixture of manure: alleyways, cross-over areas, around waterers, corners, or the ends of scrape lanes. The last location for sampling consists of the so-called cow concentration areas (2 samples). This can be a sick cow pen, if more than 2-3 cows are using these pens at one time. If the pens are empty or there are only 1 or 2 cows in the pen or there are no sick cow pens, two extra samples are collected from manure concentration areas. Each collected sample consists of 4 subsamples to maximise the sensitivity of these samples; each subsample should be a mixture of manure from as many mature cows as possible. Veterinary practitioners received formal training on proper sample collection techniques. The information on sample collection is also described in a supporting environmental sample description sheet. The environmental fecal samples are analysed using fecal culture and IS900 PCR confirmation.

Results

As of January 1 2012, 201 herds were enrolled and environmental sample results are available from 177 farms. Average herd size overall was 138 cows. Herd size was larger in herds with increasing number of positive results (Table 1).

Table 1. Number of culture positive test results per farm enrolled in AJDI.

PCR result	0 +ve	1 +ve	2 +ve	3 +ve	4 +ve	5 +ve	6 +ve
Frequency	136	15	8	4	2	7	5
Percentage	76	8	5	2	1	4	3
Average herd size	129	176	155	204	190	188	181

At the farms with at least one positive environmental sample, the largest proportion of positive samples originated from manure storage areas and manure concentration areas (Table 2).

Table 2: Positive samples per location on environmentally tested positive farms.

Location	# of positive samples (total number)	% of positive samples
Manure concentration areas (alleys, scraper lines)	61 (128)	48
Manure storage areas (manure pits, piles, lagoons)	40 (68)	59
Cow concentration areas (sick cow, calving pens)	6 (29)	21

Discussion

Currently (January 1, 2012), 201 farms are enrolled in the AJDI, which is approximately 35% of the Alberta Dairy industry. Within this cohort culture results of 177 farms were available and 41 farms or 23% of the farms have at least one positive environmental sample. Participating herds will be sampled for 3 consecutive years. We anticipate that this prevalence estimation is still an underestimation of the herd prevalence in the first round of samples as often the early adopters of a program are less likely to be test positive for the disease under investigation.

With the recently published estimation of herd sensitivity being 40% (2) and assuming specificity being approximately 100%, the true herd prevalence might be as high as 58% in our first sampling round. Still 23% of the herds being infected is a significant percentage of the industry which warrants the overall objective of the AJDI to reduce within herd prevalence of MAP and ultimately also reduce the prevalence of MAP-positive herds. Remarkable is the positive association between herd size and number of MAP-positive environmental samples. This needs to be investigated in more detail, but can be related to either farm size specific management practices or the likelihood of an environmental sample to be positive for MAP depending on farm size.

Conclusion

The prevalence of MAP-positive herds in Alberta is high and larger herds tend to be more often positive in environmental sampling. The sampling strategy will be evaluated for use as an economic valuable monitoring tool in control programs and the location of preferred sampling seems to be the manure storage areas.

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ANTIBODY SURVEILLANCE FOR *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) AT A SHEEP-GUANACO INTERFACE

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The guanaco (*Lama guanicoe*) has inhabited Tierra del Fuego Island as a free-ranging wild species since the Pleistocene era. The introduction of sheep to the island about 100 years ago decimated the guanaco population with numbers dropping from 7 million to 600,000. The co-existence of these two ruminant species provides a natural laboratory for the study of shared infectious diseases. On the Chilean side of Tierra del Fuego (Rusfin area), *Map* has been isolated from guanaco (Salgado *et al.*, 2009). Sheep from a large farm share free-range grazing with the guanaco. The farm annually rounds up thousands of adult sheep into its central facilities over a period of time of several months, potentially increasing the risk of infectious disease transmission from sheep to guanaco.

To investigate *Map* infection prevalence at this domestic-wild ruminant species interface, blood samples were taken from 150 sheep belonging to a large farmed flock. Blood samples were also taken from 150 guanacos hunted in the same area.

The hunting activity was restricted to a small group of adult animals (2,000). From both animal species, only blood serum samples were taken for paratuberculosis diagnosis.

For *Map*-antibody detection a commercial Johne's disease ELISA was used for sheep serum samples. The same kit was adapted for use in camelids by using an goat anti-llama IgG conjugate in place of the kit's anti-bovine IgG conjugate (Parreño *et al.*, 2001). To determine proportion differences of ELISA-positive individuals between the two animal populations, a proportion test for two samples using the Z statistic was used, with a confidence interval of 95%. The software Statistix 8,0 was used.

Six sheep and fifteen guanacos were ELISA test-positive, suggesting the infection is present in both species. This result is consistent with the isolation of *Map* from guanaco fecal samples in the same geographic area (Salgado, 2009), and would suggest that the infection is still present and active in both species. The proportion of positive results in the sheep sub-sample population (6/1590) was statistically different ($P < 0.05$) from the proportion of positive results in the guanaco sub-sample population (15/150) (Table 1).

Table 1. Differences in the proportion of ELISA positive results between sheep and guanaco sub-population samples in the Russfin area, Tierra del Fuego island

Variable	SHEEP	GUANACOS	PROPORTION TEST
ELISA positive-result	6/150	15/150	P = 0.045

The higher rate of ELISA positive results in guanacos could be due to many factors, such as a higher sensitivity of the adapted ELISA for the guanaco species in this trial or a higher life expectancy than in sheep, etc. However, there is a tendency to label such results as evidence of a wild animal "reservoir" of infection, implying that infected wild animals are sources of infection for domestic animal populations. It is equally plausible that the reverse is true; domestic animals constitute the reservoir that threatens the health of wild animal population. It is also conceivable that the *Map* recovered from guanaco feces are merely environmental contaminants originating from domestic animals and merely passively passing through the guanaco without actually causing infection with attendant pathology and immune response. Meanwhile, no information about *Map* infection prevalence in sheep of Isla de Tierra del Fuego is available; this domestic ruminant species can not be eliminated as a source of *Map* infection for guanacos. Then, it can be concluded that in the sheep-guanaco interface of Tierra del Fuego island, the *Map* infection would be present and in an active status.

ACKNOWLEDGEMENTS

This study has been funded by WCS-Chile

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PRESENCE AND GENOTYPES OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN DAIRY CATTLE HERDS OF AGRA, MATHURA AND FIROZABAD REGIONS OF NORTH INDIA USING MICROSCOPIC EXAMINATION, DIRECTS IS900 PCR AND IS1311 PCR-REA METHODS

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Mycobacterium avium subspecies *paratuberculosis* (MAP), the cause of Johne's disease in ruminants has been emerged as major animal pathogen with significant zoonotic concern, worldwide. Information about the presence of MAP in herds of dairy cattle is limited in India. Present study was aimed to investigate the presence of MAP and their genotypes in dairy cattle of Agra, Mathura and Firozabad region of North India.

A total of 43 fecal samples were collected from dairy cattle herds located at Agra (n=22), Mathura (n=19) and Firozabad (n=2) region of North India. All the fecal samples were processed for detection of *Mycobacterium avium* subspecies *paratuberculosis* using microscopic examination, and direct IS900 PCR. However, representative fecal samples (n=32) were also processed for culture on Herrold's Egg Yolk medium. All the isolates recovered on HEY medium were subjected to molecular identification and genotyping using IS900, ISMav02 PCR and IS1311 PCR-REA, respectively. Out of 43 fecal samples, 12 (29.9%) and 9 (20.9%) samples were positive for the presence of acid fast bacilli and MAP using microscopic examination and IS900 PCR, respectively. Region-wise, 31.8, 26.3, 0.0 and 18.1, 22.7, 0.0% animals were positive for MAP from Agra, Mathura and Firozabad region using microscopic examination and direct IS900 PCR test, respectively. Statically, there was substantial agreement (Kappa value-0.687) between microscopic examination and IS900 PCR method using Kappa test. Of the 32 fecal sample, 3 (9.3%) were positive for the growth on HEY medium. All the culture isolate were confirmed as MAP using IS900 and ISMav02 PCR, and genotyped as 'Bison type' by IS1311 PCR-REA. Present study reported moderately higher presence of MAP in dairy cattle using microscopic examination and direct IS900 PCR and indicated the need for national JD control program to secure optimum productivity from animals and minimize the human exposure to MAP. 'Bison type' was found as the major genotype in dairy cattle, therefore, indigenous diagnostic and vaccine based on 'Bison type' genotype of MAP can be used to restrict the disease in dairy cattle.

Acknowledgement: Authors are thankful to Indian Council of Medical Research, New Delhi for providing the financial support for this work and Post Doc fellowship to Dr. A.V. Singh.

INTERSPECIES SHARING OF NATIVE 'INDIAN BISON TYPE' GENOTYPE OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* BETWEEN A COLONY OF DOMESTICATED RABBITS (*ORYCTOLAGUS CUNICULUS*) AND A SHEEP FLOCK

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Mycobacterium avium subspecies *paratuberculosis* infection has emerged as major animal pathogen and is endemic in domestic and wild ruminants and non-ruminant species in India. The study investigated status of MAP infection in a colony of domesticated rabbits (*Oryctolagus cuniculus*) co-habited within organized farm of sheep at SRRC, of CSWRI, Manavannur, Tamil Nadu, India. Johne's disease is endemic in the sheep flock. Fecal samples (77) were collected from the colony of rabbits between 2009 and 2011 and screened for the presence of MAP, using microscopy and IS900 PCR. PCR positive DNA samples were further genotyped using IS1311 PCR-REA. Of the total 77 samples, 26 (33.76%) and 6 (7.8%) were positive for MAP using microscopy and IS900 PCR, respectively. IS900 positive DNA samples were genotyped as 'Indian Bison type'. This genotype has also been recovered from the sheep flock located at SRRC and domestic and wild ruminants and human population in India. Prevalence of similar genotype *Oryctolagus cuniculus* (domesticated rabbit) and ruminants is suggestive of inter-species sharing of MAP and raises serious concerns of rabbits acting as non-ruminant reservoir for MAP.

INVESTIGATION OF PARATUBERCULOSIS IN QUEBEC (CANADA) BASED ON MOLECULAR, MICROBIOLOGICAL, SEROLOGICAL AND PATHOLOGICAL ANALYSIS

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Paratuberculosis is serious ailment of ruminants whether small or large and disease is considered important in both developed and under developed world for economic and public health concerns. In Canada, there is an ongoing paratuberculosis control program and numerous related studies are undergoing with respect to dairy cattle. However, small ruminant population has largely been ignored. Small ruminant industry offers employment to considerable population of Quebec. Hence the present investigation was carried out to study the presence of disease in small ruminant herds. The two major objectives of the present investigation are; a) to explore/ design/ adopt/ optimize diagnostic methods for future studies and b) to investigate the presence of disease in small ruminants herds of Quebec. Animals from small ruminant herds from across Quebec were sampled for tissues (MLN, Intestine), blood, serum and feces. Molecular, serological and bacteriological tests were either designed or modified to suit the requirements of our laboratory. Gross and histo-pathological investigations were carried as confirmatory measures. Results show that paratuberculosis is a noticeable disease in small ruminants of Quebec. The complete results of the present study will be presented at colloquium.

COW-LEVEL ASSOCIATION BETWEEN SERUM VITAMIN D (25-HYDROXYCHOLECALCIFEROL) CONCENTRATION AND MAP-ANTIBODY SEROPOSITIVITY

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Besides its role in bone metabolism, vitamin D plays an important part in the modulation of the immune response (1). Vitamin D deficiency has been observed in patients with Crohn's disease, tuberculosis (2) as well as other diseases. A pilot study at the University of Minnesota (n = 41 dairy cows) found that MAP-seropositive cows tended to have lower serum 25-OH vitamin D3 (25-OH-D3) concentrations, the accepted marker of vitamin D status, than test-negative herd mates (P= 0.09). In addition, the 25-OH-D3 concentration was associated with the S/P ratio of the cow -- the higher the ratio, the lower the 25-OH-D3 concentration. This might indicate that a progression of infection might be associated with reduced vitamin D levels. Whether this is due to impaired absorption of vitamin D through the MAP-infection damaged intestines, lack of sunshine exposure through year-round indoor housing or a higher turnover rate of internally produced 1,25(OH)D3 for the modulation of an out-of-control immune response or lower vitamin D receptors is currently unknown. This preliminary study had limited statistical power and had not measured the intake of vitamin D through nutrition and sunshine exposure nor other factors such as stage of lactation, which influence the serum 25-OH-D3 levels. Therefore, currently a study is conducted in 5 Minnesota dairy herds, which will compare the Vitamin D3 sero-levels between MAP-antibody seropositive and ELISA-negative herd mates after accounting for their nutritional vitamin D intake, breed, predominant hair color, age, stage of lactation, milk production and farm location. Results will be presented at the conference.

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BAYESIAN LATENT CLASS PREVALENCE ESTIMATION OF HERD LEVEL INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

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The study aimed to estimate the true herd level prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in mixed species pastoral farmed livestock in New Zealand. A stratified-random sample of 238 single- or multi-species farms was selected from a postal survey population of 1,940 farms. The sample included 162 sheep flocks, 116 beef cattle and 99 deer herds from both main islands. Twenty clinically normal animals from each species flock/herd present on farm were randomly selected for blood and pooled faecal culture (PFC), sampling one pool of 20 animals from sheep flocks and two pools of 10 animals each from beef cattle and deer herds. Pooled faeces was tested by Bactec culture with mycobactin-J dependent confirmation. To increase flock/herd sensitivity (HSe), blood serum of all 20 animals from culture negative herds and flocks was tested by ELISA (sheep and cattle) or ParalisaTM (deer). Additionally, blood and faeces from up to five clinical suspect animals was collected, if present at sampling. Thus, the apparent herd status was based on four test protocols made up by the combination of the two tests with optional sampling of suspect animals. Results were adjusted for lack of sensitivity and specificity of tests through latent class Bayesian statistical modelling. True prevalence was corrected for sampling fractions to present prevalence in the total survey population stratified by island. Overall 164/238 farms (68.9%) tested positive. The highest true prevalence was observed for sheep flocks (0.68, PCI 0.60-0.75), followed by deer (0.62, PCI 0.39-0.82) and beef herds (0.31, PCI 0.23-0.39). Farms with two or more species tended to have higher true prevalence. True flock/herd prevalence estimates of MAP infection in sheep and beef cattle were significantly higher in the North Island, whereas for deer it was higher in the South Island. HSe ranged from 0.52 to 0.99 and herd level specificity (HSp) from 0.43 to 1.00, depending on the combination of test, sample type and species sampled. HSe increased by 11-40% when testing PFC-negative herds/flocks by Elisa, and by 8-20% when testing additional clinical suspect animals by PFC. HSp decreased due to using Elisa/ParalisaTM. The HSp decrease was small in cattle herds (1-2%) due to few positive tests, but was large in sheep (18%) and deer (56%). Bayesian latent class analysis of survey data provided biased-adjusted estimates of prevalence and accuracy of the detection of MAP-infected herds.

A BAYESIAN ASSESSMENT OF THE DEPENDENCE OF INFECTION PREVALENCE AND CLINICAL INCIDENCE OF PARATUBERCULOSIS ON JOINT GRAZING OF SHEEP, BEEF CATTLE AND DEER

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The objective of this study was to evaluate the effect of grazing cattle, sheep and deer either jointly or separately on the risk of infection with *Mycobacterium avium* subsp. *paratuberculosis* (Map) and the farmer-observed incidence of clinical paratuberculosis (Ptb). Randomly selected normal and, if available, suspect animals from 238 single- and multi-species farms (162 sheep flocks, 116 beef cattle and 99 deer herds) were tested by pooled faecal culture (PFC). Individual blood samples from PFC negative herds/flocks were tested by ELISA. A herd/flock was classified as positive if either the PFC or the ELISA tested positive. At sampling, a questionnaire was applied to obtain information about the population at risk, the use of pasture, and the number of Ptb cases observed in the last four years. Bayesian latent class logistic and poisson regression models were developed for each species to assess the effect of co-grazing different species on the odds of herd/flock level Map-infection and risk of clinical Ptb-incidence. Each model was adjusted for lack of test accuracy. The contact with others species at grazing, either simultaneously or sequentially, island and herd size were regressed on herd/flock infection status (logistic) and on the annual Ptb case incidence (poisson). The odds of Map-infection in separately grazed sheep flocks was similar as in sheep grazed with deer herds (odds ratio, OR=1.01), but greater when sheep were grazed with beef cattle (OR=1.59). The clinical Ptb incidence in sheep was lower when grazed with beef cattle (relative risk, RR=0.57) and higher when grazed with deer (RR=1.37). Map-infection of beef cattle herds was less likely when grazed with deer (OR=0.70) and more likely when grazed with sheep (OR=1.40) than when grazed in isolation of other species. However, the clinical Ptb incidence of beef cattle was lower when grazed with sheep (RR=0.48) than when grazed with deer (RR=0.93) or in isolation. Finally, deer herds had a Map-infection OR=2.58 when they grazed with beef cattle and OR=1.53 when they shared pasture with sheep, indicating both species as source of infection in deer. However, the risk of clinical cases decrease when deer grazed jointly with sheep (RR=0.53) and increases when they shared pasture with beef cattle (RR=1.61). Results from this study show that the co-grazing of multiple susceptible species has an effect over the chances of test positive and the clinical incidence.

PARATUBERCULOSIS INFECTION PREVALENCE AND DISEASE INCIDENCE IN FARMED DEER IN NEW ZEALAND

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Robust data on Paratuberculosis (Ptb) infection and clinical disease prevalence is required to inform management and control. This paper summarises several studies, principally on red and red/wapiti deer to establish the likely infection prevalence and clinical and subclinical disease incidence in farmed deer in New Zealand.

Herd infection prevalence nationally was estimated in 2009 from a study of lymph node infection (n=4/farm, 57 farms) at slaughter to be 59% (CI 41-78%). An on-farm faecal culture and serum elisa survey (2008) estimated herd prevalence to be 61.6% (n=99). Annual clinical disease incidence on infected farms (n = 96) in a 2005 on-farm survey averaged 1% (range 0.04 – 11.9%). Mean incidence (and range) was 1.2% (0.1-21.5) in weaners (<12 months), 2.0% (0.2–20) in yearling hinds, 2.0% (0.2–13) in yearling stags, 0.5% (0.1–1.7) in adult hinds and 0.8 (0.2–2.4) in adult stags. A 2007 postal survey showed 40% of farms (n=342) had farmer-diagnosed clinical Ptb, with a mean incidence of 0.9%. A 2008-9 on-farm survey (n = 99 farms) estimated a mean clinical disease rate of 0.32% on infected farms (range 0–6.6%). A 2007 postal survey of fallow deer farms (n=20 responses from 52 farms) identified no clinical Ptb in this species.

Ninety two percent (n=128 study 1, 2006), and 71% (n=42, study 2, 2011) of abnormal mesenteric lymph nodes (ALN) (≥ 55 mm circumference or any circumference with pathology) were culture positive. Data from a national surveillance database shows 0.69% (CI 0.688 – 0.692) (n=2.08 million) of deer were observed with ALN. The culture prevalence in 251 normal mesenteric lymph nodes (NMLN; n=37 farms) slaughterhouse sampled at random in 2009 was 45%. A similar study in 2011, showed 42.2% (n=199) culture prevalence in NMLN. By inference, the infection rate in deer on infected herds could be 70-75%, but this remains to be determined.

These data establish that Ptb has an apparently high infection prevalence but low clinical and subclinical disease incidence rate in farmed deer herds in New Zealand.

PERSPECTIVES ON EPIDEMIOLOGY: HIGHER STANDARDS IN EPIDEMIOLOGIC RESEARCH ON *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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The epidemiology of paratuberculosis primarily concern diagnosis, occurrence and transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections. Latency of MAP infections makes studies of these three features challenging, because the target condition may vary among researchers and decision makers. A central challenge may be that we disagree on what we believe we study. The target condition and consequently the diagnosis become blurred when the case definition changes from study to study. Any subsequent use of this diagnosis e.g. for prevalence estimation or transmission studies may become useless. Ultimately, reporting from such studies may not be meaningful.

A structured approach to design of diagnostic test accuracy studies (Nielsen et al., 2011) along with reporting guidelines (Gardner et al., 2011) has been proposed for diagnostic test evaluations (DTEs). Use of these procedures should aid in DTEs, but other types of MAP studies also calls for more stringency in study design and reporting.

A similar approach can be used for other types of epidemiological studies on MAP, i.e.

- 1) define the purpose of the study,
- 2) define the target condition of relevance to this study,
- 3) specify a case definition that correlates with this target condition,
- 4) design the study in relation to the above-mentioned features, and
- 5) report the study using the terminology and definitions laid out in Gardner et al. (2011)

Lack of correlation between the study purpose and the target condition is frequent in the epidemiological literature on MAP. For example, risk factor studies reporting the risk that a herd is infected given some factors on within-herd management factors are frequent. Introduction of MAP to a herd is usually hypothesised to occur with introduction of animals infected with or carrying MAP. Therefore, it could be questioned why a factor such as group housing is even considered as a potential risk factor. Group housing generally have little to do with introduction of MAP, but this practice may merely be a proxy for some other features of the farm. Thorough considerations on the study objective would have revealed discrepancies between the target condition and the purpose of the study. Just because data are available does not mean that it makes sense to study all associations possible.

Epidemiological research often requires large samples, but often these sample sizes cannot be achieved because of costs. Consensus definitions and reporting would enable combining available data e.g. through aggregation of data in meta-analyses to make the best of the data published, when stand-alone studies have insufficient statistical power. Data from such studies could be reported to a central database. Because of the long incubation period, longitudinal studies would be preferable. Establishment of such a database might enable future progress in epidemiological research on MAP, although standards for inclusion of data would be required.

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**PUBLIC HEALTH AND *MYCOBACTERIUM AVIUM* SUBSP.
PARATUBERCULOSIS IN THE ENVIRONMENT**

KEYNOTE ON: ETIOLOGICAL RELATION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* AND HUMAN CROHN'S DISEASE: HISTOLOGICAL, MOLECULAR COMPARISON AND NEW EVIDENCE OF THE PATHOGENESIS

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Introduction

Paratuberculosis (Ptb), caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is a chronic enteritis that affects many ruminants and other wild animals worldwide. The clinical disease is called Johne's disease (JD), however this is used as synonym for paratuberculosis, a *Map* infection. After oral ingestion and invasion into Peyer's patch via M-cells, *Map* infection has very long incubation period (3-6 years). Antibody level is increases in late stage of infection. Ptb is a great concern in animal health and in etiology of human Crohn's disease (CD). Crohn's disease (CD) is a type of intractable inflammatory bowel disease, causing a wide variety of symptoms and reduce QOL of the patients. Half of the patients require surgery within 10 years of diagnosis, and the postoperative recurrence may be 44-55% after 10 years. Since the first report of isolation of *Map* from Crohn's disease (CD) patients in 1986, much suggestive evidence has been accumulated as a causative agent of CD. Although there are many other etiological hypotheses of CD, it is still an intractable disease of unknown etiology. Author talks short review of our studies on the topic.

Comparative studies of Ptb and human IBD: In recent studies, we detected *Map*-specific insertion sequence IS900 of DNA in tissue sections surgically removed from lesions of patients with CD (29 samples), ulcerative colitis (UC) (17 samples), and non-inflammatory bowel disease (IBD) (20 samples). We then compared the histopathological findings of 29 CD and 17 UC cases with those of 35 cases of bovine Ptb, since few comparative pathological studies of human IBD and Ptb have been conducted. The QPCR examination indicated positive results in 13.37% of CD cases, 3.57% of UC cases, and 10% of non-IBD cases. Human CD tissues typically exhibited destructive full thickness enteritis with severe lympho-plasma infiltration and scattered additional granulomas; UC lesions exhibited much less inflammation than CD lesions. Non-IBD control samples did not exhibit pathological changes. Human CD and UC lesions were very different from Ptb lesions that are characterized by predominant granuloma formation. Immunohistochemistry for *Map* antigen and acid-fast staining were negative in all human IBD cases but were always positive in Ptb cases. Our present comparative study strongly suggests that we reconsider the previous hypothesis that "*Map* infection" causes CD, even though human intestines were considered to have been exposed to the *Map* antigen-complex containing the DNA.

Experimental model of CD by *Map* antigen

To resolve the etiological question, we hypothesized that *Map* antigen molecules cause immuno-mediated colitis rather than *Map* infection, since live *Map* have not isolated from CD lesions frequently, but *Map* DNA, a part of the antigen-complex has detected more frequently. In addition, the facts that infection of *Map* in dairy industry is pandemic and contamination of dairy foods with *Map* antigen-complex (heat killed *Map*) is common. To test the effect of the antigen in induction of colitis, we prepared experiments by using a *Map* antigen from cultured *Map* and made a new mice model by using similar manner to previously reported TNBS induced CD-like colitis. The experimental model revealed serious destructive (necrotizing) and full-thickness CD-like colitis. The colitis lesions were very similar to TNBS-induced colitis and human CD.

Conclusion

Contribution of *Map* antigen in the pathogenesis of CD was not proposed yet and present results provide reasonable explanation of the epidemiological correlation of incidence of paratuberculosis and CD. Our comparative studies revealed etiological relation of *Map* and CD, but CD lesion was not considered as infectious. The experimental mice studies provide new insights into the pathogenesis and prophylactic approach of CD and strongly suggest the urgent needs of the eradication Ptb. At the least, dairy foods

that may be contaminated with Map antigen worldwide should be avoided by family member of patients with CD or children as soon as possible, because of their possible genetic predisposition.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education No. 23240061 and No. 20228005 (to H. O.), and the Bio-oriented Technology Research Advancement Institute (BRAIN).

ISOLATION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* FROM CROPS, SOIL AND FEED AFTER MANURE SPREADING ON DAIRY FARMS WITH JOHNE'S DISEASE

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Introduction

Manure disposal on dairy farms infected with MAP can be a challenge. The purpose of this study was to determine the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in samples of soil, crops, and ensiled feeds following manure spreading on farms with Johne's disease.

Methods

The study was conducted on 10 dairy farms known to have cows infected with MAP. The farms were selected because stored manure is periodically spread on crop fields used to raise feed for the adult cattle. Manure was sampled from the storage site and tested for MAP by HEYM culture and by quantitative real-time PCR. Approximately 1 to 4 months following manure spreading, samples of soil and/or crop were tested for MAP by HEYM culture and qrtPCR. For ensiled feeds, samples were also collected 2 to 9 months later and tested as above.

Results

Of the 10 farms, 7 had stored manure samples positive for MAP on both culture and PCR, and 3 had manure that was positive on PCR only. Of the 10 farms, 7 had soil samples tested, and 6 were positive by PCR but none by culture. Six farms had crops tested, 3 of which were positive by PCR and none by culture. Eight farms had silage samples tested, of which 3 were positive by PCR only. In general, Ct values from PCR corresponded with low concentrations of MAP that are usually below the limit of detection by culture.

Discussion

Our findings suggest that although MAP can be found in soil samples following manure spreading, contamination of harvested feeds and silage is less frequent. When faced with the decision about where best to spread manure, fields used for production of crops intended for harvest are preferred to grazing pastures.

VARIABILITY IN ENVIRONMENTAL SURVIVAL OF OVINE AND BOVINE STRAINS OF MAP ACROSS CLIMATIC ZONES OF NSW

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The longer *Map* survives in the environment the greater the risk and rate of disease transmission. In the temperate regions of NSW, Australia, where Johnne's disease reached high prevalence among sheep flocks by the 1990s, Whittington et al. (2004) showed that survival of the S strain of *Map* ranged from 2 weeks in exposed (unshaded) locations to 55 weeks in full shade. Because the prevalence of ovine JD across NSW decreases from the temperate south east to the drier north west, we compared the environmental survival of S and C strains of *Map* across these environments.

Sheep and cattle faeces containing known concentrations of *Map* were placed on soil in open polystyrene boxes at 4 sites in NSW. These were Bathurst (temperate, high prevalence of ovine JD), Armidale (temperate, low prevalence of JD), Condobolin and Broken Hill (arid, low prevalence of JD). At each site boxes were either shaded (70%) or left unshaded (4 replicates per treatment). Faeces were sampled fortnightly for 6 months and monthly for the next 6 months, and cultured using BACTEC.

The maximum survival was 14 weeks. The initial concentration of *Map* was lower in cattle faeces compared to sheep faeces and this was reflected in the survival times observed. At all sites and for both strains, survival was significantly greater in faeces in the shaded treatments. Survival time for the 2 temperate sites was greater than for the 2 arid sites. Reduced diurnal temperature range in shaded compared to unshaded locations is more likely to explain increased survival than reduced exposure to UV radiation.

It is possible that poor environmental survival of *Map* may contribute to the low prevalence of paratuberculosis recorded in sheep at Condobolin and Broken Hill, but not at Armidale.

Reference

Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA, 2004. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl Environ Microbiol. 70:2989-3004.

FREQUENCY OF MYCOBACTERIA REACTIVE CD4 T-CELL CLONES IN INTESTINAL BIOPSIES OF CROHN'S DISEASE PATIENTS

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We have previously demonstrated the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) reactive T cells in Crohn's disease patients. To address whether these T cells are likely to be disease relevant, we decided to look at the frequency of mycobacteria reactive CD4 T-cell clones in intestinal biopsies from patients with active disease. The biopsies were obtained by colonoscopy from adult patients with Crohn's disease (n = 5) and ulcerative colitis (n = 2). Duodenal biopsies from celiac disease patients (n = 3) were also obtained. Celiac patients have disease relevant CD4 T cells responding to gluten in the intestinal mucosa, and we wanted to compare frequencies of antigen specific cells in the various conditions. CD4 T cells were isolated and expanded in wells containing 0.1 - 100 T-cell clones/well. The cells were screened for responses to MAP and *E. coli* for inflammatory bowel disease patients, and for responses to gluten in celiac disease patients. A frequency of MAP reactive T cells ranging from 0.49 - 1.88 % was found in Crohn's disease patients while the frequency of *E. coli* reactive T cells was 0.07 - 0.19 %. In comparison no MAP reactive clones were detected in ulcerative colitis patients. The numbers of MAP reactive T cells were similar to that of gluten responsive T cells in celiac disease patients (0.52 - 1.82 %). Six MAP reactive T-cell clones from three patients were expanded further. One of these clones appeared specific for antigens of *M. avium*. The T cells established from Crohn's patients all produced IFN- γ and clones from two patients produced IL-17. Similar frequencies of intestinal tissue resident T cells specific for MAP in Crohn's disease and gluten in celiac disease argue that MAP reactive T cells contribute to the chronic intestinal inflammation in Crohn's disease.

DEVELOPMENT AND OPTIMISATION OF A MULTIPLEX SNP GENOTYPING ASSAY FOR *MYCOBACTERIUM PARATUBERCULOSIS*

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Summary

Previous studies based on whole genome sequencing have demonstrated limited genetic diversity within *Mycobacterium avium* subsp. *paratuberculosis* (MAP) strains isolated from human hosts [1]. In contrast, animal derived MAP strains are genetically more diverse. In order to understand the possible zoonotic transmission of MAP a robust genotyping platform that discriminates different strain types is required. A total of 15 SNPs located in 12 loci that have previously been shown to discriminate different MAP strain types were selected for this assay [1]. This paper describes the development and optimisation of this platform based on Luminex xTAG® technology. This assay can be used to examine the geographical distribution and host specificity of different MAP strains.

Materials and Methods

The SNP genotyping assay was developed and optimised on a reference panel of isolates (4 human, 1 bovine and 1 ovine) with known genotypes. Loci containing SNPs were amplified using PCR. Initially, all PCRs were trialled as a singleplex reaction to ensure that amplification would occur. PCR conditions were designed so that they were identical for each locus. PCR was achieved in reactions containing 12.5 µL GoTAQ Green Master Mix (Promega), 0.2 µM each of forward and reverse primer, 5 µL of template DNA and nuclease free water to a total volume of 25 µL. Reactions were subjected to the following thermal cycle: 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. Following singleplex reactions, selected targets were trialled as multiplex reactions. PCR conditions were as described for the single-plex reactions. When multiplexed, it was noticed that some preferentially amplified compared to other loci. When this occurred, primer concentration of the most abundant bands was reduced by 2-4 fold. Table 1 describes final PCR conditions.

Table 1 Outline of all PCR reactions used within this study.

Mastermix	Locus	Primer conc	Forward primer (3'-5')	Reverse primer (3'-5')
Three-plex 1	MAPK_0066	0.2 µM	CCAACATCCAAAAGAACG	CCCATCTTGATCTTGTGC
	MAPK_1136	0.15 µM	CTTTTCGACCAGCTCAGG	GGCACCGATTGATGTAG
	MAPK_1923	0.2 µM	CTTATCGTATTGCTGGCTTT	CGTCAGCAGATAATCAGTCA
Three-plex 2	MAPK_0755	0.2 µM	CGCAGCCAGATCATTAAAC	GCTCGCTGATGCAGTACA
	MAPK_3670	0.15 µM	GCCCTGGAGATCATTGAC	GTCGTAGGAAGCCCAGAC
	MAPK_4303	0.15 µM	GGTCGCTGTACTCCTACAC	GGGGTAGTCGCAAAACAC
Duplex	MAPK_4099	0.05 µM	GTCGACGTGGTGGTGAGTAA	ACGATCCGCGTCGAAAAC
	MAPK_3924	0.1 µM	CGGCATGGAAGTGTCTG	CACTTGTTCTCCTGGTGATG
Singleplex	MAPK_0117	0.2 µM	AGTCCTGCGAGCTGTCCTT	GATAATTCCCGACCGACTGA
Singleplex	MAPK_3057	0.2 µM	GACTACGACGCGCTCAAA	GTCCGAGGCGAAGAACAG
Singleplex	MAPK_3946	0.2 µM	GGAACAACCTACCAGAACCTG	TCTTTGGCGGTGTAGTTC
Singleplex	MAPK_4333	0.2 µM	CGATCTGGCACGGTCAAC	GTTCCACTTGCCGTCACC

Primers that bind specifically to the site of the SNP were designed, one that binds to the match and one to the mismatch, therefore there were two primers for each loci. In this situation, one of the two primers will bind depending on which SNP allele is present. Each primer was designed so that the match/mismatch with the SNP was located on its 3' end. On the 5' end of the primer, a 24 nucleotide tag was added (Table 2). This tag sequence corresponds to a unique and complementary anti-tag oligonucleotide, which is pre-coupled to a unique Luminex xTAG bead set. This tag anneals to an anti-tag sequence, which is attached to the bead. A total of 5 µL of the initial PCR product was purified using 2 µL of ExoSAP-IT (USB). The allele specific primer extension (ASPE) reaction was performed in a total volume of 20 µL reactions consisting of 1x PCR buffer (Invitrogen), 1.25 mM MgCl₂ (Invitrogen), 25 nM of each ASPE primer, 0.75 units Platinum Tsp DNA polymerase (Invitrogen), 5 µM each of dATP, dGTP

and dTTP (Invitrogen), 5 µM Biotin-14-dCTP (Invitrogen), 5 µL of the cleaned PCR product, and nuclease free water to 20 µL. The reaction was then subjected to the following thermal cycle: 96°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds, and finally held at 4°C. Approximately 1500 beads (3 µL) of each bead set required was combined with the appropriate amount of 2x Hybridisation Buffer (0.4 M NaCl, 0.16% Triton X-100, 0.2 M Tris, pH 8.0) for 1x concentration in the total volume of the well. The total volume was adjusted to 45 µL per well by adding the appropriate amount of nuclease free water. ASPE reaction product (5 µL) was then added, and the plate was then subjected to the following conditions in a thermal cycler: 96°C for 90 seconds followed by 37°C for 30 minutes and held at this temperature until ready to proceed. The hybridisation reactions were transferred to the pre-wetted filter plate and the supernatant removed by vacuum filtration. It was then washed twice again by vacuum filtration with 100 µL 1x Hybridisation Buffer. The reactions were then resuspended in 75 µL of 1x hybridisation buffer containing 2 µg/mL streptavidin-R-phycoerythrin. The plate was then incubated at 37°C for 15 minutes and analysed at 37°C on a Luminex analyser (Bio-Plex 100 system based on the Luminex 100 system, BioRad) according to the system manual. The relative intensity (RI_{allele}) of each allele at each locus was calculated. The allele was called if the RI_{allele} was $\geq 65\%$. This value correlates with an approximate 2-fold increase in fluorescence intensity.

Table 2. ASPE primers used in this study. The lower case bases represents the tag sequence that anneals to the anti-tag sequence located on the Luminex xTAG bead. The upper case bases represent the target specific primer

Name	Primer and Tag (5'-3')
MAPK_0066-A-LUA43	ctttcaattacaatactcattacaGCTACTCGGTGGTGCCCT
MAPK_0066-G-LUA59	tcatcaatcaatcttttctacttCTACTCGGTGGTGCCCC
MAPK_0117-T-LUA10	atcacatcacatacaaatctacaTGTTGCGGGCCAGGGTTTTT
MAPK_0117-DEL-LUA22	aatcctttttactcaattcaatcaTGTTGCGGGCCAGGGTTTTT
MAPK_0117(2)-G-LUA43	ctttcaattacaatactcattacaGGGTGCGCCACCGCGG
MAPK_0117(2)-C-LUA59	tcatcaatcaatcttttctacttGGGTGCGCCACCGCGC
MAPK_0755-A-LUA6	tcaacaatcttttacaatcaaatcGGCGCACAAGGCGGTCAA
MAPK_0755-G-LUA18	tcaaatctcaaatactcaaatcaGCGCACAAGGCGGTGAG
MAPK_1136-G-LUA18	tcaaatctcaaatactcaaatcaGCTCAGGACGCAGGTGATG
MAPK_1136-C-LUA6	tcaacaatcttttacaatcaaatcGCTCAGGACGCAGGTGATC
MAPK_1136(2)-G-LUA22	aatcctttttactcaattcaatcaAACACCGTGGCCCCGCGG
MAPK_1136(2)-C-LUA10	atcacatcacatacaaatctacaAACACCGTGGCCCCGCGC
MAPK_1923-C-LUA88	ttacttcactttctatttacaatcTGAACGACAAGCTGCCAAC
MAPK_1923-G-LUA55	tatatacacttctcaataactaacTGAACGACAAGCTGCCAAC
MAPK_3057-A-LUA59	tcatcaatcaatcttttctacttGCGCCTTCGGAGTCGAT
MAPK_3057-C-LUA43	ctttcaattacaatactcattacaGCGCCTTCGGAGTCGAG
MAPK_3670-INS:G-LUA88	ttacttcactttctatttacaatcGCGGTGCTCGACGGGC
MAPK_3670-LUA55	tatatacacttctcaataactaacCGCGGTGCTCGACGGC
MAPK_3924-CC-LUA43	ctttcaattacaatactcattacaCCAGAAGCTCACCGACGCC
MAPK_3924-INS-LUA59	tcatcaatcaatcttttctacttCCAGAAGCTCACCGACGCG
MAPK_3946-CA-LUA6	tcaacaatcttttacaatcaaatcGGAGACCTACCTGGGGGTG
MAPK_3946-INS-LUA18	tcaaatctcaaatactcaaatcaGGAGACCTACCTGGGGGGT
MAPK_4099-A-LUA6	tcaacaatcttttacaatcaaatcGGCTGCGTGAGCTGTGCTGA
MAPK_4099-G-LUA18	tcaaatctcaaatactcaaatcaGCTGCGTGAGCTGTGCTGG
MAPK_4303-A-LUA10	atcacatcacatacaaatctacaGACGAGGGCCCAAGGTTTTT
MAPK_4303-DEL-LUA22	aatcctttttactcaattcaatcaGACGAGGGCCCAAGGTTTTT
MAPK_4333-G-LUA10	atcacatcacatacaaatctacaGACAACGACGGCACGCG
MAPK_4333-A-LUA22	aatcctttttactcaattcaatcaGACAACGACGGCACGCT
MAPK_4333(2)-DEL-LUA59	tcatcaatcaatcttttctacttTCAACCCGACCTCGACCTG
MAPK_4333(2)-A-LUA43	ctttcaattacaatactcattacaGTCAACCCGACCTCGACCTT

Results and Discussion

All PCR products produced in this study were of the predicted size and demonstrated no non-specific amplification. A total of three multi-plex reactions were developed consisting of two three-plex reactions

and a single duplex reaction (Table 1). Successful multiplex reactions were optimised following primer dilution experiments. By diluting the primer concentration of the preferentially amplified products, a more even ratio of amplicons could be obtained. All other reactions for the remaining loci were conducted as singleplex reactions containing 0.2 μ M each of forward and reverse primer. The SNP assay detected the same genotype as the whole genome sequencing for all isolates in the reference panel. This finding suggests this will be a suitable platform for assessing the genotypes of unknown isolates. There was one locus (MAPK_4333(2)) for which only one allele was observed. This SNP was previously identified in an unpublished study and was demonstrated to occur in a single bovine isolate from Western Australia. DNA from this isolate was unavailable for this study and it therefore remains to verify that this assay truly discriminates this SNP. Nevertheless, this SNP was maintained in this panel in the event that this rare SNP was present in future isolates. In conclusion this paper describes an optimised SNP genotyping platform that can be used to examine genetic diversity of MAP isolates from humans and animals. Our current research is focused on using this platform to genotype a large number of animal isolates from Australia.

Reference

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MAP SPECIFIC SEROREACTIVITY IN THREE DIVERSE EUROPEAN GROUPS OF PATIENTS, WITH AND WITHOUT IBD

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Introduction

Chronic MAP infection is implicated as a contributor in some human inflammatory bowel diseases (IBD). Extensive MAP reservoirs in animals and the environment ensure constant MAP exposure to humans however the epidemiological spread and degree of human reactivity to MAP relative to disease, has not been widely studied. This study evaluates MAP specific antigens screens for MAP serological reactivity in three European populations.

Methods

Sera from adult Dutch (n=573), adult Italian (n=66) and paediatric UK (n=40) patients with and without IBD were screened by combined IgG/M/A ELISAs for seroreactivity to eight MAP specific antigens developed through the ParaTBTools EU 6th program project.

Summary of Data

Five of the 8 MAP antigen ELISA's showed that > 80% of patients with IBD, in both Italian and UK groups representing both adult and paediatric populations, demonstrated significant ($p < 0.0001$) increases in MAP specific seroreactivity over controls. In contrast, 6 of the 8 MAP antigens in the panel tested in Dutch cohorts failed to reach this significance suggesting that either exposure or reactivity within various European regions may be considerably diverse.

Conclusions

ParaTBTools MAP antigen ELISAs may have diagnostic potential to identify MAP seroreactivity associated with IBD patients and be useful to screen for MAP exposure in human populations. The epidemiology of MAP reactivity however appears complex possibly as a result of variability in population exposure.

ANTIBODIES RECOGNIZING *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* EPITOPES CROSS-REACT WITH THE B-CELL ANTIGEN ZINC TRANSPORTER 8 (ZNT8) IN SARDINIAN TYPE 1 DIABETES PATIENTS

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The environmental factors at play in the pathogenesis of type 1 diabetes (T1D) remain enigmatic. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is transmitted from dairy herds through food contamination. MAP causes an asymptomatic infection which is highly prevalent in Sardinian T1D patients compared with type 2 diabetes (T2D) and healthy controls. Moreover, MAP elicits humoral responses against several mycobacterial proteins. We here report that antibodies (Abs) against one of these proteins, namely MAP3865c, recognized two transmembrane epitopes homologous to the β -cell antigen zinc transporter 8 (ZnT8) in 52-65% of T1D patients, but only in 5-7% of T2D and 3-5% of healthy controls. There was a linear correlation between titers of anti- MAP3865c and anti-ZnT8 Abs recognizing homologous epitopes, and pre-incubation of sera with ZnT8 epitope peptides blocked recognition of the corresponding MAP3865c peptides. These results demonstrate that Abs recognizing MAP3865c epitopes cross-react with ZnT8, possibly underlying a molecular mimicry mechanism which may precipitate T1D in MAP-infected individuals.

RECOVERY OF VIABLE *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* (MAP) FROM PASTEURIZED COMMERCIAL MILK IN BRAZIL

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Despite extensive research and large and important advances in the past few decades, the etiology of Crohn's disease (CD) remains indefinite. Due to the similarity of clinical signs and histopathological findings between the CD and paratuberculosis, associations between the two diseases have been made. The most probable transmission route of MAP from animals to humans is milk and dairy products. The aim of this study was to investigate the presence of MAP, by culture, in commercial pasteurized milk samples in the region of Viçosa, Minas Gerais State, Brazil. Forty eight commercial pasteurized milk samples were collected over 12 months. Milk samples (40 ml) were centrifuged at $2,500 \times g$ for 15 minutes and the pellet was suspended in 0.9% HPC. After 24h, the samples were centrifuged again and the pellet was suspended in antimicrobial solution and inoculated on Herrold's egg yolk medium with and without mycobactin J. Colonies similar to MAP was observed in 1/48 (2%) inoculated tube with mycobactin J and no colonies were observed in the correspondent tubes without mycobactin J. IS900 nested PCR was used to confirm MAP from this sample and fragments of similar size to that expected (563bp and 210bp) were observed. The fragment of 210bp was sequenced in triplicate. The genetic analysis revealed 97-99% of identity between the sequences obtained in this study and the MAP strain k-10 sequence deposited in the Genbank. This study provides evidence that MAP is present in commercial pasteurized milk in the Minas Gerais State. This result has become very important since human exposure to MAP is a potential risk for Crohn's disease. This is the first report of MAP presence in pasteurized milk samples in Brazil.

Financial support: CAPES, FAPEMIG and CNPq.

PRESENCE OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) IN HUMAN INTESTINAL BIOPSY TISSUES IN BRAZIL USING IS900 NESTED PCR

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Since the first isolation of MAP from intestinal tissue of human patients bearing Crohn's disease (CD), the possibility of an infectious etiology for CD has always been widely discussed and MAP has attracted the interest of many researchers because of the similarity between paratuberculosis and CD. Conflicting results exist about the presence of MAP in CD tissues. The aim of this study was to investigate the presence of MAP, by IS900 nested PCR, in human intestinal biopsy tissues in the Minas Gerais State, Brazil. Intestinal mucosal biopsies were collected from a total of 20 patients, comprising 4 with CD, 7 with ulcerative colitis (UC) and 9 with non-inflammatory bowel disease (IBD). MAP was identified in 1/4 (25%) patients with CD, in 1/9 (11%) patients with non-IBD and in 0/7 patients with UC, by amplified fragments of similar size to that expected (563bp and 210bp). Sequencing of fragments of 210bp confirmed the presence of the MAP: the genetic analysis revealed 97-99% of identity between the sequences obtained in this study and the MAP strain k-10 sequence deposited in the Genbank. Like other reports in this field, MAP was detected more frequently from patients with CD compared with those with UC or non-IBD in the present study. These data contribute to the evidence that MAP might be associated to CD, however, the pathogenic role of MAP in CD remains controversial and inconclusive. This is the first report of MAP DNA presence in human intestinal biopsy tissues in Brazil.

Financial support: CAPES, FAPEMIG and CNPq.

DETECTION OF MAP IN ENVIRONMENTAL SAMPLES OF DAIRY HERDS IN SOUTHERN CHILE

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Paratuberculosis is widely distributed in dairy cattle in southern Chile (1). ELISA test and faecal culture are the two more frequently diagnostic tests used. The aim of this study was to determine the infection status of dairy herds by culture of environmental samples. Duplicate environmental samples from three different locations were collected in 20 dairy herds (120 samples) of southern Chile following the procedure recommended by the USDA (2). To detect the presence of MAP, the liquid culture medium 7H9-AODC and the automated BACTEC MGIT 960 system were used. Those samples signaling positive in the BACTEC system were confirmed through the PCR test using specific primers for MAP (IS900). In addition, all MGIT positive tubes were subcultured on solid medium (HEYM w/M J and ANV) and suspicious colonies further confirmed by IS900. The BACTEC MGIT 960 system detected 64 (53.3%) positive samples of which only 30 (46.9%) were confirmed as MAP by the PCR test. Of the 64 MGIT positive tubes, 33 (51.6%) developed typical MAP colonies on HEYM but only 26 (78.8%) were PCR confirmed as MAP. Altogether 33 environmental samples resulted positive for MAP, 10 (30.3%) from cow alleyways, 14 (42.4%) from common areas, and 9 (27.3%) from manure storage pools. MAP was detected in 9 (45%) of the 20 dairy herds examined. These results confirmed that paratuberculosis is widely distributed in dairy herds of southern Chile and that culture of environmental samples is a good and a cost effective alternative to the conventional faecal culture to determine the infection status of a herd. Combining liquid and solid culture can enhance the sensitivity of the BACTEC to detect MAP in environmental samples. (Project DID/UACH Nr I-2009-02).

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PERFORMANCE OF A PEPSIN-HCL DIGESTION PROTOCOL FOR THE DETECTION OF MAP IN RAW MEAT

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Introduction

A reliable and sensitive method for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) detection in muscle would be an important tool to measure the level of humans exposure via cattle meat. Pepsin-HCl digestion may release MAP and consequently result in higher sensitivity of culture, but may affect PCR negatively. The objective of this study was to establish a culture method with improved sensitivity and specificity without affecting the sensitivity and specificity of PCR.

Methods

To evaluate relative analytical sensitivity of the new protocol we used cheek muscle from a single cow from a herd considered as free of paratuberculosis and from a seropositive, high-shedder animal. For the diagnostic sensitivity evaluation, we examined cheek muscles from 48 cow carcasses randomly selected at a slaughterhouse. All samples were analysed for the presence of MAP by culture and IS900 realtime PCR. For the analytical sensitivity we prepared and tested 5 replicates of undiluted, 1:10 and 1:100 dilutions of infected masseter muscle in un-infected muscle. Furthermore, field samples were prepared and tested in duplicate. In brief, specimens were trimmed in 4 aliquots of 3g. Samples were processed as recently described in a prevalence study (1) as the traditional method and including a digestion step in Pepsin (0.5%) and HCl (0.2%) at 37°C for 30 min. For digested samples the procedure was modified as follow: after digestion and centrifugation at 3000g/30', the pellet was resuspended in saline solution and adjusted to pH 7.0 (at this point a subsample was collected for direct PCR). Samples were then decontaminated and cultured in 7H9+ liquid medium followed by PCR testing of 200µl at 2, 4, 6 and 8 weeks. Direct PCR results were compared with the standard and new culture methods.

Results

Analytical sensitivity

In laboratory-prepared samples (Table 1) we determined that the Pepsin-HCl digestion was significantly more sensitive than the previous method on 7H9+ culture, allowing the detection of at least ten fold lower MAP amount. Furthermore, results of the new method were available much earlier, having a positive PCR result at the same MAP concentration two weeks in advance. Moreover, all samples tested negative to MAP presence by direct realtime PCR.

Table 1. Frequency of MAP detection in 10-fold dilutions of naturally-infected meat samples processed by pepsin-HCl or traditional method and tested by realtime PCR at 2, 4, 6, and 8 weeks of culture (between brackets: mean Ct value and SD).

Dilution	Pepsin-HCl digestion				Traditional method			
	Weeks of culture				Weeks of culture			
Infected/ Uninfected	2	4	6	8	2	4	6	8
1:1	3/5 (35.1±0.9)	5/5 (25.0±2.8)	5/5 (21.6±1.6)	5/5 (20.5±0.6)	0/5	1/5 33.6	2/5 (26.6±1.8)	2/5 (19.1±1.9)
1:10	0/5	2/5 (25.0±1.7)	5/5 (22.9±2.0)	5/5 (20.0±1.7)	0/5	0/5	0/5	0/5
1:100	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Diagnostic sensitivity

One of the samples was positive by the standard culture protocol, and a different one by the new method. None of these were positive by PCR. The old protocol resulted in 7/48 PCR positive and the new protocol in 6/48 positive. Agreement between repeated PCR runs were: $\text{Kappa}_{\text{Old}}=0.20$ (95% CI: 0; 0.60); $\text{Kappa}_{\text{New}}=0.45$ (95% CI: 0; 0.91). Agreement between parallel interpretation of the old and the new PCR method was: $\text{Kappa}_{\text{OldNew}}=0.20$ (95% CI: 0; 0.56). Both culture methods resulted in 2/48 positive samples.

Table 2: Direct PCR results in raw meat of regularly slaughtered cows processed by Pepsin-HCl or traditional method

		Pepsin-HCl		
		Positive	Negative	Total
Traditional	Positive	0	7	7
	Negative	6	35	41
	Total	6	42	48

Discussion

Despite the absence of significant differences in results from clinical samples, possibly due to very low contamination levels in regularly slaughtered cattle, we detected a higher analytical sensitivity introducing the Pepsin-HCl digestion step over the standard culture method. In addition, we obtained a marked reduction in time to detection of the pathogen, indicating that Pepsin-HCl digestion is effective in releasing MAP cells from the tissue without affecting its viability. PCR results did not appear to be influenced by the inclusion of the Pepsin-HCl digestion step. These results were probably due to the lower sensitivity of the direct PCR against culture in 7H9+, as previously demonstrated for faecal samples. In conclusion, we demonstrated that MAP detection by culture can be improved by Pepsin-HCl digestion, as previously shown in sheep (2). This treatment is widely used for the detection of *Trichinella* spp. in muscle. It is easy to perform at low cost and therefore suitable for MAP detection in beef samples.

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MICRO-FILTRATION TREATMENT OF BOVINE COLOSTRUM INFECTED BY *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP): PRELIMINARY RESULTS

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Colostrum administration can be an important critical point in the transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to the calf.

MAP is detected both in colostrum and milk of infected cows.

The calf is highly susceptible to the disease because of the immature immune system and the favourable habitat to MAP survival.

Therefore, colostrum decontamination protocols are badly needed for regular colostrum administration to calves in order to rule out the risk of MAP infection.

In this study we describe the validation of a decontamination protocol based on microfiltration of MAP-infected colostrum.

Colostrum quality was tested in terms of immunoglobulin content before and after the treatment.

The sterilizing microfiltration protocol adopted in this study was shown to remove MAP at higher concentrations than those usually present in bovine colostrums without a significant reduction of the immunoglobulin content.

ASSOCIATION BETWEEN HERD INFECTION LEVEL AND THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) IN BULK TANK MILK USING REAL-TIME PCR IN SMALL HOLDER DAIRY FARMS IN SOUTHERN CHILE

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In southern Chile, a social important spectrum of dairy producers is categorized as subsistence farmers and most of their cows are fed with direct milk consumption from their dams for at least six month. Since variable *Map* quantities have been informed in milk of cattle affected both clinically and subclinically (Giese and Ahrens, 2000), direct detection of the bacterium in milk represents a diagnostic opportunity and a sensitive PCR using milk samples could be an attractive alternative.

The study was carried out in 132 small dairy herds in southern Chile. To determine herd infection level, individual fecal samples were collected from 2,385 lactating cows. In the laboratory, the individual fecal samples were pooled by 5 for *Map* culture detection using the BACTEC MGIT 960 system. In parallel, milk for PCR testing was sampled from the milk buckets or bulk milk tank from each herd. The *Map* DNA extraction procedure for PCR on milk consisted in centrifugation, pooling of pellet and cream fractions to be subjected to enzymatic digestion plus the use of a commercial DNA extraction kit based on mechanical disruption, proteinase K, and column purification. The procedure was followed by real-time PCR. *Map* was culture-detected in 25% of the herds. Within the infected herds, distribution of pool test-positive results varied by herd ranged between 70-100% (high herd infection level), and 30-69% (moderate), and 12-29% (low). All herds categorized as high and medium herd infection level showed positive milk PCR results (Table 1).

Table 1. Real time PCR results with milk samples related with herd infection status

HERD	N° ANIMALS	POOLS	POSITIVE/TOTAL	INFECTIOUS STATUS	INFECTION LEVEL	REAL TIME PCR
1	40	8	3/8	INFECTED	MODERATE	POSITIVE
74	35	7	7/7	INFECTED	HIGH	POSITIVE
84	18	3	2/3	INFECTED	MODERATE	POSITIVE
88	6	1	1/1	INFECTED	HIGH	POSITIVE
126	23	5	4/5	INFECTED	HIGH	POSITIVE

The results of the study are consistent with what has been informed in the literature. In this regard, sick clinical cows could shed about 100 CFU/mL in milk; meanwhile subclinical animals could shed between 2 to 8 CFU/50 mL or even less (Giese and Ahrens, 2000). However, the fecal contamination of the teats could represent the most important presence in milk, which depends on the amount of *Map* shed by fecal material, as well as the hygiene management (Herthnek, 2008).

Although the dilution effect should be taken in mind, the ability of the real-time PCR using milk samples to detect *Map* genome in bulk tank is directly related to the level of *Map* herd infection. More epidemiological studies are needed in this regard. Milk PCR could become a practical sample to identify the most infectious animals in a herd.

Milk samples for Real Time PCR could represent a practical type of sample for identification of those herds with a high level of infection and the most infected animals within them.

ACKNOWLEDGMENTS

This study has been funded by an UACH-COLUN agreement.

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DOES SOIL SLOPE FAVOR MOVEMENT OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP) IN GRASSLAND SOIL?

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Details regarding the fate of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) after manure application on grassland are unknown. Soil type is a major factor influencing microbial transport and attachment in soils. This is partly due to differences in absorptive properties of soil colloidal materials, where organic matter and clay particles have the greatest effects on movement, as a result of microbial adsorption to negatively charged surfaces. It has been shown that faster microbial movement occurs in coarse soil with large pore spaces than in finer textured soils, where pore size is significantly smaller. Bacterial surface properties also influence bacterial movement or attachment to soil particles, where cell surface charge and hydrophobicity allow reversible attachment to soil particles through electrostatic forces. The majority of studies investigating bacterial movement in soil have been carried out using intact soil and disturbed soil in lysimeters, the former being more accurate in predicting pathogen movement under natural soil conditions. In the case of *Map*, two studies, both attempting to study the transport of this pathogen through saturated aquifer (Bolster *et al.*, 2009) and the adsorption of the bacteria to soil particles, have been published (Dhand *et al.*, 2009). In both studies, factors affecting organism transport and attachment were analyzed in columns of specific soil types. More recently Salgado *et al.*, (2010) reported the first study under field conditions on the fate of *Map* in soil after dairy manure application. It was concluded that the bacterium moves slowly through soils, though faster through sandy soils as compared to loamy soils. *Map* may be adsorbed to soil particles and adsorption is possibly adversely affected by a more acidic soil pH. The organism tends to remain on grass and in the upper layers of pasture soil, representing a clear hazard to grazing livestock for infection transmission.

Information available on the fate of *Map* and its transport in soil indicates that the bacterium attached to soil particles might be retained in the upper soil layers, rather than being leached through the soil. Thus, soil slope then becomes an important parameter to understand the risks of *Map* dispersion after dairy cattle manure application and direct animal grazing. To evaluate the effect of soil slope on *Map* dispersion to surface water after application of contaminated manure, a field experiment design consisting of 6 plots (1x2 m) with two different slopes was assayed (3% and 15%). The soil surface in each plot was inoculated with spiked cow manure (equivalent rate of 70 t ha⁻¹) containing >10⁶ CFU of *Map*/g. After a natural rainfall event equivalent to 40 mm, water moving along the soil surface in favor of the slope (surface runoff) was collected using PVC tubes located on the two lower (downhill) sides of the plot, at surface level but protected from direct rainfall.

Six control plots no manure application was included. Runoff water samples were taken from all plots for *Map* detection using the BACTEC MGIT 960 system, followed by real-time PCR confirmation. *Map* concentration in runoff water as well as the time to detection (TTD) in the culture system was assessed by methods described by Sung *et al* (2007). Only water runoff samples from all manure-treated plots were *Map* positive. *Map* concentration and TTD data reported by the MGIT 960 system in runoff water were highly correlated to the slopes levels, where the 15% slope showed on average a higher *Map* concentration and a shorter TTD. This data confirm that an increasing soil slope results in a greater movement and dispersion of *Map* in the environment after application of contaminated manure.

ACKNOWLEDGEMENTS

This study has been funded by FONDECYT, project N° 11100200.

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MOLECULAR AND SEROLOGICAL PREVALENCE OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN FIRST LARGE SCALE SCREENING OF HUMAN POPULATIONS AND ASSOCIATION WITH VARIOUS PATHOLOGICAL AND PHYSIOLOGICAL CONDITIONS

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Molecular and serological prevalence of *Mycobacterium avium* subspecies *paratuberculosis* and its association with pathological conditions was studied in the human population of Agra and Mathura region of Uttar Pradesh. In all 10101 clinical samples (3263 blood, 6737 serum and 101 stool) were collected daily for 173 days (23rd Dec., 2010 to 14th June, 2011) from 14 pathology laboratories of Agra (8) and Mathura (6). Samples were screened by PCR, Indigenous ELISA kit and microscopy. IS900 PCR positive DNA were genotyped using IS1311 PCR-REA. Of 1849 and 1414 blood samples, 8.4 and 6.9% were positive in IS900 PCR from Agra and Mathura regions, respectively. While of 1492 and 5245 serum samples 14.7 and 27.4% were positive for Anti-MAP antibodies by ELISA kit from Agra and Mathura regions, respectively. Of 101 stool samples 5.9% were positive for acid fast bacilli and were also confirmed in IS900 PCR. Genotyping of representative IS900 PCR positive DNA showed 'Indian Bison Type' as most prevalent genotype in the region. Of various pathological conditions for which samples were submitted, 21.2% diabetic, 22.7% thyroid, 33.6% TB and 41.9% typhoid patients were positive for anti-MAP antibodies. While 4.1% diabetic, 7.4% liver disorder and 12.8% of normal individuals were positive in IS900 PCR. MAP was reported in equal percentage in male and female population however, it was more prevalent in the age group above 40 years. Presence of MAP in pathological conditions and normal individuals indicated exposure to MAP.

DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN CHILD FOOD OF ANIMAL ORIGIN

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Introduction

Paratuberculosis is a chronic disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) an acid fast, aerobic intracellular bacillus (Ayele et al., 2001). The disease is manifested by weight loss and intermittent diarrhoea caused by chronic granulomatous enteritis. Though paratuberculosis is not considered a zoonotic disease, many studies indicate an association between the pathogenesis of Crohn's disease of man and human exposure to MAP (Chamberlin et al., 2001; Hermon-Taylor & T. Bull 2002). The specific pathogen has been detected in a large variety of food products whereas its ability to survive pasteurization has been confirmed by many groups (Hammer et al 2002; Grant IR et al 2002; O'Reilly et al., 2004; Shankar et al., 2009; Ellingson et al., 2005; Ayele et al., 2005).

Because of the concern associated with the possibility of the association of human exposure to MAP and the causation of Crohn's disease in addition more recently, to other diseases of autoimmune nature (Schwartz, 2000; Hermon-Taylor, J., 2000; Bull, T. J., 2003), we investigated for the first time, the presence of the specific pathogen in selected food products of animal origin, aimed for consumption by children in Greece.

Materials and Methods

The sampling plan was designed to include the maximum possible number of the most popular brands (commercial labels) and/or types of products. Within this context we collected seven hundred and fifty samples of pasteurized milk (n=100), yoghurt (n=100), cheese (n=100), cold cuts (n=100), desserts/canned food (n=100), ice creams (n=100), bakery products (n=100) and powdered milk (n=50), from 14 retail points of Attica, Greece. Samples were collected and tested between October 2010 to July 2011. The samples were homogenized by stomacher blender (Kleinfeld Labortechnik, Germany). A portion of 25 gr was collected from the core of each sample (cheese, cold cuts, bakery products), 10g (yoghurt, desserts/canned food, ice creams), 50ml of pasteurized milk and 200mg of powdered milk was used for DNA extraction using chemical, (Nucleospin Food, MACHEREY-NAGEL) and mechanical lysis using glass beads, (SIGMA). The product was incorporated into a Real Time PCR for the detection of IS900 using TaqMan technology (ROCHE) in LIGHT CYCLER 2.0 ROCHE, (Kim et al., 2002, 2004).

Results

Only five of the 750 samples tested (0.66%) reacted positive by RT-PCR. Of these, 2 corresponded to samples of yoghurt (2%), 1 of milk (1%), and 2 (2%) of cheese.

Conclusion

The level of positivity of the targeted food samples to MAP in Greece was relatively small. The respective percentage in other countries varies from 0% Canada and Ireland, (UK (1.7%), Czech (1.6%), Argentina (5.5%) and in India, (66%) in similar types of food, (Grant IR et al 2002; O'Reilly CE 2004; Shankar H 2009; Ellingson J 2005; Ayele W 2005). An association with a specific product label could not be established since the samples that produced a positive result were of different brands. It can be concluded that human exposure to *M. avium* subsp. *paratuberculosis* through the consumption of food products of animal origin aimed for consumption by children is probably not significant.

ISOLATION OF *MYCOBACTERIUM* SP. FROM PATIENTS WITH INFLAMMATORY BOWEL DISEASE

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Abstract

The long held belief that a mycobacterial sp. is responsible for Crohn's disease is based on a series of factors. These include the similarity in appearance of Crohn's disease to intestinal tuberculosis and Johne's disease of cattle. *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative organism of the latter. Debate exists over the role of *M. paratuberculosis* in Crohn's disease as the organisms are difficult to culture and distinguish from other *Mycobacteria* sp.

Aim: To obtain isolates of *M. paratuberculosis* in human cases of Inflammatory Bowel Disease (IBD). All intestinal mucosal biopsies of patients presenting with IBD were crushed before spreading onto solid Middlebrook 7H10 media. Any isolates were identified by microscopy and PCR screening for *IS900* and *f57* markers. A total of 69 samples have been processed over a year. Five of the initial slopes have showed growth after 8 - 40 weeks of incubation. Three isolates are from patients with UC and two are from those with Crohn's disease. The first isolate is an acid fast rod with a rough colony appearance that is mycobactin independent. Further analysis revealed the presence of *IS900* and *f57*, both markers for *M. paratuberculosis*. Sequence analysis of these markers confirmed this isolate to be *M.*

paratuberculosis. The second isolate was also an acid fast rod that formed chains with a smooth colony appearance. More material is required for further analysis on this isolate. The three remaining isolates were not acid fast and had rods to coccoid appearance. These isolates will be characterised further once more confluent growth is obtained. The *M. paratuberculosis* isolate reported here represents a rare isolate obtained from the human gut. Of the unidentified isolates, the colony morphology, length of required incubation and acid fast stain indicate a mycobacterial species. Further characterisation of these human isolates, particularly comparative analyses to bovine isolates of *M. paratuberculosis*, may determine the possible pathogenic role of gut mycobacteria in humans.

Introduction

The long held belief that mycobacterial sp. are responsible for Crohn's disease is based on many factors. These include the similarity in appearance of Crohn's disease to intestinal tuberculosis and Johne's disease of cattle¹. *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative organism of the latter². Debate exists over the role of *M. paratuberculosis* in Crohn's disease as the organisms are difficult to culture and distinguish from other *Mycobacteria* sp.

Therefore our aims were to identify the occurrence of *M. paratuberculosis* in human cases of Inflammatory Bowel Disease (IBD).

Methods

Intestinal mucosal biopsies of patients presenting with IBD were crushed before spreading onto solid Middlebrook 7H10 media. The slope was enriched with a sterile supernatant of a growing culture of bovine *M. paratuberculosis*. Slopes were left for 18 months at 37°C. Cultures were identified by microscopy and PCR screening for *IS900* and *f57* markers. In addition, 30 biochemical tests were performed on growing isolates. Antibiotic MIC profiles were determined using the agar dilution method.

Results and Discussion

Over a year, 69 samples have been processed. Five of the initial slopes showed growth after 8 - 40 weeks of incubation. Three isolates were from patients with Ulcerative Colitis & two from patients with Crohn's disease. The first isolate (43525) is an acid fast rod. It has a rough colony appearance & is mycobactin independent on Middlebrook 7H10 agar but not when tested on Lowenstein-Jensen media. Further analysis revealed the presence of *IS900* & *f57*, both markers for *M. paratuberculosis*. Sequence analysis of these markers confirmed this isolate to be *M. paratuberculosis* (Table 1). The second isolate (44796) was also an acid fast rod, 0.46-1.22 µm in length by 0.23 & 0.46 in width that formed chains with a smooth colony appearance. The three remaining isolates were not acid fast & had a rod to coccoid

appearance. Unfortunately, these isolates & 44796 did not grow upon subculture & therefore further testing was not possible.

The MIC ($\mu\text{g/mL}$) was different for 43525 compared to ATCC19698 for ethambutol, clarithromycin, clofazamine & streptomycin. Further, the MIC for streptomycin was influenced by the presence of mycobactin J, although this isolate appears to be mycobactin independent when grown on this media (Figure1).

Table 1: 30 Identification tests were compared, this table shows a selection of these. Isolate 42535 was initially tested on Middlebrook 7H10 agar and found to be independent (see figure 4), but was verified on Lowenstein-Jensen media & was found to be dependent.

Test	43525	ACTC19698
Acid Fast stain	acid fast bacilli	acid fast bacilli
Colony morphology	Rough	smooth
Growth Rate 1^o culture	40 weeks	NA
Growth 2^o culture	4-8 weeks	4-8 wks
Lowenstein-Jensen Growth	+	+
Catalase >45	-	-
Thermostable catalase	present	minimal
IS900	+	+
IS900 sequence	100%	100%
F57	+	+
F57 sequence	100%	100%
Mycobactin dependent	yes*	yes

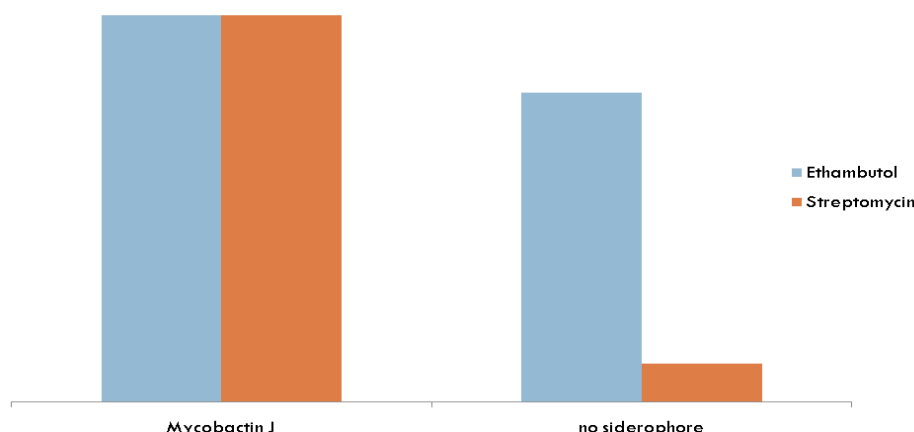


Figure 1: The MIC values of 43525 when grown with and without mycobactin J

Conclusion

The *M. paratuberculosis* isolate reported here is a rare pure isolate from the human gut. Of the unidentified isolates, the colony morphology, length of required incubation & acid fast stain, indicate a mycobacterial species. Further, characterisation of these human isolates, particularly comparative analyses to bovine isolates of *M. paratuberculosis*, may determine the role of gut mycobacteria in humans. The role of mycobactin in identification of this species appears to be media specific & has an impact on antibiotic susceptibility.

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Acknowledgements

We wish to acknowledge R Chiodini for discussion on mycobactin dependency .

EFFECTS OF PARATUBERCULOSIS VACCINATION ON TUBERCULOSIS INFECTION IN GOATS

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Most countries subjected to eradication campaigns of bovine tuberculosis (TB) have imposed a ban on the use of mycobacterial vaccines in cattle. However, *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-based vaccines are often allowed in small ruminants. The aim of the study presented herein was to assess the effect on current TB diagnostic tests in MAP-vaccinated goats before and after TB-infection with *Mycobacterium caprae*. Additionally, the existence or not of cross-protection in front of TB due to the vaccination was also evaluated.

To study these effects, 10 goats were vaccinated against MAP (week 0) and 10 were maintained as unvaccinated control group. All animals were challenged with *Mycobacterium caprae* at week 14. Simple and comparative skin tests were performed at weeks 0, 14 and 26, whereas interferon- γ (IFN- γ) assays by using different antigens were performed throughout the experiment. Goats were sacrificed at week 28. Subsequently, pathology scoring and bacterial enumeration were calculated.

The standard IFN- γ assay using avian and bovine tuberculins, showed a predominant avian response in the vaccinated group at weeks 4 to 12, but 60% of animals were bovine reactors at week 14, however avian reactors returned at week 16. The challenge with *M. caprae* changed radically the IFN- γ response, which showed predominant bovine reactors at weeks 18 to the end. Cross-reactions with bovine tuberculin observed in the vaccinated group at week 14 disappeared in 100% of cases when using the *M. tuberculosis* complex-specific antigens ESAT-6/CFP-10 and Rv3615c used as DIVA (Differentiation of Infected and Vaccinated Animals) reagents, and the sensitivity of the assay was maintained. Regarding the comparative skin test performed at week 26, 90% of the animals reacted positively. Additionally, postmortem analysis indicates a slight cross-protection in terms of reduction of pathology degree and bacterial burden in some animals and specially in containing extrapulmonary dissemination.

The results suggest a partial protection due to the vaccine, and a low interference on current TB diagnosis which disappears completely when using new DIVA reagents.

CLINICAL INVESTIGATIONS ON PARATUBERCULOSIS IN CAMELS IN AL-HASA, KSA

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The aim of this study is to focus on paratuberculosis as one of the most serious diseases affecting camels causing large losses in milk and meat production with special reference relation between age and clinical signs.

Methods used in this study are the general parameters of clinical examination, staining of intestinal smears with Ziehl–Neelsen stains and histopathology. ELISA test for serology. Data, at the Veterinary Teaching Hospital of the college of Veterinary Medicine and Animal resources, king Faisal University 200 camels (136 females and 64 males) were found clinically diseased suggesting paratuberculosis. The clinical signs observed were decreased milk production (60 %), intermittent diarrhea (98 %), dehydration (75%), emaciation (96%), intermandibular edema(46%). Gross pathological examination of these cases revealed thickening of the intestinal wall up to three or four times normal thickness, with corrugation of the mucosa which extend in some cases to the rectum. Mesenteric lymph nodes were moderately large and oedematous. The diagnosis was confirmed by staining rectal smears by Ziehl–Neelsen stains. Histopathological lesions were diffuse granulomas characterised by extensive macrophages and epithelioid cells infiltration into the mucosa and submucosa of small intestine, and colon, with numerous acid-fast organisms. Multinucleated giant cells as well as lymphocytes and few number of eosinophils were also observed. The ileocecal lymph node as well as the other mesenteric lymph nodes showed sinus histiocytosis, infiltration of macrophages and epithelioid cells containing acid-fast bacilli. ELISA test was another mean for diagnosis confirmation.

Conclusion: Paratuberculosis in camels increasing with time rapidly that needs a rapid solution, severe clinical disease appear to found in camels aged 9-18 months. Further studies are needed such as isolation and molecular characterization in order to set a suitable prevention and or vaccination program.

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HETEROGENEITY OF SUBSPECIES *MYCOBACTERIUM AVIUM* *PARATUBERCULOSIS* FROM GENOTYPE TO PHENOTYPE

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Background – In the subspecies *M. avium* ssp. *paratuberculosis* (Map) two groups, known as Cattle (C) and Sheep (S), have been defined by genotyping. Recent studies show that Map C and S have different phenotypes with respect to infection of macrophages and iron metabolism. Map is adapted to the gastrointestinal tract of ruminant, but the mechanism of entry is currently unknown. In this study, we investigated the phenotype of the Map-host interaction, involving the virulence factor heparin-binding hemagglutinin (HBHA), for both groups of Map. HBHA is described in *M. tuberculosis* as a major adhesin required for extrapulmonary dissemination of the tubercle bacillus. Method – A large collection of Map isolates (types C & S) were genotyped by MIRU-VNTR and RFLP-*IS900*. The polymorphism of the *hbha* gene was investigated by fragment analysis using GeneMapper technology. Structure-functions properties of recombinant HBHA (types C & S) were analyzed by Heparin- Sepharose chromatography and SPR analysis based on Biacore technology. Results – In silico analyses of both types of Map have revealed two forms of *hbha*. This observation, showing that *hbha* is distinct according to the group, was confirmed using GeneMapper on 83 Map strains (65 Map C & 18 Map S) with various genotypes. We found that Map type C produces HBHA with a short C-terminal domain, while that of type S presents a long C-terminal domain, similar to that of HBHA produced by *M. tuberculosis*. The purification of HBHA from Map type C and S by Heparin-sepharose chromatography highlighted a correlation between their affinities to heparin and the length of their C-terminal domain confirmed by Biacore analysis. Conclusion – We show for the first time that the types C and S of Map may be distinguished by the type of HBHA they produce, which differs in size and adherence properties. Thus, HBHA participates in the genotypic and phenotypic differences observed between the C and S types of Map.

11-ICP SUMMATION

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At the invitation of 11-ICP organizers, I provided the summation lecture as the last presentation of the four day meeting. I attended the sessions, discussed scientific reports with colleagues, and assembled my thoughts as a PowerPoint presentation which is available at <http://johne.org>. This text was written after the presentation to complete the 11-ICP Proceedings. The thoughts expressed here are my own and not those of the International Association for Paratuberculosis, the University of Wisconsin or any other organization with which I am affiliated. I highlight a few key research papers from the meeting because I think they are important to the science of paratuberculosis and to the main message of my summation. I apologize to the many other excellent presenters, posters and abstracts that I did not have time to mention.

Members of the paratuberculosis scientific community, affected animal producers, and veterinary practitioners are deeply indebted to Dr. Richard Whittington, and the many committee members who diligently worked to organize and orchestrate this high caliber meeting. We are also very thankful for the financial support provided by the many commercial sponsors, in particular the 11-ICP Platinum Sponsors: Animal Health Australia, New South Wales Department of Primary Industries, and Pfizer Animal Health. The Conference Secretariat, Concept Event Management, did a wonderful job of meeting coordination and technical support.

Fueled by large multi-disciplinary, multi-agency, and multi-national projects such as the Johne's Disease Integrated Project (JDIP) in the U.S. and ParaTB Tools in the E.U., the past decade has witnessed a surge in paratuberculosis research effort and publications. A literature analysis by Kaevska & Hruska (*Veterinari Medicina* 55:43-54, 2010) found that 5,149 authors from 1,465 institutions authored 2,305 papers published from 1995 to 2009. Veterinarians, animal producers, and society at-large should rightfully ask what all this work has accomplished, beyond advancing our careers.

What do our "customers" see?

- Increasing numbers of MAP-infected animal species.
- Increasing numbers of MAP-infected herds and flocks.
- Increasing numbers of MAP-infected countries.
- Increasing numbers of MAP-contaminated foods of animal origin.

To be fair, the paratuberculosis research community can justifiably claim:

- Sequencing of multiple MAP genomes.
- Improved knowledge of MAP pathogenesis details.
- New and validated diagnostic tests for MAP infection.
- Elegant computer models of MAP spread within animal populations.
- Greater understanding of the epidemiology and ecology of MAP.
- Reams of educational materials for producers; in print and on the web.
- Thousands of publications.

Obviously, I am being intentionally provocative. However, I believe that there is a fundamental reason underpinning the huge gap between the abundance of research products and our failing efforts at MAP containment. I call this, the "ParaTB Enigma": We do not acknowledge MAP as a zoonotic pathogen, and yet we behave as if it IS a zoonotic pathogen. Specifically, we use the zoonosis issue to justify eradication campaigns; to justify national control programs; as leverage when asking governments to subsidize control programs; and as a basis for expecting owners of low-prevalence herds make sizeable investments to control a problem that can't be economically justified. By "we" I mean to include researchers, producers, processors, regulators, and everyone dealing with paratuberculosis. The zoonosis question is the "elephant in the room" that nobody wants to candidly discuss. And yet, it is the primary issue driving much of what we do.

Failure to acknowledge MAP as a zoonotic agent has consequences readily illustrated with data on the Johne's Disease Voluntary Control Program in the U.S. Over the past decade, the U.S. has spent

roughly US\$166,384,000 in efforts to control paratuberculosis in cattle on the premise that it was an important animal health problem. No mention was made openly (to producers in particular) that it might be a zoonosis. In the years when funds were plentiful and freely flowed to producers, U.S. cattle herd owners enrolled in the program. Then, as funds gradually diminished, the number of participating herds also diminished (data provided by the USDA-APHIS-VS). My assessment of this is that producers do not, in fact, see paratuberculosis as a production-limiting disease, i.e. one worth investing money to control, except in herds where the MAP infection prevalence has reached high levels.

Two countries are providing interesting national control experiments for dairy cattle herds using a somewhat different approach. Denmark's "Operation Paratuberculosis" began in earnest in 2006. The program has been transparent with the public about the zoonotic potential of MAP and the program is driven by a producer-processor partnership, The Danish Dairy Board. This agency is privately funded rather than being government (tax payer) subsidized. Early uptake by producers is encouraging. However, while motivated by the zoonosis concern, the Danish program fails to exclude MAP-infected cows, or their milk, from the food supply. It also fails to provide financial incentives, premiums for milk, to producers with low MAP prevalence or test-negative herds. Although Danish dairy producers might be more altruistic than those in the U.S., I predict that in time Danish dairy farmers will see this program as a financial drain on their business with little or no compensation and gradually drop out of the program as did their American counter-parts.

The Canadian program, first launched in the province of Ontario in 2010, is similar to the Danish program. It is focused on dairy cattle herds and relies heavily on the milk ELISA for MAP infection diagnosis. The funding model is somewhat different with program costs primarily shared by producers and the government. The major difference between the Canadian and the Danish program, however, is that cows detected as "high ELISA-positive" are "permanently removed" from herds, i.e. they do not enter the food chain nor can they cycle back into other dairy herds. These are bold steps forward, in my opinion, because this comes closer to acknowledging MAP as a potential zoonosis, and it invokes an action consistent with this acknowledgement.

As the MAP epidemic continues expanding, affecting ever larger numbers of animals, herds and countries, a steady rise in the MAP bioburden in foods of animal-origin is inevitable. How much longer can we let this continue? Prior published research has previously demonstrated MAP in infant formula products of multiple manufacturers in the E.U. At the 11-ICP, Botsaris et al. (p. 85) from Cypress reported testing 35 samples of infant formula from 11 producers finding that 9.4% contained live MAP (culture-positive samples) and 21.9% had MAP DNA (PCR-positive indicating live or dead MAP). Given the high infection susceptibility of neonatal animals to MAP infection and the growing frequency of finding MAP in patients with Crohn's disease, including pediatric cases, it seems to me that immediate actions are warranted to insure infant formula is produced only from milk originating from MAP-free animals.

Adding to the urgency of the infant formula concern is the finding by Dr. Momotani (p. 379) in Japan who, using a mouse model, showed that MAP antigens alone (dead MAP) can induce intestinal inflammation closely resembling Crohn's disease. These data, if confirmed by other investigators, indicate that food manufacturers cannot make MAP-contaminated raw milk safe for human consumption by heat treatment alone.

Pasteurization has been the manufacturing process the dairy industry most heavily depends on to kill human pathogens that may be found in raw milk. Although heretofore considered controversial, most laboratory studies have shown that MAP is harder to kill with heat than other milk-borne pathogens. Multiple studies, the first published in 2002, attempting isolation of MAP from retail HTST pasteurized (72C x 15 sec) milk have recovered viable MAP. At the 11-ICP, yet another study (poster by Carvalho et al. from Brazil) reported that 2.7% of retail HTST milk samples tested MAP-positive by culture. This makes a total of five independent studies all from different countries indicating that retail HTST-pasteurized milk harbors viable MAP. Studies from India indicate alarmingly high rates of MAP recovery from retail milk, i.e. 67% of samples.

Elise Lamont, from the laboratory of Sri Sreevatsan at the University of Minnesota, reported findings that explain the ability of MAP to resist killing by pasteurization. The Minnesota research team convincingly demonstrated that MAP produces spores; bacterial forms well-known to resist killing by physical and chemical factors. Their findings appear both in the 11-ICP Proceedings (p. 257) and in the January issue of PLOS One. Spore formation nicely explains the much earlier results of Phillip Hammer et al. from the Institute for Dairy Chemistry and Technology in Kiel, Germany who in 2003 reported that

MAP could withstand temperatures up to 90C for 60 seconds (P. Hammer et al. 2002. Kieler Milchwirtschaftliche Forschungsberichte 54:275-303; in my opinion, the most extensive investigation of MAP's ability to survive pasteurization under commercial conditions). The spore-forming ability of MAP, if confirmed by others, radically changes food industry strategies for insuring food products are free of MAP. On-farm control efforts and herd certification become even more vital, just as described by TAFS in its position paper on paratuberculosis <http://www.tafsforum.org/paratuberculosis.html>.

The chain of evidence connecting MAP in animals to human disease is strengthening. Over the past decade, viable MAP have been reported in infant formula, pasteurized retail milk, cheese, and muscle meat from infected animals. Epidemiological studies in Japan have shown that the alarming rapid rise in incidence of Crohn's disease there is associated with a change in diet characterized by greater intake of animal and milk protein. Using more refined diagnostic tests, MAP is consistently found in patients with Crohn's disease more often than controls. Several studies report that Crohn's patients are more often serologically positive for MAP than controls. At the 11-ICP, Ingrid Olsen (p. 383) demonstrated that Crohn's patients harbor MAP-reactive CD4 T-cells, indicating that MAP is triggering a cellular immune response of the type leading to the kind of granulomatous inflammation of the intestine characterizing Crohn's disease.

Summarizing the state of knowledge about MAP as a possible zoonosis, these are the things we know, i.e., things about which there is little debate:

- MAP is an obligate pathogen; the reservoir is infected animals.
- MAP causes a disseminated infection in animals; ante mortem contamination of meat.
- MAP has been cultured from raw milk and uncooked meat.
- MAP can survive high temperature processing methods such as pasteurization.
- MAP has been recovered from retail food; milk and cheese in particular.
- Milk and meat consumption, a so called "western diet", is linked with Crohn's disease.
- Human and animal-origin strains of MAP are the same; a shared genetic finger print.
- Humoral and cellular immune responses to MAP are common in Crohn's patients.

Rod Chiodini, founder and past president of the IAP, and the first person to isolate MAP from Crohn's patients, published a review of the evidence of MAP involvement in Crohn's disease in January 2012 (R. Chiodini et al. Crit. Rev. Microbiol. 38(1):52-93). He summarizes his assessment using Hill's Criteria for establishing causality (Table 14 in his publication). Of the 10 criteria, Chiodini judged the strength of evidence as strong by seven criteria, moderate by one and conflicting for just two.

The One Health perspective acknowledges the dynamic interplay between infectious agents in animals, humans, wildlife, and our ecosystems. Paratuberculosis is a perfect model of this concept. It appears that societies the world over are facing a versatile zoonotic pathogen adept at infecting both animals and humans, persisting in contaminated environments for extended periods, and circumventing existing food processing measures designed to limit human exposure to food-borne pathogens. It is time for the community of paratuberculosis researchers to openly acknowledge that there is sufficiently strong evidence that MAP is a zoonotic agent that we must invoke the Precautionary Principle*. This means taking measures to "limit to as low as reasonably achievable (ALARA)" human exposure to MAP. It is time to end the ParaTB Enigma and behave in ways consistent with the scientific evidence. Specifically, we must control MAP on farms with the specific goal of producing raw food products with little or no MAP contamination. When MAP control programs are designed to improve animal health and welfare as well as protect consumers, animal agriculture can justifiably expect program cost sharing with food processors and the general public. Openness, transparency, and cost-sharing are essential for rational design and implementation of programs aimed at preventing both animal and human infections by MAP on a global basis "from teat to tongue".

*Toward the end of my presentation I asked the audience of roughly 300 to choose one of the following:

A) MAP is not zoonotic.

B) MAP is zoonotic.

C) MAP is potentially zoonotic; i.e. requiring measures to limit to levels "as low as reasonably achievable" in raw farm products (precautionary principle).

One person chose "A", several chose "B", and the vast majority selected "C": my informal, unscientific poll of paratuberculosis experts in the world.

I challenge the International Association for Paratuberculosis to: 1) establish a working group to define research priorities consistent with the zoonotic status of MAP, and 2) establish scientifically sound and feasible regulations for international animal trade to limit further spread of MAP. Don't wait for OIE or any national governments to do this; they are too bound up in politics.

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