Proceedings of the 10th International Colloquium on Paratuberculosis

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Proceedings of the 10th International Colloquium on Paratuberculosis

University of Minnesota

Minneapolis, Minnesota

August 9-15, 2009

Program and Abstracts

10th International Colloquium on Paratuberculosis

Organized by

International Association for Paratuberculosis

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Thank you to the following people who provided invaluable assistance to scientific committees by helping review abstracts: Carol Nacy, John Hermon-Taylor, Kris Huygen, Irene Grant, Ray Sweeney, Polychronis Kostoulas, Ian Gardner, Margaret Good, Steve Hendrick, Torsten Eckstein, Luiz Bermudez, and Søren Nielsen.

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Ramon Juste

Dear IAP members and other delegates,

As we meet here in Minneapolis-Saint Paul for this 10th edition of the International Colloquium on Paratuberculosis (ICP), I want to greet and welcome you to an edition that is historical for marking the 20th anniversary of the incorporation of International Association for Paratuberculosis (IAP). The IAP is the now adult child of the tallest figures of the early times of 20th century study of paratuberculosis, the late Richard Merkal and Rod Chiodini. They, with the support of the other founders created a structure that nowadays is the international forum for technical discussion on a subject that is growing in importance and whose backbone is the more than 160 members from 34 countries.

The colloquia are the regular meetings of the IAP for which we spend two years preparing the materials and looking forward. For the first time we have not just a seat for the next one, the 11th ICP that will be held in Sidney hosted by Richard Whittington, but two bids for the following one. Here, in Minneapolis-Saint Paul, we expect to meet 300 researchers interested in paratuberculosis in order to discuss around 200 presentations on the hottest topics in the field that will also be analyzed in the 10 keynote and perspectives talks. This is an exciting program that will keep the delegates stuck to their chairs and for which we thank Scott Wells and his team.

We also thank the Local Organizing Committee for the excellent facilities that they have been able to put at our disposal. I encourage you all to take advantage of them, to actively participate in the sessions, and to enjoy this opportunity to visit the United States of America.

Best regards,
Ramon A. Juste
President of the IAP

Trevor Ames

Welcome to the campus of the University of Minnesota. The College of Veterinary Medicine is honored to be an integral part the 10th International Colloquium on Paratuberculosis. The scientific program is exciting and it promises to provide you and your colleagues with rich content and the advancement of knowledge and practices. Congratulations and thanks to all who contributed to the planning and organization of a stellar colloquium. To all attendees, please take advantage of all the opportunities provided and enjoy your time with us. I trust you will find our University and the metropolitan area of Minneapolis and St. Paul invigorating and memorable.

Regards,
Dr. Trevor Ames, DVM
Dean
College of Veterinary Medicine
University of Minnesota

Scott Wells

- Planning Committee Chair

We are pleased to present the proceedings of the 10th International Colloquium on Paratuberculosis (ICP) held at the University of Minnesota West Bank campus from August 9-14, 2009 in Minneapolis, Minnesota. Sponsored by the International Association for Paratuberculosis, the ICP attracted over 300 scientists and administrators from universities, research organizations, and industry as well as state veterinary representatives and producers. The scientific program of the ICP included oral and poster presentations from international speakers invited to give plenary lectures and dedicated scientists reporting new results in a number of focused scientific sessions. An extension outreach workshop for veterinarians and animal producers on August 10, sponsored by Johne's Disease Integrated Program, focused on field application of the more basic research conducted around the world.

Regards,

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John Bannantine and Srinand Sreevatsan

Thank you for attending the 10th International Colloquium on Paratuberculosis. The Scientific Committee has been working hard behind the scenes to get abstracts reviewed and assembled into the book you are now holding. In total, we received 210 abstracts representing the latest science in Johne's disease. We hope you will find the science stimulating with plenty of time built in for conversations with colleagues during the breaks.

We have decided to run two sessions concurrently at this meeting in order to maximize the 4.5-day timeframe. These sessions, Pathogenomics & MAP Biology as well as MAP Control Programs, will be held on Wednesday morning August 12th. Although not finalized at this writing, we hope to have audio CDs for at least these two sessions in case attendees want to hear the talks from the session they had to miss.

Finally, we have made an effort to elevate the exposure of poster presentations at this meeting. Poster titles will be displayed on-screen during session breaks and the perspectives speakers and/or conveners may mention a few as well. The posters will be displayed throughout the meeting and a session dedicated to them will be held on Tuesday afternoon August 11th.

In closing we would like to especially thank Søren Nielsen, past Scientific Committee chair (8th ICP), for assisting us with the abstract database and abstract submission system.

We hope your time in Minneapolis will be memorable!

John Bannantine
Srinand Sreevatsan
Scientific Committee Co-Chairs

Welcome to Minneapolis and the University of Minnesota

10th ICP Daily Schedule of Events: Scientific Program and Social Events

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Sunday, August 9, 2009

1200 Noon	IAP Board Meeting: Terrace Room, Middlebrook Hall
1400 - 1700	Conference Registration: Willey Hall atrium
1745 (5:45 p.m.)	Conference Opening Session: Willey Hall 175

Paratuberculosis and Crohn's Disease: Already beyond reasonable doubt
Presented by Tim Bull

Where are the Weapons of Mass Destruction - - - the MPTB?
Presented by Herb Van Kruiningen

You are cordially invited to the 10th ICP social reception following the opening conference session. Guides will walk from Willey Hall to Hansen Hall-Carlson School courtyard where you can enjoy a summer's evening while sampling a variety of hors d'oeuvres along with a complimentary wine and soft drink bar.

Social guest registrants are welcome.

Casual attire.

Monday, August 10, 2009 – All sessions held in Willey Hall 175

0800-0830	COFFEE and TEA service in Willey Hall atrium (complimentary hot breakfast served in Middlebrook Hall for all attendees regardless of lodging choice)
0900-0930	Diagnostics & Genotyping **Keynote: Richard Whittington** – Diagnostic methods for identification and characterization of M. avium subsp. paratuberculosis: advances, constraints and opportunities.
0930-0950	**#229**: Detecting Johne's Disease heavy shedders in dairy cows. Luis A Espejo, Scott J Wells
0950-1010	**#205**: Sensitivity of environmental sampling for paratuberculosis. Maarten F Weber, Kees van Maanen, Thea von Bannisshet-Wijsmuller, Theo J G M Lam
1010-1030	**#231**: Rapid, sensitive detection of MAP in milk using a phage-based method. George Botsaris, Maria Liapi, Charalambos Kakogiannis, Christine Dodd, Catherine Rees
1030	Refreshment break
1040-1100	**#101**: Using liquid and agar-based methods to investigate the bacteriostatic activity of lactic acid bacteria and their metabolites against Mycobacterium avium subsp. paratuberculosis. John Anthony Donaghy, Jane Johnston, Michael Rowe

1100-1120	**#107**: Multilocus short sequence repeat analysis of Mycobacterium avium subsp. paratuberculosis isolates from dairy herds in northeastern United States of a longitudinal study indicates low shedders are truly infected. Abani K Pradhan, Aagje J Kramer, Rebecca M Mitchell, Robert H Whitlock, Julie M Smith, Ernest Hovingh, Jo Ann S Van Kessel, Jeff S Karns, Ynte H Schukken
1120-1140	**#105**: MIRU-VNTR genotyping of Mycobacterium avium subspecies paratuberculosis revealed Type-specific differences at two component regulatory systems. Elena Castellanos, Beatriz Romero, Sabrina Rodriguez, Javier Bezos, Lucia de Juan, Ana Mateos, Lucas Dominguez, Alicia Aranaz
1200 -1230	**Perspectives: Ivo Palvik** – Genotyping perspectives
1230-1430	LUNCH – Willey atrium and outdoor courtyard
1430-1500	Host Response and Immunology **Keynote: Judy Stabel** – Unraveling the Host Response to Mycobacterium avium subsp. paratuberculosis:One Thread at a Time.
1500-1520	**#172**: Whole genome association study for Holstein susceptibility to MAP infection Brian W Kirkpatrick, Xianwei Shi, Michael Collins, George E Shook
1520-1540	**Merkal Award - #33**: No interference of heat shock protein 70 subunit vaccination against bovine paratuberculosis. Wiebren Santema, Selma Hensen, Victor Rutten, Ad Koets
1540-1600	**#68**: Neutralisation of interleukin-10 from CD14+ monocytes enhances gamma interferon production in peripheral blood mononuclear cell from mycobacterium avium subsp. paratuberculosis infected goats. Kari Roste Lybeck, Anne Kristine Storset, Ingrid Olsen

1600-1630	Refreshment break: sponsored by Trek Diagnostic Systems
1630-1650	**#75**: Interleukin 17 and interleukin 23 gene expression differentiate severe pathology in Johne's disease. Mark William Robinson, Rory O'Brien, Frank Griffin
1650-1710	**#119**: The early IL-10 response in ovine Johne's disease. Kumudika de Silva, Douglas Begg, Deborah Taylor, Richard Whittington
1710-1730	**#173**: Local and systemic roles for bovine gamma-delta T cell subsets during the early immune response to Mycobacterium avium subspecies paratuberculosis (Map) infection. Brandon Lee Plattner, Elise Huffman, Jesse M Hostetter
1730-1750	**#190**: Development of new live vaccines for paratuberculosis. Desmond M Collins, Gabriella M Scandurra, Geoffrey W de Lisle
1750-1800	**#23**: Role of Mycobacterium avium subsp. paratuberculosis in the pathogenesis of Crohn's disease. William C Davis, Andrew J Allen, Mary Jo Hamilton, Gaber S Abdellrazeq, Heba M Rihan, George M Barrington, Kevin L Lahmers, Kun T Park, Srinand Sreevatsan, Christopher Davies
1800-1830 (6:00 -6:30)	**Perspectives talk: Kris Huygen** – Host Response and Immunology Perspectives

We invite you to enjoy an open evening and discover Minneapolis's charm and variety. Maps and information for restaurants within walking distance or short taxis rides are provided in your conference bag.

Tuesday, August 11, 2009

0800-0830	COFFEE and TEA service in Willey atrium(complimentary hot breakfast served in Middlebrook Hall for all attendees regardless of lodging choice)
0900-093	**Epidemiology - Willey Hall 175** **Keynote: Ynte Shukken** - Elimination of *Mycobacterium paratuberculosis* from dairy farms: fact or fiction?
0930-0950	**Merkal award - #145**: Multinomial regression analysis of individual host factors and paratuberculosis test results. Franziska Gierke, Mario Ziller, Heike Köehler
0950-1010	**#149**: A meta-analysis of the effect of dose and age-at-exposure on shedding of Mycobacterium avium subspecies paratuberculosis (MAP) in experimentally infected calves and cows. Rebecca Mans Mitchell, Graham F Medley, Michael T Collins, Ynte H Schukken
1010-1030	**#50**: Assessment of the age at occurrence of MAP infection adjusting for the latent infection period in Danish dairy cattle. Polychronis Kostoulas, Søren S Nielsen, William J Browne, Leonidas Leontides
1030-1100	Refreshment break: sponsored by Antel Biosystems
1100-1120	**#55**: Birth clusters of animals infected with Mycobacterium avium subspecies paratuberculosis in a New York State dairy herd. Fieke Vangenugten, Ynte H Schukken, Rebecca Mans Mitchell, Susan M Stehmann

1120-1140	**#88**: Influence of between-calves contacts on Mycobacterium avium paratuberculosis (Map) transmission in a dairy herd. Clara Marce, Pauline Ezanno, Henri Seegers, Dirk U Pfeiffer, Christine Fourichon
1140-1200	**#200**: Use of multiple tests to determine the status of UK dairy herds with respect to Mycobacterium avium subsp. paratuberculosis. Alasdair Cook, Alberto Vidal-Diez, Mark Arnold, Robin Sayers, George Caldow, Adrian McGoldrick, John Donaghy, Samuel Strain, Ian Gardner
1200-1220	**#104**: Relation between faecal shedders and environmental contamination with Mycobacterium avium subsp. paratuberculosis on an experimental farm. Susanne Eisenberg, Mirjam Nielen, Jeroen Hoeboer, Dick Heederik, Ad Koets
1230-1345	Conference Group Photo (everyone) followed by LUNCH – Willey Hall atrium and courtyard
1400-1430	**Perspectives talk: Ian Gardner**
1430-1440	**JDIP Travel Awards**
1445-1730 (2:45-5:30)	POSTER SESSION – Willey Hall atrium

Once again, we invite the attendees of the 10th ICP to enjoy an open evening and discover Minneapolis. Maps and information for restaurants within walking distance or short taxis rides are provided in your conference bag.

PLEASE NOTE: There are two concurrent Wednesday morning sessions due to the afternoon Mississippi riverboat ride.

0800-0830 COFFEE and TEA – Willey Hall atrium

Concurrent Session A – Room 125 Willey Hall : Wednesday, August 12

0900-0930 **MAP Control**

Keynote: Jason Lombard – Results from the US JD Demonstration Herd Project: Key findings for disease control.

0930-0950 **#238**: Passive MAP fecal shedding in dairy cattle.
Robert H Whitlock, T Fyock, Y Schukken, J Van Kessel, J Karns, E Hovingh, J Smith

0950-1010 **#202**: Milk quality assurance for paratuberculosis: effects of infectious young stock.
Maarten F Weber, Huybert Groenendaal

1010-1030 **#141**: Control of paratuberculosis by vaccination - a systematic review and meta-analysis.
Heike Köehler, Franziska Gierke, Tassilo Seidler, Mario Ziller

1030-1100 Refreshment break

1100-1120 **#235**: Update on a Voluntary Johne's Disease Control Program in Ontario and Western Canada.
Ulrike Sorge, David Kelton, Kerry Lissemore, Ann Godkin, Steve Henrick, Scott Wells

1120-1140 **#198**: Use of small experience groups to enhance knowledge and increase motivation of farmers in the process of controlling paratuberculosis.
Peter Raundal, Lene Trier

1140-1200 **#214**: JDConsult: An example of immersion learning.
Jeannette McDonald, Michael T Collins

1200-1230 **Perspectives talk: Søren Nielsen** – Control of paratuberculosis at the regional and global level - trends and progress.

Concurrent Session B – Rm 175 Willey Hall: Wednesday, August 12

0900-0930	**Pathogenomics and MAP Biology** **Keynote: Adel Talaat** – Genomic Perspectives on the Pathogenesis of M. avium subsp. paratuberculosis.
0930-0950	**#45**: Identification of genes expressed by Mycobacterium avium subsp. paratuberculosis in subclinical Johne's disease and in vitro infected macrophages. Sajan George, Wayne Xu, Zeng Jin Tu, Jeremy Schefers, Srinand Sreevatsan
0950-1010	**#89**: Mycobacterium avium subspecies paratuberculosis isolates recovered from infected goats in the central Spain contain deletions of mce genes and an increased capacity for persistence in macrophages. Elena Castellanos, Alicia Aranaz, Lucas Dominguez, Lucia de Juan, Ana Mateos, Richard Linedale, Tim J Bull
1010-1030	**#117**: Adsorption of MAP organisms to soil particles. Navneet Kumar Dhand, Jenny-Ann LML Toribio, Richard Whittington
1030-1100	Refreshment break
1100-1120	**#160**: Hidden gems in the Mycobacterium avium subsp. paratuberculosis genome. John P Bannantine, Adel M Talaat, Michael L Paustian, Srinand Sreevatsan
1120-1140	**#222**: Atypical structural features of two MAP P60 family members. Kasra X Ramyar, Cari K. Lingle, William J. McWhorter, Samuel Bouyain, John P Bannantine, Brian V Geisbrecht
1140-1200	**#150**: Structural characterization of lipid 550/760 from Mycobacterium avium subspecies paratuberculosis. Danielle R Cogswell, Donald L Dick, Christopher D Rithner, Julia M Inamine, Torsten M Eckstein
1200-1230	**Perspectives talk: Luiz Bermudez** – MAP biology perspectives

Wednesday afternoon conference social agenda:

Excursion on the Mississippi River by Paddleboat

1230 – 1330 (1:30) Lunch will be served at Willey Hall prior to traveling by bus to St. Paul Harriet Island to board the Padelford Mississippi River Boat. Comfortable attire is recommended. The riverboat has both open air and enclosed spaces for your comfort and viewing while traveling on the Mississippi. The trip is a very pleasant 3 hour ride. Clean, modern restrooms are available onboard. A cash bar will be open during the river boat tour and complimentary Hors D'oeuvres will be provided. Social guest registrants are welcome.

1330 (1:30)	Board buses in parking lot behind Willey Hall and Anderson Library (escort will be present at Willey Hall) The travel time is approximately 30 minutes to the dock. The Padelford staff will be present to assist you in boarding the boat which is also wheelchair accessible.
1730 (5:30)	Board buses to return to Willey Hall. Travel time back to the University west bank campus will be approximately 45 minutes due to rush hour traffic.

Once agian, we invite the attendees of the 10th ICP to enjoy an open evening and discover Minneapolis. Maps and information for restaurants within walking distance or short taxi rides are provided in your conference bag.

Thursday, August 13	All Sessions held in Willey Hall 175
0800-0830	COFFEE and TEA service in Willey Hall atrium(complimentary hot breakfast -Middlebrook Hall for all attendees regardless of lodging choice)
0900-0920	**#59**: CD4 T cells from intestinal biopsies of Crohn's disease patients react to Mycobacterium avium subspecies paratuberculosis. Ingrid Olsen, Stig Tollefsen, Claus Aagaard, Liv Jorun Reitan, John P Bannantine, Peter Andersen, Ludvig M Sollid, Knut E A Lundin
0920-0940	**#174**: Sharing of 'Indian Bison Type' Mycobacterium avium subspecies paratuberculosis between goatherds endemic for Johne's disease and their animal attendants. Ajay Vir Singh, Shoor Vir Singh, Jagdeep Singh Sohal, Pravin Kumar Singh, M C Sharma
0940-1000	**#135**: Isolation of Mycobacterium avium subsp. paratuberculosis from muscle tissue of naturally infected cattle. Marta Alonso-Hearn, Elena Molina, Marivi Geijo, Patricia Vazquez, Iker Sevilla, Joseba M Garrido, Ramon A Juste
10:30-10:50	Keynote Ramon Juste
1050-1110	**#207**: Nicotinic and Salicylic acids and a & ß nicotinamide adenine dinucleotide (NAD) cause dose dependant enhancement, and iso-nicotinic acid (INH) and para-amino-salicylic acid (PAS) cause dose dependant inhibition of M. avium subspecies paratuberculosis (MAP). Robert J Greenstein, Liya Su, Sheldon T Brown
1110-1140	**Perspectives talk: Carol Nacy** – The Public Health Perspective
1140 – 1240	**IAP GENERAL MEETING**
1240 - 1400	LUNCH

Diagnostics and Genotyping

Convenors: Beth Harris and Ray Sweeney

Diagnostics and Genotyping Keynote Lecture

Diagnostic methods for identification and characterization of *M. avium* subsp. *paratuberculosis*: advances, constraints and opportunities

Richard Whittington

Faculty of Veterinary Science, University of Sydney

There are two overarching themes for diagnosis of Johne's disease – detection of the pathogen, and detection of host responses directed against the pathogen. Despite much progress in the last 10 years, globally there is poor standardization of culture methods; unfortunately it is against these that all other pathogen detection methods need to be compared. Solid medium culture prevails in many countries despite the proven advantages of liquid culture systems. Furthermore, rapid detection methods based on PCR have proliferated in the literature but remain research tools in most instances. Why is this so? Meanwhile, new facets of immune recognition in the first contact between host and pathogen have been described, leading to glimmers of hope for early detection of infection. Through the genome sequence of Mycobacterium avium subsp. paratuberculosis, unique antigens have been identified and these provide new opportunities for inclusion in diagnostic test platforms to lift sensitivity and specificity of immunological tests. What constraints need to be overcome to bring new diagnostic methods to bear on control of Johne's disease? This brief review proposes that proper understanding of the pathogenesis of Johne's disease is the biggest hurdle and as requested by the conference organizers, will touch on some current research close to home to illustrate this.

#229

Detecting Johne's Disease heavy shedders in dairy cows

Luis A. Espejo, Scott J. Wells

Department of Veterinary Population Medicine, University of Minnesota, USA

Cows that shed high concentrations of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in feces into the environment (heavy shedders) can be identified through fecal culture, though this method is costly and requires a long period of incubation. Other methods of detection are available, including pooling of fecal samples and use of fecal PCR, though efficacy for detection of heavy fecal shedders within pooled samples has not yet been demonstrated.

The objective of this study was to identify alternative methods to identify Map heavy shedders in dairy herds. Fecal samples were collected from dairy cows in 7 herds participating in the Minnesota Johne's Disease Demonstration Herd Project. Pooled environmental fecal samples and individual cow fecal samples were collected. Samples were tested using fecal culture and fecal PCR at the Minnesota Veterinary Diagnostic Laboratory. In addition, individual cow fecal samples were pooled in groups of 5 and 10 individual samples per pool and tested using both assays. Heavy shedding cows were defined using fecal culture (greater than 50 colonies per tube).

Preliminary results of fecal culture indicated that the apparent cow-level prevalence was 4.8%, and 18.8% of them were heavy shedding cows. Compared to fecal culture of individual fecal samples, the sensitivity for detection of at least one heavy fecal shedding cow in environmental samples was 100% for fecal culture and 25% for fecal PCR. The sensitivity for detection of at least one heavy fecal shedding cow in pools of 10 cows per pool was 100% for fecal culture and 80% for fecal PCR. The sensitivity for detection of at least one heavy fecal shedding cow in pools of 5 cows per pool was 100% for fecal culture and 88% for fecal PCR. In summary, fecal PCR is an alternative method to fecal culture on pooled fecal samples for detection of heavy shedders.

#205 Sensitivity of environmental sampling for paratuberculosis

Weber MF[1], van Maanen C[1], von Bannisseht-Wijsmuller T[1], Lam TJGM[1]

[1] *GD Animal Health Service, Deventer, The Netherlands*

INTRODUCTION

Environmental sampling (ES) has been recommended in the U.S. for classification of dairy herds as infected or not infected (Collins et al., 2006), based on studies in US dairy herds (Raizman et al., 2004; Lombard et al., 2005; Berghaus et al., 2006). However, the diagnostic test characteristics of ES in Dutch dairy herds were unknown. Therefore, the aim of this study was to quantify the relative sensitivity of ES in comparison to the test-scheme of the initial assessment of the Dutch Bulk Milk Quality Assurance Program (BMQAP), i.e. individual milk samples of all lactating cattle followed by confirmatory fecal culture.

MATERIALS AND METHODS

In 246 herds with ≥ 1 milk-ELISA-positive cattle in the initial herd assessment of the BMQAP, a pooled sample from the slurry pit and a pooled fecal sample from cow alleyways were collected. At the same point in time, individual fecal samples were collected from all ELISA-positive cattle. All samples were cultured on modified Löwenstein-Jensen media (Kalis et al., 1999; Kalis et al., 2000).

RESULTS

In 216 (88%) of the 246 herds, ≥1 milk-ELISA-positive cattle were found to be individual fecal culture-positive. In the remaining 30 herds, all ELISA-positive cattle were individual fecal culture-negative. These 30 herds were excluded from our analyses because they were considered test-negative in the initial assessment of the BMQAP.

The pooled sample from the slurry pit was culture-positive in 199 (92%) of the 216 herds with ≥1 cattle being both ELISA- and fecal culture-positive. The pooled sample from the cow alleyways was culture positive in 189 (88%) of the 216 herds. In 205 (95%) of the 216 herds, at least one of these pooled samples was culture-positive. Therefore, the relative diagnostic sensitivity (exact 95% CI) of culture of a pooled sample from the slurry pit, a pooled sample from the cow alleyways, and parallel testing of a pooled sample from the slurry pit and a pooled sample from the cow alleyways was 92% (88%, 95%), 88% (82%, 92%) and 95% (91%, 97%), respectively.

DISCUSSION

ES was found to have a reasonably high relative sensitivity in comparison to the test-scheme of the initial assessment of the Dutch BMQAP. However, if ES would have been used as a pre-screening prior to individual milk sampling, 5% to 12% of herds would have tested false-negative. It is yet unknown whether the infection in these herds could be detected by repeated environmental samplings. Furthermore, the relative specificity of ES in ELISA-negative herds and the relative sensitivity of ES as compared to the test-regimen for surveillance of certified herds are yet unknown. Finally, in small to medium sized herds, the costs of ES exceed the costs of testing by milk-ELISA. We conclude that further research is needed to solve these issues.

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Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE, 2004. The distribution of Mycobacterium avium ssp. paratuberculosis in the environment surrounding Minnesota dairy farms. J. Dairy Sci. 87, 2959-2966.

#231 Application of a rapid and sensitive combined phage-PCR method for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in raw milk

George Botsaris[a], Maria Liapi[b], Charalambos Kakogiannis[b], Christine Dodd[a] and Catherine Rees[a]

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[b] *Cyprus Veterinary Services, 1417 Athalassas Av, Nicosia, Cyprus*

ABSTRACT

The objective of this study was to evaluate a new combined phage-PCR method by investigating levels of MAP in milking herds in the UK and throughout the Republic of Cyprus. Bulk milk samples were collected from 54 UK farms and 225 dairy farms in Cyprus and were tested using both the phage assay and a conventional culture method for the presence of MAP. The identity of MAP cells detected was confirmed in both cases by IS900 PCR. None of the UK samples were culture positive whereas 1 was phage-PCR positive. In Cyprus MAP 50 of the 225 samples were MAP positive using the combined phage-PCR and MAP was cultured from 2 samples, despite the fact that the animals tested were not displaying clinical symptoms of Johne's disease. Total viable count was also determined as an indicator of the general hygiene status of the samples. Comparison of MAP status with TVC suggests that these were not introduced by faecal contamination. Typing of the two MAP strains isolated from cow's milk in Cyprus by culture was performed using REA IS1311 PCR and identified one of these to be an S strain and the other C strain. The results confirm that the phage-based detection test is more sensitive than conventional culture and demonstrate the efficacy and practicality of the phage-based test as a routine rapid method for the detection of viable MAP in milk.

INTRODUCTION

Detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk currently relies on culture, immunoassays and molecular techniques. The culture based techniques require a very long incubation period of about 3 months and a chemical decontamination step which reduces the number of competitive microbes, but also reduces the number of viable *Mycobacteria* present in a sample, limiting the sensitivity of growth-based assays. Immunoassays have a very low sensitivity in milk and the molecular techniques have the disadvantage of not being able to differentiate live from dead cells. A new combined phage-PCR assay for the detection of MAP in milk was described by Stanley et al. (2007) based on the commercially available *FASTplaque*TB™ assay for detecting tuberculosis in human sputum samples. This test detects the ability of a bacteriophage (D29) to infect MAP and provided successful identification of viable cells in milk in less than 24 hours. The advantage here is that only viable cells will support phage replication, providing live-dead differentiation, no chemical decontamination of samples is required and large volumes (up to 50 ml) can be sampled. Genotyping is then performed by amplification of IS900 sequences from in the cells detected directly from the bacteriophage plaques (see Rees and Dodd, 2007). The aim of the work presented here was to apply the new method on real samples to assess the potential application of the test for the detection of MAP in milk and also compare the results with conventional culture for MAP. A viable count was also performed on the samples to investigate any potential relationship between the presence of MAP in milk with TVC which was used as a general indicator of the overall hygienic status of the samples.

MATERIALS AND METHODS

Sampling

For the UK samples 54 BTM samples were provided from the VLA in Sutton Bonington, UK. For the Cyprus survey samples were collected from from each of 225 registered dairy farms in Cyprus.

Combined phage PCR assay

BMT samples were processed by a modified version of the method described by Stanley e*t al.* (2007). In the assay bacteria in the milk samples are collected by centrifugation and the cell pellets resuspended in 2 ml of Media Plus. Here a second wash step was included using the same centrifugation conditions and then finally the pellet was resuspended in 1 ml Media Plus.

Culture examination and identification of mycobacterial isolates

Samples (30 ml) of BTM were decontaminated for 5 h with 0.75% hexadecyl-pyridinium chloride (HPC; Merck KGaA, Germany) and three samples were cultured on Herrold's Egg Yolk Media (HEYM) containing 2 μg ml^{-1} of Mycobactin J; two slopes were purchased from Becton Dickinson (New Jersey, United States) and one slope was prepared as described previously (Ayele et al., 2005). Slopes were monitored after the first week of incubation to identify either contaminated cultures or those with fast-growing mycobacteria and then observed at two week intervals until there was visible colony growth: incubation was carried out for not less than eight months at 37°C. From all primary cultures, presumptive colonies were stained using Ziehl-Neelsen (Z-N) for the presence of acid-fast bacilli (AFB). AFB isolates were identified as MAP by PCR as described previously (IS*900*, Whittington *et al*., 1998 and *F57*, Coetsier et al., 2000). IS*1311* REA-PCR was used to distinguishing between cattle and sheep strains of *MAP* with PCR products being cleaved with *Hinf*I (Marsh et al. 1999).

Total viable count (TVC)

TVC was performed on Milk Count Agar (MCA) using standard methods. Milk was diluted in MRD (Maximal Recovery Dilluent) and the dilutions were plated on MCA and incubated aerobically at 30 °C for 3 days.

RESULTS AND DISCUSSION

The results from the two surveys are presented in Table 1. From a total of 54 samples analyzed in the UK survey 19 (35.2%) were positive using the phage assay which indicates the presence of *Mycobacteria* spp, but one of these samples (1.9%) was identified as MAP following PCR genotyping. A duplicate of the samples collected was cultured by the VLA and none of the samples were positive for MAP. A recent UK survey reported that 75% of dairy herds were sero-positive but seroprevalance was only 0.12 and therefore levels of MAP expected in BTM would be low (Woodbine et al., 2009). From a total of 225 samples analysed for the Cyprus survey 218 (96.9%) were *Mycobacteria*-positive using the phage assay and 50 of these (22.2%; 95% CI: 17.1% - 28.0%) were identified as MAP by IS900 PCR. In this case only two of the samples (0.9%) were culture positive. From the 225 cows' BMT samples that were collected during this survey 50 (22.2%; 95% CI: 17.1% - 28.0%) were found to be MAP positive using the combined phage-PCR assay. The results of Slana et al. (2009) reported that 28.6% (95% CI: 95% CI: 22.5% – 34.3%) were MAP positive by IS900 qPCR, showing a good agreement with the level of prevalence recorded in the two different surveys undertaken.

All BTM samples collected in the Cyprus were also cultured after decontamination and only two of them (0.9%) produced colonies on HEYM slopes that were identified as MAP-positive by both IS900 and F57 qPCR. Both of the samples from which viable MAP were cultured were also phage-PCR positive. The two confirmed MAP isolates were analysed using IS1311 REA PCR and one of these was identified as a MAP cattle strain and the other as a sheep strain.

Table 1 UK and Cyprus MAP survey Results

Survey	Total Samples	Phage Assay			Phage - PCR			Culture		
		No.	Pos.	%	No.	Pos.	%	No.	Pos.	%
UK	54	54	19	35.2	54	1	1.9	54	0	0
Cyprus	225	225	218	96.9	225	50	22.2	225	2	0.9

The TVC results (Table 2) are separated into groups according to microbial load. No relationship was seen between the TVC and samples being phage-PCR MAP positive. This confirms that the MAP were not being introduced into the milk by faecal contamination and that in the Cypriot study we can deduce that MAP is being shed into the milk by animals that do not display any clinical symptoms of the disease (clinical herd data was not available for the UK samples). Characterization of the 2 culture isolates identified one as an S strain and the other as a C strain. The presence of the S strain in the cattle herd population is interesting from an epidemiological perspective since the presence of this strain has also been found in the Cypriot sheep and goat populations (Liapi et al., 2009). The extended culture times required to isolate this organism may explain why it was not previously cultured from bovine milk samples.

Table 2 MAP and Viable Count Results

Survey	Total Viable Count Data			Phage Positive samples			IS900-PCR positive samples		
	⩽ Log4 cfu/ml	Log4-Log5 cfu/ml	>Log5.01 cfu/ml	⩽ Log4 cfu/ml	Log4-Log5 cfu/ml	>Log5.01 cfu/ml	⩽ Log4 cfu/ml	Log4-Log5 cfu/ml	>Log5.01 cfu/ml
UK	21	7	26	15	2	2	1	-	-
Cyprus	105	95	25	102	93	23	25	21	4

Prospective analysis of the phage-PCR data revealed a strong relationship between the pfu number and the probability of that sample being shown to contain MAP by PCR genotyping. Figure 1 shows the distribution of the pfu count of the MAP IS900 positive and negative samples. An area of overlap exists between 15 pfu/50 ml (PCR-positive sample with the lowest plaque count) and 75 pfu/50 ml (highest PCR-negative sample). However all samples with >75 plaques were found to contain MAP. The *FASTplaque*TB assay gives a cutoff value of 20 pfu per sputum sample. If a cutoff is applied in the overlap region to this data, a test with a high Sensitivity (Sn, 98%) but a lower Specificity (Sp, 63.4%) was gained.

Fig.1: Relationship between plaque number and IS900 PCR results

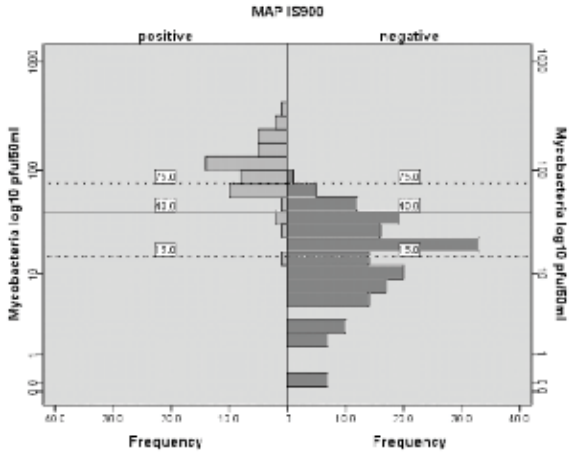
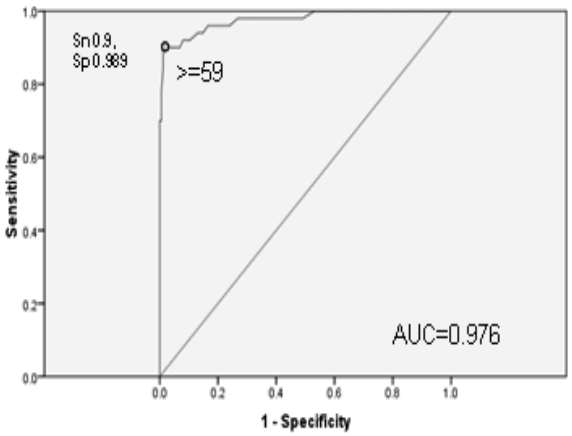


Fig. 2: ROC analysis of plaque cutoff



To better determine the most appropriate cutoff value Receiver Operating characteristic (ROC) analysis was used (Figure 2) and showed that a cutoff off 59 pfu/50 ml gives Sn and Sp values of 90% and 99%, respectively. This cutoff value can be refined as more data is generated, but shows the clear relationship between the number of plaques and presence of MAP in a sample. As high levels of Mycobacteria should not be present in hygienically collected milk, this relationship is to be expected if infected animals are shedding MAP. Hence in this case plaque numbers alone can be used to indicate MAP infection without the need for PCR confirmation.

The Cyprus cattle population is free from bovine tuberculosis however, since any Mycobacteria shed into milk will result in a plaque, if this were to be repeated in a country with endemic levels of TB, the plaque results alone could not be used as an indication of MAP infection. For instance in this work 26 other isolates were recovered on culture and identified as *Mycobacteria* spp. by Z-N stain and species-specific PCR (Tevere et al., 1996) and these cells would have given rise to IS900 PCR-negative plaques. While the relationship between infection and plaque number is expected to be maintained irrespective of the identity of the *Mycobacterium* being shed into milk, a multiplex PCR genotyping assay would then be required to confirm the identity of the organism being detected (see Stanley et al., 2007).

CONCLUSION

The combined phage PCR assay was successfully applied to real BTM samples and shown to be able to sensitively and specifically detect MAP within 24 h in a population that is free from TB.

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#101

Using liquid and agar-based methods to investigate the bacteriostatic activity of lactic acid bacteria and their metabolites against *Mycobacterium avium* subsp. *paratuberculosis*

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Mycobacterium avium subsp. *paratuberculosis* (Map) may not be completely inactivated by pasteurisation and other sub-pasteurisation heat treatments applied during the processing of milk and manufacture of other dairy products respectively. Consequently, the inactivation of Map may rely on the product's manufacturing process or intrinsic properties, *inter alia* the presence lactic acid bacteria (LAB) and their concomitant metabolic products such as lactic acid, hydrogen peroxide, acetic acid and antimicrobial peptides. The objective of this study was to develop assays to investigate the *in vitro* inhibition of Map by commercial probiotic LAB, cheese LAB and their metabolites.

LAB supernatants, cultured in MRS broth and milk was screened for bacteriostatic activity by supplementation into BACTEC 12B medium inoculated with Map cells (approx. 10^{4-5} cfu/ml). The effect on Map growth was monitored throughout a 12 wk incubation period. An agar plate screening assay was developed to monitor the effect of LAB and their metabolic products on Map growth.

Two commercially available probiotic strains elicited a substantial inhibitory effect (50-60 day growth delay) on Map. Concentrated supernatant from a number of Cheddar cheese LAB isolates exhibited bacteriostatic activity towards Map in the liquid culture and agar-screening assays. This effect was not demonstrated by D, L, D/L lactic acid (0.1-10%), H_2O_2 (0.1-1%), nisin or low pH (HCL; < 2.5) conditions. The agar screening assay could also be used for the determination of minimum inhibitory concentrations of antibiotics as part of culture method development.

The results of this study suggest the *in vitro* inhibitory effect of some lactobacilli on Map growth may be due to factors other than lactic/acetic acid production. Irrespective of the mechanism of inhibition, the possibility exists for the inclusion of such strains in dairy products for the *in situ* inhibition of Map or furthermore their use as biotherapeutic agents against Map.

#107 Multilocus Short Sequence Repeat Analysis of *Mycobacterium avium* subsp. *paratuberculosis* Isolates from Dairy Herds in Northeastern United States of a Longitudinal Study Indicates Low Shedders are Truly Infected

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ABSTRACT

The objectives of this study were to evaluate whether low shedders of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were pass-through animals or whether they were truly infected. We also evaluated whether these animals were possibly infected by the super-shedders. The MAP isolates were obtained from a longitudinal study that involved three different herds in the northeastern US. The shedding levels of animals at each culture-positive occasion were determined. Selected isolates were collected from all animals that were culture-positive at the same time super-shedders were present in the herds and from super-shedders. Using a multilocus short sequence repeat (MLSSR) approach we found 15 different strains from a total of 142 isolates analyzed. The results indicated herd-specific infections; a clonal infection in herd C with 89% of animals sharing the same strain, different strains in herds A and B. In herd C, 100% and in herd A, 17 to 70% of cows shed the same strain as that of contemporary super-shedders at a given collection date. About 82% of available tissue samples were culture-positive indicating a true infection. Taken together the results of MAP strain-typing and shedding levels, we conclude that at least 50% of low shedders have same strain as that of a contemporary super-shedder. The results of this study indicate that very few cows had characteristics of a possible pass-through animal; many more cows were actively infected. The sharing of same strain of low shedders with the contemporary super-shedders suggests that low shedders may be infected as adults by the super-shedders.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*, MLSSR analysis, longitudinal study, low shedders, truly infected

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of paratuberculosis (or Johne's disease), a debilitating chronic gastroenteritis in ruminants (Möbius et al., 2008) and is characterized by a very long incubation period (up to several years). Shedding of MAP can occur without showing clinical signs. Recently, using further serial dilutions to determining the range of shedding in animals, super-shedders were identified and defined as cattle shedding more than 10,000 MAP/g of manure (Whitlock et al., 2005). It has been indicated that super-shedders represent the greatest risk to spread of Johne's disease (Whitlock et al., 2005). Although several molecular subtyping techniques have been previously applied to investigate genetic variation in MAP, MLSSR approach has recently gained much attention and been applied in different studies (Amonsin et al., 2004; Harris et al., 2006). While only a limited number of cross-sectional studies have used this method with a restricted set of isolates, it has

been recognized that the use of longitudinal studies using several herds in multiple states is essential to apply MLSSR technique in understanding the epidemiology of Johne's disease (Harris et al., 2006). In this study, by using MLSSR straintyping technique in combination with observed MAP shedding levels, we aimed to evaluate: (i) whether low shedders of MAP were passive shedding (pass-through) animals or whether they were truly infected and (ii) whether these animals were possibly infected by the super-shedders.

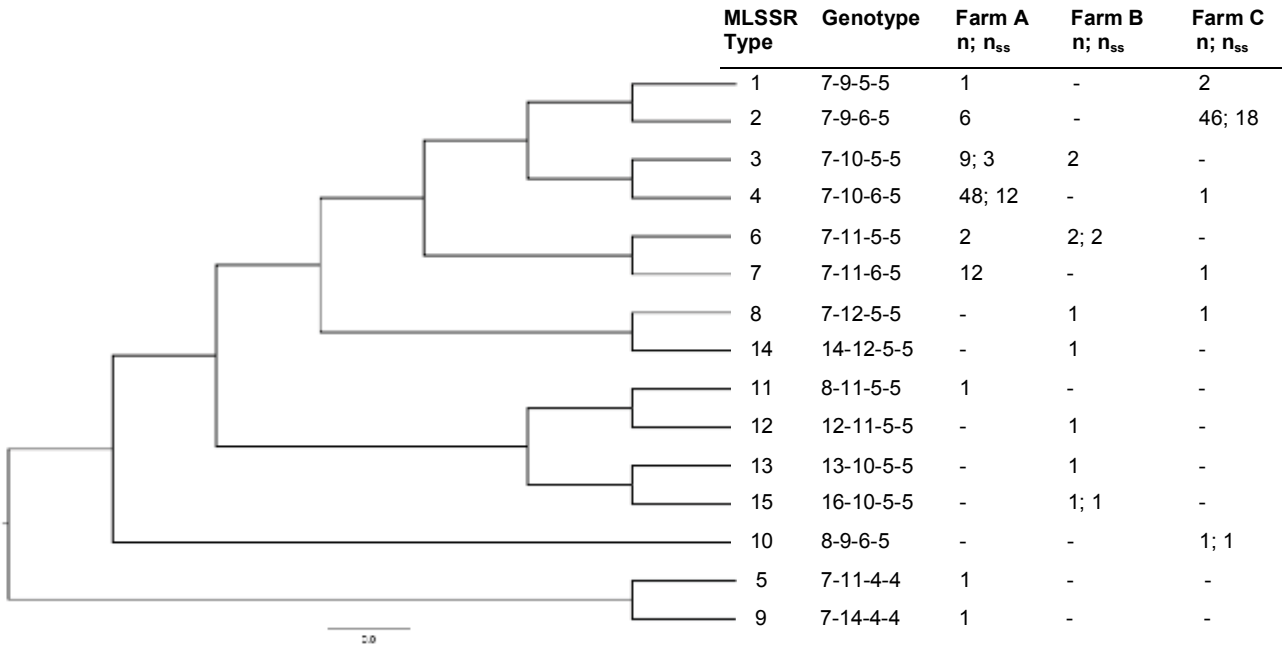
MATERIALS AND METHODS

Isolates used in this study were obtained from three commercial dairy farms in the northeastern United States: farm A in New York State, farm B in Pennsylvania, and farm C in Vermont (Pradhan et al., 2009). In this study, selected isolates were collected from all animals that were culture-positive at the same time super-shedders were present in the herds, from super-shedders, and from slaughter house samples available for these animals. We used the following criteria to evaluate the infection status of animals with positive fecal culture test results: (i) to be considered passive shedding (pass-through), an animal may shed only once with low level of shedding (i.e., ≤ 21 cfu of MAP/g) with subsequent fecal samples testing culture-negative, shedding the same strain as contemporary super-shedder(s) and subsequently *culture-negative* tissues and (ii) to be considered being truly infected, an animal shed low level of shedding, which may occur more than once and subsequently *culture-positive* in tissues at slaughter.

DNA from bacterial cells was extracted using QiAamp DNA Mini kit (Qiagen Inc., Valencia, CA, USA). For MLLSR analyses, loci 1, 2, 8, and 9 that were associated with the highest Simpson's diversity indices and identified as the most discriminatory and informative SSR loci were used for fingerprinting (Amonsin et al., 2004; Harris et al. 2006). PCR amplification was performed using the previously published primers (Amonsin et al., 2004). The amplification conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. Electrophoresed gels were stained with ethidium bromide for 2 min followed by de-staining in water for about 1 hr. The gels were visualized for quality check through UV transillumination with the Molecular Imager Gel Doc XR system and Quantity One software (Bio-Rad, Hercules, CA, USA). The PCR products were purified with a PureLink PCR Purification kit (Invitrogen Corp., Carlsbad, CA, USA). The PCR amplicons were sequenced at the Cornell University Life Sciences Core Laboratories Center. For each locus, allele numbers were assigned to reflect the number of copies represented in the SSR sequence. On the basis of the unique combination of alleles for each locus, MLSSR types were then assigned.

RESULTS AND DISCUSSION

We found 15 different strains (i.e., genotypes or MLSSR types) on three farms from a total of 142 isolates (from fecal and tissue samples; 81, 9, and 52 isolates from farms A, B, and, C, respectively); 9 types on farm A, 7 types on farm B, and 6 types on farm C (Fig. 1). The results indicated herd-specific infections; a clonal infection on farm C with 89% of isolates shared the same strain (type 2) and different strains on farms A and B (Fig. 2). On farm A, type 4 was the most predominant one with about 59% of isolates belonged to this strain. On farm B, we found a variety of strains (7 types) from a limited number of isolates (9 isolates) and all these isolates were obtained from the animals that were purchased from different sources.



MLSSR Type	Genotype	Farm A n; n_{ss}	Farm B n; n_{ss}	Farm C n; n_{ss}
1	7-9-5-5	1	-	2
2	7-9-6-5	6	-	46; 18
3	7-10-5-5	9; 3	2	-
4	7-10-6-5	48; 12	-	1
6	7-11-5-5	2	2; 2	-
7	7-11-6-5	12	-	1
8	7-12-5-5	-	1	1
14	14-12-5-5	-	1	-
11	8-11-5-5	1	-	-
12	12-11-5-5	-	1	-
13	13-10-5-5	-	1	-
15	16-10-5-5	-	1; 1	-
10	8-9-6-5	-	-	1; 1
5	7-11-4-4	1	-	-
9	7-14-4-4	1	-	-

Fig. 1. Dendogram (showing genetic relationships among all MAP isolates) was generated by the unweighted pair-group method with arithmetic averages (UPGMA) with the PAUP program. On each farm, the numbers of isolates (n) belong to a particular type and the number of isolates from super-shedders (n_{ss}) belong to a particular type (if any) are shown to the right of the dendogram ('n' includes 'n_{ss}').

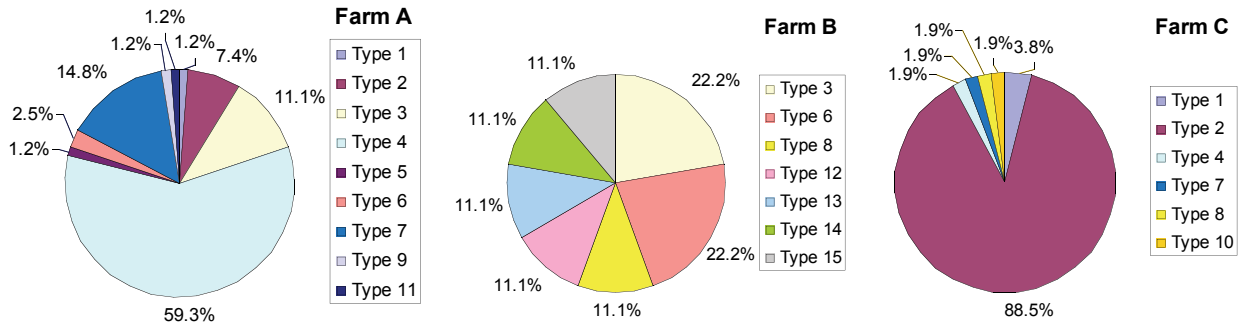


Fig. 2. Pie-charts showing the percentage of each type from a total of 81, 9, and 52 isolates (fecal and tissues) on farms A, B, and C, respectively.

We also determined the shedding level (cfu/g) over time for individual animals at each culture-positive occasion. For example, in the first visit on farm A, animal 693 was the only animal with super-shedder status, with an observed shedding level of 462,000 cfu/g, which was shedding strain type 4 in the feces. The same type was found in the fecal samples of five other animals on that visit. In the first visit on farm C, one animal with super-shedder status (cow 152) was present in the herd with the observed shedding level of 1.26 millions MAP/g; type 2 was found in her fecal sample and the same type was found in her fecal sample at slaughter as well as in 4 tissues collected. During that first visit, all 17 culture-positive animals on farm C were shedding the same strain (type 2).

In the presence of super-shedders in herds, 57.1, 16.7, 20.0, and 70.0% of animals other than identified as super-shedders shed the same strain as that of contemporary super-shedders on farm A. Although this scenario did not occur on farm B, 100% of culture-positive animals on farm C other than identified as super-shedders shed the same strain as that of contemporary super-shedders during first, third, and fourth visits to the farm. For the available tissue data, tissues from about 82% of cows other than super-shedders were culture-positive for MAP, indicating a true infection. For low shedders in three herds, about 65% of animals for fecals and 56% for fecal and tissues, shared only the same strain as super-shedders. On farm C, animals 491, 493, and 495 were purchased from other sources; animal 491 was truly infected, which may be a case of adult infection. In few occasions we found multiple strains in different samples during their life time in some animals. It was indicated that while it is reasonable to speculate that coinfection with multiple genotypes is possible, it was recognized that this may be rare event (Harris et al., 2006).

To conclude, taken together the results of MAP strains and observed shedding levels, at least 50% of low shedders have the same strain as that of contemporary super-shedders. The results of this study indicate that very few cows had characteristics of a possible pass-through animal; many more cows were truly infected. Sharing of the same strain of low shedders with the contemporary super-shedders suggests that low shedders may be infected as adults by the super-shedders. The use of SSR strain-typing combined with observed shedding levels provided a unique opportunity to get a better insight into herd infection dynamics of MAP.

ACKNOWLEDGEMENTS

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#105

MIRU-VNTR genotyping of *Mycobacterium avium* subspecies *paratuberculosis* revealed Type-specific differences at two component regulatory systems

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In this report, we genotyped a collection of Spanish *Mycobacterium avium* subspecies *paratuberculosis* isolates with a combination of six mycobacterial interspersed repetitive units (MIRU) and variable number tandem repeats (VNTR) loci; MIRU-2, MIRU-3 (Bull et al., 2003), VNTR-25, VNTR-32, VNTR-292 (Thibault et al., 2007) and VNTR-259 (this study). This research forms part of the EU project ParaTBtools.

For this purpose we screened 70 *M. a. paratuberculosis* Type II and III isolates, obtained from domestic livestock (goats and cattle) and two isolates recovered from wild animal species (fallow deer- *Dama dama*- and mouflon- *Ovis orientalis musimon*) throughout 22 different Spanish localities in a nine-year period.

The selected combination of MIRU-VNTR loci differentiated 17 different allelic variants within this population of *M. a. paratuberculosis* isolates, with a resulting discriminatory index (D) of 0.82. An interesting feature was the *M. a. paratuberculosis* Type-specific differences observed at the number of repeats at MIRU-3, located between genes *Sen*X3 and *Reg*X3, components of a regulatory system with a role in the virulence of *Mycobacterium tuberculosis* (Magdalena et al., 1998). In addition, *M. a. paratuberculosis* Type-specific number of tandem repeats was also obtained for the novel locus VNTR-259, which as in the case of MIRU-3, was located between a two component regulatory system, *AfsQ1*/*AfsQ2*, which has a role in the regulation of the antibiotic production strategies in Streptomyces (Shu et al., 2009).

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Diagnostics and Genotyping Perspectives Lecture

Genotyping perspective of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*)

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Molecular biology methods offer new opportunities to differentiate different *MAP* isolates. The aim of this presentation is to provide comprehensive information about the methods available to differentiate *MAP* at the DNA sequence level. The methods discussed in the presentation will include DNA fingerprinting, PFGE, PCR-REA, sequencing analysis, spoligotyping etc.

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Diagnostics and Genotyping

Poster Abstracts

#6 Significance of Double Map Agar Gel Immunodiffusion Precipitation Bands

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ABSTRACT

The significance of a second immunoprecipitation band in agar gel immunodiffusion test for *Mycobacterium avium* subsp. *paratuberculosis* was analyzed in a retrospective comparative study with the serum specimens' corresponding Map ELISA titters. Single, primary precipitation bands were not tightly correlated with the presence of a diagnostic Map ELISA test. Nine of the 71 (12.7%) cases in which a double band occurred had a negative UF ELISA test. Another 10 (14%) the corresponding sera ELISA readings deemed at best to be suspicious. The greatest number of cases correlated with strongly positive Map ELISA tests 39/71 (54.9%). Serial observation in three cows demonstrated a second immunoprecipitation band only developed when the corresponding ELISA titer became markedly elevated above that value at which the primary immunoprecipitation band occurred. Unlike the primary immunoprecipitation band, the second immunoprecipitation band had a positive correlation with markedly elevated Map ELISA test readings. This observation implies that second, and not the first, immunoprecipitation band identifies predominantly the same Map antigen complexes that are used in the Map ELISA test.

INTRODUCTION

Agar gel immunodiffusion test (AGID) was initially developed as a screening test for the detection of Johne's disease in flock ruminants. While AGID test has a high correlation between AGID positivity and fecal cultures and up to 100% specificity, it has only 5% sensitivity in detecting infected cows (1-3). The development of absorbed enzyme-linked immunoabsorbent assays (ELISA) tests and documentation of their great sensitivity have led to the relative abandonment of AGID in disease prevention management schema of dairy herds (1-2).

Cows with documented Johne's disease can have a diagnostic Map ELISA reading and not have a positive AGID test. However, the quantitative disparity of the amount of specific antibody required for a positive test is inconsistent with the observation that cows with Johne's disease may, on occasion, have a positive immunoprecipitation band to the test antigens and not have a diagnostic Map ELISA titer.

MATERIALS AND METHODS

Study populations: The AGID test data, derived from a five-year herd management program that was implemented between January 2001 and January 2006, were reviewed to identify the number of cows with a positive AGID test. To determine the relative frequency with which a double precipitation band occurred, the 71 cows with a positive AGID precipitation band were used as the denominator. The corresponding Map ELISA titers were tabulated for dairy cows with a single precipitation band (Table 1) and for those with two precipitation bands (Table 2). Multiple observations were available on 3 cows, in which the presence or absence of the second precipitation band varied.

The purposes of this report are to describe the relationship of the primary and second immunoprecipitation bands as they relate to their corresponding Map ELISA titers.

AGID: Petri dishes were poured with sterile saline 1% agrose prepared in 0.1 M Tris-HCL buffer at pH 10. Well distances were 8 mm. Well sizes were 4 mm for the six peripheral wells and 3 mm for the central well. The peripheral well received 45 ul of the test serum. The central well was inoculated with 35 ul of a crude protoplasmic antigen (Allied Monitor,

Missouri). Serum from a cow with documented Johne's disease constituted the positive control. Final analytical readings were done at 24 and 48 hours. The appearance of one or more clearly definable precipitation lines before or at 48 hours constituted a positive result. The AGID tests were done with both nonabsorded and *Mycobacterium phlei* absorbed sera.

Pre-absorbed ELISA test: Test sera were pre-absorbed with *M. phlei.* The ELISA results were calculated from wavelength readings at optical density (OD) 405 nm. All readings less than 1.6 OD were considered negative; readings between 1.5 and 1.9 OD were deemed suspicious/inconclusive; and readings of 2.0 to 2.5 OD were called low positive. A high positive was any reading 2.6 OD or above.

RESULTS

Frequency of double precipitation bands within the AGID test: Of the 71 individual animals identified having a positive AGID test, 13 (18%) exhibited, at one point or another, a double immunoprecipitation band.

Relationship of double precipitation bands to corresponding ELISA optical density readings: The mean ELISA optical density reading (OD) for the 13 cows whose AGID test demonstrated a double precipitation band was 3.88 with a range from 2.3 to 5.8. Only one serum had an OD reading of less than 2.6.

Single, primary precipitation bands were not tightly correlated with the presence of a diagnostic Map ELISA test. Nine of the 71 (12.7%) cases in which a double band occurred had a negative UF ELISA test. Another 10 (14%) sera were deemed to be, at best, suspicious (Table 1). The greatest number of cases correlated with strongly positive Map ELISA tests 39/71 (54.9%). Serial observation in three cows demonstrated the unmasking of a second immunoprecipitation band when the corresponding ELISA titer became markedly elevated above that value at which the primary immunoprecipitation band occurred (Table 2)

When AGID positive sera were absorbed against varying concentration of M. plea, the results for the immunoprecipitation bands remained unchanged.

Table 1. Distribution of serum Map ELISA titers among Holstein cows with a positive AGID test

ELISA Titer	Number of Cows (Percentage)
0 – 1.5 – negative	9 (12.7%)
1.6 – 1.9 – suspicious	10 (14%)
2.0 – 2.5 - positive	13 (18.3%)
Greater than 2.5 – strong positive	39 (54.9%)

DISCUSSION

The common absence of an immunoprecipitation band in the face of a high Map ELISA test result has been largely ascribed to the amount of specific antibody required for an immunoprecipitation band in contrast to the significantly smaller amount of specific antibody that is required for a diagnostic Map ELISA reading.

Unlike the primary immunoprecipitation band, the second immunoprecipitation band appears to have a positive correlation with markedly elevated Map ELISA test readings. This observation implies that the second immunoprecipitation band identifies predominantly the same Map antigen complexes that are used in the Map ELISA test.

The resultant interpretation of this data was that a better Map ELISA test could be developed using selected antigenic subsets embedded in the primary immunoprecipitation band.

Table 2. ELISA Optical Density Readings in Cows with Double AGID Precipitation Bands in One or More Sera

Date	AGID	ELISA
Cow #3917		
8/21/03	double band	5.5
8/25/03	double band	5.0
8/28/03	double band	4.1
9/2/03	single band	3.9
9/3/04	single band	3.4
9/4/03	single band	3.6
Cow #3882		
	single band	3.49
	double band	4.96
	single band	3.09
	single band	4.72
Cow #4151		
	single band	2.32
	double band	4.1

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#7

Significance of Heavy Fecal Shedding of *Mycobacterium Avium* Subspecies *Paratuberculosis* (Map): Comparison of Fecal Culture, Real-Time and Nested PCR Testing

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ABSTRACT

The potential that clumping by *Mycobacterium avium* subspecies *paratuberculosis* can influence the quantity of organisms identified by fecal culturing was analyzed in a prospective, blinded study using comparative fecal culture, hspX real-time PCR and direct and nested IS1311-based PCR testing. Of the 22 fecal samples identified as coming from "heavy shedders" by fecal culture, only 7 fecal cultures had positive correspondence with real-time and nested PCR. Clumping by Map within fecal samples can cause quantitative misrepresentation of the degree of fecal shedding within a given fecal specimen.

INTRODUCTION

A major herd management tool in controlling Johne's disease has been the ability to quantify the amount of Map present in a given fecal specimen. As a general rule, animals identified as having heavy Map fecal shedding are considered to represent a significant threat to overall herd health and are frequently culled (Collins et al., 2006).

Map differs from other pathogenic mycobacterium, such as *Mycobacterium bovis* and *Mycobacterium avium* subspecies *avium*, in that organism replication results in the tight clumping of individual mycobacterium (Harris and Barletta, 2001). Depending upon the portion selected for testing within a given fecal specimen, Map clumping theoretically introduces sampling error. No studies have been done to analyze whether sample site bias occurs.

The purpose of this paper is to present corresponding fecal culture, real-time PCR and nested PCR Map fecal test results as they relate to validating or challenging the diagnostic category of heavy shedding as defined by fecal culture.

MATERIALS AND METHODS

Study population: The fecal samples were obtained from two dairy herds that participated in the Florida Johne's Disease Dairy Herd Prevention Program. The fecal samples were sent via Federal Express next day shipment in coolers with ice packs. The number in which nested PCR data was available from the Diagnostic Laboratory of the Department of Infectious Diseases, University of Florida College of Veterinary Medicine, determined the number of fecal samples analyzed in the study.

Fecal culture tests: The fecal culture testing using the Trek® Diagnostic System was done at the Animal Disease Diagnostic Laboratory, School of Veterinary Medicine, Purdue University.

Fecal PCR tests: The direct fecal polymerase chain reaction (PCR) testing using the Tetracore® Map Diagnostic System was done at the Animal Disease Diagnostic Laboratory, School of Veterinary Medicine, Purdue University. The direct and nested fecal PCR testing using the FecaMap® Map Diagnostic System was done at the Veterinary Diagnostic Laboratory of the Department of Infectious Diseases, College of Veterinary Medicine, University of Florida.

All tests were done in accordance to the manufacturer's instructions.

The Trek®, Tetracore ® Diagnostic Systems, and FecaMap Test sampled 2, 2, and 0.25 grams of fecal material respectively.

Serum Map ELISA test: The serum Map ELISA testing (ParaChek®) was done at the Florida Diagnostic Laboratory at Live Oak, Florida.

The test results from all three veterinary diagnostic laboratories were sent as developed, directly to the USDA Office in Gainesville, Florida where the collective data was compiled and forwarded to Infectious Diseases Incorporated for secondary analysis.

Table 1. Comparative real-time and nested PCR tests on fecal specimens identified by the Trek® Diagnostic System as heavy map shedders

Cow #	Culture Date	RT Result	Nested Result	ParaChek® Result/Date
Serial Serological Observations				
550	3/2007	+	-	0/12/2006 0/2/2008
906	3/2007	-	-	0/12/2006 0/2/2008
2018	4/2007	+	+	6.04/12/2006 culled
2101	3/2007	+	+	3.57 culled
2216	6/2007	+	+	0/12/2006 culled
2854	8/2007	+	+	0.93/12/2007 0/2/20083
3079	5/2007	-	-	0/12/2006 0/2/2008
3162	5/2007	+	+	no data culled
3308	8/2007	+	-	0/12/2006 0/2/2007
3335	3/2007	+	+	0/12/2006 0/2/2008
3537	8/2007	+	-	0/12/2006 0/2/2008
3697	6/2007	-	-	0/12/2006 0/2/2008
Single Serological Observation				
4056	4/2007	-	-	0/2/2008
4094	4/2007	-	-	0/2/2008
4101	5/2007	-	-	0/2/2008
4103	5/2007	-	-	0/2/2008
4108	5/2007	-	-	0/2/2008
4115	5/2007	-	-	0/2/2008
4144	3/2007	-	+	0/2/2008
4155	5/2007	-	+	0/2/2008
4194	6/2007	-	-	0/2/2008
4200	7/2007	+	+	0/2/2008

* = positive direct 1311 PCR; + = positive test result; - = negative test result

RESULTS

Of the 327 fecal specimens analyzed by all three techniques, 22 animals were identified as heavy shedders based upon their fecal culture results.

Of the 22 heavy fecal Map shedders identified by fecal culture results alone, 7 fecal specimens had corresponding confirmative real-time and nested PCR tests, 5 specimens had either a positive real-time or nested PCR test. Ten fecal specimens (45%) failed to be confirmed by either the corresponding real-time or nested PCR tests (Table 1).

Of the 6 cows with positive correspondence between fecal culture, real-time PCR, and nested PCR, 4 were culled. Two had diagnostic ELISA titer at the time of their removal from the herd. Of the remaining two cows, one cow had an initial high suspicious titer as determined by ParaChek® Map ELISA testing that subsequently corrected. The other cow had no evidence of having had a serological response.

DISCUSSION

The three methods used to identify Map required separate test samples being taken from a given fecal specimen. The concurrence of all three tests decreases the probability of sample error due to clumping; whereas non-concurrence between the three tests argues for non-uniform distribution of organisms.

The importance of correctly identifying heavy fecal shedding of Map is two-fold:

1) animals identified as heavy shedders are considered to be primary disseminators of pathogenic mycobacterium into the environment; and
2) heavy Map replication in feces documents advanced mucosal disease and increased probability of systemic progression.

The lack of concurrence between a positive fecal culture and two sensitive PCR tests puts into question the designation of “heavy or moderate shedding” as identified by the culture. It argued that fecal culture results can be significantly colored by sampling error.

CONCLUSION

Forty-five percent of the cows identified in this study as heavy shedders by Trek® Diagnostic System were documented by real-time and nested PCR to be light fecal shedders. Decision makers may be well advised to seek additional confirmation of fecal heavy shedding status before culling an animal from the herd based upon this criterion alone.

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#9 Comparative IS*900* and IS*1311* Direct Fecal *Mycobacterium Avium* Subspecies *Paratuberculosis* Nested PCR Tests: Significance of Disparities

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ABSTRACT

To challenge the hypothesis of genomic polymorphism, two direct fecal nested polymerase chain reaction (PCR) tests based upon the IS*900* and the IS*1311* insertion sequences were constructed and tested in parallel in three United States Department of Agriculture (USDA) Laboratory Certification tests.

The sensitivities for P90-P91 and J1-J2 IS*900* direct and nested primers were 21.7% and 76.7% whereas those for the IS1-IS2 and IS3-IS4 primers were 38.3% and 86.7%. The ability of *1311* base insertion sequence primers to better identify Map in the USDA laboratory certification tests over IS*900* base insertion sequence primers argues for the existence of a degree of genetic polymorphism among culture-positive isolates of Map used in USDA's laboratory certification testing.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* is theorized to have evolved from *Mycobacterium avium* subsp. *avium* (Ma) (Frothingham, 1999; Turenne et al., 2007). Map and Ma, by genetic criteria, are classified as subsets of the same species (Harris and Barletta, 2001; Thorel et al., 1990). Research on diagnosis and epidemiological findings relative to Map has often denied the existence of closely related Map phenotypic variants more closely related to MA (Cousins et al., 1999). Some mycobacteria, more Ma-like than Map-like, contain the IS*900* insertion sequence (Bolske and Johansson, 2002; Cousins et al., 1999; England et al., 2002).

IS*1311* is present in the vast majority of pathogenic mycobacterium. A long evolutionary time span is suggested by the presence of mutations in some of the IS*1311* elements (Whittington et al., 1998). Genomic polymorphism is to be anticipated within species evolution. IS1311 is present in Ma and Map (9). Primers based upon the IS1311 insertion sequence that identify Ma variants and Map are encompassed in the direct and nested fecal FecaMap® patented primers.

The purpose of this study was to compare the respective abilities of IS1311- and IS*900*-based PCR primers for identifying Map isolates within three USDA Laboratory Certification Tests.

MATERIALS AND METHODS

Population studied: The fecal samples were obtained from two dairy herds that participated in the Florida Johne's Disease Dairy Herd Prevention Program. The number of fecal samples analyzed was determined by the number in which nested PCR data was available from the Diagnostic Laboratory of the Department of Infectious Diseases, University of Florida College of Veterinary Medicine.

Fecal culturing: The fecal culture testing using the Trek® Diagnostic System was done at Purdue University School of Veterinary Medicine in accordance with the manufacturer's instructions.

Real-time Map PCR testing: The direct fecal PCR testing using the Tetracore® Map Diagnostic System were done at Purdue University School of Veterinary Medicine in accordance with the manufacturer's instructions.

Direct fecal nested Map PCR testing: Direct fecal nested Map PCR tests were done at University of Florida College of Veterinary Medicine using the FecaMap® system in accordance with the manufacturer's instructions.

Statistical analysis: The Fisher's Exact Test was used to test whether there was any non-random association between variables of the two direct fecal, nested Map PCR test results and provided culture results. Kappa coefficient, sensitivity was estimated using Win Episcope 2.0 software (Win Episcope 2.0). Ninety-five percent confidence intervals (CI) were constructed for all estimates.

RESULTS

The direct IS*900* and IS*1311* PCR primers had a sensitivity of 21.7 and 38.3% respectively; whereas the corresponding nested PCR primers had sensitivities of 76.6% and 86.7% (Table 1). The P90-P91 primers did not identify the Ma -spiked culture as being positive whereas the direct IS*1311*, nested IS*900* and IS*1311* primers correctly identified 2 of the 3 M. avium spiked cultures as being positive respectively.

The demonstration that the IS*900* nested primers identified the *M. avium*-spiked fecal specimens in a manner comparable to the IS*1311* nested primers necessitated excluding these samples in the overall calculation of comparative sensitivity. The spiked fecal samples were deleted in the statistical comparison of direct IS*900* and IS*1311* primers. The ability of the nested IS*900* PCR primers to extend the test sensitivity to *M. avium* was also a confound variable in the comparison of the nested IS*900* vs. IS*1311* nested primers (Table 1).

Table 1. Statistical comparison of IS*900* versus IS*1311* direct and nested primers on fecal specimens with three USDA Laboratory Certification Tests

	P90-P91	IS1-IS2
Sensitivity	21.7% (13+/46-) 38.3%(23+/37-)	
Specificity	100% (19/19)	94.7% (18/19)
Kappa Coefficient	0.11	0.2
	P90-P91/J1-J2	IS1-IS2/IS3-IS4
Sensitivity	76.7% (46+/18-)	86.7% (52+/9-)
Specificity	94.7% (18/19)	100% (19/19)
Kappa Coefficient	0.2	0.76
Interpretation	good agreement	very good agreement

DISCUSSION

Given that the IS*1311* based primers, IS1/IS2, identify only 6-8 copies where as the P90/P91 primers based upon the IS*900* sequence identify 14-18 copies, there should have been no reason to anticipate that the IS*1311* base primers would exhibit superior sensitivity unless the sequences covered had greater antigenic representation.

As expected, fecal specimens spiked with M. avium were not detected by P90-P91 insertion sequence, but were detected by the IS*1311* direct primers. What was not anticipated was that the nested IS*900*-based J1/J2 in two of the three Ma spiked samples as well as the IS*1311* nested IS3/IS4 primers identified *M. avium*.

Clinical relevance is inferred by a herd study of 341 dairy cows in which IS*1311*-basedFecaMap® identified a non-IS*900* mycobacterium from a cow whose feces demonstrated heavy shedding and tested positive with the Tetracore® hspX PCR test and who was an early cull (Williams et al., 2009).

CONCLUSION

The ability of *1311* insertion sequence direct primers to better identify Map in the USDA laboratory certification tests than the IS*900* insertion sequence direct primers indicates greater antigenic representation for the 6 to 8 copies covered by the IS*1311* insertion sequence

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#16 Histopathological and immunohistochemical studies on naturally occurring paratuberculosis in goats

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Immunohistochemical and histopathological studies were carried out on spontaneously occurring paratuberculosis (Johne's disease) in 38 goats. Among these goats, focal (grade 1), diffuse lymphocytic (grade 2), diffuse mixed (grade 3) and diffuse multibacillary (grade 4) lesions were observed in 3(7.8%), 5(13.1%), 15(39.4%), 15(39.4%) goats, respectively. The focal lesions, tiny granulomas were observed in the interfollicular areas of Peyer's patches (PP), and the cortex of mesenteric lymph node (MLN). In diffuse lymphocytic lesions, besides the presence of granulomas in the Peyer's patches, jejunal and ileal mucosae were diffusely infiltrated with lymphoid cells and small number of macrophages. Small multifocal granulomas were observed in the cortex and paracortex of MLN. Goats with diffuse mixed and diffuse multibacillary lesions had shown poor body conditions and thickened and corrugated intestinal mucosae on necropsy. Histologically, in diffuse mixed lesions, mucosa showed infiltration with epithelioid cells mixed with lymphocytes. Small to large multifocal granulomas containing clusters of acid-fast bacilli (AFB) were observed in the MLN. In diffuse multibacillary lesions, intestinal jejunal and ileal villi were club shaped, at times flat and fused, and diffusely infiltrated with sheets of epithelioid macrophages loaded with clusters of AFB. In indirect immunoperoxidase test (IPT), 36 (94.7%) out of 38 goats showed positive immunoreaction, the intensity of which varied with the types of lesions. On comparison, the IPT was found to have better sensitivity than Ziehl-Neelsen (ZN) staining (84.2%). One of the most important advantages of IPT was that the positive immunoreaction could be visualized even at low magnification in all grades of lesions including focal and diffuse lymphocytic type, in which AFB were scarcely detected with oil immersion objective. Based on this study, it was concluded that a spectrum of pathology existed in caprine paratuberculosis. IPT was more sensitive than ZN for demonstration of MAP bacilli/antigens.

#17 Molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* isolates from different host and geographical origins

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The IS*900* and IS*1311* PCR and IS*1311* PCR-REA assay was used in the present study to analyse 60 MAP isolates from various species and geographic regions to assess their strain types. Five non-MAP reference strains were also included in the study. All the 35 MAP strains tested from India and the UK (27) were positive for both PCRs. The reference strain *M. avium* was negative for IS*900* and positive for IS*1311* PCR. A non-MAP reference strain *M. bovis* was found to be negative by both PCRs. All the sheep isolates (17) from CSWRI, Avikanagar farms produced 3 bands on REA (67 bp, 218 bp and 323 bp) and were identified as "bison" B type strains. MAP isolates of cattle (7), goat (8) and sheep origin (2) obtained from northern and southern parts of India and border region, were identified as 'B' type. One MAP isolate of goat origin from Bareilly region was identified as 'C' type. The MAP isolates of cattle (8), sheep (6), goat (4), crow (1), badger (1), deer (1), mouflon (1), rat (1), rook (1) and stoat (1) obtained from Scotland were found to be cattle (C) type by REA as they yielded 4 bands (67 bp, 218 bp, 285 bp and 323 bp). Three MAP reference strains (K10, ATCC 19698, 316F MTCC) were 'C' type. *M. avium* (M120/04 UK) gave M. a. avium (Maa) type REA pattern as expected. The results suggested that in India bison type strains were more prevalent than other types. In the UK cattle type strains were prevalent. Also, since most MAP strains irrespective of their host and geographic origins are of similar type on PCR-REA, the diagnostics and vaccine reagents prepared from one MAP strain (Bison type) of Indian origin may act effectively in all species of animals.

#18 Efficacy of a PCR targeting the 251 gene of *Mycobacterium avium* subsp. *paratuberculosis* for the diagnosis of Johne's disease in goats

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The diagnostic potential of a recently identified 251 gene locus specific to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been found to be 100% sensitive and specific in a study on MAP isolates (Rajeev et al., 2005). In the present study, polymerase chain reaction (PCR) using specific primers to the 251 gene of MAP was first carried out on 62 MAP strains and 5 non-MAP strains of different geographical (Indian and UK) and host origins, and then applied to clinical samples. PCR amplification of the gene was obtained for all MAP strains, but not for other non-MAP strains.

The PCR was performed on formalin-fixed paraffin embedded tissue sections from 38 naturally and 10 experimentally infected goats with different grades of pathology. All natural (100%) and 9 experimental goats (90%) were positive in the PCR. The results showed that while the PCR was specific it was also a sensitive method of diagnosis. The sensitivity of this PCR (97.9%) was found to be higher than ZN (84.2%) that detects acid fast bacilli. The assay was further carried out on 70 fresh tissue samples from 70 suspected paratuberculous goats. Positive amplifications were obtained in 26 cases (37.1%). On histopathology, all of these goats showed chronic enteritis and lymphadenitis with and without distinct granulomas formation. Acid-fast bacilli were rarely observed in the tissue sections of these goats (paucibacillary). Nucleotide sequencing of amplified 251-gene locus products from a bovine strain (accession no.EU348778) and from a goat tissue sample (accession no.EU366498) showed 100% similarity with respect to the 251-gene locus of *Mycobacterium avium* subsp. *paratuberculosis* K-10 strain.

The results suggested that 251 gene locus PCR was a highly specific and sensitive test for the diagnosis of paratuberculosis. A larger number of samples should be tested to assess it's suitability for the routine identification of MAP isolates and diagnosis of Johne's disease.

#19 Development of a multiplex PCR assay targeting IS*900* and F57 genes in the diagnosis of paratuberculosis

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The objective of the study was to develop a PCR simultaneously amplifying two genes IS*900* and F57 for identifying and confirming the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in clinical samples from animals. This could avoid subsequent confirmation of PCR products in light of the questionable specificity of the IS*900* gene. Two specific primer sets; one from IS*900* and other from F57 genes were used for the amplification of 314 bp and 439 bp PCR products, respectively. The DNA samples used in the multiplex PCR were extracted from tissues and MAP isolates maintained in the Johne's disease laboratory (GD Lab.) using standard protocols. The PCR was applied to high quality DNA from 52 MAP, 5 non-MAP isolates from various hosts and geographic origins and 45 fresh intestinal tissue samples from infected sheep and goats and suspected cases of bovine paratuberculosis. All MAP isolates (100%) were positive for both genes and all 5 non-MAP isolates were negative for both genes. Twenty six out of 30 goat tissue samples and 6 of 8 sheep samples yielded amplification of both genes, whereas only IS*900* was amplified from 4 goat and 2 sheep samples and one of 7 suspected cases of bovine paratuberculosis. These samples probably contained low numbers of MAP which could be detected only by PCR directed against a multicopy gene such as IS*900*. The results of the present study suggested that while multiplex PCR could be used for identification and confirmation of MAP isolates, a minority of clinical samples may be positive for only the IS*900* gene and additional tests may be required to confirm a diagnosis of Johne's disease in these animals.

#29 Conductometric biosensor for diagnosis of Johne's disease

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The development of non-laboratory based assays would support more frequent testing of animals for Johnes disease (JD) and could improve its control. Newly developed conductometric biosensors combine immunomigration technology with electronic signal detection and has been recently developed for the detection of *Mycobacterium avium* subsp. paratubercolosis (MAP) IgG. Optimization of this biosensor is needed to support its usefulness in diagnosis. In this study, two aspects of the previously developed conductometric biosensor were optimized for its application in JD diagnosis. After optimization, a pilot study on the biosensor's use in JD diagnosis was conducted, using samples whose JD status where unknown. A commercially available antibody detection ELISA was used as a gold standard for comparison. The biosensor's sensitivity was (71.43%) and specificity (70%) when compared to the antibody detection ELISA. There was a moderate strength of agreement (Kappa = 0.41) between the two assays. Findings from this study support the continued development of conductometric biosensors for use in the diagnosis of JD. Further optimization of the biosensor and validation with a larger sample size could support its use in JD control programs.

#31 Case study: Identification of a non-classical (bison) strain of *Mycobacterium avium* subsp. *paratuberculosis* in Cervus elaphus subsp. nelsoni (Rocky Mountain Elk)

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Tissues from an emaciated, hunter-killed elk harvested on public land in Harney County, Oregon were submitted to the Oregon State University in the fall of 2008. No other abnormalities were noted at necropsy. Histologically there were large numbers of macrophages filled with numerous acid fast bacilli expanding the lamina propria of the small intestine, and granulomas containing acid fast bacteria in the liver. Paraffin imbedded intestinal tissue was submitted to NVSL for PCR analysis, and frozen tissues were submitted for general mycobacterial culture, including bovine tuberculosis and Johne's disease. PCR on formalin fixed tissue was negative for IS*6110* (M. tuberculosis complex) and positive for IS*900* (*Mycobacterium avium* subsp. *paratuberculosis*). All general mycobacterial media without mycobactin J (MJ), and Herrold's Egg Yolk media with MJ did not support mycobacterial growth, whereas Trek ESP® media supplemented with 2ml of additional egg yolk signaled positive in 8 days. Middlebrook 7H10 media supplemented with MJ and 250ml of egg yolk/L also supported growth of the isolate after 7 weeks of incubation. The isolate failed to grow when subcultured directly onto HEY media w/MJ. Biochemically these growth patterns are consistent with sheep strains of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Confirmation of this isolate as MAP was done using commercially available MAP PCR reagents (Tetracore® and Ambion®). However, the IS*1311* restriction enzyme analysis pattern did not match sheep or cattle isolates, but was instead similar to a MAP strain isolated from bison in Montana in 2001. Therefore, this constitutes the first known report of a "bison" strain of MAP in elk in North America.

#32 Laboratory performance of fecal culture and direct PCR measured by proficiency testing

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National Veterinary Services Laboratories administers a Johne's Disease Fecal Proficiency Panel annually to diagnostic and research laboratories. In 2008, 118 panels were sent to 71 laboratories. Collectively, these data provide insight into the performance of fecal culture and direct PCR methods currently used throughout veterinary diagnostic laboratories. While some laboratories correctly classified all 25 samples for each method, interlaboratory variation within methods differed significantly: 24 of 32 (75%) laboratories passed using liquid culture; 35 of 50 (70%) passed using direct PCR; and 20 of 36 (55%) passed using solid media. Liquid media culture systems were significantly more accurate at classifying samples as positive or negative than solid media (odds ratio 2.1, 95% CI = 1.5-3.0) and more accurate than direct PCR (OR = 1.8, 95% CI= 1.3-2.4). While not significant, direct PCR tends to be more accurate than solid media (OR=1.2 95%CI=0.95-1.5). Lack of sensitivity and incorrect identification of negative samples as positive were the most common causes of misclassification. Confirmatory PCR on solid and liquid media had a false positive rate of 2.7%, whereas direct PCR had a false positive rate of 0.6%. Classification of an animal's shedding status (low, moderate, high) is currently based on colony counts from solid media. Recent research suggest that clinically important "supper shedders" may not be easily identified by colony counts on traditional solid media. Although not routinely reported by laboratories, continuous results supplied by direct PCR and liquid culture systems may have potential to classify shedding levels that are more clinically relevant. In summary, liquid culture systems currently offer the highest sensitivity across laboratories; however, strict procedures must be in place to prevent false positives during confirmatory PCR. Increased use of direct PCR and liquid media systems highlights the need to establish guidelines for reporting fecal shedding status for these methods.

#36 Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* bovine isolates in the United Kingdom

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The objective of this study was to investigate the genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* (Map) bovine isolates in the United Kingdom (UK). A panel of 204 isolates from dairy and beef (commercial and pedigree) cattle in the United Kingdom (UK) was assembled. The isolates were initially cultured from faeces samples using the ESP TREK liquid culture system and then subcultured onto Middlebrook 7H11 agar supplemented with mycobactin J. The isolates were genotyped by pulsed-field gel electrophoresis (PFGE) using SnaBI and SpeI using the standardised procedure developed at the Moredun Research Institute (www.moredun.ac.uk/PFGE-mycobacteria) and by mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis as described by Thibault et al. (J. Clin. Microbiol. 2007 45:2404-2410). A total of 15 multiplex PFGE profiles and 13 MIRU-VNTR profiles were detected. Using the combined typing data from 178 isolates, the Simpson's Index of Diversity was calculated to be 0.729. There were two predominant strain types represented by 45% and 26% of the isolates, which were widely distributed throughout the UK. The relative virulence of these strain types has yet to be investigated.

#38 Comparison of isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) with the use of specific culture media

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The aim of this study was the comparison of isolation of MAP with the use of specific culture media in sheep and goats herds in Shiraz, Fars Province, Iran. A total of 50 fecal samples from sheep and goats with clinical signs suspected to Johne's disease were prepared. Only 10 out of 50 were positive with Ziehl-Neelsen staining method. The samples were streaked on four specific media including Middlebrook 7H9 agar, Middlebrook 7H11 agar, modified Lowenstein-Jensen (MLJ) and Herrold's egg yolk agar with mycobactin J (2 slopes) and without mycobactin (1 slope). No growth on three media (Middlebrook 7H9 agar, Middlebrook 7HIl agar and modified Lowenstein-Jensen) was observed, but 5 Herrold's cultures were positive. As a result, it seems that the growth of MAP on Herrold's egg yolk agar is more efficient than the other culture media.

#51 A new marker IS*1311* PCR-REA assay for rapid differentiation of 'Indian Bison type' *Mycobacterium avium* subspecies *paratuberculosis* isolates

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Objective: Studies in last two years identified a unique molecular signature in some loci of IS*1311* element capable of distinguishing native 'Indian Bison type' *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from other non-Indian MAP isolates. Objective of the study was to design a specific test based on unique molecular signature for the specific detection and differentiation of native 'Indian Bison type' MAP.

Materials and Methods: Bioinformatics tools were applied on the IS*1311* sequences of native MAP and IS*1311* sequences retrieved from GenBank database including MAP K10 sequences to identify a restriction enzyme capable of differentiating two sets of IS*1311* sequences from 'Indian Bison type' MAP and 'non-Indian' MAP strinas based on its restriction profile. Criteria of selection of restriction enzyme was either loss or gain of new restriction site due to presence of unique molecular signature in IS*1311* sequences of native 'Indian Bison type' MAP compared to 'non-Indian' MAP.

IS*1311* sequences from MAP 'Indian Bison type' isolates recovered from different geographical regions and different host species of the country were amplified and processed for PCR-REA using the restriction enzyme selected in the above step. Sequencing of PCR products was also done in order to confirm the presence/ absence of molecular signature.

Results and Conclusions: Based on the criteria described above BsaJI enzyme was selected for PCR-REA studies. PCR-REA was successfully optimized based on this enzyme capable of distinguishing native 'Indian Bison type' MAP from 'non-Indian' MAP. Results revealed that all 'Indian Bison type' MAP isolates carried molecular signature in their IS*1311* sequences despite their origin from different species. Sequencing also confirmed results of PCR-REA. Thus PCR-REA optimized in this study may be used for rapid differentiation of 'Indian Bison type' isolates. These findings may give new directions to the paratuberculosis research in the country.

#58 Environmental fecal samples for the detection of MAP in small cattle herds

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Detection of MAP in environmental fecal samples to determine the paratuberculosis herd status of large dairy herds has recently been described. Two studies were performed at the Clinic for Ruminants at the University of Veterinary Medicine in Vienna to evaluate this new approach for the assessing of the paratuberculsois herd level in small farms.

Austrian cattle herds with and without known paratuberculosis status were sampled for MAP periodically by collecting of environmental fecal samples. Environmental samples were taken from different sites within the stable and manure storage and tested for MAP by bacterial culture and PCR (Polymerase Chain Reaction).

The correct MAP infection status was assigned in almost 70 % of the herds by fecal culture of the environmental samples. When 3 consecutive environmental samples were tested for MAP, 80 % of the farms could be assigned the correct herd infection status. All farms with a high within herd prevalence of MAP or an increasing frequency of clinical cases of Paratuberculosis could be detected by environmental fecal sampling.

These results indicate that environmental fecal samples could be used to detect the paratuberculosis herd status of small farms as a cheap alternative to other testing schemes. Repeated testing for MAP by bacterial culture seems to be the most effective way to determine paratuberculosis herd status according to these results and might be more accurate and economic than other screening methods.

#60 Evaluation of 2 extraction methods and 2 PCR assays for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in formalin-fixed paraffin-embedded tissues

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Objectives: To evaluate and compare two methods for extracting DNA from formalin-fixed paraffin-embedded (FFPE) tissues plus two PCRs for detecting extracted *Mycobacterium avium* subsp. *paratuberculosis* (MAP) DNA.

Procedure: Sections (24 mm slices) were sliced from 31 formalin-fixed paraffin-embedded tissue blocks collected from cases with ante-mortem evidence of Johne's (bovine, bison, caprine, swine) or Crohn's disease (human). The number and types of tissues per block varied but in all cases the ileum or jejunum and/or the mesenteric lymph node was included. Five negative control tissue block slices from a known uninfected animal were included as well for extraction. Five mm sections from each block were stained (haematoxylin and eosin and by Ziehl-Neelsen) to classify the tissues for the presence/absence of microscopic lesions (granulomatous infiltration and giant cells) and quantity of acid-fast organisms (scale 0-4) as the gold standard. DNA was extracted from paired sections by a boil/freeze method, which involved 2 cycles of boiling and freezing followed by centrifugation, and by a commercial DNA extraction kit (WaxFree™ DNA) specific for FFPE tissue samples a modified protocol was supplied by TrimGen. Each of the extracted samples was amplified by IS*900* nested PCR and by *hsp*X real-time PCR.

Results: Two methods of DNA extraction and two PCR protocols using different targets were compared. None of the negative control tissues were PCR positive. When the PCR results were compared with the presence of acid-fast organisms in the tissues, the sensitivity of the detection of MAP in FFPE tissue blocks was 96.8% (one false negative result) when using the boil/freeze method of extraction with the *hsp*X real-time PCR and 93.5% (one false negative and one false positive result) with the IS*900* nested PCR. When using the commercial extraction kit with *hsp*X real-time PCR the sensitivity was 90.3% (three false negative results) and with the IS*900* nested PCR 54.8% (14 false positives).

Conclusions: A simple and inexpensive method of DNA extraction of FFPE tissues is effective and easy to perform. Coupling the boil/freeze extraction method with the *hsp*X real-time PCR yields a rapid and reliable method for detecting MAP in tissues processed in paraffin.

#62 Field and interlaboratory evaluation of four commercial ELISA kits for *M. avium* subsp. *paratuberculosis* antibody detection in bovine serum

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Detection of antibodies by ELISA is an important tool for Johne's disease control. The goal of this work was to evaluate four commercial ELISA tests. We determined diagnostic sensitivity and specificity on field samples and the robustness of the kits in an interlaboratory trial. To assess diagnostic sensitivity and specificity, 92 sera from fecal culture positive animals at different level of excretion and 32 from negative herds were analysed in one laboratory. All the kits demonstrated a specificity of 100.0%. Merging inconclusive and positive results, test sensitivity was 71.7% for kit A and B and 67.4 for kit C and D. Among infected animals, ELISA sensitivity did not varied significantly with Map excretion levels with all the kit used.

For the interlaboratory trial, 30 coded samples composed by eight replicates of one negative sample and two replicates of 11 positive samples were selected and delivered with the kits to 10 laboratories throughout Italy. All the participants tested each sample in duplicates. Decoded results were analysed for reproducibility within and among laboratories and quantitative results were transformed into S/P values to compare analytical results. Kit A gave 100% of the expected results and Kit B gave almost the same outcome: just one laboratory obtained one inconclusive and one negative result in one replicate. Kit C gave the expected results for 9/11 positive samples and Kit D for 5/11 positive samples. Variations among replicates and laboratories were obtained with the remaining positive samples for these two kits. Regarding the replicates on negative sample, 5 incorrect results distributed in three laboratories for kit C and one doubtful replicate in one laboratory for kit D were detected. According to these results, two (A and B) of the four ELISA kits evaluated showed good performances and reproducibility within and among laboratories.

#66 Comparison of efficacy of conventional and a quantitative real-time polymerase chain reactions (qRT-PCR) on tissues of pathologically characterized ovine paratuberculosis

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A quantitative real-time PCR assay (SYBR green chemistry) employing IS*900* gene specific primers of *Mycobacterium avium* subsp. parartuberculosis (MAP) was developed on a MAP culture (IVRI/C-132), which subsequently served as a basis for detection of MAP genome and its quantification (in copy number) in the intestinal and mesenteric lymph node (MLN) tissues of 38 paratuberculous sheep (multibacillary-23 and paucibacillary-15). The sensitivity of qRT-PCR was found to the standard curve.

The qRT-PCR was compared be 34 copies of IS*900* gene. The number of copies in the test samples was extrapolated from with conventional PCR targeting IS*900* and 251 genes on tissue samples of these sheep. Among the multibacillary group, IS*900* PCR detected 19(82.6%), 251 PCR detected 21 (91.3%) and qRT-PCR detected all 23 (100%) sheep positive for MAP in intestine and lymph nodes of these animals. Among the paucibacillary group, IS*900* PCR detected 2 (13.3%), 251 PCR detected 4 (27%) and qRT-PCR detected all 15 (100%) sheep positive for MAP in the intestines and lymph nodes of these animals. When results of both groups were taken together, IS*900* PCR detected 21(55.2%), 251 PCR detected 25 (65.7%) and qRT-PCR detected all 38 (100%) animals positive for MAP genome either in the intestine or MLN tissues. The 251 PCR detected greater number of cases but it was not significant in comparison to IS*900* PCR. The qRT-PCR results were significant in comparison to both PCR targeting IS*900* and 251 genes. Though, the rates of detection of both MAP genes (IS*900* and 251) were greater in the intestinal than the MLN tissues, the differences were statistically non-significant. Tissues from control sheep were negative for all three PCRs. Based on the results of the present study, it was concluded that qRT-PCR was a highly sensitive test in comparison to conventional PCR for the diagnosis of paratuberculosis on infected tissues especially from paucibacillary sheep.

#84 Genetic diversity in *Mycobacterium avium* subsp. *paratuberculosis* from cattle herds in the Netherlands

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The objective of this study was to investigate the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) within and between herds in the Netherlands. Earlier research showed that MAP has a very low level of heterogeneity and it is difficult to investigate epidemiological relationships between isolates of infected herds or infected ruminant species. Recently developed PCR-based methods consisting of variable-number tandem repeats (VNTRs) of genetic elements called mycobacterial interspersed repetitive units (MIRUs) provided the possibility to study genetic variation in a simple and rapid way.

For 52 isolates originating from 36 commercial dairy herds from different regions of the Netherlands, MIRU-VNTR was applied at 11 loci. Within the group of 52 isolates, 19 different genotypes were detected. One genotype was found in 28 isolates, one genotype in 4 isolates, one genotype in 3 isolates, one genotype in 2 isolates and 15 genotypes were found only once. Eleven herds provided multiple isolates. In 5 herds a single genotype was found whereas in 6 herds more than one genotype was found.

In conclusion, this study shows that between dairy farms in the Netherlands as well as within dairy farms in the Netherlands, infected animals may shed different MAP genotypes. Although 53% of the MAP isolates were typed within one genotype, they originated from different geographical regions in the Netherlands. Further research will be done to study the possible association between the MAP genotype and host disease phenotype.

#85 Comparison of four different PCR methods for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk

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ABSTRACT

Emerging evidence suggests a role of *Mycobacterium avium* subs. *paratuberculosis* (MAP) in the development of human pathologies like Crohn's disease and type I diabetes. For this reason, the need for rapid and robust tools to detect the presence of MAP in food is increasing. Polymerase Chain Reaction (PCR) techniques are able to give rapid and specific results, but their sensitivity is generally lower than traditional culture methods. The aim of this study was to compare four different PCR methods to detect MAP on cow bulk milk samples collected from presumably infected herds. MAP DNA was extracted by Adiapure kit (Adiagene, France). The PCR were: (a) IS900 Commercial end-point PCR (kit Adiavet, Adiagene, France); (b) IS900 Nested PCR; (c) IS900 TaqMan Real time PCR; (d) f57 house-made Sybr Green Real Time PCR. Both the commercial PCR (a) and the IS 900 Real Time PCR TaqMan (c) contained an internal amplification control in order to discriminate between negative or inhibited samples. Out of 37 milk samples tested we found only one positive sample (3%) using (a), (c) and (d) methods. The Nested PCR method (b) showed eight positive samples (22%). Although we used bulk milk samples coming from presumably infected herds, the prevalence of direct detection of MAP is low in all the "one round" PCR methods. As expected, the most sensitive method is Nested PCR, although it remains difficult to apply as regards to possibility of cross contaminations.

BACKGROUND

Emerging evidence suggests a role of *Mycobacterium avium* subs. *paratuberculosis* (MAP) in the development of human disorders like Crohn's disease and Type I diabetes[1, 2]. For these reasons, the request for rapid and robust tools to detect the presence of MAP in food, especially milk, is increasing[3]. Molecular biology techniques, such as Polymerase Chain Reaction (PCR), are able to give rapid and specific results. These methods are also useful tools when the matrices are not appropriately conserved (compromised bacterial viability), or in presence of fastidious strains.

PCR assays developed for the detection of MAP have high specificity, while their sensitivity is generally lower than traditional culture methods. These assays amplify some typical MAP regions, such as f57 (one copy per genome of MAP[4]) and IS900 (12-18 copies per genome of MAP[4]). While the discovery of some IS900-like elements could make not conclusive a test based on IS900 targeting, the f57 sequence is highly specific for MAP[5, 6]. However, due to the higher number of copies, IS900 PCR tests are generally more sensitive than those based on f57[4]. In order to compare the sensitivity of PCR tests, we applied a commercial end-point PCR, a Nested PCR and two Real Time PCR assays to bulk milk samples, previously positive to serological ELISA test.

MATERIALS AND METHODS

We tested about 3000 milk samples from different Italian regions using ELISA test (ID Screen® Paratuberculosis Indirect, Confirmation Test, ID Vet, MontPellier, France).

Out of 82 positive samples, we extracted the DNA from 37 positive samples using Adiapure kit (Adiagene, France).

The PCRs were:

(a) IS900 Commercial end-point PCR (kit Adiavet, Adiagene, France), performed according to the kit manufacturing procedures. The reaction was carried out in *Mastercycler ep gradient s* (Eppendorf, Milan, Italy);

(b) IS900 Nested PCR, using p90-p91 primers[4] for the first round followed by a second round with specific primers (see Table 1). The first round of Nested PCR was performed in 25 μl final volume with 0.1 U of TAQ (Qiagen, Milan, Italy) adding two μl of DNA. The second round was performed by adding two μl of the previous run in a final volume of 25 μl. The reaction was carried out in *Mastercycler ep gradient s* (Eppendorf);

(c) IS900 TaqMan Real time PCR, performed adding two μl of DNA in 25 μl final volume, using TaqMan® Gene Expression Master Mix (Applied Biosystems, Milan, Italy) in *StepOnePlus™ Real-Time PCR System* (Applied Biosystems). Fluorescence intensity was normalized with ROX dye;

(d) f57 SYBR Green Real Time PCR, performed adding two μl of DNA in 25 μl final volume using *Power* SYBR® Green PCR Master Mix (Applied Biosystems) in *StepOnePlus™ Real-Time PCR System* (Applied Biosystems). Fluorescence intensity was normalized with ROX dye. The amplification was followed by melting curve analysis, melting the amplicon from 60 °C to 95 °C and recording the fluorescence 3.3 times per degree with a temperature ramp rate of 2.2 °C/sec.

Both the commercial PCR (a) and the TaqMan (c) contained an internal amplification control in order to discriminate between negative or inhibited samples, while the other test did not contain any internal amplification control. Primers sequences are listed in table 1.

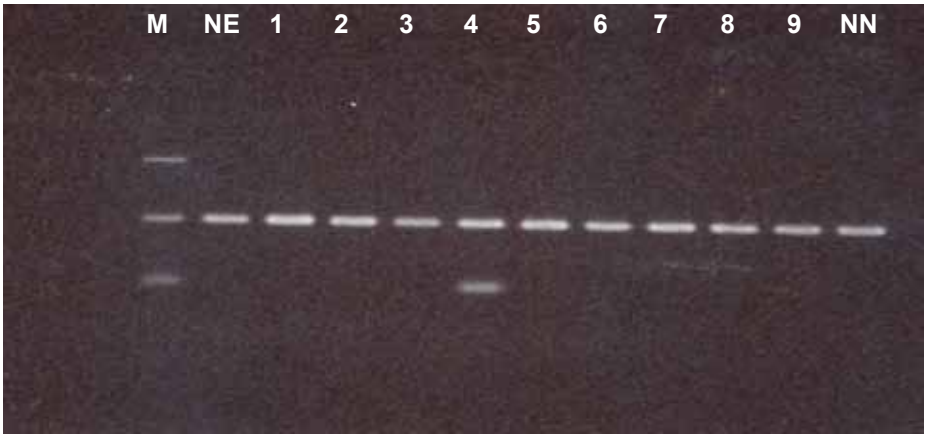
Table 1: Primers and probes sequences.

Methods	Oligo name	Sequences	Ta (°C)
a	Adiavet Kit primers	unknown	62
b	P90	5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'	62
	P91	5'-GGCGTTGAGGTCGATCGCCCACGTGAT-3'	
	Nested Forward	5'-GACGACTCGACCGCTAATTG-3'	58
	Nested Reverse	5'-CCGTAACCGTCATTGTCCAG-3'	
c	IS900 Forward	5'-CCGGTAAGGCCGACCATTA-3'	60
	IS900 Reverse	5'-ACCCGCTGCGAGAGCA-3'	
	IS 900 Probe	6-FAM-CATGGTTATTAACGACGACGCGCAGC-TAMRA-3'	
d	f57 Forward	5'-ATAGCTTTCCTCTCCTTCGTC-3'	60
	f57 Reverse	5'-CAGGGCAACAACATATTCGG-3'	

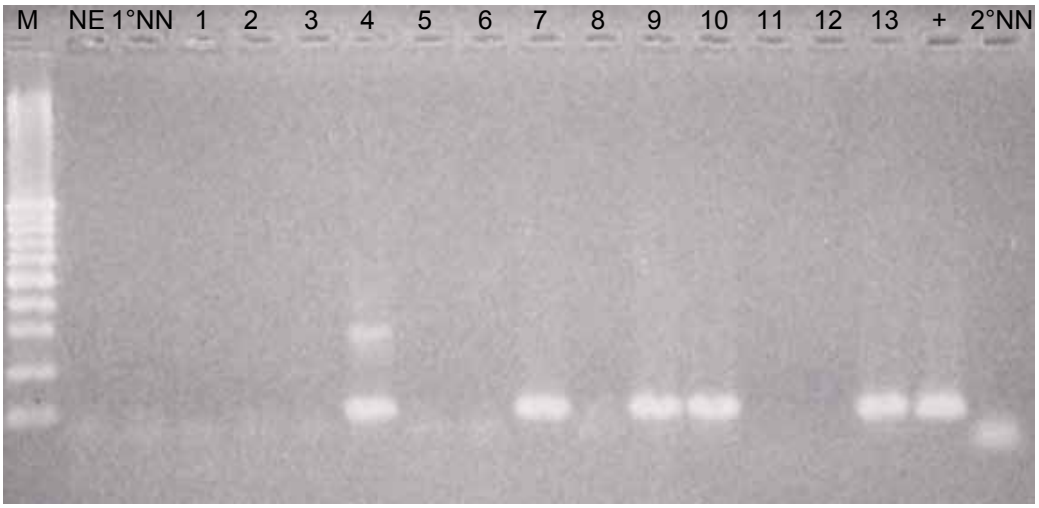
RESULTS

Out of 37 milk samples tested we found only one positive sample (3%) using (a), (c) and (d) methods. The Nested PCR method (b) showed a total of eight positive samples (22%). No inhibited samples were found with (a) and (c) methods.

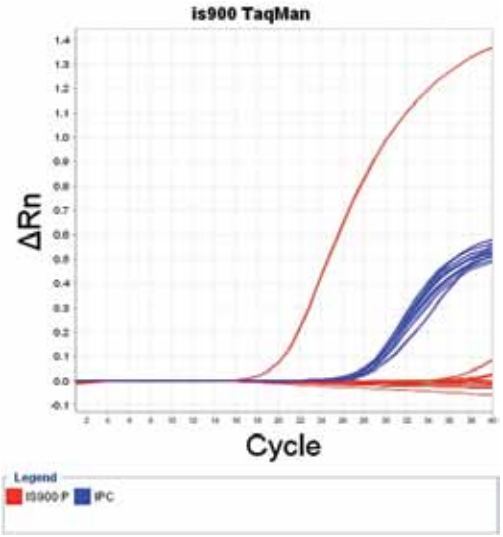
(a) Adiavet PCR. Note the internal amplification control in all samples. Only one sample (4) was positive. M: Marker; NE: negative extraction control; NN: no template control; 1-9: milk samples.



(b) Nested PCR. All positive samples (4, 7, 9, 10, 13) showed a 99 bp amplicon with the same size as the positive control. M: 100 bp Marker; NE: negative extraction control; 1°NN: no template 1st round PCR; 2°NN: no template 2nd round PCR; +: positive control (DNA from MAP ATCC 19698); 1-13: milk samples.



(c) IS900 TaqMan Real Time PCR. Note the internal amplification control. DNA from MAP ATCC 19698 was used as positive control. Data were in duplicate.

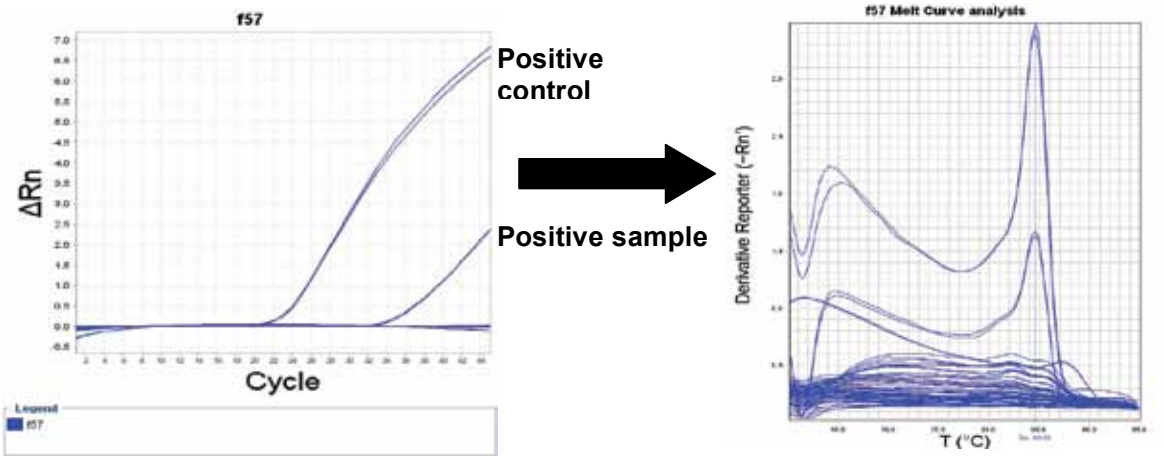


Positive control

Internal amplification control

Positive sample

(d) f57 Real Time PCR. The figure on the left shows the amplifications of the positive sample and the positive control. The figure on the right shows the melting analysis results of amplicons. Note the single peak at 84.6 °C corresponding to the single specific amplification product. DNA from MAP ATCC 19698 was used as positive control. Data were in duplicate.



CONCLUSIONS

In order to compare the sensitivity of four PCR methods for the detection of MAP, we tested bulk milk samples derived for presumably infected herds (ELISA positive). As expected the most sensitive method was Nested PCR, while conventional PCR and Real Time PCR showed lower sensitivity. Although more sensitive, Nested PCR method remains difficult to apply because is time consuming and there are high risks of cross-contamination.

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#86

Typing of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) strains isolated from different Italian regions by four Variable-Number Tandem Repeat (VNTR) methods alone or in association

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ABSTRACT

The control of Paratuberculosis requires the knowledge of the causative agent, M*ycobacterium avium* subsp. *paratuberculosis* (MAP), both in terms of epidemiology and biodiversity within different strains. One of the most widely used method for MAP typing is IS900 sequence restriction fragment length polymorphism. However it is applicable only on cultivable strains, is technically demanding and has limited discriminatory power. More recently, tandem-repeat PCR based methods overcame these problems and, at present, are considered the emerging techniques for MAP typing. The aim of this study was to evaluate the discriminatory power of four PCR typing methods. We selected ten different strains from various Italian regions. We used MIRU (3 loci), VNTR-MIRU (7 loci), MLSSR (11 loci) and MLVA (5 loci) analysis to differentiate the bacterial DNA. Both MIRU and VNTR-MIRU gave 2 clusters (DI 0.556), while MLVA and MLSSR gave respectively 5 (DI 0.667) and 9 (DI 0.978) clusters. The combinations gave different results: MIRU+MLVA and VNTR-MIRU+MLVA gave 5 clusters (DI 0.806), MLSSR+MIRU or MLSSR+VNTR-MIRU did not increase the discriminatory results of MLSSR alone (9 clusters); MLSSR+MLVA gave 10 clusters (maximum theoretic DI value i.e. 1.00). Although a limited number of strains was used in this study, our data suggest that applying a single analysis, MLSSR provides the highest DI. Moreover, MLSSR coupled with MLVA showed the best discriminatory power. Finally, the combination between MIRU or VNTR-MIRU and MLVA enhanced the indexes as compared to single analysis.

INTRODUCTION

The control of Paratuberculosis requires the knowledge of the causative agent, M*ycobacterium avium* subsp. *paratuberculosis* (MAP), both in terms of epidemiology and biodiversity within different strains.

Many methods have been proposed to type MAP: multiplex PCR for IS900 integration loci (MPIL)[1], IS900 sequence restriction fragment length polymorphism (RFLP)[2], amplified fragment length polymorphism (AFLP)[3] and Pulsed Field Gel Electrophoresis (PFGE)[4]. The most popular is IS900 RFLP, however it is applicable only on cultivable strains, is technically demanding and has limited discriminatory power.

More recently, tandem-repeat PCR based methods (TR-PCR) overcame many of these problems and, at present, are considered the emerging techniques for MAP typing.

The aim of this study was to evaluate the discriminatory power of four PCR typing methods alone or in association. We evaluated: mycobacterial interspersed repetitive units (MIRU)[5], variable number tandem repeat - mycobacterial interspersed repetitive units (VNTR-MIRU)[6], multilocus variable-number tandem-repeat (MLVA)[7] and multilocus short sequence repeat (MLSSR)[8] techniques.

METHODS

We selected ten different strains from various Italian regions. Seven samples derived from faeces, two from milk and one from ileum. Eight strains derived from cattle and two from goats. When the strain grown in culture was available, the DNA was extracted suspending one colony in 100 μl of distilled sterile water and boiling for 20 min. For the strains 9 and 10, since the culture was not possible, we extracted MAP DNA respectively from faeces and ileum tissue with DNA QIAmp Tissue Kit (Qiagen, Milan, Italy). In order to differentiate the bacterial DNA, we used the following Tandem Repeat-PCR methods:

- MIRU (3 loci);
- VNTR-MIRU (7 loci);
- MLVA (5 loci);
- MLSSR (11 loci).

All PCR reactions were carried out in a final volume of 25 μl with Taq DNA Polymerase (Qiagen). For MLSSR, we sequenced the obtained amplicons using ABI Prism 310 DNA genetic analyser (Applied Biosystems, Monza, Italy). The sequences were analysed using the SeqMan Module of Lasergene Package (DNA Star, Madison UK). Finally, the discriminatory index (DI) was calculated as reported by Hunter and Gaston[9].

RESULTS

The results for MIRU and VNTR-MIRU are shown respectively in table 1 and 2. Both methods gave two clusters (DI 0.556) with two alleles respectively at locus 2 and 292. According to Bull et al.[5], MIRU typing showed two profiles, profile I and II. Combination of MIRU and VNTR-MIRU did not improve the DI. The two profiles identified by the association of these methods have been previously described by Möbius et al.[10].

Table 1. MIRU Typing

			MIRU No. of TR motifs			
Strains	**Source**	**Matrix**	**locus 1**	**locus 2**	**locus 3**	**MIRU Profile[a]**
1	cattle	faeces	3	9	5	II
2	cattle	faeces	3	9	5	II
3	cattle	faeces	3	7	5	I
4	cattle	faeces	3	9	5	II
5	cattle	faeces	3	9	5	II
6	cattle	milk	3	7	5	I
7	cattle	milk	3	7	5	I
8	goat	faeces	3	7	5	I
9	cattle	faeces	3	9	5	I
10	goat	tissue	3	ND	ND	

[a]According to Bull et Al.[4]. ND: not detectable

Table 2. VNTR-MIRU Typing

	MIRU- VNTR No. of TR motifs						
Strains	**locus 32**	**locus 25**	**locus 292**	**locus 47**	**locus 3**	**locus 7**	**locus X3**
1	8	3	4	3	2	2	2
2	8	3	4	3	2	2	2
3	8	3	3	3	2	2	2
4	8	3	4	3	2	2	2
5	8	3	4	3	2	2	2
6	8	3	3	3	2	2	2
7	8	3	3	3	2	2	2
8	8	3	3	3	2	2	2
9	8	3	4	3	2	2	2
10	ND	ND	ND	ND	ND	ND	ND

ND: not detectable

MLVA Analysis (table 3) showed three alleles at locus 1067, two at loci 1658 and 3527 and one at loci 1605 and 3249. The combination of these alleles gave five different clusters with a DI of 0.667. According to Overduin et al.[7], the most frequent pattern was 22222. One of the isolates from milk, the strain 7, showed an unusual pattern, 22212. This pattern has never been described before, although the allele 1 at locus 3527 has been previously found in bovine strains.

Table 3. MLVA Typing

MLVA No of TR motifs

Strains	locus 1067	locus 1605	locus 1658	locus 3527	locus 3249
1	2	2	2	2	2
2	2	2	2	2	2
3	2	2	2	2	2
4	2	2	2	2	2
5	3	2	2	2	2
6	2	2	2	2	2
7	2	2	2	1	2
8	1	2	2	2	2
9	2	2	2	2	2
10	2	2	1	2	2

MLSSR technique showed the highest DI value (0.978) resulting in nine clusters. We found only one monomorphic locus (SSR 4), while other loci showed allelic frequency ranging from 0.18 to 0.72, with 2.3 as mean value.

Table 4. MLSSR Typing

MLSSR No of TR motifs

Strains	SSR1	SSR2	SSR3	SSR4	SSR5	SSR6	SSR7	SSR8	SSR9	SSR10	SSR11
	G	***G***	***CG***	***GC***	***GC***	***GCG***	***CCG***	***GGT***	***TGC***	***GCC***	***CCG***
1	8	10	5	5	5	4	6	4	5	5	5
2	8	9	5	5	5	4	6	3	5	5	5
3	7	10	5	5	5	4	5	4	4	5	5
4	7	9	5	5	5	4	6	4	5	5	5
5	7	11	5	5	5	4	6	4	4	5	5
6	7	11	5	5	5	4	5	4	4	5	5
7	7	13	5	5	5	5	5	5	5	5	5
8	>14	13	5	5	5	5	5	5	5	5	5
9	7	11	5	5	5	4	5	4	4	5	5
10	7	9	4	5	6	4	5	3	4	6	4

Combinations of the methods gave different results: both MIRU+MLVA and VNTR-MIRU+MLVA gave 5 clusters (DI 0.806), MLSSR+MIRU or MLSSR+VNTR-MIRU did not increase the DI of MLSSR alone (9 clusters), while MLSSR+MLVA gave the maximum theoretic DI value (10 clusters).

CONCLUSIONS

Although the limited number of strains used, our data suggest that the combination of simple PCR based methods such as MIRU, VNTR-MIRU and MLVA enhanced the discriminatory

indexes, compared to each single analysis. However, MLSSR, an expansive and more complex technique, also when applied as single analysis, showed higher DI values than the combination of the other techniques used in this study. Finally, MLSSR coupled with MLVA showed the best discriminatory power.

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#94 A simple liquid culture method for the assessment of paratuberculosis status in dairy cattle herds

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Automated liquid systems for culture of *Mycobacterium avium* subsp. *paratuberculosis* (Map) are more sensitive and rapid than culture on solid media, but they are expensive and require specialised equipment. In this study an inexpensive, non-automated culture method using the liquid 7H9 medium was compared with the conventional method on Herrold's medium for Map detection in dairy cattle manure. Infected herds A and B and negative herds C and D were recruited. The seroprevalence of paratuberculosis in herds A and B was 10% and 1%, respectively. A mix of manure was collected from the floor of the lactating-cow feeding area on 3 occasions at weekly intervals. Three replicates of each sample were processed for a direct realtime-PCR test, conventional Herrold's culture, and non-automated liquid culture. In the latter method, each replicate sample was inoculated into 2 tubes of 7H9 medium that were subsequently tested for growth of Map using the realtime-PCR at weekly intervals over 8 weeks. Map was detected in each sample from herd A by each method applied. Positive samples from herd B varied according to the method used: 8/9 with Herrold's, 5/9 with direct realtime-PCR and 18/18 with realtime-PCR on liquid cultures. Realtime-PCR confirmed the presence of Map at the 2nd week on 7H9 in 14/18 tubes for herd A and at the 5th week or before in 16/18 tubes for herd B. Herds C and D gave negative results in all replicates for all the methods applied. Our results demonstrated that non-automated liquid culture on herd manure increases sensitivity and reduce the time with respect to traditional culture. A protocol based on direct realtime-PCR followed by liquid culture on negative samples, confirmed at 5 weeks and 8 weeks by realtime-PCR, can be used for a semiquantitative assessment of paratuberculosis status in dairy cattle herds.

#99 Rapid assessment of the impact of heat treatments on viability of *Mycobacterium avium* subsp. *paratuberculosis* using an optimized phage assay

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Thermal inactivation studies were carried out to demonstrate the utility of a recently optimized phage amplification assay to enumerate viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk. UHT milk was spiked with 10^6-10^7 CFU MAP/ml and dispensed in 100 µl aliquots into thin-walled 200µl PCR tubes. A Primus 96 advanced thermal cycler (Peqlab, Erlangen, Germany) was used to achieve the following time/temperature treatments: a) 63°C for 3, 6 and 9 min; b) 68°C for 20, 40 and 60 s; and c) 72°C for 5, 10, 15 and 25 s. Milk samples (non-heated (time 0) and heat-treated) were centrifuged (16,000 x g for 15 min) to remove milk components inhibitory to the phage assay, and the pellet resuspended in 1 ml 7H9 broth plus 10% OADC and 2 mM $CaCl_2$. After overnight incubation of the samples at 37°C, appropriate dilutions of each sample were processed through an optimized phage assay (Foddai et al. (2009) AEM). Plaques were counted (PFU/ml) the next day and thermal D values calculated for MAP in milk at 63, 68 and 72°C; D values of 63.7 s, 10.4 s and 2.9 s, respectively, were obtained enabling calculation of a z value for MAP of 6.8°C. The plaque counts obtained using the optimized phage assay were similar to colony counts on HEYM, carried out in parallel. In contrast, phage assay results were available within 48 h rather than after a minimum of 6-8 weeks for conventional culture on HEYM. The D and z values obtained in this study are similar to previously published D and z values for MAP obtained using HEYM culture, so we have clearly demonstrated that the optimized phage assay enables rapid and accurate assessment of the impact of heat treatments on MAP viability.

#108 Evaluation of colostrum as a diagnostic sample for *M. a. paratuberculosis* antibody detection via ELISA

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Cattle infected with *Mycobacterium avium* ss. *paratuberculosis* (MAP) produce antibodies detectable in biological samples such as serum, plasma and milk. Antibody to MAP is also present in colostrum. The goal of this study was to compare antibody detection rates in colostrum vs. serum using an NVSL check-test validated non-commercial ELISA ("JTC-ELISA").

Colostrum was collected within 1-2 days of calving from 44 pregnant cows in a known infected dairy herd. Serum and fecal samples from these cows were then collected two to three weeks after calving. Serum and colostrum was frozen before processing and were tested by JTC-ELISA at dilutions 1:50 and 1:2 respectively. The fecal samples were processed fresh by the MGIT method for isolation of MAP.

Thirty-nine cows were fecal culture positive. Of these, 21/39 were both serum and colostrum JTC-ELISA positive (54%); 11/39 were colostrum ELISA positive but serum ELISA negative (28%) and 7/39 were ELISA negative for both sample types (18%). Five cows were both fecal culture and serum JTC-ELISA negative. Colostrum from two of these cows was interpreted as JTC-ELISA positive. Antibody was thus detected in colostrum from 34 of the 44 cows tested from this infected herd. The range of optical density ELISA results were statistically similar for serum and colostrum samples (i.e., 0.00 – 1.13).

This preliminary trial demonstrated that colostrum may be a useful sample for MAP infection surveillance. The JTC-ELISA was more sensitive with colostrum than serum samples. Factors needing further study to limit false-positives and establish assay specificity include ELISA interpretation algorithms for colostrum, optimization of sample collection timing, and trials with herds of varied (and zero) infection prevalence as previously established by fecal culture, serum ELISA and/or milk ELISA.

#109 A sample and efficient method for DNA extraction from *Mycobacterium avium* subsp. *paratuberculosis* cultured in an automated broth culture system

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Liquid culture of Mycobacteriu*M. avium* subsp. *paratuberculosis* (Map) has shown advantages over culture on solid media to detect Map infection. PCR represents a rapid and efficient means of confirming Map in broth culture. There are several commercial kits and proposed methods of harvesting Map DNA and removal of inhibitors, most of which are costly, laborious and time consuming. This study proposes a simple and efficient method of harvesting DNA from Map cultured in liquid medium (MGIT).

A lysis buffer and proteinase K, together with mechanical cell disruption, was used as cell lysis followed by ethanol and differential centrifugation for removing inhibiting substances from the liquid medium. The method was evaluated comparing its performance on clinical samples with a commercial DNA extraction kit based on bead beating, proteinase K, and column purification and a protocol described in the literature, also based on mechanical disruption and phenol chloroform purification. Moreover, spiked samples in serial dilutions were used for confirming the shortest time to detection (TTD) by the MGIT reader for a positive sample. Finally, analytical sensitivity of the method was calculated spiking clean MGIT tubes with a manually counted bacterial concentration of approximately 1,000 bacteria/ml of medium.

The proposed method could accurately confirm more samples than the commercial kit and the published protocol by real-time PCR. In addition, it showed lower Ct values and minimal inhibition. At different concentrations of spiked MGIT medium the proposed extraction protocol confirmed a positive signal 12 to 17 days after culture. The analytical sensitivity was assessed to less than 10^4 Map/ml medium.

These results indicate that the new extraction method combines reliability and efficiency with simplicity. The lack of inhibition and the high analytical sensitivity suggest an effective Map DNA harvest and an effective removal of inhibitors from both the specimen and the MGIT medium components.

#110 Detection and molecular confirmation of *Mycobacterium avium* subsp. *paratuberculosis* from intestinal tissue and faecal material of hares (*Lepus europaeus*)

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Evidence of natural infection with *M. avium* subsp. *paratuberculosis* (Map) has previously been reported in the European hare (*Lepus europaeus*), with low infection rate and no corresponding histopathological lesions. This could indicate that no active infection but passive transmission has occurred. Low diagnostic sensitivity could be one explanation for this discrepancy. The aim of this study was to detect Map infection in wild hares using the BACTEC MGIT 960 system, improving resuscitation and growth of Map.

A total of 385 hares captured in different areas in southern Chile were randomly selected and sampled for Map from the ileum, mesenteric lymphatic nodes and faeces. All positive MGIT tubes were confirmed by a robust real-time PCR system for IS*900* and F57 targets, including effective mechanical cell disruption, DNA extraction and removal of PCR inhibitors.

Map was detected and confirmed in 22 (5,7 %) ileum, 21 (5.5 %) lymphatic node, and 16 (4.2 %) faecal samples. In 10 hares Map was simultaneously detected and confirmed in tissue and faeces by IS*900* and F57. There was no difference between the proportion of positive results in any type of sample (Fisher = 0.089). All positive samples (59) showed on average 33 cycle threshold values (Ct), where the Ct cut-off value is above 40 for a negative result. The analytical sensitivity of the real-time PCR system used here has been estimated to be <104 organisms/ml. Therefore the degree of infection in a hare may be conjectured as at least a low-medium shedding individual. In addition, hares with positive culture in all samples suggest an active infection. The combined use of the IS*900* and F57 genetic Map markers assures no false positive results.

Although this study should be complemented with histopathological examination, these findings confirm Map-infection in this wildlife species at a higher rate than reported elsewhere in earlier studies.

#124 Culture independent determination of *Mycobacterium avium* subsp. *paratuberculosis* viability by propidium monoazide F57 real time qPCR

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For the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the cultivation is considered to be a gold standard. This technique can detect exclusively only viable cultivable MAP cells. Other techniques as PCR derived methods can detect all MAP despite of the viability. Determination of all viable MAP cells despite of their cultivability can reveal new possibilities in the direct detection of MAP.

We have introduced and standardised the method for the MAP viability profiling based of the usage of propidium monoazide (PMA) and subsequent real time qPCR analysis. PMA enters inside dead MAP cells and incorporates to the DNA. This incorporation prevents the DNA polymerase to elongate such DNA. By the analysis of PMA treated and untreated portions of a single sample, the percentage of viable and dead cells can be determined.

We have exposed two different MAP isolates (collection strain CAPM 6381 and field isolate 8819) to temperatures from 60°C to 90°C for 30 seconds. Viability after the heat shock was determined independently by culture on solid media and by PMA F57 real time qPCR. Both MAP strains were not able to survive 80°C for 30 seconds, 70°C for 30 seconds significantly reduced viability in cultivation and in PMA F57 real time qPCR as well. The newly introduced method is reliable and faster and in comparison with cultivation it can determine not only the viability of MAP, but also the absolute amount of MAP cells.

#125 Detection of Mycobacterium avium subsp. paratuberculosis DNA isolated from archival formalin-fixed, paraffin-embedded tissue of Crohn's disease patients

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ABSTRACT

The objective of our study was to examine the archival formalin-fixed, paraffin-embedded tissues of Slovenian patients with Crohn's disease (CD) for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (Map) by IS*900* PCR.

One hundred and sixty-four intestinal tissue specimens, taken from 33 CD patients, and 39 control specimens, taken from individuals with intestinal cancer, were processed. After the paraffin removal, a commercially available kit was used to extract DNA. For the detection of Map, specific primers were used to amplify a 298 bp fragment of IS*900*. Map was found in six of 164 (4%) specimens obtained from four patients. Three specimens belonged to a single patient and the other three to the remaining patients. In total, four out of 33 (12%) CD patients had Map DNA detected in their tissues. All of the control samples tested negative.

With the method described in this study we could detect the IS*900* sequence of Map in the formalin-fixed and paraffin-embedded tissues of CD patients. Our findings contribute to the current knowledge about Map being a possible etiological agent of CD in Slovenian patients.

INTRODUCTION

Crohn's disease (CD) is a chronic, transmural inflammatory bowel disease of unknown etiology. Current theories implicate the role of genetic, microbial, immunologic, environmental, dietary, vascular, and even psychosocial factors as potential causative agents. It has been suggested that patients have an inherited susceptibility for an aberrant immunologic response to one or more of these provoking factors. The leading infectious candidate is *Mycobacterium avium* subsp. *paratuberculosis* (Map), in part because it causes a very similar disease, Johne's disease, in animals, including primates (Chacon et al., 2004; Shanahan, 2002).

For the detection of Map in CD patients, various specimens including fresh or formalin-fixed intestinal mucosa obtained by biopsy or surgical resection, blood etc. have been analyzed using different techniques, e.g. PCR, *in situ* hybridization, cultivation of Map and cultivation of Map followed by PCR (Bull et al., 2003; Feller et al., 2007; Romero et al., 2005; Ryan et al., 2002; Schwartz et al., 2000). Different detection methods and diverse clinical material may be one of the reasons for conflicting results about the detection rate, ranging from 0 to 100% (Feller et al., 2007; Quirke, 2001). Considering solely the results obtained from archival, formalin- fixed and paraffin-embedded tissue, similar incongruence is observed (Baksh et al., 2004; Cheng et al., 2005; Ryan et al., 2002).

The present preliminary study was carried out to determine whether Map DNA, as an indicator of formerly present live bacteria, could be detected in the archival formalin-fixed, paraffin-embedded gut tissues of Slovenian patients with CD by IS*900* PCR.

MATERIALS AND METHODS

Patients and specimens. A total of 203 formalin-fixed, paraffin-embedded archival specimens obtained from the Institute of Pathology, Faculty of Medicine, Ljubljana were included in the study. Among them, 164 were taken from 33 CD patients and 39 from patients with colorectal carcinoma without inflammatory bowel disease (IBD). The latter represented a control group. All 33 CD patients had an established diagnosis of CD based on clinical presentation,

endoscopic criteria and histopathology findings. Most patients had a history of long-standing CD. Patients underwent surgery for different reasons, usually stenosis. The disease was localized in the ileum in three patients (9%), in terminal ileum in 15 (45%), in cecum in two (6%), in ascending colon in five (15%), in transverse colon in two (6%), in descending colon in two (6%) and in rectosigmoid in four (12%) patients. In the control group, tumour was localized in cecum in three patients (8%), in ascending colon in one (3%), in transverse colon in 10 (26%), in descending colon in 2 (5%), in sigmoid colon in seven (18%) and in rectum in 16 (41%).

Specimens of the individual patients which showed the most pronounced lesions (transmural infiltration, cryptitis, crypt abscesses, epitheloid granulomas) were selected for further analysis. All specimens were stained according to Kreyberg trichrome method and histologically examined.

DNA extraction. Formalin-fixed, paraffin-embedded specimens were one to 19 years old. Five 6-µm sections were cut from each tissue block. The microtome was cleaned with xylene (Merck) and a new knife was used for each tissue block. All five sections were transferred into a 1.5-ml tube. Tubes were coded and processed blind. A total of 1200 µl of xylene was added to each tube and vortexed vigorously to remove the paraffin. After centrifugation at full speed for 5 min at room temperature, the supernatant was removed by pipetting. 1200 µl of absolute ethanol was added to the pellet to remove the residual xylene, mixed gently by vortexing and removed by careful pipetting after centrifugation at full speed for 5 min. Ethanol washing of the pellet was repeated once again. The tissue was then air-dried. DNA was extracted from all samples using the commercially available High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics).

Detection of Map-specific IS900. For the PCR assay, IS*900*-specific primers described previously, namely Av1 (5'-*ATG TGG TTG CTG TGT TGG ATG G*-3') and Av2 (5'-*CCG CCG CAA TCA ACT CCA G*-3'), were used to amplify a 298 bp fragment of the IS*900* gene (3). A 50-µl PCR reaction mixture contained 1× Expand High Fidelity reaction buffer containing 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide, 0.2 mM of each dNTP, 200 µM of each primer and 3.5 U of Expand High Fidelity *Taq* polymerase (Expand High Fidelity PCR System, Roche Diagnostics). The amplification conditions were as follows: 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 3 min, with a final extension at 72°C for 7 min. Amplification products were detected by electrophoresis in 1.5% agarose gels stained by ethidium bromide and visualized by UV transilluminator and camera (Bio Imaging System, Gene Genious, Syngene). Each batch was run with a process control, i.e. archival formalin-fixed, paraffin-embedded sections from cattle with Johne's disease, and with a positive PCR control (DNA from Map strain ATCC 43015). Negative PCR samples were tested for inhibition by spiking 200 ng of DNA positive control to one of the specimens of each CD patient and control patient after getting negative PCR Map results. Sterile PCR-grade distilled water was used as negative PCR control.

Statistical analysis. The Statistical Package for the Social Sciences (SPSS), v.17.0.0. for Windows, was used for the statistical analysis (t-test). Statistical significance was accepted at $p<0.05$.

RESULTS

Out of 33 CD patients, 19 (58%) were female and 14 (42%) were male. Patients were 15 to 59 years old with the mean age being 34 years. Paraffin-embedded tissue sections were cut from 164 blocks, with two to 5 specimens belonging to a single patient, except for one patient where 25 specimens were processed. In the control group, there were 39 patients, 21 (54%) male and 18 (46%) female patients. Patients were between 41 to 89 years old with the mean age of 70 years. From each patient one formalin-fixed, paraffin-embedded specimen of intestinal resection margin of surgical resection was processed.

All controls were negative to Map by IS*900* PCR (Fig. 1). Map was detected in six of 164 (4%) specimens obtained from four patients between 26 and 41 years old. Three specimens belonged to a single patient and the other three to the remaining three patients. In total, Map DNA was detected in the formalin-fixed, paraffin-embedded tissue from four of 33 (12%) CD patients. Four positive samples were obtained from terminal ileum, one from rectum and one from mesenteric lymph node taken from the ileal resection specimen. Statistical significance was observed when comparing IS*900*-specific DNA detection rate between CD and non-IBD group (p=0.022).

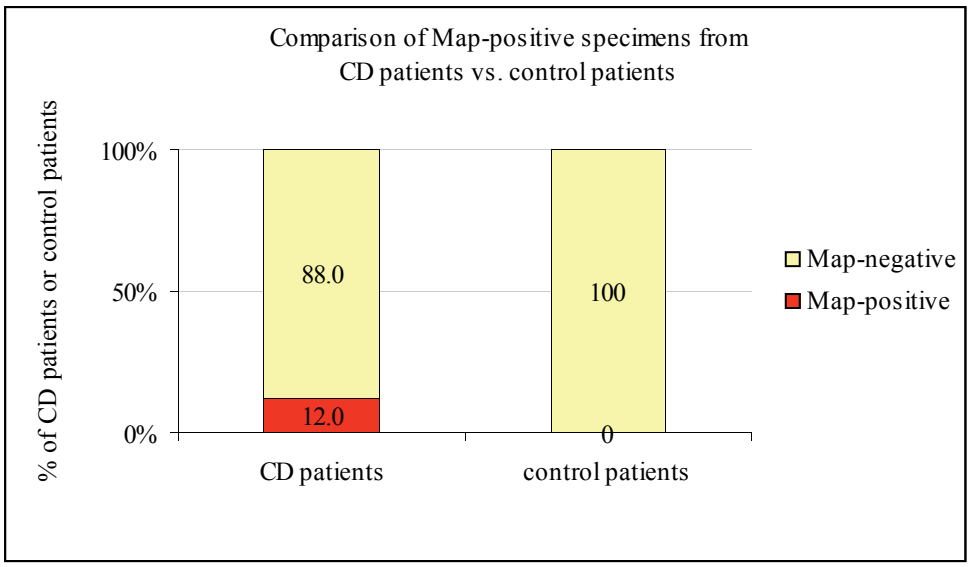

Fig.1: Comparison of Map-positive CD patients vs. control patients

DISCUSSION

Extraction of nucleic acids from formalin-fixed, paraffin-embedded tissues enables different retrospective studies. The technique has been used to detect Map as a possible infectious agent in the pathogenesis of CD (Baksh et al., 2004; Cheng et al., 2005; Ryan et al., 2002).

In this study we were able to detect Map DNA in the formalin-fixed, paraffin-embedded intestine samples from Slovenian CD patients using IS*900* PCR. Not all CD patients tested positive to Map, but no Map DNA was detected in control specimens. The detection of Map in archival formalin-fixed and paraffin-embedded tissue sections is especially challenging. The target DNA may be fragmented or cross-linked to proteins, which leads to poor or no PCR amplification. This may be the reason for some potentially false negative results in our study. Moreover, extraction of Map DNA may also fail due to very low Map concentrations in CD patients and due to strong, and even for mycobacteria unconventional cell wall (Hermon-Taylor, 2001). To overcome this limitation, more specimens of the same CD patient were analyzed.

Individuals without Map infection are 17-times less likely to have IBD than Map-infected individuals (Scanu et al., 2007), which is in accordance with our Map-negative non-IBD control group. Since this is a preliminary study, all IS*900*-positive specimens are still to be confirmed by sequencing.

In conclusion, our findings contribute to the current knowledge about Map being the infectious agent proposed to play the role in the etiology of CD, also in Slovenian CD patients.

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#130 *Mycobacterium avium* subsp. *paratuberculosis* detection in faeces and tissue samples by quantitative real time PCR and by cultivation

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Due to the fact that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is primarily an intestinal pathogen, the most common matrix that is used for MAP detection by cultivation is faeces (and after slaughter of animal is an intestinal mucosa). For culture, it is necessary to perform decontamination to eliminate the growth of other bacteria. Although MAP is quite tolerant to sample decontamination procedures, the sensitivity of culture is reduced. Methods based on PCR are suggested as an alternative tool for the diagnosis of paratuberculosis.

We focused on the introduction and optimization of an effective and reliable method for the DNA isolation of MAP from faeces and from tissue samples. The developed techniques of the DNA isolation are based on a slightly modified commercialy available kit. Detection of isolated DNA is made by two independent quantitative real time PCR (qPCR) assays targeting two MAP specific elements: multicopy element IS*900* and single copy element F*57*. Both qPCR assays incorporate an internal amplification control amplified with the same primers as the targets and the identical probe is used in both assays.

Faecal and tissue samples of different animal species suffering from paratuberculosis were collected over a period of last 16 months and tested for the presence of MAP. More than 1691 faecal samples and 361 tissue samples were examined by qPCR and simultaneously by culture. Based on these results we determine the dependence of concentration MAP DNA in faecal sample and the possibility to detect MAP DNA in tissue sample.

#131 Seroprevalence of paratuberculosis in cattle in Slovenia

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ABSTRACT

Paratuberculosis is a common disease of ruminants in Slovenia. Because of the lack of data about the prevalence since 2001, the purpose of this work was to estimate the seroprevalence of paratuberculosis in cattle herds in Slovenia.

Animals older than two years in 20% of herds originating from different regions of Slovenia were tested for the presence of antibodies to *M. avium* subsp. *paratuberculosis* in 2008. A total of 38,374 sera from 6,780 cattle herds were examined in ELISA test. A total of 228 (0.59%) animals from 188 (2.77%) herds were positive.

Currently, the seroprevalence of paratuberculosis in cattle herds in Slovenia is almost the same as it was in 1997 (2.77% vs. 2.84%). Compared to the majority of European countries, the herd prevalence is rather low. This could be partly attributed to a small number of animals per herd.

INTRODUCTION

Paratuberculosis is a chronic infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). The significance of the disease in cattle breeding lies in great economic losses caused by the infected herds. In the USA, losses due to paratuberculosis are estimated at over 1.5 billion dollars per year (Cocito et al., 1994). The first case of paratuberculosis in Slovenia was detected in 1961 in imported Jersey cows. No other cases were reported until 1993, when paratuberculosis was found in a sheep flock. Since then, several outbreaks of the disease in cattle, goats and sheep have been documented.

The estimation of prevalence is necessary for taking the appropriate measures to control the disease. Therefore, a systematic screening of paratuberculosis in Slovenia began in 1995. In order to estimate the prevalence and geographic distribution of paratuberculosis in Slovenia, 5-20% of cattle in all herds were tested in 1996 and 1995, respectively. In the following two years, 3% and 5% of cattle in all herds were tested by ELISA (Ocepek et al., 1999). In 1999, 10-15% of cows and pregnant heifers were tested, while in 2000 and 2001, the monitoring was limited only to breeding cattle herds because of their significant involvement in the selection for reproduction and animal trade (Ocepek et al., 2002).

Since 2001, no data on paratuberculosis prevalence in Slovenia were available. Therefore, the purpose of this study was to estimate the current seroprevalence of paratuberculosis in cattle herds in Slovenia.

MATERIALS AND METHODS

Animals older than two years were tested in 2008 for the presence of antibodies against Map in 20% of cattle herds originating from all regions of Slovenia. A total of 38,374 sera from 6,780 herds were examined with an in-house ELISA test using a protoplasmic antigen (Allied Monitor). All positive samples were retested and confirmed by a commercial screening and confirmation ELISA kit (ID.VET).

Along with the estimation of the current seroprevalence, the comparison of the current and past prevalence data on paratuberculosis in Slovenian cattle was made.

RESULTS

A total of 228 (0.59%) animals from 188 (2.77%) herds tested positive for paratuberculosis. The majority of the positive herds originated from the central and the eastern part of Slovenia as shown on Figure 1. In the herds with more than one seropositive animal, almost a half

(47%) of the positive animals were of Black and white (Holstein-Friesian) breed which represents 19% of cattle in Slovenia.

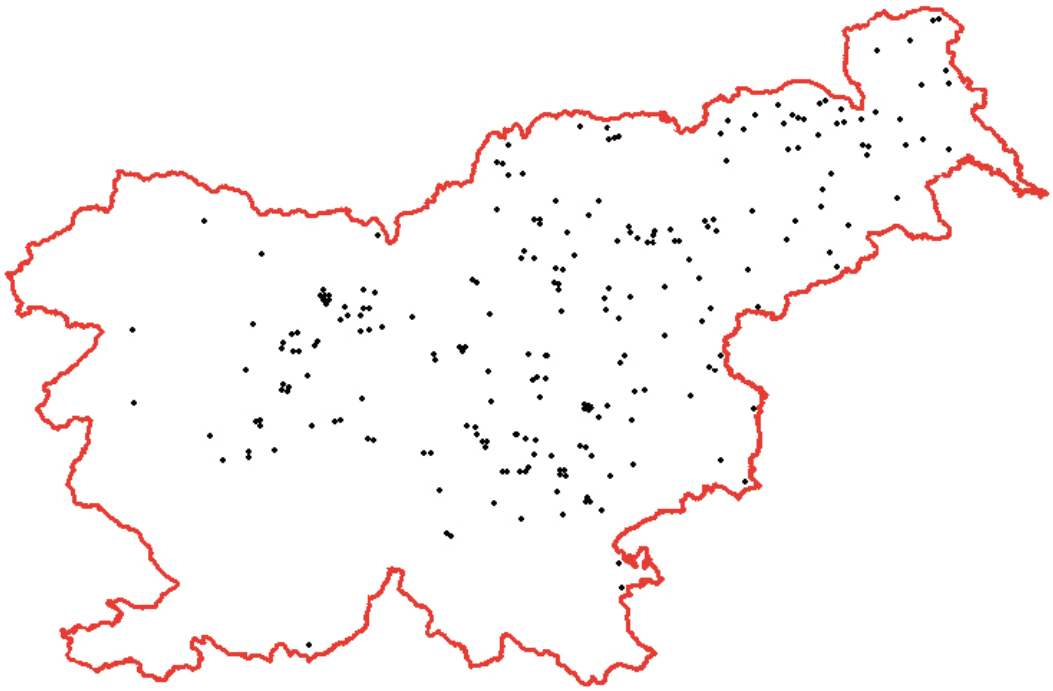

Fig. 1: Geographic distribution of paratuberculosis-positive herds in Slovenia.

Comparison of the data obtained on Map-positive sera from the current and previous studies on paratuberculosis prevalence in cattle in Slovenia is shown on Figure 2. In the last decade, the prevalence remained approximately at the same level.

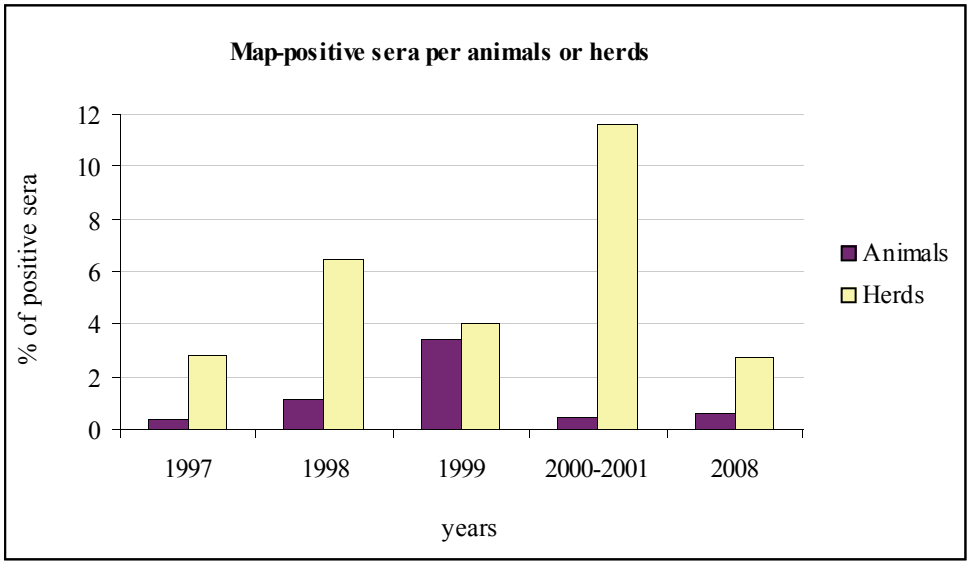

Fig. 2: Comparison of the prevalence data from the current study with the data collected in the previous studies.

DISCUSSION

The herd prevalence in Slovenia is similar to the prevalence estimated ten years ago and is rather low compared to the majority of European countries (Nielsen and Toft, 2009). This

could be partly attributed to a small number of animals per herd, i.e. the family-farm breeding strategy. In 2008, the tested herds comprised on average 5.67 animals (aged >2 years) in contrast to the period 2000-2001 when the average was 31 animals (aged >2 years) per herd. The relatively good present situation could change markedly in the near future due to unlimited trade of animals in the European Union. Moreover, in-country animal trade originating from big dairy-cattle herds with Black and white (Holstein-Friesian) breed, which is most commonly affected, can also contribute to the spread of paratuberculosis.

Differences in the prevalence on both the animal and the herd level, observed over the years, reflect also the different tested populations, the number of animals and herds included in the test and the use of ELISA kits with different sensitivities.

In general, our findings contribute to the current knowledge on paratuberculosis prevalence in the European countries. Because of the increasing trade and changes in animal breeding strategy (decreasing numbers of herds and increasing number of animals per herd), the decision and policy makers should prepare efficient measures for surveillance and control of the infection.

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#137 Comparative evaluation of the specificity and % recovery capabilities of different magnetic capture approaches for *Mycobacterium avium* subsp. *paratuberculosis*

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The objective of this study was to compare the performance of two types of commercially-available anti-MAP magnetic beads - Pathatrix-PM50 (polyclonal antibody coated beads, Matrix MicroScience, UK) and AnDiaTec ParaTub-S® (monoclonal antibody coated beads, AnDiaTec GmbH, Germany) - and three types of in-house coated magnetic beads - M280-Dynabeads coated with polyclonal sheep anti-rabbit IgG (original IMS approach) and Pierce paramagnetic beads covalently linked to MAP-specific peptides aMp3 and aMptD (Stratmann et al. 2002, 2004) – in order to identify the best magnetic capture approach for isolation of MAP. MAP broth samples (10^4 CFU/ml) were processed as follows: addition of 10 µl beads, 30 min capture at room temperature, magnetic separation for 10 min, three washes in PBS-0.05% Tween 20, and final resuspension in 1 ml 7H9/OADC broth before culture on Herrold's egg yolk medium containing 2 µg/ml mycobactin J (HEYM). Percentage recovery was calculated on the basis of before and after counts (CFU/ml). Specificity for MAP was assessed by performing magnetic separation on broths of five other Mycobacterium spp. Pathatrix-PM50 beads and Pierce magnetic beads coated with either aMp3 or aMptD showed the highest % recoveries of MAP (close to 100%). However, >10% non-specific binding was recorded in all cases. The inclusion of a blocking step before magnetic separation reduced non-specific binding to ≤1%, although this also resulted in a slight reduction in MAP recovery. In-house polyclonal antibody-coated Dynabeads and AnDiaTec ParaTub-S beads achieved much lower % recovery of MAP (≤10%). Results indicate that not all magnetic capture approaches for the isolation of MAP perform equally well. We observed that bead characteristics (composition, size, surface area) as well as the nature of coating antigen (polyclonal or monoclonal antibody, or peptide) impact specificity of capture and % recovery.

#142 Identification of new antigens of *Mycobacterium avium* subsp. *paratuberculosis* by cross-immunoproteomic approach

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We have identified in a previous study, combining proteomic and reverse genomic approaches, twenty-five new *Mycobacterium avium* subsp. *paratuberculosis* (Map) antigenic proteins (1). From the ten first proteins studied, a cocktail of three has been selected using a panel of 69 sera (21 positive / 48 negative) from cattle categorised by faecal culture, Pourquier and ID-Vet seroassays. Based on this panel, this cocktail yielded a more efficient diagnostic ELISA assay than the most widely used European paratuberculosis commercial seroassay (Pourquier test). However, when expanding performance assessment to a larger serum reference panel (81 positive / 50 negative), responses of positive reference sera appeared unpredictable and the sensitivity of the cocktail dropped significantly from 94.74% to 19.67%.

The aim of this study was therefore to identify, by cross-immunoproteomic approach, new specific antigenic targets which would complement the cocktail, and increase its sensitivity. In this context, 5 Pourquier and faecal culture positive sera, that failed to react with the antigenic cocktail, were used to probe 2-D Western blots of the secretome and cellular protein extract of Map. As expected, all failed to detect any 3 of the cocktail proteins. Yet, 7 antigenic proteins complementary to the original cocktail were detected, identified and characterized.

In parallel, we have grown MAP in different culture conditions (acid pH, hypoxia, presence of nontoxic concentrations of NO and nutrient starvation) presumed to mimic *in vivo* latency conditions. Antigens present in these culture filtrates and extracts may have a potential for early diagnosis and Johne's disease control. Interestingly, among the candidates previously identified by cross-immunoproteomic approach, few appeared be over-expressed in latency conditions.

With the prospect of serological mass screening for paratuberculosis control, this study indicates the necessity to use a cocktail of complementary antigens. Moreover, the development of latency models opens perspectives for the discovery of novel early diagnosis antigens.

Reference: 1. Leroy, B., et al., Proteomics, 2007. 7: 1164-76.

#146 Analysis of volatile organic compounds in sera - a future prospect of paratuberculosis diagnosis?

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Objective: Paratuberculosis (paraTB) in the early stages of disease is difficult to diagnose due to the low sensitivity of established direct and indirect diagnostic methods. Alternative diagnostic methods, which indicate host-pathogen interaction, may have some value in improving the sensitivity of diagnosis. Therefore, an electronic nose was evaluated for the ability to discriminate the infection status of animals through analysis of the volatile organic compounds (VOC) present in sera. Electronic noses consist of an array of non-specific gas sensors and require complex data analyses. In this study, the ability of the e-nose technology to discriminate the sera of paraTB infected- *Brucella* infected- and healthy cattle was assessed.

Materials and Methods: A total of 117 sera from cattle, naturally infected with paraTB (n=43) or brucellosis (n=26) and from corresponding control animals (2 x 24) were analysed randomly and blinded to their infection status using a ST 214 e-nose (Scensive Ltd, Leeds, UK). Samples were collected under field conditions on different farms. Sensor responses were analysed using multifactor ANOVA, linear regression and homogeneity testing for biological and methodological variation as well as principle component analysis (PCA) to identify the most powerful factors.

Results: Sensor responses of the device were influenced by methodological and biological factors. Despite of that, significant differences between populations of paraTB infected, Brucella infected, and healthy animals were detected. However, due to the methodological variation, discrimination between single individuals was not possible.

Conclusions: This particular e-nose cannot be used as a diagnostic tool for paraTB or Brucella detection and classification. Nevertheless, the data indicates that there are differences in the sensor responses dependent upon disease status, and therefore suggests that analysis of volatiles may be exploited in future for the identification of disease specific biomarkers for paraTB and other infectious diseases.

#147 Increasing the throughput of Johne's testing with PARACHEK® 2

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Prionics AG, Switzerland

The PARACHEK® is the original Johne's absorbed ELISA. It is able to detect antibodies against *M. paratuberculosis* in serum and milk prior to the onset of clinical signs. Prionics AG has now developed the PARACHEK® 2 which is more user-friendly and enables automation. It contains a one component substrate and incubation times were adapted for user-friendliness.

To evaluate the performance of both tests a set of negative and positive cattle and sheep serum samples and a set of negative and positive milk samples from cattle were tested. The samples derived from animals with a known fecal culture status for MAP. These samples were tested with both the PARACHEK® and the PARACHEK® 2. The agreement is expressed by the Cohen's Kappa coefficient and interpreted using the Landis and Koch table. The agreement between the two assays is almost perfect with kappa values of 0.88 (ovine serum), 0.90 (bovine serum) and 0.91 (bovine milk).

The PARACHEK® 2 also has two options for detection, a kinetic protocol for a high plate to plate reproducibility or an end-point protocol enabling automation. The PARACHEK® 2 was automated on a Beckman Coulter Biomek® FXP Laboratory Automation Workstation equipped with a 96-well plate washer and a plate reader which allows for a throughput of up to 16 plates in one working day (8.5 hours) starting from serum or milk samples. The results of the fully automated system were compared to manual processing of the samples. The agreement is almost perfect with a kappa value of 0.96.

These results demonstrate that the PARACHEK® 2 can be easily run on an automated system with the same excellent performance as with manual processing of the samples using the original PARACHEK® and thus enabling laboratories to save time and freeing staff for other work.

#155 Comparative characterization from German isolates of *Mycobacterium avium* subsp. *paratuberculosis* using SSR-, IS*900*-RFLP- and MIRU-VNTR-analysis

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Objective: The objective of the present study was to detect the heterogeneity of a widespread panel of Map isolates from German cattle herds and red deer using different techniques to be able to evaluate the methods and to compare the results with genotypes originating from other countries of the world.

Material and Methods: A set of about 120 Map isolates were genotyped by restriction fragment length polymorphism analysis based on IS*900* (IS*900*-RFLP) using BstEII and PstI digestion, by mycobacterial interspersed repetitive unit – variable number tandem repeat (MIRU-VNTR) typing based on 8 markers, and by short sequence repeat (SSR) analysis at locus 1 (G residue), 2 (G residue), 8 (GGT residue), and 9 (TGC residue). The results were analyzed separately and in combination.

Results: Results of the three typing techniques were not associated, the discriminatory power was different. A combined analysis of these results enhances highly the discriminatory power of typing. Using all three methods unique genotypes were detected for all studied isolates originating from epidemiological unrelated herds. Different as well as identical genotypes were found inside of individual herds.

Conclusion: Results allow different epidemiological interpretations depending on the used typing technique. SSR based on locus 1, 2, 8, and 9 can not replace the other techniques. The significance of single or combined typing techniques concerning epidemiological conclusions will be further clarified based on the randomly selected large strain panel from Germany.

#162 Comparison of faecal culture, ELISA and IS*900* PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle

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Comparative efficacy of faecal culture, Enzyme-linked immunosorbent assay (ELISA) and IS*900* Polymerase chain reaction (PCR) assay of faecal samples was investigated in 40 clinically suspected cases of Johne's disease in dairy cattle. The sensitivity of faecal culture, ELISA and PCR assay in this study was 52.5% (21/40), 87.5% (35/40) and 90% (36/40) respectively. All isolates appeared only on the mycobactin J supplemented Herrold's egg yolk medium (HEYM) at 8-16 weeks post-inoculation, were acid-fast and were positive for IS*900* PCR yielding a single amplicon of 217 bp. Of the 40 serum samples tested by the indigenous ELISA kit using soluble protoplasmic antigen from 'Bison type' genotype of Map of goat origin developed by Central Institute for Research on Goats (CIRG), Makhdoom, 17 (42.5%) were strong positive, 17 (42.5%) were positive, 1 (2.5%) was low positive and 5 (12.5%) were negative. A total of 28 faecal samples out of 40 were positive by IS*900* primary PCR assay for *Mycobacterium avium* subsp. *paratuberculosis* (Map) yielding an expected product of size 217 bp. Twelve faecal samples, which gave negative results in the primary PCR, were subjected to secondary PCR assay. Of the 12 samples, 8 gave positive results in the IS*900* nested PCR (nPCR), which yielded a PCR product of 167 bp, proving better sensitivity of nPCR assay than single amplification PCR. The chi-square test showed a highly significant difference between faecal culture and ELISA and faecal culture and PCR ($P< 0.01$) whereas the analysis indicated no significant difference between ELISA and PCR assay ($P> 0.05$). Among the tests employed in the present study, IS*900* PCR assay and ELISA showed highest sensitivity. This study suggests that IS*900* PCR or ELISA based detection of Map could be used as a potential diagnostic tool for rapid and effective Johne's disease surveillance.

#167 Analysis of *Mycobacterium avium* subsp. *paratuberculosis* antigens used in an in-house enzyme-linked immunosorbent assay for Johne's disease

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We developed a novel enzyme-linked immunosorbent assay (ELISA), called EVELISA, for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in cattle which showed a higher sensitivity than current ELISA test. We previously reported that the use of heat-killed M. flavascens for pre-absorption of cross-reactive antibodies improved specificity of the EVELISA. Further, we showed that EVELISA antigens can also be used for detection antibodies against MAP in a microfluidic system (the 9th International Colloquium on Paratuberculosis). The EVELISA uses surface antigens removed from the bacteria with 80% ethanol followed by gentle vortex agitation. To test the hypothesis that the high sensitivity of EVELISA was due to MAP-specific cell surface antigens, we used thin layer chromatography (TLC) to compare the extract of MAP against that of a series of bacteria that are either closely related to MAP or known to cause false positive antibody reaction in commercially available ELISAs. Surface antigen extracts were fractionated using the Folch wash method followed by cold-acetone precipitation and loaded onto aluminum-backed silica-gel-60 plates, then developed with a series of solvents, including ceric sulphate/ammonium molybdate, ninhydrin and α-naphthol solution. MAP-specific molecules were detected in the chloroform fraction of the Folch wash and were not precipitated by the cold acetone treatment. Fractions obtained after the Folch wash and acetone precipitation were tested for anti-MAP antibody detection in an ELISA format. We found that none of the fractions could achieve the level of EVELISA sensitivity, indicating that the cocktail of antigens in EVELISA was the key for EVELISA's high sensitivity. The protein components contained in the aqueous fraction are being analyzed by using polyclonal antibodies produced against the MAP extract.

#186 Identification of *Mycobacterium avium* subsp. *paratuberculosis* in fecal specimens by culture, real-time PCR, and nested PCR

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Direct fecal *Mycobacterium avium* subsp. *paratuberculosis* (Map) real-time PCR based on heat shock protein gene (*hsp*) combining DNA extraction procedures with PCR has been commercially available (Tetracore VetAlert™ Johne's Real-Time PCR, Rockville, MD). Direct fecal Map nested PCR based on insertion sequence (IS)*1311* has been recently developed (FecaMap®, Infectious Diseases Incorporated, Bellevue, Nebraska). These two PCR tests were compared to culture for direct detection of Map in fecal specimens obtained from two dairy herds in Florida. All three tests were applied to 327 fecal specimens and the results were analyzed and compared. Direct fecal real-time PCR was performed at Purdue University and direct fecal nested PCR was carried out at University of Florida. Two hundred and sixty-eight fecal specimens were negative for Map in all three tests. Of these negative fecal cultures, 14 were positive in both the real-time and nested PCR tests. Twelve real-time PCR tests positive for Map occurred in the absence of confirmation by the fecal culture or nested PCR. Seven positive nested PCR tests were recorded without correspondence in the fecal culture or real-time PCR. Fifty-nine fecal specimens were culture positive for Map. The results of direct fecal real-time and nested Map PCR agreed with those of culture in 13 fecal samples. The overall correlation of culture with direct fecal real-time PCR and direct fecal nested PCR was 24/59 (40.8%) and 20/59 (34%), respectively. When the data were corrected for the 14 presumed false negative cultures, direct fecal real-time and nested Map PCR identified 38/73 (52%) and 34/73 (47%) cultures positive for Map, respectively. The results of direct fecal real-time and nested Map PCR are comparable and reflect a difference in the amount of sample used in the test, as the nested PCR used 1/10th the amount of feces processed for the real-time PCR.

#187 Comparison of a quantitative and a semi-qantitative method to investigate survival of *Mycobacterium avium* subsp. *paratuberculosis* in bovine monocyte-derived macrophages

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Intracellular growth and survival of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is dependent on the bacterial genotype. Until recently we lacked methods to differentiate MAP strains beyond the level of host specificity. Techniques such as multilocus short sequence repeat (MLSSR) strain typing enable more precise differentiation between host-specific isolates. This allows investigating the characteristics of MAP in *in vitro* infections. In this study we report the comparison of two quantitations systems (fluorescent microscopy and real-time quantitative PCR (qPCR)) evaluating *in vitro* survival and growths of MAP in bovine monocyte-derived macrophages (MDMs) by using data from a single experimental setup.

MDMs for these experiments were obtained from Johne's disease-positive (n = 3) and age and stage of lactation matched Johne's disease-negative (n = 3) multiparious cows from the same farm. MDMs were challenged *in vitro* with four MAP strains of different host specificity (bovine, ovine) and were harvested at 2 hours, 2 days, 4 days and 7 days following infection. For each time point we measured simultaneously the number of both host cells and bacteria applying a semi-quantitative approach by using fluorescence microscopy and a quantitative approach using qPCR.

Overall, there was a high level of agreement between the bacterial and cell numbers assessed by the two divergent methods: Host infection status (test-positive or test-negative) did not influence bacterial survival irrespective of MAP strain used while bovine-specific MAP strains showed significantly better survival in bovine MDMs compared to ovine-specific strains. Throughout these analyses compared to the fluorescence microscopy assays qPCR proved to be more consistent between samples.

It may be concluded that both methods are appropriate to investigate MAP infections *in vitro*. For the investigation of larger data sets the qPCR is preferred not only because of the higher precision of results but also to reduce the work load.

#188 The utility of fecal culture, direct fecal real-time PCR, and direct fecal nested PCR in the determination of *Mycobacterium avium* subsp. *paratuberculosis* infectivity

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The present study was conducted to evaluate the utility of fecal culture, direct fecal real-time PCR, and direct fecal nested PCR in determining the status of *Mycobacterium avium* subsp. *paratuberculosis* (Map) infectivity in dairy herd. Eight hundred and twenty-seven (827) fecal samples were collected from two dairy herds participating in Johne's Disease Demonstration Herd Program. Fecal culture was carried out by using Trek ESP system with IS*900* PCR confirmation. Direct fecal Map real-time PCR uses heat shock protein gene (*hsp*) as the target in PCR (Tetracore VetAlert™ Johne's Real-Time PCR, Rockville, MD, USA). Direct fecal Map nested PCR is based on IS*1311* (FecaMap®, Infectious Diseases Incorporated, Bellevue, NE, USA). The percentages of fecal samples positive for Map were 13.5% by culture, 11.6% by real-time PCR, and 21.8% by nested PCR. Using positivity by direct fecal culture as the gold standard, 35 samples were positive for Map by real-time PCR with 83.3% accuracy and 51positive for Map by nested PCR with 77.3% accuracy. Using positivity by culture or both PCR as the gold standard, 112 samples were positive for Map by culture with 97.3% accuracy, 49 positive for Map by real-time PCR with 85.0% accuracy, and 65 positive for Map by nested PCR with 78.8% accuracy. Using positivity by culture or direct fecal Map real-time PCR or direct fecal Map nested PCR as the gold standard, 112 samples were positive for Map by culture with 81.7% accuracy, 96 positive for Map by real-time PCR with 76.8% accuracy, and 180 positive for Map by nested PCR with 87.0% accuracy. The results indicated that using positivity for Map by culture or both PCR as the gold standard is a more accurate tool in determining the status of Johne's disease infectivity in dairy herd.

#191 Potentiating day-old blood samples for detection of interferon-gamma responses following infection with *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

The interferon gamma (IFN-γ) test measuring specific cell-mediated immune responses in whole blood can be used for diagnosis at an early stage of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. A major obstacle for the practical use of IFN-γ testing is the recommended maximum 8 hour time interval from blood sampling to culture.
The objective of the study was to assess options for use of day-old blood samples for early-stage diagnosis of MAP infections. Bovine interleukin 12 (IL-12) can induce, and IL-10 reduce, IFN-γ production. Therefore, addition of recombinant IL-12 and anti-IL-10 antibodies could result in enhanced production of IFN-γ in samples exposed to MAP antigens.
Whole blood samples were collected from heifers in a Danish dairy herd known to be infected with MAP. The samples were collected on three sample dates. On each date the blood samples were stimulated with johnin purified protein derivative (PPDj) and recombinant antigens as fresh samples, as day-old samples potentiated with bovine IL-12, and as day-old samples treated with anti-bovine IL-10 antibody. The correlations between IFN-γ responses in the three types of samples and on different sampling dates were then investigated. The highest correlations were seen between fresh samples and day-old samples added anti-IL-10 antibodies. There was little difference between addition of IL-12 and anti-IL-10 antibodies. However, between-sample day variation was high for both types of day-old samples, irrespective of treatment type. Optimisation of the IFN-γ test on day-old samples for diagnosing MAP infected herds would facilitate the large scale use of this diagnostic test.

INTRODUCTION

Early MAP diagnosis can be achieved by measurement of specific cell-mediated immune responses to MAP antigens by the IFN-γ test. The IFN-γ test is a proliferation assay in which whole blood samples are cultured with MAP antigens and released IFN-γ is measured in the supernatant by enzyme linked immunosorbent assay (ELISA) (Wood et al., 1989). The large scale use of the IFN-γ test for routine diagnostics is limited by the short timeframe recommended of less than 8 hours from blood sampling at the farm to culture with antigens in the laboratory. To circumvent this short time frame, the use of day-old blood samples potentiated with recombinant bovine IL-12 have previously been investigated (Jungersen et al., 2005). Potentiation with IL-12 demonstrated to rescue antigen specific IFN-γ responses of day-old samples. Another study demonstrated that the IFN-γ response can be enhanced by adding anti-IL-10 antibodies to whole blood samples stimulated with PPDj (Buza et al., 2004). Here, we further explored the use of day-old blood samples in the IFN-γ test and addition of either of IL-10 antibodies or recombinant bovine IL-12.

MATERIALS AND METHODS

Blood samples were collected 3 times with 4 and 5 week intervals from the same 30 heifers 15-24 months of age in a Danish dairy herd known to be infected with MAP. On each sample date the whole blood samples incubated for 20-22 hours at 37ºC in 5% CO_2 with PPDj, a negative (PBS) and a positive control (Staphylococcal enterotoxin B; SEB). All samples were incubated with antigens as fresh samples, as day-old samples potentiated with recombinant bovine IL-12, and as day-old samples treated with anti-bovine IL-10 antibody (MCA2110, AbD Serotec, UK). The day-old samples were stored overnight at 4ºC from the day of blood collection, but otherwise followed the same protocol as the fresh samples. Following overnight culture, the culture plates were centrifuged and the supernatants collected and

stored at -20ºC until further analysis. The antigen specific IFN-γ production in supernatants were determined by an in-house ELISA as described elsewhere (Mikkelsen et al., 2009). The level of IFN-γ (pg/ml) was calculated using linear regression on log-log transformed reading from the two-fold dilution series of a reference standard with known IFN-γ concentration.
Samples were excluded if the IFN-γ value in the SEB-stimulated sample was below 1500 pg/ml or the PBS-stimulated value was higher than 250 pg/ml. The correlations between IFN-γ responses in the three types of samples and on different sampling dates were then investigated.

RESULTS

In Figure 1 the IFN-γ (pg/ml) responses to PPDj-stimulation in fresh samples and day-old samples + IL-12 from all 3 sampling dates are compared. The greatest correlation was observed at the first sample date (R^2 = 0.72) whereas lower correlations were observed at the second (R^2 = 0.16) and third (R^2 = 0.21) sample date.

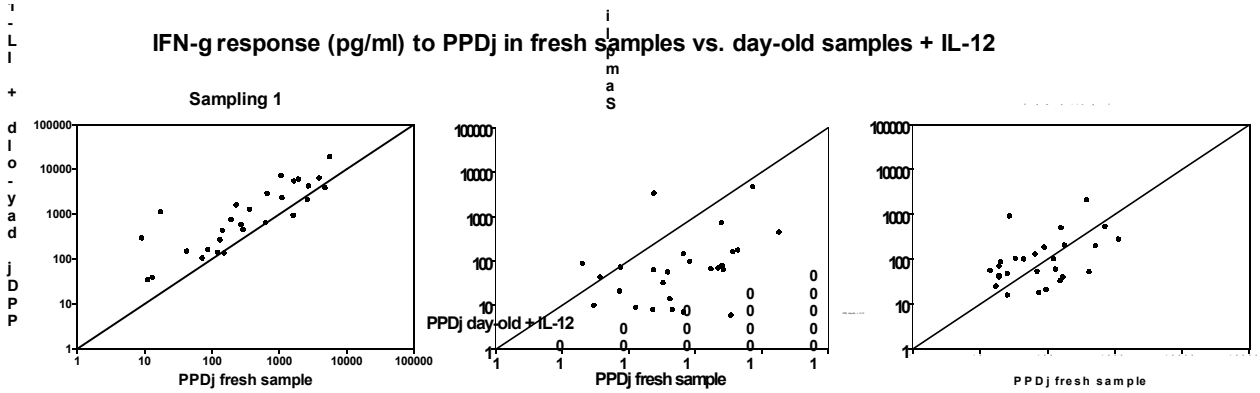

Figure 1. Correlations between IFN-γ responses to PPDj stimulation in fresh samples and day-old samples + IL-12 potentiation. Results represent data from 28 samples at sampling 1 and 27 samples at sampling 2 and 3, each from 30 animals, since some samples were excluded in accordance with the exclusion criteria.

In Figure 2 the correlations between IFN-γ (pg/ml) responses to PPDj-stimulation in fresh samples and day-old samples + anti-IL-10 antibodies form all 3 sampling dates are presented. Again, the greatest correlation was observed at the first sample date (R^2 = 0.67), and lower correlations at the second (R^2 = 0.09) and third (R^2 = 0.35) sample date.

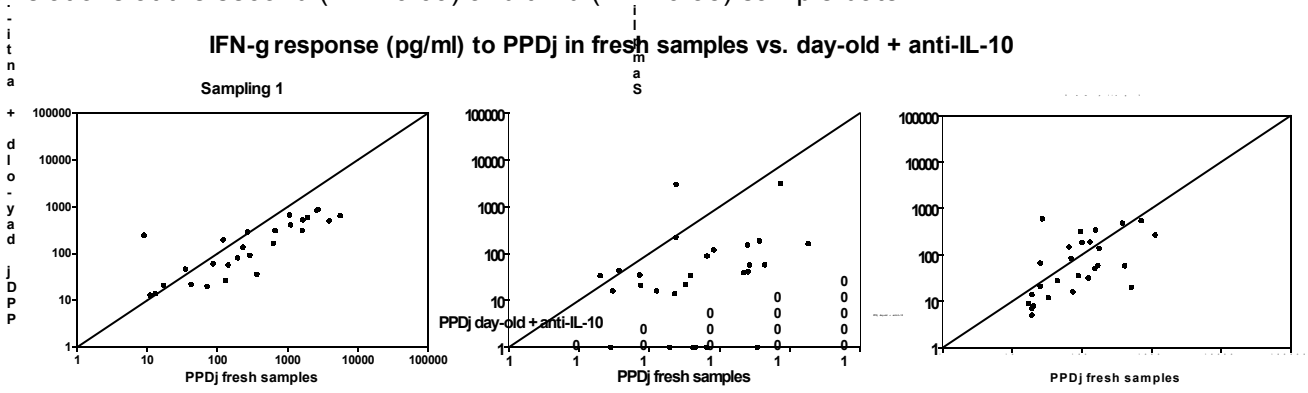

Figure 2. Correlations between IFN-γ responses to PPDj stimulation in fresh samples and day-old samples + anti-IL-10 antibodies. . Results represent data from 27 samples at all three sampling dates, since some samples were excluded in accordance with the exclusion criteria.

The highest correlations were observed between fresh samples and day-old samples potentiated with IL-12, but there was little difference between addition of IL-12 and anti-IL-10 antibodies. The between-sample day variation was high for both types of day-old samples irrespective of treatment type. We have previously observed fluctuating IFN-γ responses in

samples from the same animals on different sample days; hence repeated testing is required for validation of an optimized IFN-γ protocol. The fluctuations may be caused by variation in a number of factors, such as stress response of animals during sample collection, number of leukocytes in the blood samples or perhaps the fluctuations are higher when using a complex antigen mixture such as PPDj compared to well-defined antigens. The variation in IFN-γ responses to these novel antigens remains to be further investigated and the application of a day-old protocol using other antigens needs to be further explored

Acknowledgements
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#192 Novel antigens used to detect cell-mediated immune responses over time in *Mycobacterium avium* subsp. *paratuberculosis* infected cattle

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ABSTRACT

Early stage *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection of cattle can be detected by measuring specific cell mediated immune responses, using the interferon gamma (IFN-γ) test. Available IFN-γ tests are using purified protein derivatives of MAP (PPDj) which are crude products consisting of undefined antigens with possible cross reactions toward other environmental bacteria. The objective of the study was to optimize the IFN-γ test using different types of novel antigens for stimulation. Fourteen novel antigen candidates were selected for testing, including 4 peptides of the ESAT-6 family and 10 hypothetical proteins: 4 latency proteins, 3 secreted proteins, 2 proteins not present in *Mycobacterium avium* subsp. *avium* (MAA) and 1 from an immunological hot spot region. To determine variation of IFN-γ responses, three repeated tests was done with 4 and 5 week intervals on the same 30 heifers from a known MAP infected herd.

Determination of cut-off for each antigen was based on samples from a non-infected herd, including 60 heifers. Based on PPDj stimulations, more than 50% of the heifers tested MAP positive at the first two samplings, whereas only 20% tested positive at third sampling. The resulted showed that PPDj detect a high percentage as MAP positive animals, as this crude antigen mixture is expected to induce non-specific IFN-γ production. However, the tested latency antigens, some secreted proteins and some peptides of the ESAT-6 family detected a comparable high percentage of animals as MAP positives. By combining novel antigens higher specificity might be obtained.

INTRODUCTION

Early MAP specific cell-mediated immune responses can be measured using the IFN-γ test (Wood et al., 1989). The IFN-γ test is a whole-blood proliferation assay, in which blood is cultured overnight with MAP antigens followed by collection of supernatant. The IFN-γ level in supernatants is then detected by an IFN-γ specific enzyme linked immunosorbent assay (ELISA). Available whole IFN-γ tests for MAP diagnosis are using PPDj, which is a crude undefined extract of MAP antigens. Lack of standardized PPDj is a major concern, as the preparation of PPDj and therefore the antigen composition varies between laboratories. In addition, PPDj are known to cross-react with environmental mycobacteria such as MAA leading to low specificity of the IFN-γ test. To induce the specificity of the IFN-γ test, well-defined and MAP specific antigens are needed. In this study 14 novel antigen candidates were selected for testing with the aim to increase specificity of the IFN-γ test. The novel antigen candidates included peptides of the ESAT-6 family (van Pinxteren et al., 2000), latency proteins (Leyten et al., 2006), secreted proteins (Cho and Collins, 2006), proteins not present in MAA and a protein from an immunological hot spot region.

MATERIALS AND METHODS

Blood samples were collected 3 times with 4 and 5 week intervals from the same 30 heifers 15-24 months of age in a Danish dairy herd known to be infected with MAP. On each sample date the whole blood samples incubated for 20-22 hours at 37ºC in 5% CO_2 with the 14 novel antigens, PPDj, a negative (PBS) and a positive control (Staphylococcal enterotoxin B; SEB) in parallel cultures. The 14 novel antigens candidates included 4 peptides of the ESAT-6 family (MAP160, esxH, esxK and esxU) and 10 hypothetical proteins: 4 latency proteins (MAP2487c, MAP2768c, MAP3273c, and MAP3701c), 3 secreted proteins (MAP217, MAP1662 and MAP2888) 2 proteins not present in *Mycobacterium avium* subsp. *avium*

(MAA) (MAP87 and MAP3776) and 1 from an immunological hot spot region (MAP3783). Following overnight culture, the culture plates were centrifuged and the supernatants collected and stored at -20ºC until further analysis. The antigen specific IFN-γ production in supernatants were determined by an in-house ELISA as described elsewhere (Mikkelsen et al., 2009). The level of IFN-γ (pg/ml) was calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference standard with known IFN-γ concentration.

Samples were excluded if the IFN-γ value in the SEB-stimulated sample was below 1500 pg/ml or the PBS-stimulated sample was higher than 250 pg/ml. Based on these exclusion criteria, one heifer was excluded at first and second sampling and two heifers were excluded at the third sampling. To determine the cut-off for each of the 14 novel antigens and PPDj, blood samples were collected from 60 heifers 15-24 months of age from a non-infected herd. Samples from the non-infected herd heifer were excluded, if the IFN-γ value in SEB-stimulated sample was below 1500 pg/ml or the PBS-stimulated sample was higher than 75 pg/ml. The remaining samples were used to calculate the cut-off for each antigen. The cut-off for each antigen was calculated as: mean IFN-γ response + (1.96 × standard deviation of IFN-γ response).

RESULTS

Figure 1 shows the percentage of positive calves detected by each antigen at the three sampling dates.

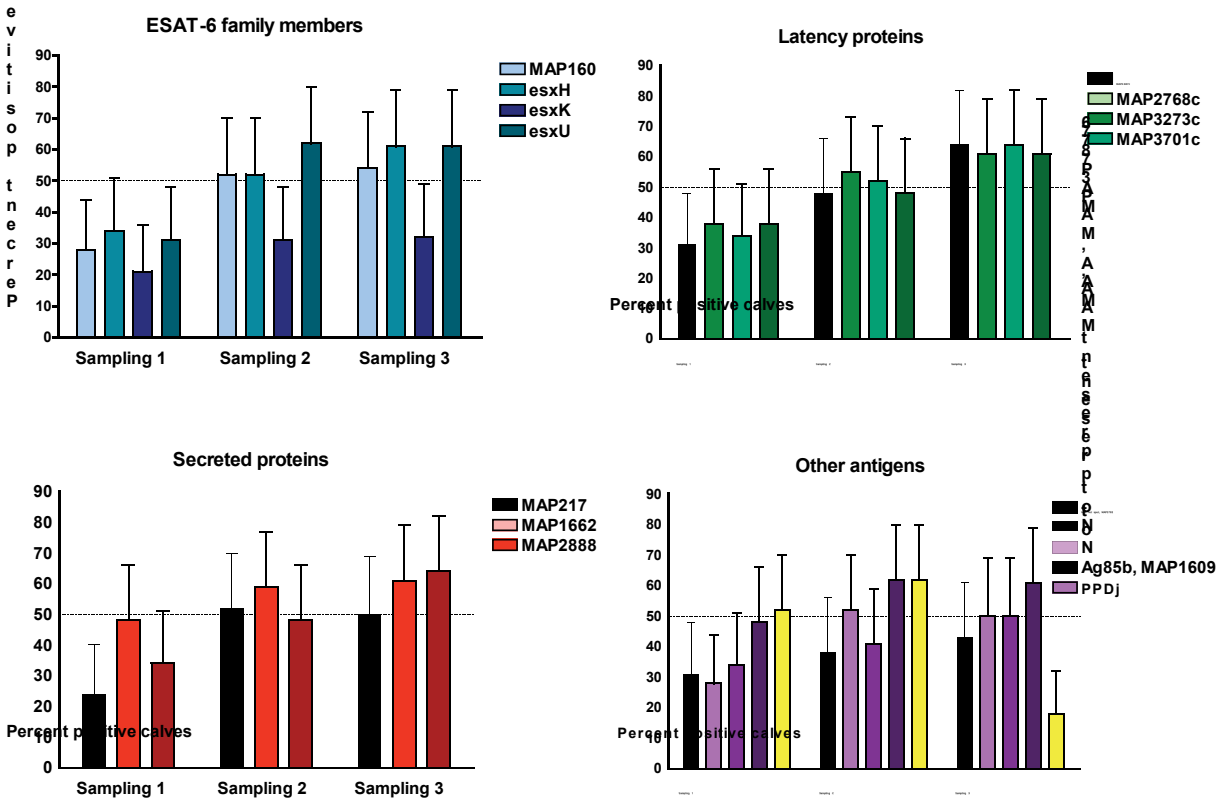

Figure 1. Percent positive calves detected by each antigen by the IFN-γ test. The novel antigens tested were divided into four groups: ESAT-6 family members, latency proteins, secreted proteins and other antigens. Cut-off for each antigen, to distinguish between test-positives and test-negatives, was calculated based on results from a negative herd. The same 30 heifers were tested at the three samplings. Data presented here are from 29 heifers at sampling 1 and 2, and 28 heifers at sampling 3, after exclusion of invalid samples.

PPDj detected 50% of the heifers as MAP positives at the first sampling, 60% at the second sampling and less than 20% on the last sampling date. In comparison the antigen 85B (Ag85B), which share high homology with other mycobacteria such as MAA, detected 50% as positive on the first sampling and 60% as positive on the second and third sampling. In general, the antigens detected the highest percentage of heifers as positives on the second and third sampling with exception of PPDj. The groups of latency proteins and secreted proteins detected 50% to 60% as positive on the second and third sampling. Similarly, the three ESAT-6 family peptides (MAP160, esxH, esxU) detected 50% to 60% as positive on the second and third sampling. The two protein antigens selected as not present in MAA, detected 30% to 50% of the heifers as positives, whereas the protein antigen selected from an immunological hot spot region detected 30% to 40% of the heifers as positive.

DISCUSSION AND CONCLUSION

The IFN-γ responses fluctuated between the three sampling dates, which indicate the importance of repeated test for evaluation of novel antigen performance in the IFN-γ test. Surprisingly, PPDj detected less than 20% as positive on the third sampling, but 50% to 60% at the first two sampling dates. For the majority of the antigens, the highest percentage of positive animals was detected at the second and third sampling dates. There is no evident explanation for the low percentage of animals detected by PPDj on the third sampling date and this result do not agree with the result for the majority of the antigens. On the other hand, the observed fluctuations may emphasise the need for an alternative to PPDj in the IFN-γ test. For optimization of the IFN-γ test well-characterised antigens should be included to induce specificity to MAP and reduce cross-reactions to environmental mycobacteria. To obtain high specificity of the IFN-γ test a combination of perhaps three novel antigens should be included. The optimal combination of novel antigens to be included in a MAP specific IFN-γ test remains to be selected.

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Technicians Abdellatif El Ghazi and Sardar Ahmad have done most of the ELISAs. This study was co-funded by the European Commission within the Sitxh Framework Programme, as part of the project ParaTBTools (contract no. 023106 (FOOD)).

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#196 Inter- and intra-subtype genotypic differences that divide *Mycobacterium avium* subspecies *paratuberculosis* strains

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Mycobacterium avium subspecies *paratuberculosis* (Map) strains are of two major types known as Sheep type (also called type I) and Cattle type (type II), but an Intermediate subtype (type III) that seems to cluster within the Sheep group according to several genotypic and phenotypic features has been described also. This study was undertaken to genotype a panel of type I or III small ruminant isolates from different origins with known pulsed-field gel electrophoresis (PFGE) profiles and to compare them with a panel of type II isolates and a well documented type II isolate collection. Methods used included the multiple-locus variable-number tandem repeat analysis (MLVA) which is based on genetic elements called mycobacterial interspersed repetitive units (MIRUs), the analysis of large sequence polymorphism (LSP), the single nucleotide polymorphism (SNP) based analysis of *gyr* genes and the IS*900*-restriction fragment length polymorphism (IS*900*-RFLP) analysis. Seventeen type I or III isolates and 24 type II isolates were analyzed. Eight different IS*900*-RFLP profiles were identified among type I or III isolates, some of them not previously published. Five belonged to the Intermediate type (or type III) and 3 were of the Sheep type (or type I). Some novel types were found amongst the 6 MLVA genotypes identified in these isolates. No amplification was observed for type I strains (pigmented) in LSP20 while type III isolates (pigmented and non-pigmented) gave a negative result, compared to the positive result recorded for type II strains. LSPA4 was positive for all type I or III isolates and negative for all type II isolates. Pigmented type III isolates have been found. Our results suggest that the techniques used correlate well. These findings support the division of Map strains into type II (Cattle lineage including Cattle types) and Type I and III (Sheep lineage subdivided in Sheep and Intermediate types).

#197 *Mycobacterium avium* subsp. *paratuberculosis* detected and quantified using different DNA extraction and real-time amplification methods in artificially inoculated fecal samples from cattle

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NEIKER-Tecnalia, Spain; Vacunek, Bizkaia, Spain

The aim of this study was to identify the best combinations of DNA extraction and real-time amplification methods available using different genomic targets for a sensitive and specific PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map). Homogenized fecal samples from a paratuberculosis-free cow were spiked with 10 to 107 bacteria per gram as assessed by direct microscopic count in a Neubauer Improved chamber of two Map cultures (K10 reference strain and 764 field isolate). Serial dilutions were plated to assess viable Map cells in the inocula. Six different commercially available DNA extraction kits were employed on triplicate samples of each level of inoculation with both reference and field strains. DNA extracts from all kits were submitted to two triplex real-time PCR amplification assays with an internal amplification control to rule out inhibition. Two extraction kits included their own PCR method that was used with extracts from their respective kit only. Map targets used in these assays were the multi-copy insertion elements IS*900*, IS*Mav2*, ISMAP02 and the single copy element F57. In addition, a quantitative real-time PCR kit based on sequence F57 was used to quantify the mycobacterial DNA present in extracts. Culture of inoculated feces to compare isolation and PCR detection sensitivities is also in progress. Two extraction methods have been discarded because of low efficiency. Two appeared to have the highest sensitivity according to quantification results. The quantitative PCR showed an excellent correlation between Map estimated number of copies and actual cell concentration. Most combinations of extraction and amplification reliably detected 10,000 cells per gram of feces according to direct counts or, 1,240 CFUs and 223.75 CFUs per gram according to plate counts both for field and reference strain inocula.

#204

Fecal culture for *Mycobacterium avium* subsp. *paratuberculosis*: ESP culture system II with para-JEM broth versus modified Löwenstein-Jensen media culture

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ABSTRACT

The aim of this study was to compare the culture of *Mycobacterium avium* subsp. *paratuberculosis* from 2050 individual fecal samples from Dutch cattle herds in the following two culture methods: on a conventional solid agar, using modified Löwenstein-Jensen media (LJ), and in the TREK ESP para-JEM Culture System II. All samples cultured in the ESP-system were tested by Ziehl-Neelsen staining (ZN) and IS900 PCR after 42 days incubation. LJ-cultures were read at 8, 12 and 16 weeks, and suspect colonies were tested by IS900 PCR.

Overall, 12% of the samples were positive by both ESP-system and the LJ culture method and 79% were negative by both methods. A further 4% of the samples were positive by ESP culture only whilst 5% were positive by LJ culture only. The agreement between both methods was reasonable (*kappa* = 0.68).

INTRODUCTION

Culture of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) on conventional solid agars is time-consuming due to the slow growth of *Map*. Therefore, systems with a reduced incubation time, such as the TREK ESP para-JEM Culture System II, may allow earlier detection of *Map* shedders in certification-, surveillance and control programs for *Map*. However, the diagnostic test characteristics of the TREK ESP para-JEM Culture System II in routine diagnostic samples from Dutch cattle herds were unknown. Therefore, the aim of this study was to compare results of the ESP culture system II (TREK diagnostic systems, *para*-JEM reagent liquid media) with a modified LJ media culture on routine diagnostic fecal samples from Dutch cattle herds.

MATERIALS AND METHODS

A total of 2050 individual fecal samples were tested in-parallel with both a modified LJ media culture method and the ESP culture system II.

For the culture on LJ-media the samples (2 gram) were decontaminated using a 4% sodium hydroxide solution and malachite green-oxalic acid suspension in two steps. After centrifugation the sediment was treated overnight with Neomycin and amphotericin B. From each sample 4 tubes of LJ-media (with mycobactin) were inoculated with 0.2 mL from the white intermediate layer. The tubes were incubated for up to 16 weeks and inspected every four weeks. When suspect growth was observed, growth of *Map* was confirmed by *IS900* PCR.

A modified sedimentation-centrifugation method as described by Stable et al. (1997) was used for the decontamination of samples cultured in the TREK ESP system. Briefly, the samples (2 gram) were diluted in 35 ml sterile destillated water, and after shaking and sedimentation 5 ml of the supernatant were removed. For decontamination, 25 mL 0,9% HCP-BHI (hexadecylpyridinium chloride in brain heart infusion broth) was added and incubated overnight. After centrifugation and discharging the supernatant the sediment was treated overnight with an antibiotic mixture of neomycin and amphotericin B. One flask Para-Jem (TREK diagnostic systems) was inoculated with 1mL of the samples after adding the supplements and antibiotics according to the manufacturer. The flasks were then incubated in the ESP TREK system which measures the gas pressure every 20 minutes. After 6 weeks of incubation, all samples, detected or not yet detected by ESP system, were further investigated via Ziehl-Neelsen staining and the IS900-PCR.

RESULTS

In 601 (29%) of 2050 samples, the ESP-system indicated growth of *Map* based on the optical signal which indicates the reduction of gas-pressure in culture flasks. However, only 29% of these 601 samples could be confirmed using both the Ziehl-Neelsen staining of the culture material and the IS900-PCR. Moreover, in 8% of the 2050 samples the ESP-system did not indicate growth of *Map* whilst the samples were Ziehl-Neelsen-positive and IS900-PCR-positive. Overall, 340 of the 2050 samples (17%) cultured in the ESP liquid culture were ZN-positive and PCR-positive.

In 352 (17%) of the 2050 samples, suspected colonies were found in the LJ-culture that were subsequently confirmed as *Map* using the IS900-PCR. Approximately 60% of these samples were found positive at 8 weeks of incubation.

Overall, 254 samples (12%) were positive by both ESP liquid culture (defined as acid-fast bacilli using the ZN-staining and PCR-positive as confirmation) and the LJ solid culture method, and 1612 (79%) were negative by both methods (Table 1). A further 86 samples (4%) were positive by ESP culture only, whilst 98 samples (5%) were positive by LJ culture only. Agreement beyond chance between both methods was reasonable (κ = 0.68; 0,64-0,72).

Table 1: Comparison of the ESP liquid culture after 6 weeks of incubation and the LJ culture after 16 weeks of incubation.

	LJ positive	**LJ negative**	**Total**
ESP positive (ZN+/PCR+)	254	86	340
ESP negative	98	1612	1710
Total	352	1698	2050

Kappa = 0,68 (0,64 – 0,72)

DISCUSSION AND CONCLUSION

In total 2050 samples were investigated in-parallel with both a solid phase culture method using Löwenstein-Jensen media and the liquid media ESP TREK system.

The detection rates of both methods were comparable for the detection of *Map* in feces. there was a reasonable agreement between both methods. However, to obtain a comparable detection rate with the ESP system, it is necessary to test all samples after 6 weeks of incubation using the Ziehl-Neelsen staining and IS900-PCR, which adds considerably to the costs of the system. The usage of only the optical signal of the ESP system leads to both false-positive and false-negative results; therefore a further confirmation is to be advised. The advantage of the ESP culture system is a considerable lower time-to-detection then with the conventional culture method on Löwenstein-Jensen media.

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#210 Production and evaluation of an *Mycobacterium avium* subsp. *paratuberculosis* Purified Protein Derivative for use in in-vivo and in-vitro diagnostic testing

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Purified protein derivatives (PPD's) were prepared from the cultured filtrate of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) ATCC strain 19698. The production of PPD has historically been problematic because of the difficulty with maintaining optimal floating cultures yielding defined immunogenic components. To obtain more consistent and potent PPD preparations, production methods must be re-evaluated for process improvements and culture selection. PPD production was conducted using *Mycobacterium avium* subsp. *paratuberculosis* (MAP) ATCC strain 19698 and two recent bovine field isolates utilizing both Povitsky bottles and Erlenmeyer flasks. Traditional production consisted of floating culture incubation at 37°C, live organism inactivation by autoclaving 30 minutes at 121°C, and coarse filtration to remove cellular debris. Proteins were subsequently precipitated by adding trichloroacetic acid to a final 4% concentration in the suspension. Floating cultures were readily maintained from the ATCC 19698 culture, but similar suspension cultures were difficult to grow using the field isolates. Culture production in Erlenmeyer flasks was superior to that of Povitsky bottles as determined by floating culture mat density, time required for growth, and consistent media suspension characteristics. SDS-PAGE evaluation of four production lots did identify specific protein bands, but was difficult to discern due to protein smearing. Rabbit antiserum raised against NVSL Johnin Lot 1 was utilized to evaluate four ATCC 19698 production lots by immunoblot. Immunoblot results from the four new production lots indicated reactivity equal to or greater than Johnin Lot 1. Results indicate that the current production procedures are capable of producing Johnin PPD equivalent to the current NVSL Johnin Lot 1, but a need exists to further evaluate processing procedures, with a goal of producing a PPD which is better defined. The antisera raised against Johnin Lot 1 will be used to screen a MAP DNA expression library to identify clones expressing specific immunogenic proteins in PPD.

#211 Culture of mycobacteria other than paratuberculosis (MOP) without antimicrobials in MGIT Para TB Medium with and without egg yolk and in MGIT 960 Medium

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Introduction: This study assessed MAP culture in BD BACTEC MGIT 960 mycobacterial culture media in the absence of selective agents.

Methods: Twenty-two MOP were seeded at < 200 cfu/culture into MGIT Para TB Medium with (PTB+EY) and without (PTB) 0.5ml egg yolk, and clinical MGIT 960 medium (MGT), N=3 cultures per condition. Each media type received its respective growth supplement but no antibiotics. Cultures were incubated in MGIT 960 instruments with medium-specific software. Results were instrument-generated detection times or assessments of negative for growth after 42 days. Mean days-to-positive (mDTP) were analyzed statistically by ANOVA-type General Linear Model. Growth performance was also subjectively rated as comparable between test conditions for a given organism for mDTP < 48 hours apart.

Results: Overall MOP detected most rapidly in PTB+EY and most slowly in MGT, as mDTP pairwise comparisons from MGT were significantly longer vs. PTB ($P = 0.0052$) or PTB+EY ($P < 0.0001$), and longer from PTB vs. PTB+EY ($P = 0.0009$). Subjectively, 10 species had comparable mDTP among the three growth conditions: *M. abscessus*, *M. asiaticum*, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, *M. simiae*, *M. smegmatis*, and *M. szulgai*. Only *M. marinum* and *M. phlei* had their shortest mDTP in MGT. *M. avium*, *M. bovis*, *M. celatum*, *M. flavescens*, *M. gordonae*, *M. malmoense*, and *M. terrae* had their longest mDTP in MGT. Only M. gastri had its longest mDTP in PTB+EY. The three failures of a medium to support growth were all with PTB (*M. marinum*, M. ulcerans, and M. xenopi).

Conclusions: PTB+EY yielded 100% detection and the shortest mDTP of the three test conditions. Detection in MGT was 100% but mDTP were the longest. PTB yielded shorter mDTP than MGT but failed to support growth of three MOP.

#212 Paratuberculosis: novel antigenic targets for early diagnosis

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Johne's disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map) affects domestic and wildlife ruminants worldwide but there are currently neither efficient treatment nor vaccine, and diagnostic tests require improvement in terms of sensitivity and specificity. Recently, combining advanced proteome and reverse genomic approaches, we identified twenty-five new antigenic targets of Map. Five of them have already shown promise in ELISA for serological screening (Leroy et al., 2007). In this project, we focused on IFN-γ release assay because this test has the advantage over serology to allow for early detection of infection at subclinical stages. We further evaluated the diagnostic potential of five antigens with this test. The coding sequences of MAP3547c, specifically detected by sera from cattle naturally infected with Map in 2-D Western blot, and of four reverse genomic targets, Ag 5, Ag6, Ag7 and Ag8, were cloned into the pQE-80L expression vector (Qiagen) and purified from transformed Top-10F' E. coli cells as His-tagged recombinant proteins. They were screened for specificity on 32 cattle originating from 2 culture-confirmed herds infected with *M. bovis* and for sensitivity on 102 cattle aged 3 to 68 months from a culture-confirmed Map-infected herd. Heparinized whole blood samples were stimulated within 8 hours of collection with each antigen at 5 µg/ml along with controls, and incubated for 20 hours with 5% CO2 at 37°C. IFN-γ release was measured in culture supernatants using a bovine IFN-γ sandwich ELISA (Invitrogen). Applying the manufacturer's cutoff for positivity, preliminary results indicate that single antigen sensitivity ranges from 0 to 97% and specificity from 91% to 100% (95% CI). The potential to combine single antigens to improve diagnostic performance, and cut-off optimization, are further discussed.

#233 Use of Phage Amplification Assay to rapidly enumerate viable MAP and other Mycobacteria

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The FASTPlaqueTB™ assay is a commercial diagnostic test for the rapid detection of viable *Mycobacterium tuberculosis* from human sputum samples. The end-point of the assay is the development of plaques following successful infection of a viable target cell by a bacteriophage. The phage used in this assay is D29 which has a broad host range in the Mycobacterium genus. The objective of this work was to investigate whether this rapid detection method could be used to enumerate numbers of viable MAP cells when working with laboratory cultures. Mycobacterium cells grown on agar slopes were resuspended in Media Plus to provide an inoculum. Samples were processed through the phage assay using a modified protocol that allowed the number of plaques to be determined at a range of dilutions of the samples being tested. The results showed a direct correlation between viable count and plaque number. The method was further used to rapidly monitor heat inactivation of MAP cells and to determine the MIC of the antibiotic rifamycin. Using the modified phage assay data was gained in 24 h demonstrating that the assay can successfully be used to monitor cell number and replace culture techniques for some physiological studies of this slow growing organism.

#242 Application and field validation of real-time PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* from bovine fecal samples

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Background: While the real-time PCR has been widely used for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map), a few issues, including the sensitivity, specificity, reproducibility and standardization of real-time PCR analysis, must be addressed before it's routine usage in clinical laboratories. We investigated some of these issues for the development of diagnostic system for Johne's disease using quantitative real-time PCR (qPCR). Methods: Fecal DNA samples were prepared by commercial DNA extraction reagents with bead-beating. For specificity testing, 775 fecal samples were collected from farms which have been confirmed Map-free status by a routine culture and ELISA tests. Sensitivity and reproducibility were validated in collaborative studies with 23 prefectural animal health centers by performing the qPCR with the same reference fecal samples and purified Map DNA which were supplied from our lab. Furthermore, 3,782 fecal samples were collected from 23 prefectures and tested with both the qPCR and the culture. **Results**: In a validation of sensitivity and specificity of qPCR, it detected 0.001 picogram purified Map DNA and 2 copies of IS*900*, and no cross-reactions were observed with any other Mycobacterium species including closely rerated Mycobacterium sp. st.2333. Fecal DNA samples from Map-free farms were all negative in the qPCR. In reproducibility tests using reference fecal sample and Map DNA provided, the most of the results reported from collaborative centers were almost concordant with the values we have expected. Comparative analysis of 3,782 fecal samples of unknown Map status yielded results that 194 samples (5.1%) were positive in bacterial culture and 327 (8.6%) were positive in the qPCR. Of 194 samples with culture positive, 169 were also positive in the qPCR (a concordance of 87.1%). **Conclusion**: The combination of validated fecal DNA preparation and the qPCR is a reliable diagnostic tool in terms of rapid and appropriate JD-control measures.

#243 Evaluation of a Johne's disease bulk milk ELISA as a herd screening tool

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A genomic study for the Johne's Disease Research Consortium requires extensive screening to identify New Zealand dairy cows with advanced MAP infection. We therefore need efficient tools to target and screen herds with higher Johne's disease risks.

One such tool may be a bulk milk ELISA test - which is simple and cheap to administer using dairy company vat samples. To investigate the performance of the 'Pourquier Paratuberculosis ELISA screening kit' as a vat test, we sampled 154 dairy herds (case herds) that have recorded Johne's disease cows over several years on the LIC National Dairy Cow Database, as well as 278 random herds from across New Zealand as controls.

A single vat test revealed a clear right shift of test results amongst the case herds with 14% > 0.1 S/P ratio compared to 1.4% of control herds. 64 (42 case and 22 control) herds across the range of vat test results (including most herds >0.1 S/P) were then selected for pooled-individual ELISA testing of herd-test milk samples, targeting 2nd + lactation cows. Herds with S/P ratios up to 0.05, >0.05 – 0.10 and greater than 0.10 were grouped into low, mid and high vat test herds.

The vat test result showed a very high correlation with individual cow reactor prevalence (R^2 = 0.83). Mean apparent reactor prevalence differed significantly between low, mid versus high vat test herds ($p<0.01$), ranging from 0.7%, 2.0% to 5.3%. However, the mean prevalence within all three vat test groups appeared higher amongst case herds than control herds.

The preliminary results of this investigation clearly demonstrate that the ELISA vat test will be a useful adjunct to clinical history for the identification of high-risk Johne's herds.

#246 Results from the 2008 Johne's Serologic Proficiency Test and Update on the 2009 Johne's Milk ELISA Proficiency Test

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The 2008 Johne's Disease Serologic Proficiency Test panel consisted of 0.25 ml of sera from 21 animals. Selected serum samples were used in duplicate to constitute the total of 25 serum samples per test panel. The Prionics Parachek™ Test was successfully utilized by 44 laboratories and 51 individuals. Three laboratories failed the first round of testing, but passed their retests. The IDEXX Herdchek™ ELISA Test was successfully utilized by 53 laboratories and 63 individuals. Five laboratories failed the first round of testing, but four successfully completed their retests. The fifth laboratory failed the first retest using the IDEXX test and submitted results for their second retest using the Prionics test. This laboratory failed again by missing one critical sample. Of other participating laboratories using alternative testing methods, three laboratories utilized the Pourquier Test to complete the test with a passing score and one of these laboratories also submitted a successful Complement Fixation Test. One laboratory failed using an in-house procedure, but had passed using the Prionics test. The 2009 Johne's Disease Milk ELISA Proficiency Test has been sent to 39 laboratories with results due May 1, 2009. Participation in the Milk ELISA Proficiency Test has increased from last year when 36 laboratories participated.

#248 A Simple Internal PCR Control for *Mycobacterium avium* subsp. *paratuberculosis* Constructed by PCR Techniques

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Aims: Polymerase chain reaction (PCR) is routinely used to confirm the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in the diagnosis of ovine Johne's disease (OJD). In a recent study to overcome specificity issues with the current PCR we also developed an internal amplification control (IAC) to monitor the integrity of individual reactions. This is important given the opportunity to introduce PCR inhibitory substances within samples. Unlike other internal controls that require cloning and other molecular manipulations, this IAC only requires PCR capability for its production.

Method: The IAC was constructed in a two step PCR process that amplified a region of the *Mycobacterium avium* subsp. avium (MAA) genome that is not present in the MAP genome using composite primers made of an MAA region and an MAP region. The IAC was then incorporated in to a multiplex PCR that included a new MAP specific target to increase specificity. The analytical sensitivity of the IAC and multiplex PCR was established prior to evaluation on DNA samples that had been previously examined for OJD.

Results: The IAC had no adverse effects on the analytical sensitivity of the MAP specific multiplex PCR. The new PCR test was successfully used to determine the presence/absence of MAP in 25 faecal samples with known OJD status and simultaneously determine the integrity of each reaction.

Conclusion: We present a new test multiplex PCR for MAP that incorporates an IAC. The procedure used to produce the IAC is simple and highly adaptable to other PCR-based diagnostic tests.

#251 Diagnosis of paratuberculosis in cattle from a dairy region in Columbia

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ABSTRACT

In Colombia serum and fecal samples from 307 asymptomatic lactating Holstein cows over three years of age from 14 herds with no previous diagnosis of paratuberculosis were taken. All Serum samples were analyzed with a lipoarabinomannan based-ELISA (ELISA A). Positive and doubtful samples in ELISA A were analyzed with a protoplasmic antigens based-ELISA (ELISA B), including pre-absorption with *Mycobacterium phlei*. Fecal samples from animals positive in ELISA A were analyzed using a nested IS*900*-PCR and a F57 / IS*Mav2*-real-time PCR. Fecal samples of animals from ELISA A-seropositive herds were decontaminated with 0.75% Hexadecylpyridinium Chloride and cultured on Herrold´s Yolk Agar medium. The same samples were decontaminated later with 4% NaOH and 5% oxalic acid and cultured on Lowestein-Jensen medium. Ten percent (31/315) of the samples and 70% (10/14) of the herds were positive with ELISA A. Only two animals of two different herds were positive with ELISA B. Six fecal samples were positive with PCR and only one was simultaneously positive in the two PCR types. Serological and PCR results did not always coincide. Cultivation was negative for paratuberculosis in all samples inoculated. However *Mycobacterium engbaekii* was isolated from LJ medium. Results confirm the presence of paratuberculosis in dairy herds in Colombia and demonstrate the limitations of available diagnostic tests for detection of subclinical infections, the determinant influence of ELISA type used, the low bacterial shedding in the cattle feces examined, the possible roll of other mycobacteria and the effect of conservation on the diagnosis of paratuberculosis.

INTRODUCTION

Paratuberculosis and the circulation of its causal agent *Mycobacterium avium* subsp. *paratuberculosis* (MAP) among dairy herds and wild animals in South America have been already demonstrated. However a consistent prevalence for the whole subcontinent or for some countries has not been yet estimated. In Colombia updated information about the presentation of paratuberculosis for dairy cattle is limited. Although the disease has been diagnosed in sheep and in cattle, more information about this disease in Colombia is needed. The objective of this study was the diagnosis of paratuberculosis in dairy cattle from herds with no previous diagnosis of paratuberculosis, using serological, cultural and molecular methods in a dairy region in Colombia.

MATERIALS AND METHODS

Three hundred seven Holstein lactating cows over three years old from 14 herds belonging to 9 districts of a municipality in a dairy region in Colombia were sampled. None of the herds had a previous diagnosis of paratuberculosis and had no clinical cases at the moment of sampling. Blood and fecal samples were taken and frozen at -20°C until analysis at the Justus-Liebig-University Giessen (Germany). For screening all serum samples, a commercial lipoarabinomannan (LAM) based indirect ELISA without pre-absorption (ELISA A) was used. For verification of positive and doubtful ELISA A samples, a commercial test based on detection of antibodies to protoplasmic MAP antigens with pre-absorption with *Mycobacterium phlei* (ELISA B) was used. A herd was considered seropositive if at least one animal tested positive with the ELISA A. DNA isolation from fecal samples of ELISA A seropositive animals was carried out with a commercial DNA preparation kit (High Pure PCR Template Preparation Kit, Roche). DNA samples were tested with an IS*900* nested PCR (Bull et al., 2003). Samples were also tested with a real-time PCR that amplify the F57 and the IS*Mav2* molecular targets (Schonenbrucher et al., 2008). All fecal samples from herds that tested positive in ELISA A and/or in PCR were decontaminated and cultivated using two

different procedures: Hexadecylpyridinium Chloride (HPC) solution and Herrold´s Yolk Agar medium (HEYM) with Mycobactin J and ANV and 4% NaOH and 5% oxalic acid and Lowestein-Jensen medium (LJ) with Mycobactin J and PACT. Slants of the two media were incubated for maximum 20 weeks and checked at 1-2-week interval. Colonies with mycobacterial morphology were stained by using the Ziehl-Neelsen staining method and were sub-cultivated on HEYM and on LJ, respectively. Bacteria were also further analyzed using the PCR methods described above. MAP negative bacteria were analyzed to determine its identity using a PCR to amplify the 16S rRNA gen using universal primers. PCR products were sequenced. Sequences were compared with the public sequence database RIDOM for similarity-based species identification.

RESULTS

Districts, herds' sizes and number of animals sampled in every herd, as well as serological and PCR results are presented in Table 1 and Table 2, respectively. Ten percent (31/315), 268 (87%) and 8 (2.6%) of the samples were positive, negative and doubtful, respectively with ELISA A (Table 1). Seventy percent (10/14) of the herds were considered positive, when having at least one ELISA A-seropositive animal. From 39 positive and doubtful samples in ELISA A, two animals (5.1%) belonging to two different herds (herds 9 and 10) and two different districts (districts E and F) were also positive with ELISA B, 37 (94%) were negative and none was doubtful (Table 1). All doubtful results in ELISA A were negative with ELISA B.

Six fecal samples from the 31 serological positive animals with ELISA A were positive in the nested PCR (Table 2). One positive animal in the real-time PCR were also positive in the nested PCR (Table 2). Only 16% and 6.5% of the ELISA A-positive animals were positive in the nested PCR and real-time PCR, respectively. Samples from herds in which just one positive animal were detected with ELISA A, were always negative in the PCR tests (Table 2). Cultivation of feces was negative for MAP in all inoculated samples in all inoculated media. However atypical mycobacteria were isolated from LJ medium. HEYM showed lower contamination compared to LJ medium. Atypical mycobacteria isolates were confirmed as acid-fast rod-shape bacteria in the Ziehl-Neelsen stain and were identified as *Mycobacterium engbakii* (99.78% of similarity in RIDOM) by sequencing of the amplified 16S rRNA gen.

Table 1. Paratuberculosis ELISA A-test results of herds from a dairy region in Colombia

Herd	District	Herd size	Samples	Elisa A			Elisa B[1]		
				positive	negative	doubtful	positive	negative	doubtful
1	A	102	20	3	17	0	0	3	0
2	B	75	19	0	19	0	nd[2]	nd	nd
3	C	146	21	3	17	1	0	4	0
4	D	274	29	1	28	0	0	1	0
5		108	19	0	18	1	0	1	0
6		81	25	3	19	3	0	6	0
7		116	23	1	22	0	0	1	0
8	E	96	20	4	16	0	0	4	0
9		144	22	6	13	3	1	8	0
10	F	226	23	5	18	0	1	4	0
11	G	80	20	1	19	0	0	1	0
12		76	20	0	20	0	nd	nd	nd
13	H	77	21	4	17	0	0	4	0
14	I	40	25	0	25	0	nd	nd	nd
			307	**31**	**268**	**8**	**2**	**37**	**0**

[1] Performed only to positive and doubtful samples with Elisa A
[2] Not determined

Table 2. Paratuberculosis PCR results of herds from a dairy region in Colombia

Herd	District	Elisa A-positive	Nested PCR[a]		Real-Time PCR[b]	
			positive	negative	positive	negative
1	A	3	1	2	1	2
2	B	0	nd	nd	nd	nd
3	C	3	2	1	0	3
4	D	1	0	1	0	1
5		0	nd	nd	nd	nd
6		3	0	3	0	3
7		1	0	1	0	1
8	E	4	1	3	0	4
9		6	1	5	0	6
10	F	5	0	5	1	4
11	G	1	0	1	0	1
12		0	nd	nd	nd	nd
13	H	4	0	4	0	4
14	I	0	nd	nd	nd	nd
		31	**5**	**26**	**2**	**29**

[a] Performed only to positive samples with Elisa A
[b] Performed only to positive samples with Elisa A
[c] Not determined

DISCUSSION

Our preliminary hypothesis was the diagnosis of paratuberculosis in a higher proportion of animals by using of indirect and direct methods. In the same way it was expected a higher concordance between the different tests used. However confirmation of all ELISA A-seropositive animals with ELISA B was not achieved. Differences in antigens LAM in ELISA A and protoplasmic antigen in ELISA B is a possible explanation of the poor concordance between both tests. In addition the use of the pre-absorption phase in the ELISA B could reduce the number of false positives, but also the detection of true positives (McKenna et al., 2005). On the other hand, a cross-reaction with other mycobacteria could have played an important role in the positive results obtained with the ELISA A. The capacity of other mycobacteria to interfere with pre-absorbed ELISA serological test in cattle has been demonstrated (Muskens et al., 2007).

The poor agreement between PCR and serology contradicted the proposed idea that the combined use of ELISA and fecal PCR has the potential to increase the overall sensitivity for the diagnosis of paratuberculosis infection in dairy cows. The low shedding rate of MAP organisms in subclinical animals can make real time-PCR equal or less sensitive than ELISA (Wells et al., 2006). On the other hand, although some primers use in the PCR systems have shown adequate results (e.g. F57), others have shown limitations (e.g. IS*Mav2*). In addition, single-round PCR has been evaluated as very reliable, but the used of nested PCR assays could lead to high contaminations risks (Mobius et al., 2008).

Regarding the cultural results, negative samples from ELISA-positive herds could be explained by the low or still absent MAP shedding rate in seropositive animals, by the low sensitivity of culture or by the culture of samples from false positive animals in ELISA A. In fact, in herds without previous diagnosis or clinical cases of paratuberculosis, the positive predictive value of an ELISA test is expected to be low (Muskens et al., 2003). ELISA can predict better the presence of culture positive animals when these are heavy shedders. The sensitivity of the ELISA is higher when a heavy instead a low shedder is tested. Negative results or false positive results in a cross-sectional study have a low probability of deliver a positive culture, if just a single sampled is planned (Sweeney et al., 2006).

Finally the conservation of serum and fecal samples at -20°C for several weeks could have affected ELISA and culture results. Serum-ELISA results can change from positive to negative (Alinovi et al., 2009) and fecal samples can undergo a significantly reduction of

viable microorganisms, changing the status from positive to negative, in animals with a extremely low number of detectable MAP organisms (Khare et al., 2008).

CONCLUSION

Results confirm the presence of paratuberculosis in dairy herds in Colombia and the limitations of the currently available diagnostic tests for the detection of subclinical paratuberculosis infections in dairy cattle. They demonstrate the determinant influence of the ELISA type used, the low bacterial shedding on the feces of the examined cattle, the roll of other mycobacteria and the possible effect of conservation on the diagnosis of paratuberculosis. Further paratuberculosis research in Colombia is needed.

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#254

Programmes on Paratuberculosis in Europe

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) have caused infections in European ruminants for more than a century, and some herds experience significant economical losses due to reduced milk production, reduced weight at slaughter, premature culling, loss of genetic potential and increased death rates. However, infections may pass unnoticed for several years in individuals as well as in herds. Because of these variable effects, and because other infections may cause greater losses or may have greater concern from a public health view, control of MAP infections does not always have a high priority. Furthermore, the prevalence of MAP infections may in some areas be low and the impact has therefore yet to be noticed, whereas the prevalence in other countries is high and considered to be a significant burden.

Several countries have implemented measures to approach MAP infections (Kennedy and Benedictus, 2001; Kennedy and Nielsen, 2006). These measures include control, surveillance and certification. Although some schemes are widely known, a number of other initiatives take place without having been described in widely available literature. This paper summarises approaches to MAP infections and go over the main points of efforts used to manage MAP in Europe.

TERMINOLOGY: AIMS IN MANAGEMENT OF MAP INFECTIONS

There are basically three main types of aims in management of infectious agents: i) control; ii) eradication; or iii) do nothing. Control can be defined as “any effort directed toward reducing the frequency of existing disease to levels biologically and/or economically justifiable or otherwise of little consequence” (Martin et al., 1987). Eradication can be defined as “the purposeful reduction of specific disease prevalence to the point of continued absence of transmission within a specified area by means of a time-limited campaign” (Yekutiel, 1980). Notice that real eradication is removal of MAP globally, whereas it is here used to also define regional removal of the agent. Basically, the word “eradication” is derived from the Latin word “radix”, meaning “root”, and “eradication” is the removal of the “root of the disease” i.e. for paratuberculosis it is MAP. Reasons to choose eradication over control are discussed by Payne (1963) and Yekutiel (1980).

The term “elimination” is by some used to describe the elimination of disease, but not pathogen (Payne, 1963). “Limen” means threshold and “elimination” is therefore a reduction to the limit/threshold where the root (pathogen) is present, but there is no disease. “Elimination” is by others considered to be “destruction of an infectious disease and its causal organism from a region of the world” (Anon., 2009a). “Doing nothing” is considered all other approaches not leading to control or eradication.

Differences in terminology emphasise the need to be specific in the description of different efforts. Furthermore, the above-mentioned differences suggest that distinction between “disease” and “root of disease” is important. The target condition may differ from one area or decision maker to another. Specification of the target condition is therefore important, especially for chronic infections, where there can be huge differences between being infected and being diseased.

EFFECTS OF MAP INFECTIONS

MAP infections usually develop over several years, and the incubation period may range from longer than the productive life-time to less than 2 years, in naturally infected animals. Furthermore, the effect of infection in the individual animal may vary significantly. This variation may be due to differences in management practices, e.g. intensive or extensive management, feed constitution, housing density etc. although these aspects are still poorly

described. The effects on animal and herd level are also different, because we are dealing with an infection, i.e. animals may become infectious. This means that an animal can be directly affected (e.g. reduced milk yield, weight loss, diarrhoea, death), or she can be indirectly affected through lost value at trading, simply because she is infected and carry MAP, and, hence, at risk of becoming MAP-affected in the future. On the herd-level, she can be a burden because she can transmit MAP to susceptible herd mates and thereby become a long-term liability to the herd-level productivity.

TARGET CONDITIONS

Different stages of infection (target conditions) are of interest from different perspectives considering the different effects at different time-points in the pathogenesis. Examples of different target conditions are: a) non-infected; b) MAP-infected; c) MAP-infectious; and d) MAP-affected. The latter is by some referred to as “clinical paratuberculosis”, but this may be an unfortunate term, because “clinical” is often perceived as “observed” and the degree of observation usually differs greatly among observers. For example, in small herds, animals are often more closely observed whereas in large herds, individuals may not be observed directly on a regular basis. The target condition or case definition can therefore vary among decision makers and countries, but a clear definition of the target would be preferred to ease communication.

COLLECTION OF INFORMATION

A questionnaire was circulated to European members of the International Association for Paratuberculosis or veterinary authorities in the following European countries: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Switzerland, Slovakia, Slovenia, Spain and Sweden, requesting the recipient to respond to the questionnaire or forward it to persons involved in programme management. The questionnaire was designed to collect information on: A) Existence of a national or a significant regional approach on paratuberculosis, and the species covered by the programme; B) Reasons for establishment of the programme if one exists, or reasons for lack of a programme; C) Objectives of the programme; D) Organisations/institutions driving, managing and funding the programme; E) Strategies used to reach the objectives; and F) Supplementary information.

Based on the information collected, the countries were divided into four categories: I) non-responders; II) countries with a national approach or regional approaches for the whole country; III) countries with some regional approaches; and IV) countries without a national or a significant regional approach. Furthermore, the countries were divided into categories based on the objectives specified and the target condition of “paratuberculosis”. Limited information was given for most countries.

PROGRAMMES ON PARATUBERCULOSIS

A map illustrating significant activities on MAP infections are shown in Fig. 1. Many responders provided limited information about the programme and it’s extent, and the map may not be very accurate and should be interpreted with care.

PROGRAMME OBJECTIVES AND TARGET CONDITIONS

The specified objectives of the different programmes are summarised in Table 1. There is considerable overlap between several objectives, e.g. reducing the prevalence of infected animals should reduce the spread of MAP and eliminate MAP shedders. However, the objectives given in the table were kept as close to the original specifications as possible. The information is categorised graphically in Fig. 2 illustrating the diversity of programmes in the European countries based on the information available.

Fig. 1. European countries specifying to have national or regional approaches (green), or with some regional control activities (yellow-grey). Some countries did not specify any significant activities (red), whereas others did not provide information (grey) or were not included in survey (black).

Table 1. Objectives and potential future aspects for control of MAP infections in European countries specifying to have programmes on MAP. The objectives are categorised as "passive" (red), "active control measures" (yellow) and "active eradication measures" (green)[§]

Objective	Austria	Belgium	Croatia	Czech Republic	Denmark	Estonia	France	Germany	Greece	Italy (Lodi and Milano)	Italy (Veneto)	Luxembourg	Netherlands	Spain	Sweden
Report disease								X							
Provide financial support to affected farmers									X						
Identify herds with MAP				X											
Monitor prevalence of "clinical paratuberculosis"						X									
Monitor herds for MAP (high) shedders													X		
Monitor/establish prevalence			X								X				
Standardise control measures								X			X				
Prevent clinical losses				X			X		X					X	
Increase farmers' awareness and stimulate control										X					
Eliminate MAP (high) shedders from dairy herds													X		
Remove sero-positive animals			X												
Reduce spread of MAP	X					X		X		X	X				
Reduce economical losses	X	X			X	X		X	X						
Reduce prevalence of MAP infected animals					X							X		X	
Certification of animals with high genetic merit							X			X					
Secure trade with neighbouring countries		X								X					
Document freedom of MAP disease										X					X
Take precautionary measures on food safety		X											X		

[§]"Eradication" in this context means removal of MAP from an area. Some of the objectives may fit into several categories.

ADDITIONAL INFORMATION ON SPECIFIC PROGRAMMES

Information on different programmes is often available in the language(s) of the specific country only (see list of additional country specific information in the back). Some of this information plus information provided in the questionnaires is briefly summarised here.

Austria

"Clinical Paratuberculosis" became notifiable in Austria in 2006, and the program is focused on animals with clinical disease. The government covers cost for laboratory examinations and provide compensation for culling of clinically affected animals. Management measures to reduce transmission of MAP are also implemented on farms with MAP affected animals.

Belgium

A voluntary programme on paratuberculosis in dairy cattle was established in Belgium in 2006, aiming at including 10% of producers in 2006 and 30% of producers by 2009. These goals have not been met. The programme is run by the dairy industry, farmers' organisations and animal health services.

In programme herds, all animals > 30 months of age are tested annually using antibody tests on milk or blood samples. Testing is paid by farmers, but he receives a bonus per animal tested as follows: 2 EUR per test-negative animal, and 3 EUR per test-positive animal that is slaughtered.

Denmark

Denmark also has a voluntary programme in dairy cattle. Approximately 29% of the herds and 40% of the cows were enrolled in the programme by June 2009. The programme is run as a risk-based control programme (Nielsen, 2009). All enrolled herds are tested 4 times per year using an antibody test, and cows are classified into High- and Low-Risk cows, and specific measures should be established to reduce potential MAP transmission from High-Risk cows. All costs are paid by the farmers.

France

Different programmes exist in different regions of France, but they are all voluntary. On a national level, a programme exists to certify animals with a high genetic potential. On a regional level, different control schemes are in place, for example in the main dairy region in France, Bretagne, where around 20.000 cattle farms and 2 million cattle are considered "active in paratuberculosis control". Control activities include different test-schemes, where test-positive animals are culled within a specific time interval, and measures to reduce MAP transmission to calves are implemented.

Germany

Paratuberculosis is notifiable in Germany. However, there is no obligation to test animals with clinical signs of this disease. Owners and veterinarians often refrain from submitting samples to diagnostic laboratories in order not to know if an animal or herd is paratuberculosis positive. The reason is that farms reporting cases may be held responsible for the damage caused by knowingly selling animals from paratuberculosis positive herds to another farm, whereas those that do not know their status are not, irrespective that the prevalence can be similar in the reporting and the non-reporting herd.

The Federal Ministry for Consumer Protection, Food and Agriculture in 2005 made guidelines to standardise control measures, to reduce the prevalence of clinical disease and thereby economic losses, and to prevent spread of MAP. The implementation of the guidelines lies with the individual German states. Financial support can be obtained through infectious disease insurance programmes in some states if specific requirements are fulfilled.

The Netherlands

A bulk milk quality assurance programme was established in the Netherlands in 2006, and by 2008 approximately 75% of the herds had voluntarily joined the programme. The aim of the programme was to reduce the concentration of MAP delivered to milk factories. All herds are tested at minimum every 2nd year with a highly specific but in-sensitive antibody ELISA. Tested herds are classified in three categories: A) Herds with only test-negative animals; B) Herds with test-positive animals, where all test-positive animals are removed from the herd; and C) Herds with test-positive animals remaining in the herd. The Dutch dairy industry has made participation compulsory for farmers delivering milk to their factories from 2010. Only herds in category A and B can deliver milk from January 1, 2011.

Spain

There is no national approach to MAP infections in Spain, but in the Basque Country, there are initiatives to reduce the prevalence and reduce losses associated with the infections in cattle. Two main tools are used in two different regions: vaccination in the Gipuzkoa region, and test-and-culling using serological and faecal test schemes. Furthermore, the use of vaccination may be extended to other regions within the next 5 years.
Several other initiatives are in place in other parts of the country, but with less specific programme objectives

Sweden

Sweden claims that the prevalence of MAP infections is low. They aim at providing test-information to ascertain freedom of MAP infections, but struggle with providing test-information that can certify herds and animals free of MAP.

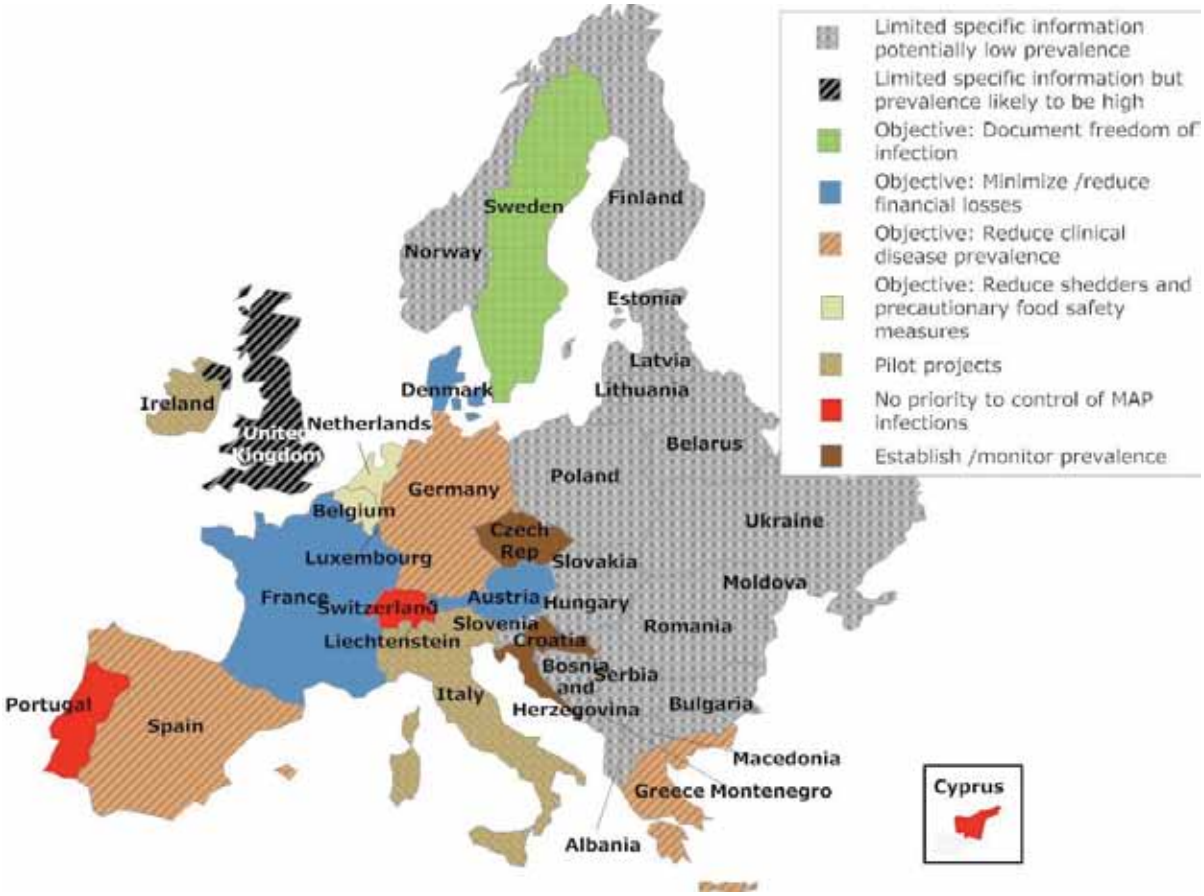

Fig. 2. Objectives and approaches to MAP in different European countries

REASONS FOR NOT HAVING A PROGRAMME

Reasons for not having a programme are specified in Table 2. The primary reasons seem to be that MAP infections have lower priority than other infectious diseases, economical

constraints and perceived low prevalence. A few countries are considering introducing programmes.

DISCUSSION

Compiling information on programmes dealing with infectious diseases in Europe is a challenge. The terminology appears to differ significantly, and the lack of a clear definition of "paratuberculosis" and differences in target conditions further confuse the interpretation of the information collected. However, the information collected suggests that a variety of initiatives exist, although there is limited information on the actual participation in these initiatives. Successful initiatives have still to be effectively documented. Lack of such documentation may be the reason that so many different initiatives exist. Furthermore, MAP infections are of low priority in several countries, and the lack of documented successful initiatives may also be an explanation for that. Such documentation will be pivotal for future standardisation of approaches leading to control or eradication of MAP.

Table 2. Reasons for lack of programme on MAP in European countries specifying not have a programme

Country	Reasons for lack of programme
Cyprus	Apparent high prevalence of herds with MAP infected animals, but cattle are not reported affected by MAP infections. Sheep and goats are reported affected by MAP infections, but losses are considered limited. Vaccination is used if problems are experienced. MAP infections are not of high priority because problems are considered limited
Finland	Not considered a problem based on some pilot projects. Only 5 beef herds and no dairy herds were identified with MAP in 1992-2008. At the present, surveillance is based on clinical signs and the veterinarians have to report further, if paratuberculosis is detected at a farm". Poor diagnostics are a discouragement in the establishment of a programme
Hungary	MAP infections are not of priority
Ireland	MAP infections were not considered a problem until after introduction of "The Single Market" in the European Community (European Union) in 1992. MAP infections were not really recognised until about 2000. A programme may be established in the future.
Lithuania	No specific programme on MAP, but a general infectious disease programme is in place for breeding herds. Breeding establishments should do regular MAP testing. The prevalence is considered low, but a survey may be carried out in near future to establish the prevalence
Poland	Economical constraints and a general belief that there is no disease in the country
Portugal	A prevalence study suggested that although many herds /flocks of ruminants (cattle, sheep and goats) were infected, the animal level prevalence was so low that priority should be given to other infections, primarily *Brucella melitensis*.
Slovakia	Economical constraints
Slovenia	Prevalence is considered so low that MAP infections are not considered a problem by cattle breeders and the government
Switzerland	Diagnosis is too complicated Exact implications in human disease are not known Resources currently bound to BVD eradication and bluetongue virus control A future programme is a possible target

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#276

Elimination of *Mycobacterium avium subspecies paratuberculosis* from dairy farms: fact or fiction?

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ABSTRACT

In this key note paper, we discuss whether elimination of *Mycobacterium avium* subspecies *paratuberculosis (MAP)* from dairy farms is a realistic option for modern dairy farms. Longitudinal observational studies have shown that farms may have low apparent prevalence of MAP for long periods of time. This is remarkable as it may be expected that a low prevalence would result in infection fade out from a substantial proportion of the farms. We present data that would indicate that the true MAP prevalence on dairy farms is much higher. Data from tissues collected at slaughter in cows from longitudinally studied herds show an apparent prevalence of infection in culled cows of approximately twenty-five percent. The reasons for this high prevalence are discussed. The impact of this high prevalence for control programs is evaluated using both mathematical, economical and molecular tools. We conclude that elimination of MAP for most dairy farms is more fiction than fact.

INTRODUCTION

Johne's disease or paratuberculosis is an infectious disease in cows, caused by *Mycobacterium avium* subspecies *paratuberculosis (MAP)*. In many countries, the prevalence of MAP in dairy herds has been estimated, and these estimates are usually very close. Most studies show an apparent prevalence of infected cows in herds in the 5-10% range (Woodbine et al. 2009, VanSchaik et al. 2003). Given this low estimated prevalence, it is surprising that no reports have been published on long term infection free herds.

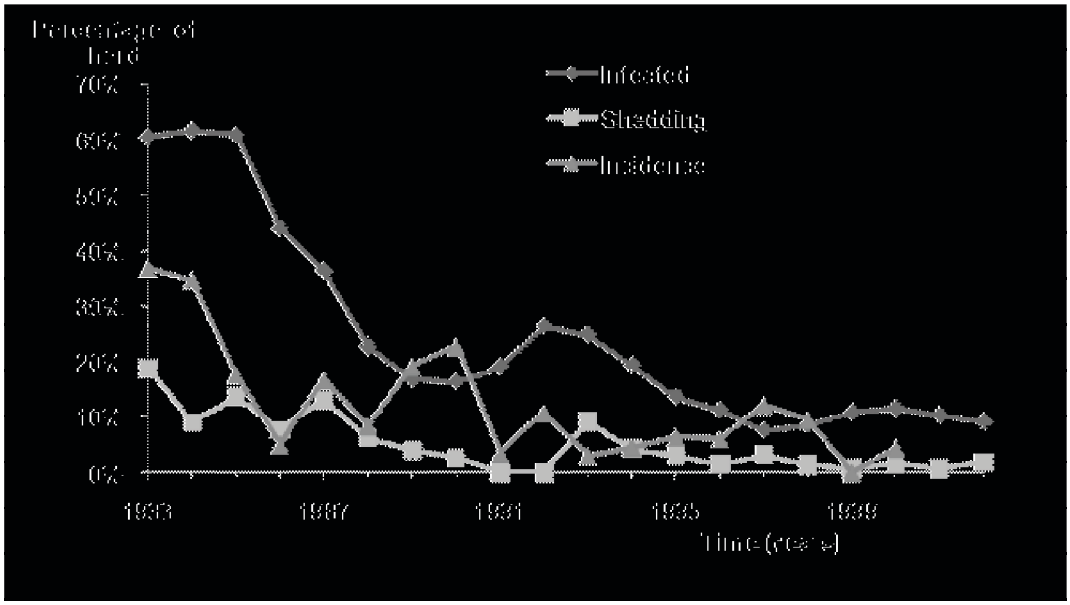

Fig. 1. Prevalence of shedding, infected animals and the incidence of MAP infection during a 20-year longitudinal study in a dairy herd going through a MAP control program (from Benedictus et al. 2007).

Most studies that report on the implementation of control programs, report a dramatic decrease in incidence and prevalence, but never a full elimination of the infection. An example of such a control program in a well monitored dairy herd is shown in Figure 1

(Benedictus et al, 2007). Clearly, the Johne's disease eradication programs based on the known MAP transmission routes were not successful in eliminating Johne's disease from the herd. There are at least two lessons to be learned from the data shown in Figure 1. First, there are likely more routes of MAP transmission than we currently recognize. Second, the low incidence and prevalence that were observed after implementation of control programs (after 1995 in Figure 1) are unlikely to be correct, as culling of a few infected cows would lead to elimination of MAP infection in many herds going through such a control program.

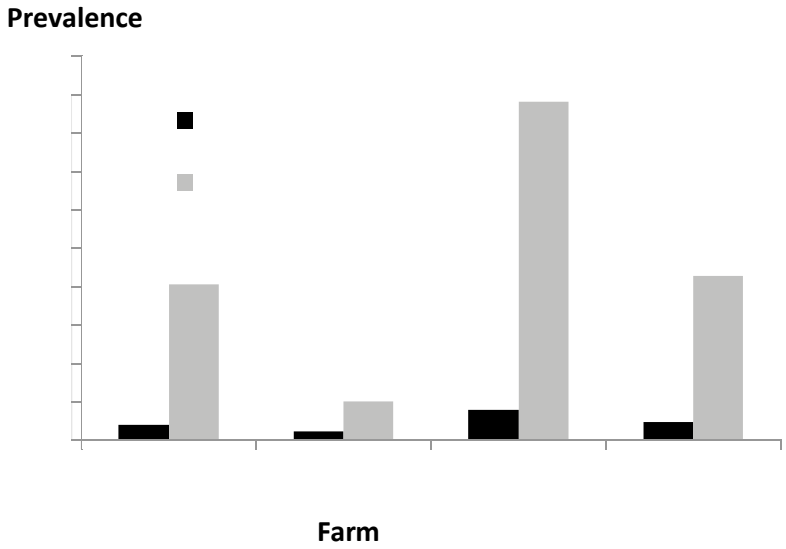

Fig. 2. Prevalence of MAP infection in three herds. Prevalence in fecal samples and prevalence in tissue samples at slaughter is shown (RDQMA project, unpublished data).

Recent studies have provided evidence for other previously not recognized routes of MAP transmission. A study by VanRoermund et al. (2007) showed that infected calves may be infectious to their peers in birth cohorts (VanRoermund et al., 2007). These calves would be shedding infectious organisms and because of their close contact with susceptible individuals, transmission of infection would occur. A hallmark of such calf-to-calf transmissions would be the presence of clusters of infected animals when sorted by day of birth as shown by Benedictus et al. (2007) and Pradhan et al. (2009) (Benedictus et al. 2007, Pradhan et al. 2009). A second little recognized transmission route was recently proposed by Pradhan et al. (2009). They argued based on molecular typing of MAP strains that adult animal infection is an important route of transmission in dairy herds. Animals shedding very high numbers of bacteria (so called 'super shedders') were particularly able to infect adult animals. It was observed that In the presence of super-shedders in dairy herds, approximately 50% of animals other than those identified as super-shedders shed the same strain as that of contemporary super-shedders. When these low shedders were followed through to slaughter, about 60% of these suggestive adult infected cows showed a tissue infection with the same strain as super-shedders.

Estimates of true prevalence of MAP in dairy herds vary widely, mostly because of uncertainty in the 'gold standard' definition of infection status. Often fecal culture results are used as the gold standard, but it is also widely recognized that fecal culture results severely underestimate true infection status. Recent studies by the Regional Dairy Quality Management Alliance (RDQMA) provide strong evidence for a much higher prevalence of MAP infection compared to the prevalence obtained from fecal culture. In this longitudinal multi-site study, animals in three herds have been followed and tested from birth to slaughter. Results are presented in Figure 2 and show that the MAP infection prevalence, as estimated by culture of intestinal lymph nodes and the intestinal tract, is at least 10 times as high as the MAP prevalence estimated by fecal culture. Although these data need confirmation from

other projects and investigators, the much higher prevalence of infection in dairy herds would explain the inability of current control programs to eliminate MAP from dairy farms.

Recent economic models and economic data obtained from observational studies (Groenendaal and Wolf 2008) show that control programs for MAP on dairy farms are generally only cost effective when best management practices particularly with regard to calf raising were practiced. Extensive test-and-cull strategies alone were shown to be ineffective and costly for producers (Groenendaal and Wolf 2008). Milk production loss linked to MAP infection was studied across a number of longitudinally followed populations (Nielsen et al. 2009, Smith et al. 2009). In both of these studies, animals known to be infected with MAP but shedding low bacterial numbers, did not show an important milk production loss relative to uninfected controls. Only when cows started to shed larger numbers of bacteria or when MAP ELISA values were increased for a prolonged period of time was a discernable effect on milk production present (Smith et al. 2009, Nielsen et al. 2009). These data would also indicate that test-and-cull strategies maybe costly when applied across all MAP infected animals.

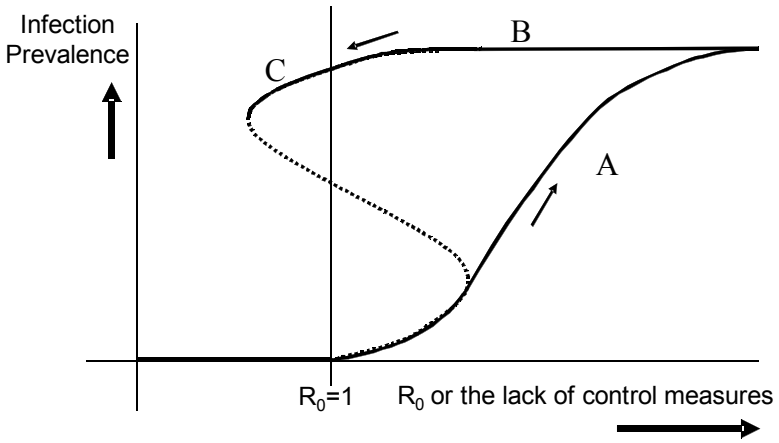

Fig 3. The relationship between R_0 and the prevalence of infection in a herd. In this graph a backward bifurcation is presented.

Mathematical modeling to study MAP infections in dairy herds has been proposed (Lu et al. 2008). With these mathematical infection models, MAP infections in herds can be simulated and studied. These models try to reflect the biology and epidemiology of MAP as realistic as possible, but often relatively simple models are used to study simulated infections. Despite their simplicity, mathematical models have shown to be useful in obtaining a better understanding of MAP transmission on dairy farms. Some important results include the prediction that calf-to-calf transmission may play an important role in infection maintenance, the quantification of the importance of super shedders in herds and a full understanding of the value of test-and-cull control programs in dairy herds (Lu et al. 2008). These mathematical models also provide a more generic insight into MAP infection dynamics in dairy herds. The basic reproduction ratio or R_0 of an contagious disease is defined as the number of secondary infections after the introduction of a single infectious individual in a susceptible population (herd). A threshold value of 1 for R_0 distinguishes successful control measures ($R_0 < 1$) from non-successful or a lack of control measures ($R_0>1$). In figure 3, the relationship between the R_0 value and the endemic infection prevalence is shown. When the R_0 value is below 1, the endemic prevalence is stable at zero. With increasing R_0, or a lack of control measures, the endemic infection prevalence increases in a sigmoid fashion (line A). Under normal circumstances, prevalence would decrease again along the same sigmoid curve (line A) when infection control measures are implemented (R_0 will be smaller). However, with some endemic infections prevalence will initially not, but remain high despite the reduction in R_0 value to values below the threshold value of 1 (Line B). Under these circumstances there is a situation possible where there is a high prevalence of infection in a management situation where no introduction of infection would be successful (Line C). This

phenomenon is defined as backward bifurcation (Figure 3). There are a number of reasons to believe that the backward bifurcation phenomenon is present in the case of MAP infections in dairy herds. First, environmental contamination and MAP survival in the environment may lead to a backward bifurcation; second, the presence of dose dependency in the likelihood of calfhood shedding status and the subsequent increased rate of development into a super shedder will also result in a backward bifurcation. These findings would imply that in endemically MAP infected herds, the effort to control the infection will be much more elaborate than what may have been predicted based on introduction of the infection in a dairy herd. This appears to be the case in many dairy herds in virtually all countries with reported MAP prevalence data.

DISCUSSION

Is elimination of MAP from dairy herds fact or fiction ? To answer this question we need to consider a number of arguments. The key arguments are that 1) within herd prevalence is much higher than measured using current diagnostic tools, 2) several currently underestimated transmission routes of infection appear to be important for infection maintenance on dairy farms, 3) aggressive test-and-cull based control programs are not economically feasible in modern dairy farms and 4) endemically infected dairy herds will need to employ extensive MAP control programs to overcome the impact of backward bifurcation in MAP infection dynamics. Based on these arguments, combined with observed data across all dairy herd populations it appears that MAP elimination from dairy farms is more fiction than fact.

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Host Response and Immunology

Convenors: Paul M. Coussens and Kris Huygen

Host Response and Immunology Keynote Lecture

Unraveling the Host Response to *Mycobacterium avium* subsp. *paratuberculosis*: One Thread at a Time

Judith R. Stabel

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The study of host immune responses to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is complicated by a number of factors, including the protracted nature of the disease and the stealthy nature of the pathogen. Improved tools for the measurement of immunologic responses in ruminant species, particularly the bovine, a key target species for MAP infection, has allowed the dissection of host immunologic responses to infection to some extent. Noted as one of the more fastidious mycobacteria, infection with MAP is often characterized by periods of subclinical infection extending for 3 to 5 years. Many animals will clear the infection during this period but it is almost impossible to distinguish by current methods animals that have cleared the infection from those that remain infected but are able to control the progression of disease. Escalation of paratuberculosis to a more clinical state, marked by diarrhea and weight loss, is thought be caused by immune dysfunction. Due to the intracellular nature of MAP, the macrophage is the first defense against infection. However, MAP is able to disarm host defense mechanisms and survive within cells of key target tissues. This disarmament is likely due to the provocation of immune mediators that may be immunosuppressive in nature. Cytokines such as IL-10 and TGF- β have been shown to mediate immunosuppression due to their inhibitory effects on CD4+ T cell activation and IFN- γ production. However, the dynamic between macrophages (APCs), T cells, and potentially B cells cannot be so easily explained, as a multifactorial dysregulation of host immunity is likely.

#172 Whole genome association study for Holstein susceptibility to MAP infection

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The objective of this study was to identify genetic markers associated with host susceptibility to MAP infection in Holstein cattle. Associated SNPs were identified by genotyping 521 Holstein cows, infected with MAP, and comparing allele frequency of these positives with an estimate of the general Holstein population allele frequency. The population allele frequency estimate was derived from genotypes of 6,283 Holstein sires used by artificial insemination (AI). The 521 infected cows were actually 233 and 288 animals from two resource populations of about 5,000 head each collected independently. Population 1 was comprised primarily of daughters of twelve Holstein sires, used heavily within the US dairy cattle population during the time preceding sample collection (1999-2003). Samples were obtained from 300 cooperating commercial dairy herds throughout the US. Population 2 consisted of dairy cattle from six, large cooperating dairy herds in Wisconsin, with all animals in the herds tested for Johne's disease as part of the research project. In both cases, only the animals with the highest ELISA scores (categorized as positive or strongly positive, versus low positive, suspect or negative) and/or positive fecal culture tests (Population 1) were genotyped. Genotyping was performed with the Illumina Bovine SNP50 Bead Chip, providing genotypes for 54,001 SNP markers. Data from the two resource populations was analyzed both in separate and combined analyses. The most significant markers from the individual and combined analyses (n=229) were used in a stepwise logistic regression analysis to identify a set of eleven markers that could be used as a predictor of genetics for Holstein cattle susceptibility to infection by MAP.

#33

MERKAL AWARD LECTURE

No interference of Heat shock protein 70 subunit vaccination against bovine paratuberculosis

Wiebren Santema, Selma Hensen, Victor Rutten, Ad Koets

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Vaccination against bovine paratuberculosis with a whole bacterin vaccine is limited due to interference with tuberculosis diagnostics and therefore not registered in e.g. Europe. In earlier studies we showed the protective effect of Hsp70/DDA subunit vaccination against bovine paratuberculosis (Koets et al., Vaccine, 2006). In the current study, potential interference of this vaccination with immunodiagnostic procedures to detect bovine tuberculosis and paratuberculosis was studied.

Five experimental groups of six adult cows were subjected to immunodiagnostic assays (tuberculin comparative skin test, BOVIGAM interferon-gamma, Pourquier paratuberculosis ELISA) and responses before and after vaccination were compared. Control cattle were sham immunized or immunized in the dewlap with an inactivated whole cell vaccine. The three remaining groups received Hsp70/DDA and differed by the application route and frequency of vaccine administration.

Vaccination of cattle with an Hsp70/DDA subunit vaccine has little side-effects. Skin test and BOVIGAM interferon-gamma responses do not differ between non-vaccinated and Hsp70/DDA groups. The vaccinated groups seroconverted in the Pourquier ELISA, but the serological assays for paratuberculosis diagnostics can be adapted by inclusion of an Hsp70 preabsortion step that enables differentiation between Hsp70/DDA vaccinated and infected animals.

In conclusion, the Hsp70/DDA subunit vaccine may contribute to paratuberculosis control strategies, without compromising diagnosis of bovine tuberculosis or paratuberculosis.

#68

Neutralisation of interleukin-10 from CD14+ monocytes enhances gamma interferon production in peripheral blood mononuclear cells from *Mycobacterium avium* subsp. *paratuberculosis* infected goats

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Previous studies in Norway have shown that the sensitivity of the IFN-γ test in goats subclinically infected with *Mycobacterium avium* subsp. *paratuberculosis* was lower than expected. Aiming to increase the sensitivity of the IFN-γ test, we wanted to look at regulatory immune mechanisms that could affect the IFN-γ response in goats with paratuberculosis. Goats from a herd with naturally acquired paratuberculosis were examined.

IL-10 was neutralised by adding a monoclonal antibody (mAb) against IL-10 to PPDj stimulated PBMCs from 14 infected and 6 subclinically infected goats. An increase in IFN-γ production, measured by ELISA, was seen in all goats when the anti-IL10 mAb was added compared to stimulation with PPDj only. This is in accordance with previous results from other groups, and it was suggested that IL-10 is produced by regulatory T-cells.

Depletion of putative regulatory $CD25^{high}$ T cells, using magnetic beads and an anti-CD25 mAb, had no clear effect on the number of IFN-γ producing cells. The cells producing IL-10 was subsequently identified by intracellular staining and flow cytometry to be $CD14^+$ MHC class II^+ monocytes both in PPDj stimulated and control cultures. However, possible regulatory T cells in goats were seen as $CD4^+CD25^+$ T cells produced IL-10 in response to Concanavalin-A stimulation. Additional investigations revealed that adding the anti-IL10 mAb also increased IFN-γ production in unstimulated PBMCs and in PPDj stimulated PBMCs from 8 out of 17 non-infected goats. Furthermore, we observed increased numbers of both $CD4^+$, $CD8^+$ and $CD8^+$ γδTCR^+ cells producing IFN-γ after neutralisation of IL-10.

In conclusion IL-10 from CD14 positive monocytes inhibits maximal IFN-γ production in goats with paratuberculosis. However due to the unspecific reactions observed, this does not appear to be a useful way of increasing the sensitivity of the IFN-γ test for diagnosing paratuberculosis in goats.

#75

Interleukin 17 and interleukin 23 gene expression differentiate severe pathology in Johne's disease

Mark William Robinson, Rory O'Brien, Frank Griffin

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The gastrointestinal immune response to infectious agents such as *Mycobacterium avium* subspecies *paratuberculosis* must be balanced in order to effectively contain the infection while at the same time avoiding excessive tissue destruction. As such, differences in the host immune response to challenge can potentially influence disease progression and outcome of Johne's disease. Using an experimental infection model of Johne's disease in red deer (*Cervus elaphus*) we studied the immune responses during different pathological states. A group of 30 red deer were challenged with ~10^9 colony forming units of bovine strain *Mycobacterium avium* subspecies *paratuberculosis* and were monitored for Johne's disease development over the course of 1 year. Peripheral blood samples were acquired at 20 weeks post-infection and post-jejunal lymph node (PJJLN) samples following necropsy at either 20-28 weeks post-infection for clinically diseased animals or at 50 weeks post-infection for remaining animals. Animals were retrospectively grouped into 3 pathological outcomes (Infected, Paucibacillary Diseased or Multibacillary Diseased) on the basis of histology scores and tissue culture results. Gene expression of transcripts for IFNg, IL2, Tbet, IL1a, TNFa, TRAF1, GATA3, IL4, Foxp3, IL10, IL17, IL23p19, IL6 and TGFb were detected using quantitative real time PCR and data was analysed using REST software to determine significant changes in gene expression levels. Stimulation of peripheral blood mononuclear cells gave distinctive patterns of gene expression for all 3 groups with strong Type 1 responses in both the Infected and Paucibacillary Diseased groups while the Multibacillary Diseased group showed a mixed immune response with significant up-regulation of the IL23p19 gene transcript. The PJJLN gene expression profiles of the Multibacillary Diseased group exhibited a highly inflammatory profile with no evidence of a role played by T regulatory or Type 2 immune responses. Gene expression profiles provide novel diagnostic markers for different pathological states of Johne's disease and support the hypothesis that differing host immune responses direct disease progression and pathological outcome.

#119

The early IL-10 response in ovine Johne's disease

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The classic early immunological response to Mptb is mediated by T-cells with predominant interferon gamma activity. This response is curbed as clinical signs of disease appear and may, at least partly, be due to a surge in the activity of the immunosuppressive cytokine interleukin 10 (IL-10). While there is evidence of IL-10 activity in the later stages of disease its involvement at earlier time points is not known. The aim of this study was to monitor the antigen-specific IL-10 response during experimental Johne's disease. Merino lambs were left unchallenged (controls, n=20) or challenged orally with Mptb (n=40). Lymphocytes isolated from blood (every 4 months) and lymph nodes (11-13 months after challenge) were cultured in the presence or absence of Mptb antigen. IL-10 in culture supernatants was determined by ELISA. Faecal shedding and presence of Mptb in tissue samples were determined by culture in a radiometric system (Bactec). Histological lesion type was also assessed. Antigen-specific IL-10 secretion in peripheral blood of sheep exposed to Mptb was significantly higher than in control animals ($P < 0.001$) as early as 4 months p.i. and increased progressively. The peripheral blood IL-10 response in animals with multibacillary lesions tended to differ from animals with no histological lesions or with paucibacillary lesions. In ileal and jejunal lymph node cells, IL-10 secretion was significantly higher in the exposed animals ($P < 0.05$). In addition, IL-10 secretion from these cells was significantly higher in animals with no lesions or with paucibacillary lesions when compared to animals with multibacillary lesions. Our novel findings demonstrate that IL-10 activity occurs soon after exposure to Mptb and may play a role in determining disease outcome.

#173

Local and systemic roles for bovine gamma-delta T cell subsets during the early immune response to *Mycobacterium avium* subspecies *paratuberculosis* (Map) infection

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The role of gamma-delta (gd) T lymphocytes during the immune response to infectious agents continues to be explored. Elevated numbers of gd-positive cells are found as intra-epithelial lymphocytes at mucosal surfaces and in peripheral blood of some species. Peripheral blood gdT cells from both naïve and previously exposed individuals (cattle and humans) proliferate robustly and secrete cytokines when stimulated with mycobacterial antigens, suggesting a role for gdT cells during early host defense against mycobacterial disease. A significant question that remains unanswered is whether peripheral blood studies accurately reflect what occurs *in vivo* at the infection site. While gdT cells have been shown to be recruited to early mycobacterial infection sites *in vivo*, their functional role remains unclear; this is largely due to the difficulty in obtaining sufficient numbers of gdT cells from infection sites for analysis. Our hypothesis is that bovine gdT cells have subset-specific roles during the initial host response to Map infection in young calves. In this study, we investigated our hypothesis using a novel subcutaneous biopolymer gel matrix-based assay developed in our laboratory to characterize the *in vivo* cellular and cytokine responses to Map infection in both naïve and vaccinated calves. To accomplish this objective, we used multi-color flow cytometry and enzyme-linked immunosorbent assay (ELISA) to characterize surface marker expression and cytokine secretion from cells recruited directly to Map infection sites. Further, we compared our findings with gdT cell responses from peripheral blood cells during Map infection. Our results suggest that bovine gdT cell subsets are differentially recruited to early mycobacterial infection sites. Further, we show that bovine gdT cell subsets recruited to infection sites differentially secrete cytokines. Our data suggests that this initial response by gdT cell subsets to mycobacterial infection may play an important role during development of the subsequent immune response.

#190

Development of new live vaccines for paratuberculosis

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Control of paratuberculosis would be greatly facilitated by the availability of effective vaccines. However, despite 80 years of vaccine development and use, current paratuberculosis vaccines provide only limited protection, often cause unacceptable lesions at the site of inoculation, and interfere with diagnostic tests for bovine tuberculosis. In order to develop vaccine candidates, mutants of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were produced by random transposon mutagenesis and specific gene inactivation by allelic exchange. Cytokine responses, levels of apoptosis and survival of selected mutants were assessed in bovine macrophages, and virulence of the mutants was determined in mice and goats. Levels of apoptosis varied and correlated with MAP strain virulence. All three models identified the same mutant (WAg906) as being the most attenuated vaccine candidate, but some differences in the levels of attenuation were evident amongst the models when testing the other strains. While we found macrophages and murine models to be rapid and cost effective alternatives for the initial screening of MAP mutants for attenuation, the results correlated imperfectly with those from the goat model, indicating the importance of assessing the virulence of likely vaccine candidates in ruminants. Interestingly, another mutant (WAg915) that was less attenuated than WAg906, showed the greatest vaccine potential in a preliminary vaccine experiment, as animals vaccinated with WAg915 had the lowest counts in the spleen and liver for the virulent MAP challenge strain.

#23

Role of *Mycobacterium avium*. subsp. *paratuberculosis* in the pathogenesis of Crohn's disease

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Crohn's disease (CD) is a clinically defined syndrome of unknown etiology. Patients with CD develop chronic relapsing bouts of enteritis. The mechanisms of induction and persistence of inflammation appear to include modulation of the immune response through activation of the IL-23/IL-17 and IL-22 pathways that promote chronic inflammation. Factors that trigger CD are thought to include exposure to specific pathogens, bacteria present in normal microflora of the intestine, or other undefined factors that induce persistent immune mediated inflammation of the bowel. *Mycobacterium avium* subsp. *paratuberculosis* (Map) the etiological agent of Johne's disease (JD) is the pathogen most frequently implicated in CD immunopathogenesis. The finding of Map in many patients with CD supports this possibility. It remains unclear, however, how Map could be involved in CD pathogenesis. Elucidation of the mechanisms of pathogenesis mediated by Map in its natural host could provide insight into its potential role in CD pathogenesis. We developed a bovine ileal cannulation model to analyze the mechanisms of JD immunopathogenesis. The studies revealed animals become persistently infected following exposure to Map with no signs of clinical disease during the first year post infection (PI). Map elicited a prominent CD4 T cell response to Map and Map antigens 3 months PI. A similar response was observed in animals at the clinical stage of disease. The CD8 T cell response was more prominent in animals at the clinical stage of disease. Quantitative RT-PCR revealed a complex pattern of expression of genes encoding IFN-γ, IL-17, IL-22, and granulysin PI indicating the presence of CD4 T cells associated with a Type I immune response and Th17 and Th22 CD4 T cells associated with a proinflammatory response. The findings show that persistent infection with Map could play a role in CD pathogenesis.

Perspectives in Host Reponse and Immunology

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A wide range of topics will be covered in this session:

-humoral and cellular immune responses in cattle, sheep and goats and variations in this response linked to genetic susceptibility, age and polymorphism of the MAP isolates
-possible link of MAP infection with Crohn's disease
-development of new or optimization of existing experimental infection models
-discovery of new antigens
-development of new vaccines based on live attenuated whole bacteria or on sub-unit components (protein and DNA)

The interaction between the early, innate immune response and the cognate acquired immune response had not been studied in much detail so far for infections with *M. avium* subsp *paratuberculosis*. More and more results now suggest that a malfunctioning innate response, caused by an immature system in young animals and/or by genetic factors (a.o. deficient reactivity to mycobacterial peptidoglycan as observed in Crohn's patients) would lie at the basis of the deficient cell mediated immune (CMI) response observed in animals with multibacillary forms of the disease. The evidence for a role of Th2 and regulatory T cells in the decreased CMI responses, so characteristic of the clinical phase of Johne's disease, is questioned and although high levels of IL-10 can be detected, it are CD14+ MHC class II+ monocytes rather than T cells that produce this cytokine and moreover, even at early stages of infection IL-10. On the other hand, the role of another Th subset, i.e. Th17 may be more relevant, as gene expression of interleukin17 and interleukin 23 was found to be upregulated in an experimental infection model of red deer during disease progression. This Th17 population has also been incriminated in Crohn's disease and other auto-immune diseases.

With respect to vaccination, it is clear that live attenuated vaccines can be very effective, but a balance has to be found between their immunogenicity (less attenuated strains may be more immunogenic but may interfere with the immunodiagnostic tests) and their vaccine potential. Even for subunit vaccines the interference with existing diagnostics may be an issue. Characterization of immunodominant and specific antigens of MAP will be essential to overcome this hurdle.

Host Response and Immunology

Poster Abstracts

#4 NOD2 and mycobacterial recognition

Marcel Behr *McGill University, Canada*

Objective: To determine the effect of NOD2 polymorphisms on mycobacterial recognition *in vivo*. **Materials and Methods**: We obtained *Nod2-/-* mice that manifest the same loss-of-function phenotype during ex vivo stimulation with peptidoglycan (PGN) as is observed with Crohn's patients that are homozygous for the 3020insC mutation. Mice were challenged *in vivo* challenge with *M. tuberculosis* and *M. bovis*, looking at early time-points (pathology and adaptive immunity) and late-points (bacterial CFU and survival). In a second study, macrophages from Nod2-/- mice were stimulated ex vivo with mycobacteria wild-type and disrupted for namH, responsible for the modification of PGN to produce N-glycolyl muramyl dipeptide (MDP). **Results**: *Nod2-/-* mice generated a diminished inflammatory response, with less pathology, less IL-12 production and reduced adaptive immunity one month after infection. After 6 months, *Nod2-/-* mice had higher bacterial burdens and succumbed to infection sooner than wild-type controls. Using bacterial genetics and synthetic chemistry, we found that NOD2 activation depends on the presence of the mycobacterial namH and that the mycobacterial PGN (N-glycolyl MDP) is at least 10-times more potent at NOD2 activation than the commonly encountered N-acetyl MDP. **Conclusion**: NOD2 is important and specific for recognition of mycobacterial infection, *in vitro* and *in vivo*.

#27 Assessment of different strategies to determine MAP-specific cellular immune responses in cattle

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Assessment of cellular immunity in cattle against *Mycobacterium avium* ssp. *paratuberculosis* (MAP) by established methods remains unsatisfactory for diagnostic purposes. Recent studies conclude that analysis of T-cell subset responsiveness may improve diagnostic outcome. Aim of this study was to identify T-cell subsets which respond most specifically to MAP antigens. Peripheral mononuclear cells (PBMC) from 7 MAP-positive and 5 MAP-negative cows (defined by herd status, serology, fecal culture, PCR) aged 2 - 5 years were incubated in medium supplemented with whole cell sonicates (WCS) or purified protein derivatives (PPD) of MAP, *M. avium* ssp. avium, and *M. phlei* for up to 6 days. Flow cytometry was used to quantify IFN-γ production in $CD4^+$ and $CD8^+$ T-cells and to quantify CD25 and CD26 expression on $CD4^+$ and $CD8^+$ memory T-cells ($CD45RO^+$), γδ-T-cells ($TcR1\text{-}N24^+/CD2^{+/-}$), and NK-cells ($CD335^+/CD2^+$). Different gating strategies were applied to analyze lymphocyte and lymphoblast populations as well as IFN-γ and CD markers individually or in combination. WCS preparations induced more specific responses compared to PPD throughout. Compared to MAP-negative animals, $CD4^+$ lymphoblasts from MAP-positive cows responded with a significantly higher IFN-γ content after incubation with WCS-MAP. IFN-γ production by $CD8^+$ PBMC hardly differed between the groups. $CD4^+/CD45RO^+$ PBMC from MAP-positive cows responded to WCS-MAP with a significantly higher expression of CD25 and CD26. γδ-T-cells ($CD2^{+/-}$) from MAP-positive cows reacted similarly with a higher CD25 expression. $CD8^+$ and NK-cells from MAP-positive but not of MAP-negative cows responded with an enhanced CD25 expression independent of the origin of mycobacterial antigen. In conclusion, $CD4^+$ T-cells from MAP-positive cows responded most sensitively and specifically to WCS-MAP with regard to both, IFN-γ production and CD25/CD26 expression. Because early stages of MAP-infection are dominated by cellular immunity, quantifying the responsiveness of $CD4^+$ T-cells ex vivo may be a useful approach to improve early MAP-diagnosis in cattle.

#28 Intestinal MAP Infection via Peyer's Patch Inoculation in a Calf Model

Jesse Hostetter, Brandon Plattner, James Roth, Jean Zylstra, Ratree Platt, Yu-Wei Chiang, *Iowa State University, USA; Fort Dodge Animal Health, USA*

The **objective** of this project was to develop an intestinal model of MAP infection in the calf for evaluation of mucosal pathology, and local and systemic immunologic responses. **Methods**: Our approach was to directly inoculate MAP into the Peyer's patches of the ileocecal junction in 40 five week old calves. We used right flank laparotomy to identify and exteriorize the ileocecal junction. MAP suspended in saline was injected through the serosal surface of the intestine into the Peyer's patch region and the incision closed. We tested inoculum doses ranging from 10^3 to 10^9 CFU, and each dose for 7, 30, 60 and 90 days post infection with 2 calves per group. Feces was collected from each calf weekly. At each time point we necropsied calves in each dose group and collected the inoculation site, lymph nodes (ileocecal, mesenteric, prescapular), spleen, and peripheral blood. **Results**: The ileocecal valve and mesenteric lymph nodes were consistently colonized with MAP in the 10^7 and 10^9 groups from 7-90 days post infection, and this correlated with fecal shedding of the organisms. Histologically we identified consistent granulomatous lesions with numerous acid fast bacilli in the 109 group at each time point. These microscopic features were found in Peyer's patches, distal villi, and in the ileocecal lymph nodes. Using immunohistochemistry we identified mycobacteria within the villi and ileocecal lymph nodes in the 10^7 group at 30, 60, and 90 days. In the 10^7 and 10^9 groups we identified significant production of IFN-g and high expression of the activation marker CD25 in peripheral blood and lymph node mononuclear cells using antigen recall assays. **Conclusion**: These data demonstrate that inoculation of MAP into the Peyer's patches results in infection that shares features with natural MAP infection including mucosal colonization, fecal shedding, mucosal pathology, and local and systemic immunologic responses.

#64 Association between paratuberculosis infection and general immune status in dairy cattle

Pablo J Pinedo, Art Donovan, Owen Rae, Jason De la Paz, *College of Veterinary Medicine, University of Florida, USA*

The objective was to investigate the association between paratuberculosis infection as determined by serum ELISA and the general humoral and cellular immune status of adult cows in a dairy herd in Florida (USA). A total of 781 Holstein cows were tested for paratuberculosis infection by use of a USDA-licensed ELISA (IDEXX-Laboratories). Optical densities were used to obtain sample-to-positive (S/P) ratios that were converted to 4 S/P-ratio categories: negative (0-0.49); inconclusive (0.50-0.99); positive (1-3.49); strong positive (≥3.5). Simultaneously, individuals were categorized for their ability to mount a general antibody (AMIR) and cell-mediated immune response (CMIR). Ovalbumin and killed, whole cell Candida albicans were antigens used to stimulate AMIR and CMIR, respectively. AMIR response was measured using an ELISA. CMIR was evaluated by a delayed-type hypersensitivity reaction using an intradermal injection of candin (*C. albicans* antigen) in the tail skin-fold and measuring the inflammatory response 24 hours later. Levels for immune response were categorized as low, medium, or high for each of the two variables. The statistical analysis was based on logistic regression (cumulative logit model), and considered the 4 S/P-ratio categories for paratuberculosis ELISA as the dependent variable. The independent variables were AMIR, CMIR, and parity. Given the potential association among variables, two way interactions were included in the model. Parity and CMIR were significantly associated with paratuberculosis infection status. Cows with higher levels of cell-mediated immunity were less likely to demonstrate evidence of paratuberculosis infection. For each category increase in CMIR (low, moderate, high), the odds of being above any given ELISA S/P-ratio category decreased by 68%. Furthermore, older cows were more likely to show paratuberculosis infection. For each increase in parity, the likelihood of a higher level of infection increased by 75%. It is concluded that higher cell-mediated immune status was associated with a lower probability of paratuberculosis seropositivity.

#67 The γδ cells as marker of non-seroconverted cattle naturally infected with *Mycobacterium avium* subspecies *paratuberculosis*

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Early diagnosis of MAP infection is a pressing need that enables efficient intervention with the spread of MAP infection in herds. Hence, study of lymphocyte subsets and their expressed adhesion molecules could contribute in defining a distinct diagnostic marker (or markers) at the subclinical period of the infection that could in turn facilitate the development of effective diagnostic approach. In accordance with this objective, milk and blood samples were collected from two groups of cattle naturally infected with MAP and their corresponding negative controls. Group(C) comprised 3-4 year-old ELISA negative/PCR positive-cattle that were considered as subclinical seronegative low shedder group (early stage). Group (A) included 6-8 year-old ELISA positive cattle, which were considered as a clinical seropositive group (late stage). Flow cytometry of B cells, $CD8^+$, $CD4^+$ and gd cells and the adhesion molecules $CD44^+$, CD62L, LFA-1 and LPAM-1 indicated increase ingroup A. $CD4^+$ and B cells levels were higher in blood than milk of group A, and significant expression of $CD44^+$ in blood and milk and LPAM-1 in blood only. The $CD8^+$ cells count in milk was higher than blood in the late stage. The peculiar feature of the early stage (group C) was the high level of gd cells in the blood and milk, with tendency to express high level of CD62L. Compelling evidence could support the assumption that the dominant gd cells at early stage of MAP infection could be of $CD8CD2^-$ $WC+1^+$ phenotype. gd cells appear as promising markers in defining early changes of MAP infection due to their important role in priming innate and cell mediated immunity. Possible utilization of this peculiar changes in the gd cells level in the early diagnosis of MAP infection should be the subject of further research.

#70 Profiling immune responses to the PE and PPE protein families in naturally MAP infected cattle

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The PE and PPE proteins make up two families of polymorphic proteins specific to mycobacteria. These proteins are composed of conserved N-terminal domains and highly variable C-termini. While these proteins make up a significant portion of the coding capacity of pathogenic mycobacteria, their functional roles are largely unknown. Of the current hypotheses, the most widespread is that they play a role in antigenic variation. Their polymorphic nature, as well as their observed immunogenicity in mycobacterial infections makes them interesting diagnostic and vaccine candidates. Indeed, if these proteins were involved in antigenic variation, we would expect to see patterns of immune responses emerge to these proteins following disease progression. To this end, we are developing and testing a novel protein array to study bovine immune responses to the 10 MAP PE proteins, the 36 MAP PPE proteins, and a panel of previously identified antigens, including: Map0857c, Map0862, Map1087, Map1204, Map1272c, Map1637c and Map1730c. This represents the first time a protein array approach has been used to study immune responses against polymorphic protein families in their entirety. Representatives of conserved regions of each PE and PPE subfamily were produced recombinantly in *Eschericia coli* and purified by nickel affinity purification alongside all variable regions of the MAP PE/PPE repertoire, allowing demonstration of specific immune responses. Animals in different stages of disease with varying ELISA, fecal and tissue culture results (n=16) were tested in Western and dot blot assays as well as negative samples (n=8) from non-infected controls. Using this approach we identified variable immune responses between animals and categories of infection. This information will be invaluable in the identification of early disease markers and provides clues as to the role of these proteins in MAP pathogenesis and host responses.

#73 Age susceptibility of red deer *(Cervus elaphus)* to paratuberculosis

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Aim: To measure the relative susceptibility of three age classes of red deer to paratuberculosis, using experimental challenge with MAP.

Materials and Methods: Three groups of seronegative female deer (30 three-month-old weaners, 20 fifteen-month-old yearlings and 20 adults) received four oral doses of ~10^9 cfu of a bovine MAP. They were paddock-monitored daily, weighed at 1-4 week intervals, blood sampled regularly and faecal sampled over the 50 week study. Clinically affected animals were promptly euthanised and necropsied. The remaining deer were killed at the end of the study and necropsied. Gross findings were recorded, faecal samples taken for culture and samples of intestine and associated lymph nodes were taken for culture and histopathology from all deer.

Results: Ten weaners developed clinical paratuberculosis and were euthanased 20-28 weeks pi. No clinical cases occurred in the yearlings or adults ($P<0.05$). All 10 clinically affected weaners had severe gross and histopathological lesions of Johne's disease. Three weaners died of misadventure. At slaughter, gross lesions were seen in jejunal lymph nodes of 8/17 weaners, 2/19 yearlings and 0/20 hinds ($P<0.05$). The histopathological lesion severity scores of deer slaughtered 50 weeks pi averaged 4.9, 3.5 and 1.1 for the weaner, yearling and adult groups, respectively ($P<0.05$).

MAP was recovered from faeces of 13/19 weaners, 6/19 yearlings and 1/20 adult hinds 24 weeks pi ($P<0.05$) and from faeces of 3/17 weaners, 4/19 yearlings and 1/20 adult hinds at slaughter (NS). MAP was cultured from samples of the intestine and/or lymph n

Conclusion: Young deer are very susceptible to heavy oral challenge with MAP, but there is a strong age-related resistance against clinical paratuberculosis and subclinical disease, but not to infection.

#80 Experimental challenge trial of a paratuberculosis vaccine in red deer (*Cervus elaphus*)

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Aim: To test the efficacy of a paratuberculosis vaccine in young red deer, using experimental challenge with MAP, and to assess effects of vaccination and challenge on bovine tuberculosis tests.

Materials and Methods: Forty 12-week-old fawns were vaccinated with a 1ml dose of Silirum® [Pfizer] while 40 remained unvaccinated Controls. Six weeks later, all 80 calves received an oral challenge with ~10^9 cfu MAP daily for 4 days.

The deer were regularly monitored, weighed and tested for antibodies to MAP and Tb. They were skin tested with bovine tuberculin (MCT) at 17 weeks post challenge (pc), and with avian and bovine tuberculin (CCT) at 47 weeks pc. Clinically affected animals were promptly euthanised. Surviving deer were slaughtered at 49 weeks pc. All deer were necropsied, examined and samples taken for culture and histopathology.

Results: At 17 weeks pc, 95% of both groups were MCT-positive. Two weeks post-MCT, 82% Vaccinates and 35% Controls were ELISA-positive for Tb. At 47 weeks pc all deer were CCT-negative for Tb. At 49 weeks pc, 100% of Vaccinates and 57.1% of Controls were ELISA-positive for MAP. There were five clinical cases of confirmed Johne's disease; four Controls and one Vaccinate (NS). At slaughter the Controls had significantly more severe gross lesions than the Vaccinates ($P<0.05$). The Controls tended to have more severe histopathological lesions than the Vaccinates (NS). MAP was isolated from 94.6% Vaccinates and 91.4% Controls at sl

Conclusion: Vaccination reduced the severity of disease resulting from experimental challenge, but did not protect against infection, nor reduce the prevalence of faecal shedding. Vaccination and challenge caused false positive reactions in the MCT and Tb.

#82 Oral vaccination of mice with *Mycobacterium bovis* BCG vaccine in a lipid matrix protects against infection with *M. avium* subsp. *paratuberculosis*

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Objective: Existing vaccines against MAP, based on whole killed or live attenuated bacteria, can delay the onset of clinical symptoms but not the actual infection. Moreover, vaccinated cattle develop antibodies that interfere with serodiagnosis of Johne's disease and they become reactive in the PPD skin-test, used for the control of bovine tuberculosis. Furthermore, the intramuscular administration of these vaccines in oily adjuvant induces local granulomas at the injection site and poses a risk for the veterinarian. Here we have analyzed the potential of oral vaccination to overcome these hurdles.

Materials and Methods: MAP susceptible BALB.B10 mice [1] were taken off food but not water for five hours before placing them individually in a cage with water, minimal bedding and the lipid vaccine (*M. bovis* BCG Danish 1331, 10^7 CFU in lipid PK) in a cup as described before [2] . Control animals were fed with cups containing only lipid PK. Each animal was left overnight to consume the vaccine. After the paste was eaten, animals were returned to group cages with normal mouse food available. Mycobacteria specific IFN-g responses were analyzed 5 weeks post immunisation. Fifteen weeks post immunisation, mice were challenged with 1.6 x10^6 RLU of luminescent MAP ATCC 19698 and bacterial replication was monitored in spleen and liver for 12 weeks.

Results: Although only weak mycobacteria-specific IFN-g and lymphoproliferative immune responses were detected in spleen and maxillary lymph nodes of mice fed with the lipid-formulated BCG vaccine, bacterial numbers (as detected by luminometry) were significantly lower in spleen and particularly in liver at four and eight weeks post challenge.

Conclusion: Lipid based, orally delivered mycobacterial vaccines may be a safe and practical method of controlling paratuberculosis.

References: 1: Roupie V et al Infect.Immun. 2008. 76: 2099-2105. 2: Clark S et al Infect Immun. . 2008. 76: 3771-3776.

#92 Differential expression of CD5 on B lymphocytes in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

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CD5 is a cell surface molecule involved in antigen recognition and is present on all T lymphocytes and a subset of B lymphocytes. The purpose of this study was to examine $CD5^+$ expression on peripheral blood B cells from healthy, noninfected cattle and cattle with subclinical and clinical paratuberculosis. Peripheral blood mononuclear cells (PBMC) were freshly isolated or cultured for 7 days in the presence or absence of live *Mycobacterium avium* subsp. *paratuberculosis* (*M. avium* subsp. paratuberculosis), and then analyzed by flow cytometry for CD5 expression within the B cell subpopulation. Analysis demonstrated a significant increase ($P < 0.01$) in B cells in clinical animals as compared to healthy control cows and subclinically infected cows. In addition, three subpopulations within the $CD5^+$ B cell population were identified: CD5dim, CD5bright, and a minor population that was characterized as CD5extra bright. A decrease in the CD5dim B cell population along with a concomitant increase in CD5bright B cells was observed in infected cows, an effect that was highly significant ($P < 0.01$) for subclinically infected cows in cultured PBMC. *In vitro* infection with live *M. avium* subsp. *paratuberculosis* did not affect $CD5^+$ expression patterns on B cells, regardless of animal infection status. Addition of exogenous IL-10 to PBMC cultures resulted in decreased numbers of CD5bright B cells for healthy control cows, whereas, a synergistic effect of IL-10 and infection with live *M. avium* subsp. *paratuberculosis* resulted in increased CD5bright B cells for subclinically infected cows. These results suggest that differential expression of CD5bright and CD5dim subpopulations on B cells in animals with paratuberculosis may reflect a shift in host immunity during the disease process.

#95 Role of nitric oxide production in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*

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Nitric oxide (NO) is a crucial mediator in host defense and is one of the major killing mechanisms within macrophages. Its induction is highly affected by the types of cytokines and the infectious agents present. In the current study, NO production was evaluated after *in vitro* infection of unfractionated peripheral blood mononuclear cells (PBMCs) with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) after 8 hr, 3 and 6 days of culture for cows in different stages of disease. In addition, the effects of *in vitro* exposure to inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) as well as the pro-inflammatory cytokine IFN-γ were correlated with the level of NO production. Nitric oxide production was consistently higher in cell cultures from subclinically infected animals at all time points. An upregulation of NO production was demonstrated in unfractionated cell cultures from healthy control cows after exposure to MAP infection as compared to noninfected cell cultures. A similar increase in NO due to the addition of MAP to cell cultures was also noted for clinically infected cows. NO level among subclinically infected cattle was greater at all time points tested and was further boosted with the combination of both *in vitro* MAP infection and IFN-γ stimulation. Finally, the *in vitro* exposure to inhibitory cytokines such as IL-10 and TGF-β prior to MAP infection or LPS stimulation resulted in the downregulation of this inflammatory mediator (NO) in all experimental groups at all time points. In summary, a higher level of NO production was associated with cows in the subclinical stage of MAP infection.

#127 Multiplication of *Mycobacterium avium* subsp. *paratuberculosis* is higher in macrophages induced by M-CSF than GM-CSF

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Mycobacterium avium subsp. *paratuberculosis* (MAP) strain ATCC 19698 was grown in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 2 micrograms/ml mycobactin J and 12.5% OADC Enrichment for several weeks. One milliliter of bacteria culture was collected in a microtube and centrifuged. After removal of the supernatant, the pellet was suspended in 0.5 ml PBS, and then the bacteria were dispersed for 5 seconds with a homogenizer. The microtubes were allowed to settle for 3 minutes, and several suspensions were pooled and passed through a 5-micrometer-pore-size filter. Twenty milliliters of bovine blood was infected with MAP ($3x10^8$ cells). After 90 minutes of incubation, peripheral blood mononuclear cells (PBMC) were isolated by use of Ficoll-Paque PLUS density gradient centrifugation. Monocytes were collected from the PBMC by a magnetic cell separation system (MACS) with anti human CD14 antibody- coupled microbeads. Monocytes were incubated at $2x10^5$/well in 96-well tissue culture plates with 10% FCS RPMI1640 including either M-CSF or GM-CSF. One, two and three weeks later, macrophages induced by M-CSF or GM-CSF were collected into microtubes by using 0.2% EDTA and 5% FCS in PBS and genomic DNAs were extracted with an UltraClean Microbial DNA Isolation kit. Multiplication of MAP was estimated by the amount of MAP DNA measured by real- time PCR. The amount of MAP DNA phagocytosed by monocytes in blood was 14.5pg/well (day 0). The amounts of MAP DNA within macrophages incubated with M-CSF were 40.1 pg/well (day 7), 48.3 (day 14) and 23.1 (day 14). Meanwhile, DNA volumes of MAP in GM-CSF-stimulated macrophages were 29.5 pg/well (day 7), 23.7 (day 14) and 8.9 (day 21). These results suggest that MAP multiplies in high number in macrophages induced by M-CSF as compared to GM-CSF. For the next step, we will investigate cytokine profiles of these macrophages.

#133 Immunological and pathophysiological characteristics of *Mycobacterium avium* subspecies *paratuberculosis* infection in guinea pigs

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In order to elucidate the subject why profound chronic enteritis is caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map) in ruminants but not by other *M. avium* subspecies which have great similarity in genetical and antigenical characteristics, we have tried to find differences that may characterize Map infection from those of other mycobcaterial infections.

In preliminary experiments using guinea pigs, shedding of intraperitoneally inoculated Mycobacterium organisms into feces was detected with surprising speed of within 24 hours after inoculation, therefore the experiments were divided into two different observation periods of 24 hours and two months after inoculation. Four-week-old female Hartley guinea pigs were intraperitoneally inoculated with 5 different species or subspecies of live mycobacterium (Map, *M. avium* subspecies avium, *M. intracellulare M. scrofulaceum*, and *M. bovis*). After 24 hours or two months, whole intestine and principal organs were collected and performed bacteriological and histopathological examinations with bacterial culture, quantification of bacteria by realtime PCR and immuno-pathological staining using anti CD68 monoclonal antibody.

Results obtained from our experiments are as follows: 1) Common findings among all Mycobacterial infections: Granuloma formation in liver and spleen; Thickening of duodenum because of infiltration with inflammatory cells; Bacterial shedding within 24 hours after intraperitoneal inoculation. 2) Characteristics in Map and other *M. avium* subspecies infections: Jejunoileal inflammation consisted of macrophages, plasma cells, and eosinophils at 2 months postinoculation. 3) Characteristics exclusively observed in Map infection: Significantly lower number of organisms in the duodenum at 24 hours after inoculation than those of others; - Scattered distribution of $CD68^{+}$ macrophages in the lamina propria mucosae of duodenal villi contrary to focused distribution of those cells at the tip of villi in other Mycobacterial infections.

These observational data generated from guinea pig infections may help us to understand the pathogenesis of Map infection.

#134 Analysis of the vaccine potential of nine MAP specific proteins, identified by immunoproteomics and in silico bio-informatic screening, in a murine model

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Objective: Control of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is seriously hampered by the lack of adequate diagnostic tools, vaccines and therapies. In this study, we have evaluated the vaccine potential of nine MAP proteins: MAP3199, MAP2677c, MAP1693c, previously identified by immunoproteomic analysis of MAP secretome [1] and Ag5, Ag6 (not-annotated), MAP1637c, MAP0388, MAP3743 and MAP3744 identified by bioinformatic *in silico* screening of the MAP genome.

Materials and Methods: BALB/c and C57BL/6 mice were vaccinated with plasmid DNA encoding the nine selected candidates as described before [2]. Three weeks after the last immunisation, Th1 type cytokine responses (IL-2 and IFN-γ) against the respective purified recombinant proteins were tested on spleens from individual mice. Levels of antigen-specific total immunoglobulin G (IgG), IgG1, IgG2a and IgG2b antibodies were determined by ELISA. Six weeks after the last immunization, mice were challenged intravenously with luminescent MAP ATCC 19698 ($2X10^6$ CFU/mice). Mice were sacrificed and the number of bioluminescent bacteria was determined in spleen and liver homogenates at 4, 8 and 12 weeks after challenge [3].

Results: Vaccination with DNA encoding MAP1637c (predicted carboxylase) induced the strongest Th1 type immune responses, both in BALB/c and C57BL/6 mice, confirming previous findings on the potential of this *in silico* selected antigen for the serodiagnosis of bovine paratuberculosis in cattle [4]. Protective potential of the nine antigens was weaker than the one we have reported for the putative transglycosylase MAP0586c [2].

Conclusion: Plasmid vaccination, coupled to the use of bioluminescent MAP is a powerful tool for the screening of the vaccine potential of paratuberculosis antigens in mice.

References: 1. Leroy, B., et al., Proteomics, 2007. 7(7): p. 1164-76. 2. Roupie, V., et al., Vaccine, 2008. 26: p. 4783-4794. 3. Rosseels, V., et al., Infect Immun, 2006. 74(6): p. 3684-6. 4. Leroy, B., et al., Vet. Microbiol., 2009 135(3-4): p. 313-319.

#153 SNP discovery in bovine *Sp110* gene and its genetic association with infetion by MAP

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Three polymorphisms of the *Sp110* gene have been associated with tuberculosis susceptibility in humans (Tosh et al. 2006). Moreover, Ipr1 gene in mice, a *Sp110* homolog, has been reported to confer genetic resistance to *Mycobacterium tuberculosis* and Listeria monocytogenes through apoptotic pathway activation in infected macrophages (Pan et al. 2005). Therefore, we hypothesize that the bovine *Sp110* gene could be involved in the innate immunity response against *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in cattle. To test the hypothesis, a SNP discovery study on the *Sp110* gene for use on a case-control association study has been undertaken comparing 500 healthy and 350 infected Holstein-Friesian cattle.

As nucleotide variation of bovine *Sp110* gene is poorly described, we focused on SNP (single nucleotide polymorphisms) discovery by comparative sequencing. Looking for functional variation, 9980 bp out of 51532 bp of the gene were sequenced in 30 animals (15 healthy / 15 infected). This included the 300 bp of the promoter, 12 exons, and both 3'- and 5'- UTR regions of the gene. In all, 20 SNPs were detected for the first time in Holstein-Friesian cattle. The application of the selected SNP polymorphisms to the case-control samples has not yet revealed any association.

#156 Different cytokine responses after interaction between human macrophages and *Mycobacterium avium* subsp. *paratuberculosis* type II and type III strains

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Objective: The crucial process in the pathogenesis of paratuberculosis is the internalization of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by intestinal host macrophages, its survival and intracellular multiplication. The knowledge about these pathogenic mechanisms is still limited. Furthermore, despite similarities of paratuberculosis to Crohn`s disease (CD) concerning their histopathology and clinical symptoms, the putative pathogenic role of MAP in CD is unknown. The objective of the current study was to investigate differences in the cytokine responses of human monocytes after interaction with three MAP strains of different genotypes.

Materials and Methods: The human monocyte cell line THP-1 was incubated with two MAP strains of type II (reference strain ATCC19698, one bovine field isolate) and one MAP strain of type III (intermediate type) characterized by IS*900*-RFLP and MIRU-VNTR genotyping before. An *in vitro* model was established and the test conditions were standardized. As parameters for the determination of the impact of these strains on the macrophage responses, the production of mRNA of the cytokines TNF-alpha, IL-1, and IL-10 was determined by quantitative real-time PCR using GAPDH as housekeeping gene. The biological active proteins IL-1 and IL-10 were measured using ELISA, TNF-alpha by a cytotoxicity test.

Results: Differences in the expression of mRNA and the production of the proteins IL-1, IL-10 and TNF-alpha were detected after interaction of human monocytes with the three MAP-strains. These differences were determined between MAP type III and MAP type II as well as between the MAP type II reference and field strains.

Conclusion: It seems that MAP strains of diverse genotypes induce different responses of macrophages. Further investigations will show if there is a correlation of the cytokine responses of macrophages and the virulence properties of these specific MAP strains.

#157 Study of the role of CD40L expressed as adjuvant by recombinant BCG in the activation of immune responses against paratuberculosis

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CD40L, a co-stimulatory molecule preferentially expressed on activated $CD4^+$ T cells, is the ligand of CD40 on dendritic and antigen presenting cells. CD40-CD40L interaction induces the production of IL-12 and the initiation of a Th1-type immune response. Several studies show that CD40L is required for the activation of macrophages and the maturation of DCs. Moreover, CD40L enhances the capacity of $CD8^+$ T cells to produce IFN-γ and to lyse *Mycobacterium tuberculosis*-infected monocytes. In this study we attempt to improve existing Map vaccines with a recombinant BCG expressing CD40L.

We prepared the recombinant BCG strain expressing CD40L (rBCG2) by electroporation of BCG with a pGFM11/Ag85B signal sequence/CD40L extra-cellular domain construct, and another BCG recombinant strain (rBCG1) with the empty pGFM11 vector as a control. The expression of CD40L has been evaluated by Western blot. BALB/c mice were vaccinated with the live recombinant BCG vaccines. BCG persistence *in vivo* was determined by counting viable bacteria (CFU) in spleen and lungs. The immune response was evaluated by measuring Th1 type cytokine secretion (IFN-g, IL-2) of splenocytes after *in vitro* restimulation with selected immunodominant antigens and peptides. Two months post vaccination, mice were challenged with Map and protection was evaluated by Map RLU measurement on spleen and liver.

Preliminary results show normal persistence of the two recombinant BCGs. Analysis of the immune response shows an effect of CD40L 2 weeks after vaccination but not at 4 and 8 weeks. rBCG2 seems to be more protective against paratuberculosis than rBCG1. Another vaccination experiment is in progress to confirm these results. The effects of BCG-CD40L on cultured DCs *in vitro* will further be explored.

#159 Experimental infection in adult sheep and lambs with *Mycobacterium avium* subsp *paratuberculosis* (Map) at two different doses

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Objective: To establish the effect of age and doses of Map upon susceptibility to Johne´s disease in ovine.

Materials and Methods: Sixteen 1,5-month-old lambs out of 24 and 22 adult sheep (over two years old) out of 28 were experimentally challenged orally and both divided into two groups infected: a) with a high dose ($1.1x10^{10}$ CFU), b) with a lower dose ($1.1x10^{3}$ CFU) of an ovine field strain of Map. The remaining animals were used as unchallenged control groups.

Peripheral cellular and humoral immune responses were assessed as well as Map fecal shedding between 0 and 210 dpi. Sixteen challenged and five control animals were slaughtered at 120 dpi, and the remaining at 210dpi. Histological and bacteriological studies were conducted in samples of intestine and related lymphoid tissue (Peyer´s patches and lymph nodes). Animals were classified according to their lesions and granulomas were counted in 3 tissue sections from each sample.

Results: No gross lesions were observed in any of the animals. Only the high dose-challenged groups showed histological lesions associated with paratuberculosis (92.3% of the adult sheep and 100% of the lambs). However, those consisted of few small demarcated focal granulomas restricted to the lymphoid tissue (mostly Peyer´s patches) in the adults, whereas these were more numerous, larger and spreading to the lamina propia in the lambs. The tissue and faeces culture were positive in 5 high dose-challenged lambs. Cellular immune responses appeared earlier in the adult sheep than in lambs.

Conclusions: Adult sheep can be infected, as seen by the development of characteristic lesions; however, they seem to be able to control the progression of the disease. The dose of Map plays an important role in the establishment of the infection both in adult sheep and lambs.

#163 Immunohistochemical expression of iNOS in different types or paratuberculosis granulomatous lesions

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Objective: To assess the immunohistochemical expression of the “inducible nitric oxide synthase” (iNOS), an enzyme which catalyzes the synthesis of NO -a radical produced by macrophages and toxic for intracellular bacteria-, in different types of lesions associated with Map infection.

Materials and Methods: iNOS expression was evaluated in 69 samples of intestine (ileum and jejunum with and without lymphoid tissue, and ileocaecal valve) and lymph nodes, from natural an experimental cases of ovine and bovine paratuberculosis. Lesions were categorized as focal (located in the intestinal lymphoid tissue or lymph nodes,with none of scant bacteria), multifocal (focal granulomas in the lamina propria) and diffuse with abundant mycobacteria (multibacillary) or scant (paucibacillary). Immunohistochemical staining was performed using a polyclonal antibody raised against iNOS (Upstate) and intensity subjectively scored and compared with the presence of Map in the lesions, demonstrated by Ziehl-Neelsen.

Results: The most common pattern of staining observed, both in bovine and ovine samples and regardless the tissue sample, consisted of a marked immunolabelling of macrophages and giant cells forming the granulomas found in focal and multifocal lesions, associated with small amounts of bacteria, whereas in diffuse multibacillary lesions the staining was weak of negative.

Conclusions: These results suggest that iNOS may play an important role in the pathogenesis of paratuberculosis. The expression of high levels of iNOS in lesions with small amounts of mycobacteria, would suggest a higher ability of macrophages to control Map multiplication.

#171 Phagocytosis of *M. paratuberculosis* by human monocytic THP-1 cells

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Thus far conflicting experimental data originating from different experimental approaches to prove or to disprove the existence of a causative link between *M. paratuberculosis* (MAP) and Crohn’s disease in humans, have led to a lack of clarity on the role of MAP in the etiology of Crohn’s disease. To be able to assess the possible causal role of MAP a better understanding of the interaction between MAP and the human host will be essential. Whereas the interaction of M. tuberculosis and *M. bovis*, both proliferating in macrophages, with their respective hosts have been well studied, the interaction of MAP with human macrophages is less well characterized. In this present study this interaction is studied by comparing the interaction of *M. bovis* and MAP with the human macrophage using *in vitro* infection by both strains of the human monocytic cell line THP-1. Within a 96-hour time frame this infection is monitored using in parallel immunofluorescent microscopy, electronmicroscopy as well as microarray-assisted mRNA profiling of the host. The obtained data showed that in contrast to the less efficient phagocytosis of MAP, the infection by MAP elicited a much stronger and more complex host response than infection by *M. bovis*. Furthermore, whereas *M. bovis* was able to proliferate in the human macrophages MAP only showed a limited proliferation and was more susceptible to degradation.

These results indicate that MAP shows a human macrophage interaction remarkably different from *M. bovis* and support the need for further study of the interaction of MAP with the (human) macrophage.

#175 Differential activation of γδ+ and CD8+ T cells in the early innate immune response to MAP infection

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We used carboxyfluorescein succinimidyl ester (CFSE) labeling of bovine PBMCs isolated from naïve animals, antibody staining of distinct T cell subsets and 2-color FACS to show suppression of innate proliferative responses of gd^+ and $CD8^+$ T cell subsets after exposure to bovine monocytes that have taken up *M. avium* ssp. *paratuberculosis*. Consistent with differential activation, the CD8 T cell population showed little IFN-g production when compared with cells exposed to monocytes that had taken up *M. avium* ssp. avium. The $CD4^+$ subset remained unchanged after exposure to either subspecies. Results were similar when comparing T cell responses to infected allogeneic or syngeneic (autologous) monocytes. We have further shown that when taken up in bovine monocytes, *M. avium* ssp. *paratuberculosis* suppressed mRNA(s) encoding select signaling leukocyte activation molecules (SLAM) and confirmed by RT-PCR altered expression of mRNA encoding SLAM F5, F7 and F8. SLAM proteins serve as TCR co-receptors that can modulate the production of IFN-g in T cells. All nine receptors in the SLAM family (SLAM F1-9) are differentially expressed on hematopoietic cells. In T cell activation, SLAM cross-linking with its homotypic ligand on the surface of an infected cell will induce interaction of SAP with its cytoplasmic tail and induce IFN-g and IL-2. Increased macrophage SLAMF5 has previously been associated with increased T cell proliferation and secretion of IFN-g; and higher levels of SLAMF7 with increased killing by natural killer (NK) cells and increased proliferation of B cells. There is less known of effecter functions in cells where SLAMF8 is increased. The apparent suppression of SLAM molecules when comparing virulent *M. avium* ssp. *paratuberculosis* infection with that of avirulent *M. avium* ssp. avium is consistent with suppressed T cell activation and provides one potential mechanism for *M. avium* ssp. *paratuberculosis* to avoid or dampen a robust early innate response.

#184 Development of an experimental infection protocol in cattle using a low passage cultured strain of MAP

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Experimental infection of animals permits the study of disease pathogenesis over time. However, many cattle experimental infections use gut homogenates or large doses of an unknown passage strain of MAP. Though ensuring infection, these can have very different disease progression and clinical outcomes compared to natural infection.

A pilot study was performed to assess the efficacy of a low passage laboratory seed stock culture (CM00/416) of a MAP field isolate to infect calves. Highly passaged cultured strains of MAP can lose virulence resulting in low numbers of infected and clinically diseased animals.

Calves were sourced from a dairy farm confirmed to be free of Johne's disease in an area of Australia with low disease prevalence in cattle. Three doses of MAP were given over 4 weeks with a total dose of 4.8 x 10E8 viable bacteria. Calves were necropsied 2-3months post-infection and infection status was assessed by faecal/tissue culture, Mptb DNA detection in the gut tissue and associated lymph nodes, serum ELISA and histology.

Tissue culture identified MAP in the gut and lymph nodes of infected calves 2 months post-infection. Quantitative PCR (QPCR) to detect IS*900* MAP DNA was performed. This QPCR assay was designed such that it does not detect environmental mycobacteria and has a high analytical sensitivity (0.001pg of MAP genomic DNA). Mptb DNA was detectable in gut and lymph nodes of exposed calves 2-3 months post-infection. No visible macroscopic or microscopic lesions were found.

This experimental model was designed to be a reproducible and standardizable technique for inducing infection, with advantages over high dose regimes and gut or faecal homogenates that contain variable numbers of MAP and contaminants. Confirmation of infection with this low passage cattle strain via QPCR and tissue culture has enabled the initiation of a large scale experimental infection trial using this method.

#193 Development of an experimental infection model for Johne's disease using a lyophilised pure culture of *Mycobacterium avium* subspecies *paratuberculosis*

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In the first study of its type, a standardised experimental model for Johne's disease was developed utilising a low passage pure culture seed stock of *Mycobacterium avium* subspecies *paratuberculosis* (M.ptb). Repeatable infections of known duration are required for comparison and validation of new diagnostic tests and vaccines. Experimental inoculations of sheep resulted in repeatable infection rates across multiple trials, modulated by the interval between inoculation and examination. The clinical disease outcome from the pure laboratory culture was manifested later than that obtained using a gut homogenate from a naturally affected sheep but other measures of infection were similar. Immunological diagnostic assays showed most of the animals were IFN-g positive in the early stages of the infection. An increasing number of sheep became M.ptb-specific antibody positive over time and shed M.ptb in their faeces. The consistency of this experimental infection model will enable study of pathological, bacteriological and immunological responses for pathogenetic analyses, diagnostic test comparisons as well as assessment of vaccine efficacy.

#194 Expression profile of certain cytokine genes in tissues of two distinct classes (paucibacillary and multibacillary) of ovine paratuberculosis

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Expression of certain cytokines was quantified in tissues of paucibacillary (PB), multibacillary (MB) and uninfected control sheep by quantitative real-time-polymerase chain reaction (qRT-PCR) and analysed. In the small intestine, PB sheep showed significant enhancements in the expression of IL-10 (4 folds), TGF-b (4.7 folds), iNOS (3.8 folds), IFN-g (4 folds) and TRAF-1 (3.5 folds, non-significant) in comparison to similar tissues from uninfected control sheep. IL-1a (2.7 folds) expression was, however, significantly reduced ($P<0.01$). The expression of IL-10 (3.9 folds, $P=0.02$) in the mesenteric lymph node (MLN) tissue was significantly increased ($P<0.01$) as compared with the control sheep. MB sheep also revealed significantly enhanced expression of TGF-b mRNA (3.2 folds) and reduction in the expression of IL-1a (2.3 folds) in comparison to control sheep. The increased expression of TRAF-1 (3.1 folds), and decreased expression of IFN-g mRNA, (2.3 folds) in intestines of these sheep were statistically non-significant. In the MLN of MB sheep, the expression of IL-10 (4.5 folds) and TGF-b (1.9 folds) were significantly ($P<0.01$) greater, and IFN-g was significantly ($P<0.05$) down regulated (21 folds) in comparison to uninfected control sheep. When the cytokine expressions were compared between two distinctly infected groups, the MB sheep showed highly significant decrease ($P<0.01$) in the expressions of iNOS (4.7 folds) and IFN-g (9.3 folds) in small intestine and IFN-g (22.4 folds) in the MLN tissues.

It was concluded that IFN-g and iNOS were found to play important role in the induction of Th1 type immune response in paucibacillary sheep. Increased expression of IL-10, TGF-b and TRAF1 indicated their role in the inflammatory process, and progression of paratuberculosis in both the forms of disease. Decreased expression of IL-1a suggested that this cytokine might not play significant role in the early (PB) and advanced (MB) states of ovine paratuberculosis.

#199 Development of DNA constructs expressing *Mycobacterium avium* subsp. *paratuberculosis* proteins in mammalian cell line and study on their immunogenicity in murine model

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Several antigens of *Mycobacterium avium* subsp. *paratuberculosis* (Map) were studied as vaccine candidate and their immunogenicity has been evaluated. Previous studies in our laboratory revealed that 35 kDa protein (P35) expressed in prokaryotc expression vector could induce significant T cell immune response as well as secretion of Th1 associated cytokine interferon gamma (IFNγ). We have further cloned P35 (35 kDa), PE (10 kDa) and PPE (34.9 kDa) protein genes of Map alone and with murine IFNγ into the eukaryotic expression plasmid pIRES 6.1 and transfected in mammalian cell line which showed eukaryotic expression of recombinant proteins detected with hyperimmune sera raised against respective recombinant proteins in western blot and immunofluorescent assay. These monocistronic and bicistronic constructs were used as DNA vaccine in mice and their immunogenicity was studied by delayed type hypersensitvity (DTH), lymphocyte proliferation, nitric oxide (NO) determination and IFNγ assay. Significant DTH responses were evoked in mice immunized with bicistrionic constructs than the monocistrionic constructs. Also higher proliferation of the splenocytes and enhanced production of NO exposed to respective antigen was found in bicistronic groups. Significantly higher amount of IFNγ was also released in bicistronic groups. Flow cytometry analysis revealed higher $CD4^+$ and $CD8^+$ T cell response to the recombinant antigens. Studies also showed that co-expression of IFNγ with Map genes enhanced the immunogenicity. These results indicate the T cell epitopic nature of the antigens which could potentially be used in the development of effective DNA vaccine against paratuberculosis infection.

#208 MAP –Epithelial cross-talk induces phagosome acidification and Nod2 expression to Enlist IL-1β processing

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It is well recognized that the initial site of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection occurs at the epithelium; however, few studies have investigated this interaction. Prior to residing in the macrophage, MAP must first be processed by the host epithelium. Processing of MAP by the host epithelium involves a dynamic innate immune response initiated by MAP-Epithelial cross-talk, which may also be augmented by further cross-talk between host pathways as well as cell types (epithelial-macrophage). We hypothesize that chronic stimulation of *Nod2*, a pathogen recognition receptor, by MAP or its major cell wall components will result in prolonged inflammation that is sustained by toll-like receptors (TLRs) 2 and 4 in JD. We investigated Mac-T cell (bovine mammary epithelial cell line) responses to MAP strain K-10 (pWes4) and its cell wall components at 10-240 minutes. Data show early phagosome acidification by epithelial cells in response to MAP K-10 (pWes4). Phagosome acidification coincides with a strong host upregulation of IL-1β, which may serve as a chemoattractant for macrophages. The *Nod2*-caspase 1 pathway has previously been reported to be an essential signaling cascade required for IL-1β expression. Our findings indicate that MAP is capable of inducing *Nod2* expression with 240 min. *Nod2* expression appears to be preferentially upregulated by ManLAM. We propose that MAP allows phagosome-acidification in epithelial cells to induce IL-1β processing in order to cause host migration of macrophages. Thus, MAP guidance of phagosome-acidification and *Nod2* may enlist IL-1β processing in order for it to easily transverse through the epithelium and into its niche—the macrophage.

#209 Murine macrophages carrying an insertional mutation at residue 2939 of *Nod2* show a proinflammatory response to *Mycobacterium avium* subsp. *paratuberculosis* and its cell wall components

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Genetic linkage studies have identified the *Nod2* as a Crohn's Disease (CD) susceptibility locus of which $Nod2^{3020insC}$ is the most common mutation. Several studies suggest that $Nod2^{3020insC}$ may result in increased expression of proinflammatory cytokines and mediators of apoptosis in response to bacteria. We hypothesize that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) signals through $Nod2^{3020insC}$ to initiate an aberrant inflammatory response, which is prolonged and enhanced via cross-talk with Toll-like receptors (TLRs) 2 and 4. We investigated Nod2 intact and $Nod2^{2939insC}$, the murine equivalent of human $Nod2^{3020insC}$, macrophages in response to MAP (strain K-10) and MAP cell wall glycolipids at early time points (10 min., 30 min., 60 min., and 120 min.). Gene and cytokine expression profiles were determined using Quantitative Real-Time PCR (QT-RT-PCR) and ELISA, respectively. Transcriptional analyses show an 80,000-fold increase of *Nod2*, TLR4, and beta defensin-1 in K-10 stimulated $Nod2^{2939insC}$ macrophages. Nfkbib and Ticam2, which are involved in NF-κβ and TLR stimulation, were also elevated in MAP treatment at 2 hr. Previous studies have shown that optimization of beta-defensins rely on *Nod2* expression, which in turn are capable of acting as TLR4 ligands. Cytokine data show rapid and unique responses to K-10, mannosylated lipoarabinomannan (ManLAM) and lipomannan (LM). LM induced proinflammatory responses as shown by upregulation of IL-1α and IL-6, while ManLAM induces an IL-10 anti-inflammatory response at 2hr. These data suggest that initial signaling with MAP occurs via *Nod2*, which may act as a central signaling receptor that connects TLRs 2 and 4 in a sustained circuit. Establishment of a *Nod2* signaling circuit is the first step in elucidating a mechanistic role of MAP involvement in CD onset.

#215 Development of an antigen delivery system for elicitation of protective immune response against *Mycobacterium avium* subsp. *paratuberculosis* using attenuated Salmonella enterica serovar Typhimurium

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Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causing agent of Johne's disease in cattle and other ruminants has adversely affected the production and economy of dairy industry. As being intracellular pathogens similar to M. tuberculosis, MAP-infected host requires essentially T-cell immunity against MAP to eliminate its infection. Therefore to elicit T-cell immunity by a vaccine, the cytosolic antigen delivery is considered a prerequisite.

Methods: Attenuated *Salmonella* engineered to produce heterologous proteins present a versatile tool to deliver the targeted antigen(s) into cytosol of macrophage and into other antigen presenting cells using its type III secretion system. Hence we, by using homologues recombination method: λ phage mediated, have made deletion mutant of *aroA*, and *yeJ*, of serovar Typhimurium, Newport and Dublin. Further we have tested these strain to deliver MAP antigens Ag85A, Ag85B, SOD and 74F into culture medium via its type III secretion system. To evaluate delivery efficacy of MAP antigens by attenuated Salmonella, we have made several truncated constructs of Ag85A, Ag85B, SOD and 74F and fusion $Ag85A_C$-SOD^{1-72}-$Ag85B_C$ gene with sopE104 a N-terminal fragment *sopE* of *Salmonella* and *sopE* promoter and compared the delivery efficacy by attenuated *Salmonella* strains.

Results: Our primary data show that truncated fragments and fusion of two or there protective antigens; Ag85A, Ag85B, SOD and 74F were expressed well by Salmonella (delta aroA+yeJ) and its promoter, and secreted into culture medium via type III secretion system.

Conclusion: Attenuated *Salmonella* (delta aroA+yeJ) strains are efficient tool for targeted antigen delivery of MAP antigen to elicit T cell immunity. By using type III secretion system of *Salmonella* a T cell epitope or whole optimized antigen or many fused T-cell antigens can be delivered into cytosol to induce protective immunity. Thus *Salmonella* delivery system could be used to develop an effective vaccine against MAP .

#221 Comparative analysis of transcriptome changes in bovine macrophages infected with SuperShedding strains of *Mycobacterium avium* subspecies *paratuberculosis*

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Mycobacterium avium subspecies *paratuberculosis* is often spread between animals through ingestion of feces-contaminated food. This mode of contamination is of substantial concern when considering a subgroup of MAP called SuperShedders. Infection with these MAP strains can increase the fecal colony load of the animal over 1000 fold the levels observed in infection with other strains of MAP. Previous research in this area has shown that SuperShedders share many common transcriptome changes in infected macrophages when compared to the transcriptome of macrophages infected with other MAP strains. The objective of this research is to better understand what changes occur in macrophages infected with SuperShedders that leads to higher fecal colony loads. Through careful reexamination of previously collected microarray data on experimentally infected monocyte-derived macrophages, we found 78 bovine genes that were significantly differentially regulated in SuperShedder infected macrophages when compared to other MAP strains. DAVID software, a program designed to examine functional groupings, was used to study enrichment in several annotation categories. We found significant differences in lymphotoxin and glucose transport regulation. Several genes were selected from the DAVID results. These genes were tested for alternative expression via RT-qPCR validation of RNA extracted from monocyte-derived macrophages infected with 10 strains of MAP. Two strains used in this study are SuperShedders. Based on these results, we hypothesize that regulation of glucose transportation and lymphotoxin regulation play import roles in MAP proliferation and fecal colony load.

#224 Application of a rapid and sensitive combined phage-PCR method for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in raw milk

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ABSTRACT

The objective of this study was to evaluate a new combined phage-PCR method by investigating levels of MAP in milking herds in the UK and throughout the Republic of Cyprus. Bulk milk samples were collected from 54 UK farms and 225 dairy farms in Cyprus and were tested using both the phage assay and a conventional culture method for the presence of MAP. The identity of MAP cells detected was confirmed in both cases by IS900 PCR. None of the UK samples were culture positive whereas 1 was phage-PCR positive. In Cyprus MAP 50 of the 225 samples were MAP positive using the combined phage-PCR and MAP was cultured from 2 samples, despite the fact that the animals tested were not displaying clinical symptoms of Johne's disease. Total viable count was also determined as an indicator of the general hygiene status of the samples. Comparison of MAP status with TVC suggests that these were not introduced by faecal contamination. Typing of the two MAP strains isolated from cow's milk in Cyprus by culture was performed using REA IS1311 PCR and identified one of these to be an S strain and the other C strain. The results confirm that the phage-based detection test is more sensitive than conventional culture and demonstrate the efficacy and practicality of the phage-based test as a routine rapid method for the detection of viable MAP in milk.

INTRODUCTION

Detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk currently relies on culture, immunoassays and molecular techniques. The culture based techniques require a very long incubation period of about 3 months and a chemical decontamination step which reduces the number of competitive microbes, but also reduces the number of viable *Mycobacteria* present in a sample, limiting the sensitivity of growth-based assays. Immunoassays have a very low sensitivity in milk and the molecular techniques have the disadvantage of not being able to differentiate live from dead cells. A new combined phage-PCR assay for the detection of MAP in milk was described by Stanley et al. (2007) based on the commercially available *FASTplaque*TB™ assay for detecting tuberculosis in human sputum samples. This test detects the ability of a bacteriophage (D29) to infect MAP and provided successful identification of viable cells in milk in less than 24 hours. The advantage here is that only viable cells will support phage replication, providing live-dead differentiation, no chemical decontamination of samples is required and large volumes (up to 50 ml) can be sampled. Genotyping is then performed by amplification of IS900 sequences from in the cells detected directly from the bacteriophage plaques (see Rees and Dodd, 2007). The aim of the work presented here was to apply the new method on real samples to assess the potential application of the test for the detection of MAP in milk and also compare the results with conventional culture for MAP. A viable count was also performed on the samples to investigate any potential relationship between the presence of MAP in milk with TVC which was used as a general indicator of the overall hygienic status of the samples.

MATERIALS AND METHODS

Sampling

For the UK samples 54 BTM samples were provided from the VLA in Sutton Bonington, UK. For the Cyprus survey samples were collected from from each of 225 registered dairy farms in Cyprus.

Combined phage PCR assay

BMT samples were processed by a modified version of the method described by Stanley e*t al.* (2007). In the assay bacteria in the milk samples are collected by centrifugation and the cell pellets resuspended in 2 ml of Media Plus. Here a second wash step was included using the same centrifugation conditions and then finally the pellet was resuspended in 1 ml Media Plus.

Culture examination and identification of mycobacterial isolates

Samples (30 ml) of BTM were decontaminated for 5 h with 0.75% hexadecyl-pyridinium chloride (HPC; Merck KGaA, Germany) and three samples were cultured on Herrold's Egg Yolk Media (HEYM) containing 2 µg ml^{-1} of Mycobactin J; two slopes were purchased from Becton Dickinson (New Jersey, United States) and one slope was prepared as described previously (Ayele et al., 2005). Slopes were monitored after the first week of incubation to identify either contaminated cultures or those with fast-growing mycobacteria and then observed at two week intervals until there was visible colony growth: incubation was carried out for not less than eight months at 37°C. From all primary cultures, presumptive colonies were stained using Ziehl-Neelsen (Z-N) for the presence of acid-fast bacilli (AFB). AFB isolates were identified as MAP by PCR as described previously (IS*900*, Whittington *et al*., 1998 and *F57*, Coetsier et al., 2000). IS*1311* REA-PCR was used to distinguishing between cattle and sheep strains of *MAP* with PCR products being cleaved with *Hinf*I (Marsh et al. 1999).

Total viable count (TVC)

TVC was performed on Milk Count Agar (MCA) using standard methods. Milk was diluted in MRD (Maximal Recovery Dilluent) and the dilutions were plated on MCA and incubated aerobically at 30 °C for 3 days.

RESULTS AND DISCUSSION

The results from the two surveys are presented in Table 1. From a total of 54 samples analyzed in the UK survey 19 (35.2%) were positive using the phage assay which indicates the presence of *Mycobacteria* spp, but one of these samples (1.9%) was identified as MAP following PCR genotyping. A duplicate of the samples collected was cultured by the VLA and none of the samples were positive for MAP. A recent UK survey reported that 75% of dairy herds were sero-positive but seroprevalance was only 0.12 and therefore levels of MAP expected in BTM would be low (Woodbine et al., 2009). From a total of 225 samples analysed for the Cyprus survey 218 (96.9%) were *Mycobacteria*-positive using the phage assay and 50 of these (22.2%; 95% CI: 17.1% - 28.0%) were identified as MAP by IS900 PCR. In this case only two of the samples (0.9%) were culture positive. From the 225 cows' BMT samples that were collected during this survey 50 (22.2%; 95% CI: 17.1% - 28.0%) were found to be MAP positive using the combined phage-PCR assay. The results of Slana et al. (2009) reported that 28.6% (95% CI: 95% CI: 22.5% – 34.3%) were MAP positive by IS900 qPCR, showing a good agreement with the level of prevalence recorded in the two different surveys undertaken.

All BTM samples collected in the Cyprus were also cultured after decontamination and only two of them (0.9%) produced colonies on HEYM slopes that were identified as MAP-positive by both IS900 and F57 qPCR. Both of the samples from which viable MAP were cultured were also phage-PCR positive. The two confirmed MAP isolates were analysed using IS1311 REA PCR and one of these was identified as a MAP cattle strain and the other as a sheep strain.

Table 1 UK and Cyprus MAP survey Results

Survey	Total Samples	Phage Assay			Phage - PCR			Culture		
		No.	Pos.	%	No.	Pos.	%	No.	Pos.	%
UK	54	54	19	35.2	54	1	1.9	54	0	0
Cyprus	225	225	218	96.9	225	50	22.2	225	2	0.9

The TVC results (Table 2) are separated into groups according to microbial load. No relationship was seen between the TVC and samples being phage-PCR MAP positive. This confirms that the MAP were not being introduced into the milk by faecal contamination and that in the Cypriot study we can deduce that MAP is being shed into the milk by animals that do not display any clinical symptoms of the disease (clinical herd data was not available for the UK samples). Characterization of the 2 culture isolates identified one as an S strain and the other as a C strain. The presence of the S strain in the cattle herd population is interesting from an epidemiological perspective since the presence of this strain has also been found in the Cypriot sheep and goat populations (Liapi et al., 2009). The extended culture times required to isolate this organism may explain why it was not previously cultured from bovine milk samples.

Table 2 MAP and Viable Count Results

Survey	Total Viable Count Data			Phage Positive samples			IS900-PCR positive samples		
	⩽ Log4 cfu/ml	Log4-Log5 cfu/ml	>Log5.01 cfu/ml	⩽ Log4 cfu/ml	Log4-Log5 cfu/ml	>Log5.01 cfu/ml	⩽ Log4 cfu/ml	Log4-Log5 cfu/ml	>Log5.01 cfu/ml
UK	21	7	26	15	2	2	1	-	-
Cyprus	105	95	25	102	93	23	25	21	4

Prospective analysis of the phage-PCR data revealed a strong relationship between the pfu number and the probability of that sample being shown to contain MAP by PCR genotyping. Figure 1 shows the distribution of the pfu count of the MAP IS900 positive and negative samples. An area of overlap exists between 15 pfu/50 ml (PCR-positive sample with the lowest plaque count) and 75 pfu/50 ml (highest PCR-negative sample). However all samples with >75 plaques were found to contain MAP. The *FASTplaque*TB assay gives a cutoff value of 20 pfu per sputum sample. If a cutoff is applied in the overlap region to this data, a test with a high Sensitivity (Sn, 98%) but a lower Specificity (Sp, 63.4%) was gained.

Fig.1: Relationship between plaque number and IS900 PCR results

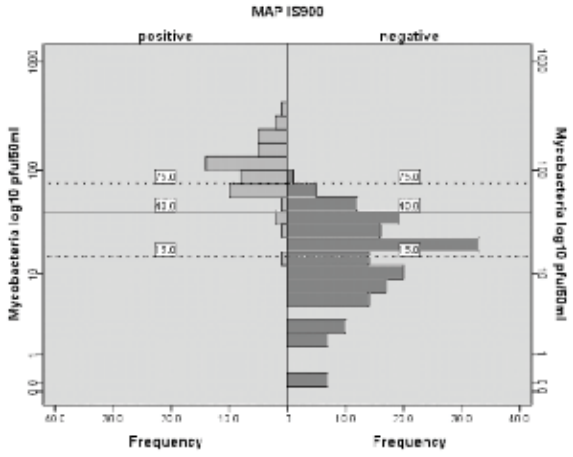
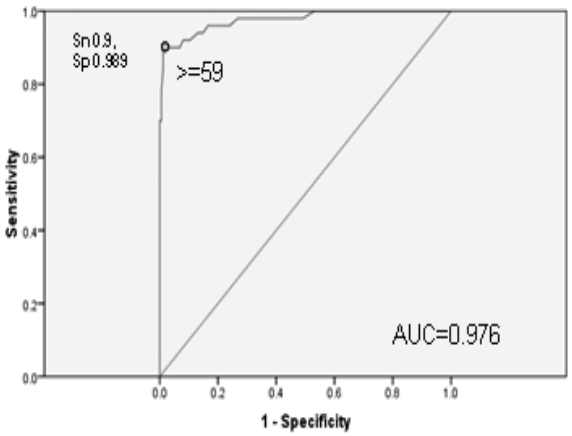


Fig. 2: ROC analysis of plaque cutoff



To better determine the most appropriate cutoff value Receiver Operating characteristic (ROC) analysis was used (Figure 2) and showed that a cutoff off 59 pfu/50 ml gives Sn and Sp values of 90% and 99%, respectively. This cutoff value can be refined as more data is generated, but shows the clear relationship between the number of plaques and presence of MAP in a sample. As high levels of Mycobacteria should not be present in hygienically collected milk, this relationship is to be expected if infected animals are shedding MAP. Hence in this case plaque numbers alone can be used to indicate MAP infection without the need for PCR confirmation.

The Cyprus cattle population is free from bovine tuberculosis however, since any Mycobacteria shed into milk will result in a plaque, if this were to be repeated in a country with endemic levels of TB, the plaque results alone could not be used as an indication of MAP infection. For instance in this work 26 other isolates were recovered on culture and identified as *Mycobacteria* spp. by Z-N stain and species-specific PCR (Tevere et al., 1996) and these cells would have given rise to IS900 PCR-negative plaques. While the relationship between infection and plaque number is expected to be maintained irrespective of the identity of the *Mycobacterium* being shed into milk, a multiplex PCR genotyping assay would then be required to confirm the identity of the organism being detected (see Stanley et al., 2007).

CONCLUSION

The combined phage PCR assay was successfully applied to real BTM samples and shown to be able to sensitively and specifically detect MAP within 24 h in a population that is free from TB.

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#228 *Mycobacterium avium* ssp. *paratuberculosis* infection causes change in the epithelial cells permeability: correlation with the cell adhesion and tight junction molecule expression

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Epithelial cells constitute the first mechanical and immunological barrier against infection of the *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Earlier, we found that cell-adhesion molecules were one of the top-scoring pathways affected by MAP infection during bovine ligated-ileal-loop experiment.

Objective: The objective for this study was to distinguish the role of epithelial cells during MAP interaction, and correlate it with cell-adhesion molecule gene expression and alteration in the tight junction and permeability of infected cells.

Materials and Methods: The T84, a human cell line of colon carcinoma cells were polarized after seeding the cells on the apical compartment of Transwell plates and adding medium to the basolateral compartment. During 7-10 days, the cells develop a transepithelial resistance (TER) that allows them to mimic the conditions of the intestinal epithelium. A method for attaining the epithelial permeability *in vitro* was achieved by the measurement of electrical physical resistance to determine the TER. These cells developed a transepithelial resistance of 500-1500W/cm2. MAP were added at a multiplicity of infection ratio of 1:10 (cells to bacteria ratio). The TER of Polarized T84 cells was measured at various times post-infection. The cells were also processed to obtain RNA for the gene expression.

Results: The results show that MAP infection caused a decrease in the TER, thus increased the permeability of the epithelial cells for bacterial invasion. The polarity of the epithelial cells was disturbed due to alteration in the tight junction. Various cell adhesion molecules (Vascular, Intracellular, Neuronal and Junctional), that play important role in the maintenance of the cellular architecture, showed a differential gene expression.

Conclusion: In conclusion, this study show that MAP infection to epithelial cells causes increased permeability by weakening of the tight junctions. Permeability of epithelial cells is also correlated with the changes in the gene expression of cell adhesion molecules.

Epidemiology

Convenors: Søren S. Nielsen and Ian A. Gardner

#145 MERKAL AWARD LECTURE

Multinomial regression analysis of individual host factors and paratuberculosis test results

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Objective: Individual factors and paratuberculosis test results of infected cattle were integrated in a multinomial regression analysis to detect the most powerful animal influences on fecal and serological test results.

Materials and Methods: 1.313 animals from 2 dairy cattle herds (A and B) were examined for fecal shedding of MAP by cultivation and confirmation of MAP by IS*900*-PCR as described before*. Culture results of infected animals were assigned to 3 groups: high, medium-low and low-no shedding. Blood and milk samples were tested with 2 paratuberculosis-ELISA-tests (nonabsorbed/LAM ELISA 1; absorbed ELISA 2). For the statistical analysis with the software R (Version 2.4.0; http://www.r-project.org) test results and host factors were grouped in classes. Reference groups were selected randomly for the multinomial regression analysis.

Results: Significant animal influences on the detection of high shedding were not established. The highest probability for medium-low shedding occurred during the 3rd lactation stage (211-315d). The most powerful influences for the detection of positive ELISA 1 blood or milk test results were animal age and a high intensity of shedding. A significantly higher chance for a positive ELISA 1 milk test result was detected in herd B. High daily milk yields (> 45 kg) were negatively associated with positive ELISA 1 milk test results. The occurrence of positive ELISA 2 blood and milk test results increased significantly when medium or high fecal shedding was detected simultaneously. ELISA 2 milk test results were less often positive when milk yields > 35 kg were recorded.

Conclusion: High shedders can be detected irrespective of the examined individual factors. There is a high probability to detect medium-low fecal shedders during the 3rd lactation stage. Paratuberculosis-ELISA-test results were modified by individual host factors. Particularly the negative association of high milk yields and milk ELISA-test results should be taken into account when milk ELISA-tests are used for herd examinations.

* Gierke, F., M. Ziller, H. Köhler (2007): Can the detection rate of faecal shedders of MAP be increased by optimising the time point of faecal sampling? Proc. 9ICP, Tsukuba, Japan, 107-110

#149 A meta-analysis of the effect of dose and age-at-exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows

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Calves artificially challenged with high doses of MAP bacteria have been reported to become infectious more quickly relative to low-dose challenge infections. However, the relationship between dose of challenge and speed of this progression has never been fully quantified. In ordinary differential equation (ODE)-based mathematical models of infectious dynamics within herds, it is common to assume a constant rate of exit from each stage (compartment) of the infection process. Studies of properties of exit rate distributions indicated that the nature and heterogeneity of infection progression are highly influential on infection dynamics and may impact the efficacy of intervention strategies being evaluated.

In this research, a meta-analysis was performed using all published and one unpublished long-term infection-challenge experiments to quantify the age- and dose-dependence of early and late shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Studies were included if sufficient experimental details and sampling frequency were reported, resulting in 197 animals from 17 studies of which 176 were infected with a known dose and 21 were exposed naturally. Parametric time-to-event models indicated that challenging calves at a younger age and with a higher challenge dose resulted in faster transitions into shedding periods and longer durations of shedding. Experimentally infected calves shed during two distinct phases, with an Early Shedding stage within 12 months of infection, a Carrier period in which no bacteria can be cultured and a subsequent Late Shedding stage. In the current analysis, the possibility that animals do not experience Early Shedding but enter an Incubation period before Late Shedding is considered. The log-normal distribution was the most appropriate underlying distribution for infection-progression events. Calves that were exposed naturally showed variable progression rates, but not dissimilar to other infection routes.

We propose that the observed infection patterns allow better understanding of low-grade endemicity of MAP in cattle. This work resulted in transition parameter estimates for future transmission dynamic modelling.

#50

Assessment of the age at occurrence of MAP infection, adjusting for the latent infection period, in Danish dairy cattle

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ABSTRACT

Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection is considerably delayed due to the long latent infection period. We developed and applied a Bayesian Weibull regression model to make inferences about the risk of MAP infection from the analysis of data from tests that detected infection with delay. Milk and fecal samples were collected (11 and 4 times/year, respectively) for 1912 cows from 8 naturally infected Danish dairy herds and tested by an indirect milk ELISA and LJ or HEYM, respectively. We used (I) a standard Weibull survival model to assess the association between age at milk seroconversion and MAP shedding and (II) the proposed model, which adjusted for the latent period of MAP infection, to assess the association between age at induction of MAP infection and MAP shedding. Estimated median infection times under (II) revealed that animals getting infected earlier in their lives were more likely to become MAP shedders, while there was no difference between shedding levels. Importantly, the proposed model captured the inverse relationship between the incidence rate of seroconversion and that of infection with time. The observed incidence rate of milk seroconversion increases, while that of infection decreases with age. Young calves are more susceptible to MAP infection and susceptibility to infection decreases with time. Ignoring the latent infection period severely affects survival estimates; therefore, the proposed model can be particularly useful in the case of chronic infections, like paratuberculosis, that have a long latent infection period.

INTRODUCTION

Standard diagnostics for *Mycobacterium avium subspecies paratuberculosis* (MAP) infection – although highly specific – lack sensitivity, hampering control of paratuberculosis in domestic ruminants. In the absence of sensitive tests, detection of MAP infection is delayed depending on the duration of the latent infection period. To infer the time-to-infection based on time-to-detection of infection data, survival analysis models should adjust for the latent infection period.

Few studies considering survival analysis models adjust for the measurement error on survival times and most of them concern retrospectively collected socioeconomic data (e.g. unemployment time), where respondents round up or off their recall times ("heaping"). These models, however, mainly consider errors that are symmetrically distributed around zero (Augustin and Wolff, 2004), which are, therefore, not applicable to infectious diseases because detection of infection is systematically delayed with non-zero mean. Other models considering multiplicative errors (Skinner and Humphreys, 1999) would not be appropriate because the latent infection period is not expected to be proportional to the age at infection. For example, a multiplicative error model would assume that a cow infected with MAP at the age of three would have a latent infection period three times that for a one year old cow.

We developed and applied a Bayesian Weibull survival model that adjusted for the latent period of MAP infection. We initially analyzed the data (I) ignoring the latent period to assess the association between age at milk seroconversion and shedding level.

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Subsequently, (II) under the proposed model, we assessed the association between time to MAP infection and fecal shedding status. The risk change over time for seroconversion or acquiring MAP infection was also estimated under I and II, respectively.

MATERIALS AND METHODS

The model

We assumed that the unobserved time to infection (tr_i) is Weibull distributed:

$$f(tr_i) = \lambda_i \times \rho \times tr_i^{\rho-1} \times e^{-\lambda_i * tr_i^{\rho}}$$

,where $\lambda_i = e^{b*z_i}$, $\boldsymbol{z_i}$ the covariate vector for the i^{th} individual, $\boldsymbol{b}$ a vector of unknown regression coefficients and *ñ* the shape parameter of the Weibull distribution. The unobserved tr_i was modeled as a function of the observed time to detection of infection (t_i) and the latent infection period (u_i):

$$tr_i = t_i - u_i$$

With appropriate prior information on u_i the model is fully specified. Extended model description with step by step explanation of the corresponding WinBUGS code is available upon request from the corresponding author.

Data

Data were collected from February 2000 to March 2003 from 1912 cows, in 8 Danish dairy herds. Briefly, milk samples were obtained 11 times/year from all lactating cows and tested by an indirect milk ELISA. Fecal samples were collected from all lactating and non-lactating cows 4 times/year and were cultured on LJ or HEYM. Fecal culture (FC) positive cows were divided in two shedding groups: FC_{High} if positive with high bacterial counts (>10 cfu/g) or many consecutive FC-positive tests and FC_{Low} otherwise. Animals not shedding or shedding undetectable levels of MAP were classified as FC_{Neg}. An animal contributed a censored observation if it was still milk-ELISA negative when it exited the study for a reason not associated with MAP infection.

Priors

For the proposed model we had to specify prior information about u_i from the induction of MAP infection to milk seroconversion. For naturally infected dairy cattle, we chose a prior value of u_i equal to 1300 days (3.5 years) with a 95% confidence interval extending from 1000 (2.7 years) to 1600 (4.4 years) days: That is a normal *N*~(1300, 4.4 x 10^{-5}).

RESULTS

Estimated model parameters under (I) the standard and (II) the proposed Weibull model are given in Table 1. The shape of the Weibull hazard – as expressed by *ñ* – and the baseline hazard – expressed by b_0 – differed between models I and II. Median time to milk seroconversion was 2638 [95%Credible intervals (CrIs): 2542; 2743], 1880 (1758; 2016) and 1722 (1594; 1869) for FC_{Neg}, FC_{Low} and FC_{High} cows, respectively. The corresponding median time to MAP infection was 1726 (1420; 2160), 356 (255; 502) and 227 (153; 339) days. The risk of MAP infection decreases with time (*ñ*=0.56 (0.51; 0.61) <1 under II), while the risk of milk seroconversion increases (*ñ*=2.67 (2.52; 2.83) >1 under I). This decrease and increase, respectively, is significantly steeper for shedders (heavy or low) than cows not shedding or shedding undetectable levels of MAP (see Figure 1).

Table 1. Posterior median (CrIs) of model parameters from (I) a standard Weibull model that ignored the latent infection period (u_i) of *Mycobacterium avium subsp. paratuberculosis* (MAP) infection and (II) the proposed Weibull model that adjusted for u_i. Estimations were based on 1912 cows from 8 Danish dairy herds naturally infected with MAP.

Variable	Level	Median (CrIs)	
		Model (I)	Model (II)
Intercept		-21.42 (-22.67; -20.28)	- 4.53 (-4.89; -4.19)
MAP shedding level	FC_{Low}	0.91 (0.70; 1.11)	0.88 (0.67; 1.09)
	FC_{High}	1.14 (0.90; 1.36)	1.13 (0.89; 1.37)
	FC_{Neg}	Ref.	Ref.
Shape parameter	ρ	2.67 (2.52; 2.83)	0.56 (0.51; 0.61)

1a

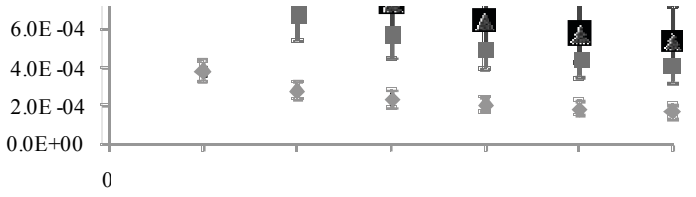

Figure 1. Posterior median (CrIs) of the hazard function (HF) – the age-specific seroconversion rate (1a) and infection rate (1b) – for heavy-, low- and non-shedders of MAP.

DISCUSSION

The proposed model allowed us to assess association between time to infection and shedding level by the analysis of available time-to-seropositivity data. Estimated median infection times demonstrated that animals getting infected earlier in their lives were more likely to become shedders in the future, further contributing to the vicious cycle of MAP

persistence in an infected herd. The latter findings are consistent with the well established need for control strategies that will, among other things, prioritize the interruption of MAP transmission during early calfhood.

Importantly, we captured the inverse relationship between the incidence rate of seroconversion and infection with time: the incidence rate of milk seroconversion increases, while that of infection decreases with age (Figure 1). Young calves are more susceptible to MAP infection and susceptibility to infection decreases with aging (Taylor, 1953), while high-parity dairy cattle are more likely to become seropositive than low-parity dairy cattle (Nielsen et al., 2002).

Our model heavily depends on correct prior specification for u_i. We, therefore, had to base our priors on adequate, relevant and scientifically justified information. Within an infected herd most animals acquire MAP early in their life. In natural infections, the immunological and infectious properties of MAP infection are not fully characterized. Susceptibility to infection decreases over time, while environmental (Tiwari et al., 2009) and genetic (Koets et al., 2000) factors, not fully conceptualized yet, play a critical role on whether initial entrance and persistence of MAP will lead to clinical manifestations, be restrained during the productive life of infected animals or even be cleared out. Most clinical cases occur between 3 to 5 years (Chiodini et al., 1984). In experimental infections, the latent period is generally shorter (Lepper et al., 1989) but can only be indicative for natural infections because of the variation in infective doses occurring during natural infections (Nielsen and Ersbøll, 2006). Specified priors captured both our prior belief and the variation of latent period occurring at natural infections.

The proposed model adjusts for the latent period of infection and can be quite useful for the analysis of chronic infections – like MAP – with a long latent period, which, if ignored, will have a severe impact on estimations.

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#55 Birth clusters of animals infected with *Mycobacterium avium* subspecies *paratuberculosis* in a New York State dairy herd

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ABSTRACT

In this longitudinal study on an eight year database of a New York dairy farm, we attempted to evaluate whether cows who eventually become *Map* shedders, were more likely born in clusters of shedders. The data set used in this study contains data of 2788 cows from one dairy farm during an intensive individual animal diagnostic testing period starting in 1995 and lasting until 2002. During this period a Johne's Control program was implemented on the farm. The SaTScan™ v 7.01 software was used to make temporary scans for the birth clusters in this herd. SaTScan™ is a software that analyzes spatial, temporal and space-time data using the spatial, temporal, or space-time scan statistics This program was designed to use windows of different size to identify statistically significant clusters of events ($p<0.05$). In this study, windows of 4, 10, 60, 90 and 120 days were used to search for birth clusters. Birth cohort clusters in a window of 10 days were the most frequent observed clusters before the implementation of the Johne's Control Program. Management changes implemented through the Johne's Control Program resulted not only in a decrease of prevalence and incidence at this farm, but also in a total elimination of birth clusters. These data indicate that birth clusters of *Map* infection are an important component of maintaining endemic infection levels of dairy farms.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*, epidemiology, longitudinal study.

INTRODUCTION

Johne's disease or paratuberculosis is an infectious disease in cows, caused by *Mycobacterium avium* subspecies *paratuberculosis (MAP)*. After an incubation period of 1.5 years to more than 5 years it can cause severe intestinal inflammation. Before cows will get clinically ill they can contaminate the environment and infect other cows in the herd with *Map* by fecal shedding. Shedding level differs from low (<10 colony forming units, CFU, per tube) to moderate (10-50 CFU/tube) and high (>50 CFU/tube).[3] (Whitlock and Buergelt 1996). Previous studies on *MAP* transmission assumed that transmission occurs from environmental contamination to susceptible animals, from cow to cow and from dam to calf (Benedictus et al, 2007). Johne's disease eradication programs based on these transmission routes were not successful in eliminating Johne's disease from a herd without culling the whole herd. This suggests that there are still unknown transmission routes for *Map* in Dairy herds.

Recent studies showed that infected calves may be infectious to their peers in birth cohorts (Van Roermund et al., 2007, Benedictus et al. 2007). These calves would be shedding infectious organisms and because of their close contact with susceptible individuals, transmission of infection would occur. A hallmark of such calf-to-calf transmissions would be the presence of clusters of infected animals when sorted by day of birth. Several types of these birth clusters may exist. The first type is a birth cluster of future shedding cows with mainly positive dams. This type of cluster confirms the hypothesis from previous studies that the chance to get infected with *Map* is higher for cows with a Johne's positive dam than for cows with a Johne's negative dam. The clustering can be seen as coincidence in time. The second cluster type is a birth cluster of future shedding cows with just one or a few positive dams. Infection of these

animals may have occurred peri-parturient (sharing maternity pen facilities, colostrums sharing) or from calf to calf in the first months of life. Infection would be transmitted by calves that were infected by their dam in the first days. The third type is a birth cluster of future shedding cows without any positive dams being present at the time the birth cluster occurred. Finding such clusters suggests a contaminated calving area or calf to calf transmission in the first days, weeks or even months of their lives.

In this longitudinal study on an eight year database of a New York dairy farm, we attempted to evaluate whether cows who eventually become *Map* shedders, were more likely born in clusters of shedders. The eventual objective of this study was to get a more in depth understanding of *Map* transmission on dairy farms.

MATERIALS AND METHODS

The data set used in this study contains data of cows present at an Upstate New York dairy farm during an intensive individual animal diagnostic testing period starting in 1995 and lasting until 2002. A total of 2,788 cows have been at this farm in this period. The Johne's status, JS, of the individual cows is based on results of annual fecal culture testing of *Map* in the herd. The fecal diagnostic tests were run in the Animal Health Diagnostic Center (AHDC), Cornell University. The laboratory used initially a Herrold Egg Yolk Media system (HEYM), and later switched to a liquid medium culture system where fecal cultures were grown for 35-42 days and then checked with an acid-fast stain. In the liquid culture system, bottles with acid-fast organisms detected were confirmed using *MAP* PCR. Semi quantitative results were provided based on days to positive culture. The specificity of the culture is assumed to be 100%, the sensitivity is estimated at 30-40%. The JS was expressed as positive, negative or unknown. A cow's JS was considered positive when fecal culture results were at least once positive for *Map*. A total of 1577 cows qualified with either a positive or negative Johne's status and were used in this study. Cows with an unknown JS were not used in the cluster calculations.

Prevalence per year was calculated as the percentage of cows with a Johne's status ever (JSever) positive of all adult cows on the farm that year. The current Johne's status (JScurrent) is the percentage of fecal shedding positive cows in that year of all the adult cows present on the farm that year. The incidence was calculated as the fraction of animals which were first tested positive in that year of all cows tested in that year.

The SaTScan™ v 7.01 software was used to scan for the birth clusters in this herd. SaTScan™ is a software program that analyzes spatial, temporal and space-time data using the spatial, temporal, or space-time scan statistics (Kulldorff 1997). This program was designed to use windows of different size to identify statistically significant clusters of events ($p<0.05$). In this study, windows of 4, 10, 60, 90 and 120 days were used to search for birth clusters. These windows were designed to provide insight into the presence of different clusters as described in the introduction above. A window of 4 days reflects the period around birth, for example the influence of the presence of a Johne's positive dam in the maternity pen (direct horizontal and vertical transmission). Assuming that cows leave the maternity pen at approximately 24 hours after calving, a window of 10 days provides information about the transmission routes of the maternity pen area (indirect horizontal transmission). A window of 60 days is assumed to be related to calf-to-calf transmission in the first 60 days after birth (direct and indirect horizontal transmission). The 60 days window is based on the assumption that calves are the most susceptible for *Map* infection during the first 60 days of their lives. Using a window of 90 days or more is based on the assumption that susceptibility of calves for *Map* continues during the first four months after birth at the farm.

During the eight years of testing for *Map* at this farm, some important management changes have taken place. Since late 1998 the farm participated in a Johne's Control Program that included different control measures to prevent infection of new born and young stock in the herd. Important parts of the Control Program were the immediate separation of new born calves

from their dam, separate colostrum collecting and feeding to the calves by the calf manager and improvements in general sanitation throughout the calf feeding program and housing. The Johne's Control Program also included the discontinuation of feeding the milking herd TMR overages to the young stock herd. These measures resulted in a decrease in Johne's prevalence at this farm. In the data analysis we therefore made a comparison between the two different time blocks. Calves born before and after January 1,1999 were distinguished and analyzed separately to evaluate influence of management changes to control Johne's disease at this farm.

RESULTS AND DISCUSSION

Figure 1 shows the change in incidence and prevalence from 1995 to 2002. Incidence of infections was initially high, but dropped dramatically after 1998 when a Johne's control program was implemented. Prevalence also dropped but less dramatically. Although it is a single farm observation, it appears that the implementation of a Johne's control strategy had a dramatic impact on incidence of new infections. Prevalence dropped also, but because of the lagging effect of incident infections from previous years, the drop in prevalence was not as steep compared to the drop in incidence. Fecal shedding prevalence was a function of overall infection prevalence and showed the same gradual decrease.

Significant clusters are presented in Table 1. We did not find any cluster with a window size of 4 days. There were a total of 8 clusters with a window size of 10 days, 4 clusters with a window size of 60 days and the same number of clusters with a window size of 90 days, finally, two clusters with a window size of 120 days were observed. The data were separately analyzed for the births before January 1, 1999 and the births after this date. These split analyses showed that all birth clusters occurred before January 1, 1999. No significant clusters of MAP infection were shown to be present after this date.

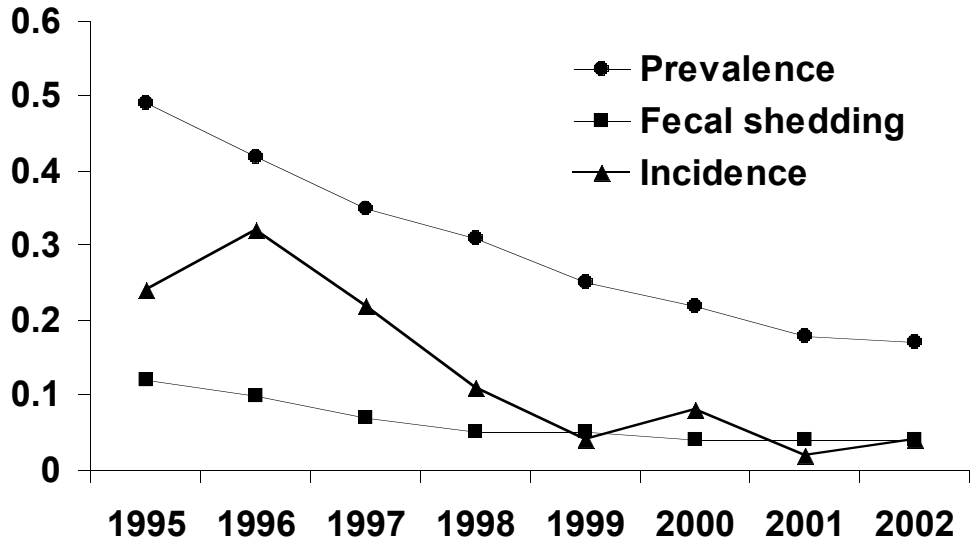

Fig. 1 Prevalence, Incidence and fecal shedding at the study farm.

Table 1. Significant MAP infection birth clusters in the time frame from 1989 to 2003. Clusters of different window size are presented. All clusters are shown and the mean number of cases and the total cluster size of the clusters are presented.

Window size	MAP infection birth clusters. cases in cluster (total cluster size)								mean
4	-	-	-	-	-	-	-	-	-
10	27 (34)	26 (33)	20 (23)	18 (20)	25 (33)	24 (32)	29 (42)	14 (17)	23 (29)
60	111 (154)	104 (166)	67 (116)	57 (110)	-	-	-	-	85 (137)
90	170 (255)	115 (209)	36 (55)	67 (133)	-	-	-	-	97 (163)
120	214 (327)	118 (238)	-	-	-	-	-	-	166 (283)

These data indicated that for the herd studies, most clusters were observed with a window of 10 days, indicating important contamination of calving pens so that multiple calves may have become infected due to a single MAP shedder. The window size of 60-120 days would indicate the presence of calf-to-calf transmission with most evidence pointing to an infectious period of 60 to 90 days. The data from this farm showed very similar results compared to previous experimental (VanRoermund et al. 2007) and observational studies (Benedictus et al. 2007).

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#88

Influence of between-calves contacts on *Mycobacterium avium paratuberculosis* (Map) transmission in a dairy herd

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Mycobacterium avium paratuberculosis (Map) can be transmitted from adults to calves. In the dairy industry, calves are rapidly separated from their dam and are not raised in contact with adults. Calf-to-calf transmission has recently been reported. However, the role played by this route of transmission has not been evaluated yet, despite there being extensive opportunity of contact as calves are usually raised together. Our objective was to assess the effect of contact structure between calves on Map transmission and to specifically assess the effect of calf isolation during the first weeks of age, while taking account of other transmission pathways. We used a stochastic compartmental model with discrete time steps to represent Map spread in a closed dairy herd after the introduction of an infected heifer. A dairy herd management system was modelled with calves being housed in individual pens before being moved to collective pens. Grazing was allowed during pasture season and after 6 months of age. Vertical and horizontal transmission from adults (classified in 3 infectious statuses) and from transiently infected calves could occur. Indirect transmission via the environment was explicitly modelled. Influence of herd size and of the time spent by calves in individual pen on contact structure was studied. Three herd sizes were considered for which the number of collective pens before and after weaning differed. The time spent in individual pen was assumed to vary from 0 to 8 weeks in compliance with EU regulation. Infection mainly occurred via vertical transmission or adult-to-calf horizontal transmission through the ingestion of faeces present in the environment. Effects of the time spent in individual pen before being moved to collective pen on the persistence of Map and on within herd prevalence suggest that it is more important to consider hygiene management than contacts in calf housing facilities.

#200

Use of multiple tests to determine the status of UK dairy herds with respect to *Mycobacterium avium* subsp. *paratuberculosis*

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A random sample of 136 UK dairy herds was recruited to a survey to estimate the proportion of these that were infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Three tests were employed: firstly, heparinised blood samples were collected from all female cattle over three years old and sera were tested using a commercial ELISA test (Pourquier); secondly, individual faecal samples were collected from these cows and combined into pools of five which were inoculated into a liquid media system (Trek ESP II) for culture for MAP and finally, bulk milk samples were collected and tested for presence of MAP DNA by PCR targeting the IS*900* insertion sequence. These tests are all imperfect and there is no “Gold Standard”. They were applied at different levels – to individual animals, to groups of 5 animals or to the entire herd. Each herd was categorised as positive (at least one positive sample) or negative for each test. Bayesian approaches have been proposed (eg Branscum et al 2004; 2005) for data from two or more imperfect tests and we applied an integrated multilevel Bayesian model to the data to estimate the herd-level sensitivity and specificity of each test. The median herd level sensitivity (HSe) and specificity (HSp) and 95% credibility intervals were estimated for each test as follows: ELISA HSe 95%-100% and HSp 47%-53%; pooled faecal liquid culture HSe 54%-69% and HSp 98%-100%; bulk milk PCR HSe 13%-51% and HSp 57%-82%. Bayesian analysis incorporates prior estimates of parameter values based on existing knowledge and results can be sensitive to these assumptions. We undertook a sensitivity analysis by varying the prior values that were used in our model. This showed that prior assumptions of the individual level specificity of the ELISA test had a limited impact on the results described above.

#104

Relation between faecal shedders and environmental contamination with *Mycobacterium avium* subsp. *paratuberculosis* on an experimental farm

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Cows and the environment of an experimental cattle farm were sampled 19 times over a period of 16 months to investigate the influence of management measures on the temporal and spatial spread of *Mycobacterium avium* subsp *paratuberculosis* (MAP). Over a 16 month period a destocked farm was cleaned, was restocked with high and medium MAP shedders and was destocked, cleaned again and disinfected. Samples were collected inside and outside the barn every 3 weeks and analysed by IS*900* real-time PCR for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). At the same moments faecal samples of all animals were collected and cultured by TREK culture system to investigate the level of bacteria shed in the environment.

Total MAP excretion of the animals did vary significantly over the 19 sampling times. The organism could be detected inside as well as outside the barn in environmental samples. Settled dust samples (reflecting a 3 week period) were more often MAP contaminated compared to floor samples. Data show that floor samples are less consistently MAP positive than samples of the cubicles and the slatted floors. Destocking and cleaning with a high pressure cleaner did not reduce MAP in settled dust and floor samples. After disinfection no MAP could be detected in settled dust samples anymore but MAP DNA was still present in some floor samples. Whether this was viable MAP will be tested in the near future.

MAP contaminated dust seems to spread undisturbed by management actions. Regular cleaning seems not to influence the contamination of the environment with MAP from settled dust. Further research on the concentration and accumulation of MAP in settled dust is needed to asses the risk of MAP infection in young animals by (long-term) accumulated dust ingestion.

Epidemiology Perspectives Lecture

Epidemiology, transmission and economics of Johne's disease: state of knowledge, knowledge gaps, and possible future directions

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Evidence-based medicine is predicated on the principle that risks and benefits of interventions can be explicitly quantified through scientifically-valid studies. These studies can then inform decisions about the economic value of interventions whether for a single herd or flock, or for national and regional programs. Current knowledge indicates that there are multiple sources of MAP infection and consequently, the recommendation lists for producers to control Johne's disease are long and often unprioritized. There are few published randomized controlled trials (RCT) or cohort studies assessing the effects, let alone the cost-benefits, of interventions. Evidence from the U.S. Johne's Disease Demonstration Herd Project and experiences elsewhere indicate that effective control is possible, but eradication is unlikely in intensive production systems such as large dairy herds. Data on the benefit-cost ratio for individual or multiple interventions or entire control program are sparse. This is a critical need for this information for dairy herds because Johne's disease is typically not a high priority disease from a financial perspective. This may change with increasing pressure from public health authorities to mitigate risks from dairy and meat products. Any expectation that interventions that are applied on farm or along the production chain can ever be linked to a reduction in Crohn's disease incidence is very unrealistic.

Investigation of the epidemiology, transmission and economics of Johne's disease offers numerous challenges, which can be broadly categorized as biological, financial and methodological. I would like to focus on the latter and offer some perspectives on the future. First, there has been inadequate use of longitudinal designs, especially RCT, to evaluate interventions and as a consequence, there are no systematic reviews of the effects and economics of interventions. Cost and feasibility are the key limitations. Second, the lack of uniformly-applied quality standards for publication of risk factor studies is symptomatic of the reality that we lag behind our human health colleagues (e.g. CONSORT and STROBE in human health). Case-control and cross-sectional studies have outlived their usefulness for bovine and ovine paratuberculosis. Third, statistical methods exist to incorporate prior knowledge into risk factor analyses and adjust for non-differential misclassification of the outcome (e.g. ELISA, culture or PCR-based test results) but these methods are infrequently used. The challenge in the next 5 years is to design, finance and implement well-planned longitudinal studies to answer critical research questions through networks and programs such as JDIP, JDRC and ParaTBTools.

Epidemiology

Poster Abstracts

#15 The natural history of *Mycobacterium avium* subspecies *paratuberculosis* as interpreted by the FUIDI #2 Map Test

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One thousand, one hundred and thirteen dairy cows within USDA's Florida Dairy Herd Demonstration Project were studied using the FUIDI #2 Map ELISA test component of the FUIDI Herd Management Schema in order to better understand the natural history of Map infection.

The FUIDI #2 test identified 110 animals as having some level of either ongoing or very recent infection. Of these 110 cows, 9 cows were designated as having significant ongoing infection and 6 cows were identified as being susp[icious for having significant infection.

Fourteen months later, 661 of the original 1,113 cows were available for re-analysis. Of the 91 cows previously identied as having low or non-diagnostic evidence of ongoing infection, 54 were available for re-evaluation. Of these 54 cows, 45 had lost all evidence indicative of active infection, 8 exhibited evidence of continuing infection, and 2 progressed into the category indicative of significant active infection.

Of the 540 previously negative cows in the FUIDI #2 test, fourteen months later 18.9% of thes cows had evidence of active infection.

Presuming the FUIDI #2 data to be correct, the natural history of infection is comparable to that observed with M. tuberculosis in the human model system: namely that disease induction is rare relative to the true incidence of infection and in the majority of cases infected dairy cows can achieve immunological governance over Map.

#22 The Prevalence of Possible *Mycobacterium Avium* Subspecies *Avium* in Fecal Sample from Dairy Cows

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ABSTRACT

A comparative study of fecal culture, hspX Map real-time PCR and nested 1311-based PCR tests was undertaken to determine the incidence of positive fecal test results using IS1311-base nest PCR primers relative to fecal culture and real-time PCR using hspX.

Three hundred sixty-eight fecal samples from the Florida Johne's Disease Dairy Herd Demonstration Project had been analyzed using fecal culture, real-time PCR and nested PCR for the detection of *Mycobacterium avium* subsp. *paratuberculosis* {Map}. Forty-one fecal specimens tested positive by the direct fecal nested Map PCR test (FecaMap®}. In 34 of the cases, the corresponding real time PCR test for Map was also positive. Mycobacterium isolates were achieved by fecal culture in 21 of the 41 cases. In 20 of the 21 cases of culture recovery of a mycobacterium, IS*900*-based primers confirmed Map. In the remaining case, fecal culture demonstrated case heavy growth and the corresponding hspX real time PCR were both positive. In the remaining 6 direct nested PCR tests, no evidence of mycobacterium growth was present. Assuming fecal culture to be 100% sensitive, the project herd false-positive incidence using IS1311 based nested primers would be approximately 1.1%

INTRODUCTION

The Linda strain of *Mycobacterium avium* subspecies *paratuberculosis* (Map) that established the IS*900* insertion sequences as the definitive marker of mycobacterium that cause Johne's diseases was deemed a centralist stain representative of the group (Harris and Barletta, 2001). It has been subsequently argued that the IS*900* insertion sequence is a vertical cut through a horizontal evolutionary process emanating from *Mycobacterium avium* subspecies *avium* in which exist polymorphic variants of these two species that can cause Johne's disease in herbivores and omnivores (Frothingham, 1999; Turenne et al., 2007). In horses, pigs, and dogs, Ma and *Mycobacterium avium* complex (Mac) are the causative agents of Johne's disease (Turenne et al., 2007). The FecaMap® and LactoMap (Infectious Diseases Incorporated, Bellevue, NE) direct and nest PCR test primers were developed using the IS*1311* insertion sequence in order to effectively span the potential spectrum of pathogenic mycobacterium as well as to provide tests applicable to avian species and zoo animals not identifiable using commercial Map ELISA tests.

Despite demonstrated pathogenicity in horses, pigs, dogs, and selected zoo animals, there is a tendency to consider Ma as an environmental mycobacterium rather than a pathogenic mycobacterium. The argument can be advanced that use of PCR tests based upon the IS*1311* insertion sequence would only result in a significant number of false-positive results.

The purpose of this study was to analyze to what degree using a IS*1311*-based PCR test would positive results be identified that were not substantiated by fecal culture or real-time PCR using hspX.

MATERIALS AND METHODS

Study population: Three hundred sixty-eight dairy cows within the Florida Johne's Disease Dairy Herd Demonstration Project constituted the study population. Selection of a cow was predicated upon prior independent analysis of its feces using the FecaMap® nested Map PCR test.

Fecal cultures: Fecal cultures were done at Animal Disease Diagnostic Laboratory, School of Veterinary Medicine, Purdue University using the Trek® Map Culture System in accordance with the manufacturer's instructions.

Real-time Map PCR tests: Real-time Map PCR tests were done at Animal Disease Diagnostic Laboratory, School of Veterinary Medicine, Purdue University using Tetracore® Map Extraction and DNA test kit in accordance with the manufacturer's instructions.

Direct fecal nested Map PCR tests: Direct fecal nested Map PCR tests were done at University of Florida College of Veterinary Medicine using the FecaMap® system in accordance with the manufacturer's instructions. The FecaMap® direct primers recognize a 242 base pair sequence of Map IS*1311* and its nested primers overlap and span a 104 base pair region within the insertion sequence.

RESULTS

Three hundred sixty-eight fecal samples from the Florida Johne's Disease Dairy Herd Demonstration Project had been analyzed. Out of 368 fecal samples, Ma/Map was identified by the IS*1311* primer of the FecaMap® test (1.1%).

Forty-one fecal specimens tested positive by the nested Map PCR test. In 34 of the cases, the corresponding real time PCR test for Map was also positive (Table 1). *Mycobacterium* isolates were achieved by fecal culture in 21 of the 41 cases. In 20 of the 21 cases of culture recovery of a mycobacterium, IS*900*-based primers confirmed Map.

In the remaining case, fecal culture demonstrated case heavy growth and the corresponding hspX real time PCR was positive. The animal was culled before the need to retest was identified. Six fecal samples identified by the IS*1311* nested PCR were not substantiated by either fecal culture or real-time PCR using hspX.

Table 1. Analysis of dairy cows in the Florida Johne's Disease Prevention Dairy Herd Demonstration Project for prevalence of Map/Ma DNA in fecal samples as determined by the FecaMap® direct nest fecal Map PCR test.

# of fecal specimens	# culture +/ # nested -	# RT PCR +/ nested +	# non-Map + cultures/ RT & nested +
368	20/41	34/41	1/1

DISCUSSION

Despite covering only 6-8 copies, the direct IS1311 direct and nested primers were demonstrated to be more sensitive in identifying Map contained within USDA's Laboratory Certification Tests.

The overall incidence of a positive IS1311-based nested Map PCR test was 1.1%. A positive nested reaction had an 85% probability of identifying Map; however the remaining 14% of positive nested PCR tests were not confirmed by their corresponding fecal real-time PCR and culture tests. More importantly, the nest Map PCR identified a non-IS*900* mycobacterium whose test profile was that of being a heavy shedder in the Trek® culture system and of testing positive in the Tetracore® PCR system. These observations coupled with early culling makes it, more likely than not that this animal had a significant mycobacterium infection. Culture data demonstrating heavy shedding would have been disregarded had diagnostic confirmation been done using IS*900* primers. To what degree other non-IS900 potentially pathogenic mycobacteria have been dismissed as being environmental contaminants is unknown. In this small series, the presence of a non-IS900 presumably pathogenic mycobacterium was calculated to be 2.4%.

CONCLUSION

IS*1311*-based nested Ma/Map primers done on fecal 368 fecal samples tested positive for 1.1% of the specimens, had an 85% positive correlation with the probable presence of Map, but had a 14% presumed false positive rate.

If IS*1311* PCR primers are used to confirm mycobacterium culture isolates derived from fecal or milk cultures, the IS*1311* primers appear to identify non-IS900, pathogenic mycobacterium.

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#25 Isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in milk samples from a dairy herd in Brazil

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Mycobacterium avium subsp. *paratuberculosis* (Map) can be shed in the milk, feces or semen of infected animals. Map infections are endemic in European and North American dairy cattle. Map infections are considered rare and of minor importance in Brazil, despite that clinical cases have been recently reported and serological estimates bear similarity to those obtained from other countries. The aim of the present study was to determine if Map were present in samples of raw and pasteurized milk. Thirty-three samples of raw milk were collected directly from the cooling tank at farms. The samples of raw milk were obtained from three dairy herds, two of them located in the state of Rio Grande do Sul and one located in the state of Rio de Janeiro, in which the pathogen was isolated from a farm with recent cases of the disease and identified. A total of 379 samples of milk sold in retail stores in Porto Alegre, southern Brazil. Monthly samples of conventional, UHT and HTST were treated and grown on HEYM for isolation of Map. Partial results show that Map could not be isolated from the 379 samples of milk sold in 5 different retail stores. Of the 33 samples of raw milk samples, one was isolated on HEYM. Based on cultural, phenotypic and molecular characteristics, the sample testing positive was deemed to contain Map.

#39 Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cows by milk nested PCR and its association with production and reproduction in Fars Province, Iran

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To evaluate the prevalence of Johne's disease and its impact on production and reproduction in dairy cows, IS*900* based nested PCR was used. Furthermore, data about production and reproduction were collected. Overall, milk samples from 252 animals of 21 dairy herds were examined. In nested PCR, 8 samples (3.2%, 95%CI: 1.35.1) were positive, corresponding to herd prevalence of 23.8% (95%CI: 6.241.4). Statistical analysis revealed no significant differences in genetic purity, total milk and lactation length between positive and negative cows. However, 305 days milk (P=0.005) and calving interval (P=0.04) was significantly different between herds with and without positive animals. Considering the effect of parity and genetic purity, 305 days milk was 8040 kg in negative herds, 520 kg higher than positive herds.

#42 Seroprevalence survey of paratuberculosis in dairy and pastoral cattle in Uganda

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Between 1999 and 2002, lesions consistent with the description of paratuberculosis were observed from abattoir specimens taken from slaughtered cattle. The aim of this study was to determine if there was serological evidence for the prevalence of the disease among the cattle population. A total of 1023 cattle were selected by a multistage sampling technique from 66 cattle herds in 4 districts and tested for antibodies against *M. paratuberculosis* using Pourquier ELISA. The tested animals were from 7 different breeds, namely, Holstein Friesians, East African Zebu, Ankole long horn, Boran, Sahiwal, Jersey and Guernsey. At least 43 heads of cattle (4.2%) were serologically positive. 24 of the 66 herds of cattle had at least one positive animal (36%) based on the manufacturer's cut off. All the breeds of cattle except Guernsey had at least one infected animal. The disease was found to be present in all the four districts surveyed. Infection was also observed in cattle under all the different types of husbandry practices. This is the first time the prevalence of Johne's disease has been determined in Uganda. Paratuberculosis has hitherto been silently spreading among the cattle population in Uganda. This study provides information that will be used in future epidemiologic studies and control programs.

#44 Genetic variation in serological response to *Mycobacterium avium* subspecies *paratuberculosis* and its association with performance in Irish Holstein-Friesian dairy cows

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Few estimates of the genetic variation in measures of susceptibility to MAP are available in the literature and even less have attempted to elucidate the genetic associations between measures of susceptibility to MAP and performance in dairy cattle. Therefore, the objectives of this study were to estimate the genetic variation in serological response to MAP in 5,280 Holstein-Friesian dairy cows, and to quantify its genetic association with performance traits measured in the first three lactations of genetically related animals. Univariate mixed linear and threshold animal models were used to estimate variance components and genetic correlations were estimated using bivariate sire linear mixed models; MAP serological response was treated as a continuous variable and dichotomous variable. The prevalence of MAP in the sample population was 4.8%. However, because of the data editing criteria used, this is not a representative statistic of the national prevalence. Estimates of heritability for MAP serological response varied from 0.07 to 0.15 depending on the model of analysis and whether serological response was treated as continuous or binary; standard errors varied from 0.024 and 0.062. Genetic correlations between MAP serological response and lactation milk, fat and protein yield were negative although not always more than two standard errors from zero; stronger negative genetic correlations were evident in older parity animals. Serological responseto MAP was not genetically correlated with milk fat concentration but was positively genetically correlated with lactation milk protein concentration and negatively correlated with calving interval. Positive genetic correlations existed between MAP serological response and somatic cell count but the correlations were not greater than two standard errors from zero. There was little or no genetic association between serological responseto MAP and survival. Results from this study corroborate previous international suggestions that selection for reduced serological responseto for MAP is possible, although this does not necessarily imply a concurrent selection for either reduced prevalence of clinical disease or increased resistance to MAP infection.

#63 Risk of detecting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in colostrum and on teat skin of Holstein cows in Johne's disease (JD) infected herds

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Objective: To describe the relationship between fecal shedding of MAP around calving time and detection of MAP in colostrum samples and on teat skin surfaces.

Methods: Feces collected within 3 d prior to actual calving, colostrum and teat swabs collected immediately after calving from 112 cows in 4 JD endemic herds, were tested for MAP using culture and PCR targeting the gene ISMAP02 in colostrum and teat swabs. Logistic models were used to evaluate the relationship between MAP fecal shedding status and MAP detection in colostrum samples or teat swabs. Attributable fractions for the proportion of colostrum and teat swabs positive for MAP attributable to fecal shedding status of the donor cows were also calculated.

Results: Odds ratio for detecting MAP in colostrum samples or teat swabs in fecal culture positive (vs. negative) cows were 2.02 ($P < 0.001$) and 1.87 ($P < 0.008$), respectively. Findings suggested that withholding colostrum from MAP fecal culture positive cows might reduce the risk of exposing calves to MAP through ingestion of colostrum by 18.2% and that limiting the chances of natural nursing by calves might reduce the risk of exposing calves to MAP present on teats of MAP fecal shedding dams by 19.5%. While the latter interventions might achieve some reduction in risk of exposure to MAP, they by no means eliminate the risk of exposing calves to MAP through ingestion of MAP present in colostrum or on bovine teats in JD endemic herds since a greater proportion of MAP detected in the colostrum samples and teat swabs had sources unrelated to the fecal shedding status of the donors.

Conclusion: Findings underscore need for adherence to practices that limit contact of calves with adult cows and to practices that promote hygienic colostrum handling to avoid possible contamination during harvest, storage or feeding processes.

#77 Characterisation of clinical paratuberculosis on New Zealand deer farms

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Aim: To characterise clinical paratuberculosis (ill-thrift and/or scouring unresponsive to treatment) in New Zealand (NZ) farmed deer herds.

Method: Data were obtained by personal interview of 174 volunteer deer farmers in 2005 with known deer paratuberculosis culture status. Subsequent postal questionnaire data were obtained to 15 November 2007 from 81 of those herds. Descriptive data and associations between clinical Ptb, season and location are presented.

Results: By 2005, 89% (96/108) of MAP positive herds had experienced ≥1 clinical case. In 2005 the median incidence in MAP positive herds was 1.2% (range 0.1% - 21.5%) in deer <12 months of age (weaner), 2.0% in deer 12 – 24 months of age (yearling) (range 0.2% - 20.0% and 0.2% - 13.2% in hinds and stags, respectively), 0.9% (range = 0.1% - 20.8%) in adult hinds and 1.3% (range = 0.2% - 8.9%) in adult stags. The incidence of reported paratuberculosis increased substantially from the 1990s to 2005 and again to 2007. The highest incidence was in South Island yearling hinds (1.4%) and the lowest in North Island adult stags (0.1%; $p<0.0001$). The incidence of clinical paratuberculosis was greatest in winter (all age classes) and spring (weaners and yearling stags). The odds of a herd with no clinical paratuberculosis cases in 2005 having clinical paratuberculosis cases in 2006-7 was 3.9 (95% CI = 1.1, 13.9) times higher than similar herds having no clinical paratuberculosis in 2006-7. In 2005 and 2007 the median within-herd incidence of clinical paratuberculosis was 0.31% to 0.54%, respectively (NS, $p>0.05$).

Conclusion: Significant clinical paratuberculosis incidence was observed in all age classes. Data supports anecdotal evidence of spreading infection in farmed deer. Accurate national data on clinical and sub-clinical incidence is required to evaluate the economic impact of paratuberculosis in deer herds.

#103 Persistence of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in field dried hay fertilized with bovine slurry from Map infected herds

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ABSTRACT

Objective: Use of Map contaminated slurry or manure to fertilize crop fields is generally considered a risk factor for the Map transmission, although data on survival of Map in crops are sparse. Therefore, the persistence of Map on crops fertilized during the autumn-winter period with slurry or manure coming from Map infected herds was studied.

Methods: Ten Map infected herds with different prevalence levels of infection were selected. Culture and PCR tests were performed on environmental samples collected from the infected farms to assess the level of Map contamination. The same tests were performed on crop samples collected at three different time-points: (1) on fresh hay before harvesting; (2) on hay after field drying; (3) on dried hay at the beginning of its use for animal feeding.

Results: The environmental samples had massive presence of Map in the manure and slurry used to fertilize the fields. The tests performed on the fresh hay samples, collected before harvesting, showed a single positive result by PCR (10%) and were always negative by culture. The hay samples collected after field drying and at the beginning of their use for animal feeding were always negative in both culture and PCR.

Conclusion: These results suggested that under the described conditions, the contamination risk for field dried hay, although possible, is of limited importance for the spreading of infection. On the other hand this must not be underestimated in uninfected herds purchasing forage.

Key words: Paratuberculosis, forages, survival, slurry, manure

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (Map), the causal agent of Paratuberculosis, can survive outside the animal for extended periods (163 days in river water, 270 days in pond water, 11 months in bovine faeces and soil, but only 7 days in urine) (1) . The fermentative processes in packed manure should, if treated correctly, result in higher temperatures than in slurry, thereby also reducing the risk of Map survival compared to in slurry (2). Map survival in bovine slurry has under experimental conditions been demonstrated to be 98 days at 15 °C, but can reach 252 days at 5 °C (7)..

Because modern management procedures of dairy manure do not provide optimal conditions for Map inactivation, control plans against Map usually recommend not to fertilize forages with slurry from Map infected herds.. Spreading faeces on fields from which forage is later harvested and fed to animals of any age group is statistically correlated to the risk of Map infection in the herd (3).

Therefore, the persistence of Map in crop fertilized during the autumn-winter period with slurry or manure coming from bovine infected herds was studied.

MATERIAL AND METHODS

A serologic survey was carried out on bulk milk samples coming from the dairy herds of North Italy, using an ELISA commercial kit (Institut Pourquier, France). Among the herds positive to the ELISA test, 10 were selected because they used to spread faeces on fields for forage use..

A visit in these 10 farms was carried out to provide information about the incidence of clinical cases of Paratuberculosis and procedures used to manage and spread manure and/or liquid slurry on fields..

The liquid slurry was stoked in lagoons (50%) or in intered containers (50%) and spread on forages without an adequate period of maturation, unlike the manure. The manure and/or the slurry were spread on the fields in autumn (45%) or winter (55%). The spread quantity varied from a minimum of 8 x 10^2 to a maximum of 5 x 10^4 Kg per hectare.

A serologic survey was carried out on individual milk samples of all the lactating cows of the herds (Kit ELISA Institut Pourquier) to confirm that the clinical cases were related to Map infections and to estimate the within-herd seroprevalence of Paratuberculosis..

Environmental samples were collected from manure, slurry, and manure scrapers when present.. All samples collected (n=32) were submitted for culture (6) and PCR. PCR was performed by bead grinding extraction (5) followed by testing using a commercial kit (Paratuberculosis Adiavet, Adiagene).

The fields fertilized with the contaminated manure/slurry in autumn/winter period were repeatedly sampled, keeping fixed the sampling area, by the collection of:

- 10 grass samples, in the end of April;
- 9 field-dried hay samples, before baling, in the end of May;
- 9 field-dried hay samples, at the beginning of the use for animal feeding, in July-August.

From the herd no.. 4, hay samples were not collected, because the grass was not used for animal feeding.

The forage (nearly 800 g of fresh grass per sample, or 400 g of dried hay per sample) was submitted to an extraction procedure, by leaving it overnight, after agitation, in sterile distilled water, enough to wash it completely (nearly 500-1000 ml). From 100 of the extract liquid we performed culture and from 10 ml we performed bead grinding extraction followed by PCR as done for the environmental samples.

RESULTS

The prevalence of ELISA positive and clinical cases in the 10 herds are shown in Table 1.. The within-herd sero-prevalence ranged from 4% to 38%. In herd 7, were the maximum sero-prevalence value was recorded (38%), the clinical case prevalence was higher than 5%.

Table 1.. Herd dimension and Paratuberculosis diffusion in the selected herds

Herd Code	Lactating cows	ELISA positive cows	Sero-prevalence	Clinical case incidence
1	24	1	4 %	0 %
2	122	11	9 %	2-5 %
3	88	13	15 %	0-2 %
4	100	14	14 %	0-2 %
5	48	8	17 %	0-2 %
6	75	13	17 %	0-2 %
7	29	11	38 %	>5 %
8	48	5	10 %	0 %
9	69	4	6 %	2-5 %
10	53	7	13 %	0-2 %

The results of the culture and PCR tests performed on manure and slurry are shown in Table 2.

Table 2.. Results of the tests (PCR and culture) performed on environmental samples

	Slurry			Manure			Scraper		
Herd Code	No.	PCR Positive	Culture Positive	No.	PCR Positive	Culture Positive	No.	PCR Positive	Culture Positive
1	1	0	0	2	0	0	1	0	0
2	3	3	3	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1
4	1	1	0				1	1	1
5	2	2	2	1	1	1	1	1	1
6	1	1	1				1	1	1
7	1	1	1	1	1	0			
8	1	0	1	1	0	1	1	1	1
9	1	1	1	1	1	0	1	1	1
10	2	2	0	1	1	0	1	1	0
Total	14	12 (86%)	10 (71%)	9	6 (67%)	4 (44%)	9	8 (89%)	7 (78%)

The environmental samples had high contamination levels, particularly in the case of liquid slurry and scrapers.

The proportions of PCR positive were for all the processed materials always higher than proportions of culture positive. This could be due to a vitality loss of Map, which would still be detectable by PCR.
The results of the forage sample are shown in Table 3.

Table 3. Results of the tests (PCR and culture) performed on forage

Herd Code	Fresh grass			Dried hay (at harvesting)			Dried hay (at use)		
	No.	PCR Positive	Culture Positive	No.	PCR Positive	Culture Positive	No.	PCR Positive	Culture Positive
1	1	0	0	1	0	0	1	0	0
2	1	0	0	1	0	0	1	0	0
3	1	0	0	1	0	0	1	0	0
4	1	0	0	n.e.			n.e.		
5	1	0	0	1	0	0	1	0	0
6	1	0	0	1	0	0	1	0	0
7	1	1	0	1	0	0	1	0	0
8	1	0	0	1	0	0	1	0	0
9	1	0	0	1	0	0	1	0	0
10	1	0	0	1	0	0	1	0	0
Total	10	1 (10%)	0 (0%)	9	0 (0%)	0 (0%)	9	0 (0%)	0 (0%)

n.e.= not examined

The only positive result recorded was from fresh grass, and it was only positive by PCR, while culture on the same sample was negative. The positive sample originated from Herd 7, where the highest sero-prevalence (38 %) was recorded.
The dry hay samples, both the first and the second sample, were always negative to the PCR and culture.

DISCUSSION AND CONCLUSIONS

Forages contaminated by Map represent a potential vehicle of Map transmission; in fact the correct management of faeces originating from infected herds represents a critical point in Paratuberculosis control plan application.
If the optimal management method of infected faeces, from a sanitary point of view, is represented by spreading them on the fields before ploughing, this practice is not suitable from an environmental point of view, because of low efficiency of N absorption by plants.. Therefore, alternative methods with a higher efficiency are adopted, such as spreading manure on actively growing plants..
In our study we have verified the contamination risk of forages on which faeces had been spread in autumn and winter.
Our results confirm that liquid slurry seems to be more contaminated if compared with packed manure, consistent with data reported by other authors in studies regarding environmental sampling in infected herds (4).
Although the limited number of samples processed must be taken into consideration, our results show that in the described conditions, the risk of forage contamination by Map, after spreading faeces from infected herds, was limited; in fact the only positive result has been recorded on fresh grass 15-30 days before harvesting, in a herd with a very high prevalence (38%).
In all the other cases, no positive results were recorded on hay, both shortly after field drying and after stocking it in bales.. This result confirms the data reported by other authors, in which the research of Map on forages contaminated with infected faeces had always given negative results (4).
We can conclude that, spreading contaminated faeces on the fields, provided that it is done at least a few months before harvesting, has minor importance in an infected herd if compared with other hygienic and managerial factors.. However, one useful precaution is to avoid the use of potentially contaminated forages to feed animals lower than 12 months, the period of higher sensitivity to the infection..
Another important aspect is represented by the purchasing of forages in negative herds, in which also a limited risk must be avoided, preferring the purchasing of forages not fertilized with bovine faeces..

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#111 Detection and molecular confirmation of *Mycobacterium avium* subsp. *paratuberculosis* in drainage water and forage after application of dairy cattle manure on agricultural soils

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Mycobacterium avium subsp. *paratuberculosis* (Map) is considered an obligate pathogen. In theory it can be eradicated by the removal of all infected animals. However, Map can survive for long periods outside the host. Land spreading of manure can cause water pollution, either through direct aerosol contamination or through runoff from manure applied on the fields. There is no published data on Map survival in soils after manure application. The aim of this study was to detect the presence of Map after application of contaminated manure onto agricultural soils.

A longitudinal study was carried out by extracting intact soil columns that were kept under controlled environmental conditions. Spiked manure with a known bacterial concentration (10^8 Map/ml) and manure from Map positive animals was applied to the soil and both drainage water and forage samples were analyzed bacteriologically. An automated liquid culture system (MGIT) was used for Map detection. All positive tubes were confirmed by a robust real-time PCR for IS*900* and F57 targets, including effective mechanical cell disruption, DNA extraction and removal of PCR inhibitors.

Preliminary results show that Map could be detected by MGIT and confirmed by IS*900* and F57 PCR in drainage water 2 to 4 months after manure application. Map presence in forage and top soil could be confirmed 8 months after manure application. Regarding the cycle threshold values (Ct) from the real-time PCR (where the Ct cut-off value is above 40 for a negative result), positive drainage water samples showed on average 33 Ct, compared with 25 Ct of positive samples from forage and top soil.

These results are suggestive of forage and top soil being a more favourable environment for Map survival than can be provided by the subsoil, implying a higher risk for grazing animals than for pollution of well water.

#113 Effect of soil type and rainfall regime on the movement and survival of *Mycobacterium avium* subsp. *paratuberculosis* due to dairy slurry application

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There are few studies on water contamination by Map and there are not published data on Map dispersion or survival after contaminated dairy slurry application to soils. International research suggest that acidic soils with high iron content, such as those found in Southern Chile, will create suitable conditions for Map survival.

Cattle production systems in southern Chile are based on pastures, with animals mostly grazing all year round. However, during the last years there has been an intensification of dairy systems with partial or total cow's housing generating high volumes of dairy slurry, which are commonly used as organic fertiliser so that these applications could be a primary source for Map dispersion to water by leaching. The aim of this research was to study the effects of rainfall and soil texture on the movement and survival of Map after the application of contaminated slurry to grasslands.

To achieve this, intact soil columns (0.95 m depth, 0.4 m diameter) were collected and placed under controlled condition to test the effect of a loamy or sandy soil texture and rainfall (1000 or 2000 mm) on Map leaching. The experiment was organised on a randomized design with two factors and three replicates. Slurry was prepared with faeces collected from Map-fecal culture positive dairy cows (1:1.5, water:faeces) and it was applied to the top of the soil columns. After rainfall application, drainage samples were collected forthnightly and cultured for Map using both solid (HEYM) and liquid (BACTEC MGIT 960 system) media. Positive cultures were confirmed by PCR using specific primers for Map (IS*900*).

Preliminary results showed that there is a lag time of two months before Map is found on the water samples, and that Map leaching would be favoured by light soil textures and increasing rainfall.

#114 Epidemiological characteristics of MAP-infected population of wild hares cropped in southern Chile

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Although infected cattle are the main source of infection, previous studies suggested that the rabbit possess the greatest risk of transmission to domestic livestock. However, in southern Chile the hare is more common than rabbits and it is considered a plague. The aims of this study were to provide evidence that hares might be infected with Map, to assess the prevalence of infected hares, and to assess association between individual characteristics and risk of infection in a hare population cropped in southern Chile.

A cross-sectional study was designed using 385 wild hares randomly selected from cropped animals in two provinces of southern Chile. Samples were stratified by geographical area based on their location at the time of capture and information about the individuals and the place of capture were collected. Faecal samples, mesenteric lymph nodes and representative tissue of the small intestine were aseptically collected once a week, transferred to sterile containers, and transported to the laboratory for bacteriological analysis by liquid culture (MGIT) and PCR confirmation. The true prevalence was estimated using a Bayesian approach, assuming a Sensitivity = 60% and Specificity = 99%. Associations between characteristics of hares and disease status were assessed by logistic regression (LR).

Apparent prevalence was 12.7%. The median of the estimated posterior for the true prevalence was 14.1% (95% credibility interval 0.0; 0.20). From LR model, we estimated that hares captured in the Osorno province were 2.1 times (95% CI 1.1; 4.1) more likely to be infected compared to those from Valdivia province. Hares younger than one year were 2.2 times (95% CI 1.1; 4.5) more likely to be infected compared to older ones.

This study provides the first evidence that Map infection is common in wild hares in southern Chile and it seems that have a heterogeneous spatially and age-risk distribution.

#136 Error-adjusted, variance partitioning coefficients to assess the association between clustering of MAP infection and calf management risk factors in Danish dairy herds

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The variance partition coefficient (VPC) measures the clustering of infection/disease among individuals with a specific covariate pattern. Failure to adjust for imperfect diagnostic tests downwards biases VPCs. Error-adjusted, covariate-pattern-specific VPCs provide insight to the groups that exhibit great infection heterogeneity and should be targeted for intervention. We developed a Bayesian discrete mixed model for the estimation of error-adjusted, covariate-pattern-specific VPCs and applied it to the identification of calf management risk factors associated with MAP infection. Details on practices regarding colostrum and milk feeding were obtained from 633 herds (64,945 animals). Individual animal records on MAP antibody ELISA status and age were retrieved from the Danish Cattle Database. We initially ran (i) a naïve model that ignored the imperfect accuracy of the ELISA and subsequently (ii) our model that adjusted for the varying with age imperfect Se and Sp. Ignoring age-dependent Se and Sp greatly biased VPC estimation. Animals fed colostrum from multiple cows were more likely to be infected compared with those fed only from their own dam. Calves suckling with foster cows were more likely to be infected than calves fed milk replacer. Error-adjusted, covariate-pattern-specific VPCs revealed that herds feeding colostrum to calves only from their own dam and milk replacer were of the least heterogeneous. Herds feeding colostrum from multiple cows and/or allowing suckling from foster cows were of the most heterogeneous. Much of the overall heterogeneity of MAP infection among herds remained unexplained after adjusting for the significant calf management risk factors. Thus, additional risk factors exist, which operate at the herd level and contribute to the between herd heterogeneity of MAP infection. Error adjusted, covariate-pattern-specific VPCs could be utilized in control programs to optimize sample sizes according to the herd-specific component of risk factors.

#138 Estimating Prevalence of Paratuberculosis in sheep. A case study: the Viterbo Province (Latium, Italy)

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ABSTRACT

The number of prevalence studies carried out among small ruminants is small and there is no studies that provided accurate and unbiased prevalence estimates.

The objective of the present study was to assess the ovine paratuberculosis prevalence in Viterbo Province.

A total of 1500 individual blood samples belonging to 30 flocks, were tested with commercial ELISA test (Pourquier).

The apparent prevalence on animal and herd level was calculated as the number of test-positive animals among the total number of animals tested (AP), or test-positive herds among the total number of herds tested (HAP). An estimate of the true prevalence (TP) was calculated from the AP by correction via the Rogan-Gladen estimator.

INTRODUCTION

The prevalence of an infection is an important tool to know the diffusion of the infection itself and to apply measures as eradication, control and surveillance. The number of prevalence studies carried out among small ruminants is small and there are no studies that provide accurate and unbiased prevalence estimates.

The objectives of the present study were to assess the ovine paratuberculosis prevalence in Viterbo Province. We met the minimum requirements of a prevalence study (Nielsen and Toft, 2009) which are descriptions of: (a) target and study populations, including the age distribution;(b) the test used, including test accuracy and protocol ; (c) study period; (d) APs which are converted to TPs.

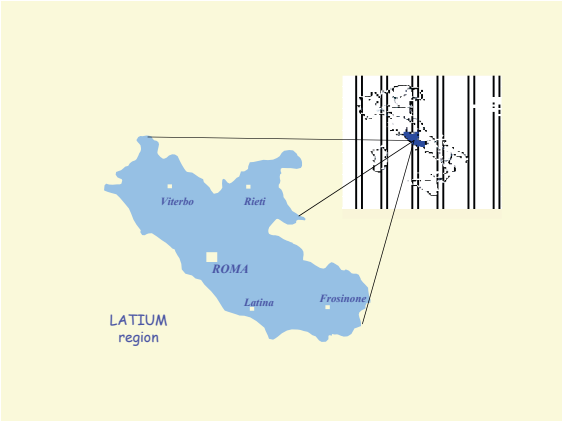
There is a distinction between true prevalence (the proportion of a population that is actually infected) and apparent prevalence (the proportion of the population that tests positive for the disease). Given point estimates for sensitivity (se), specificity (sp), and apparent prevalence (AP), one may calculate true prevalence using the following expression: ***True Prevalence = (AP+sp-1)/(se+sp-1).***

MATERIALS AND METHODS

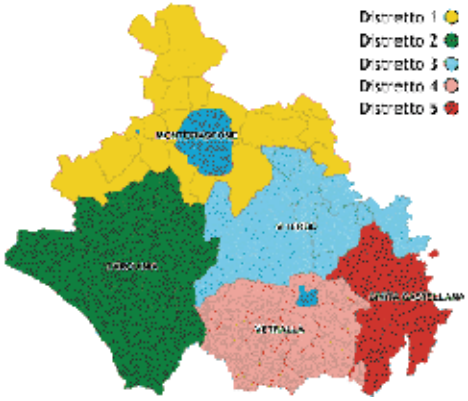
Fifty sheep flocks were randomly selected and were proportionally allocated according to the 5 sanitary districts of the province. A total of 1500 individual blood samples, 30 per flock, were tested. A commercial ELISA test (Pourquier) was used for screening and confirmation. Sera were treated according to the protocol provided by the manufacturer.

The Apparent Prevalence on animal and herd level was calculated as the number of test-positive animals among the total number of animals tested (AP), or test-positive herds among the total number of herds tested (HAP). An estimate of the true prevalence (TP) was calculated from the AP by correction via the Rogan-Gladen estimator (Rogan and Gladen, 1978). Sensitivity and specificity of the test, used for calculation of true prevalence based on apparent prevalences were, Se: 0.40 (40%) and Sp: 0.985 (98%)

Latium Region



Viterbo districts



RESULTS

At animal level the AP was in the range 1.6 -4.3 % with a total prevalence of 3%. The TP was in the range 0.26 -7.3 % with a total prevalence of 3.9% . Among the flocks the HAP was from 20% to 50% and the HTP was from 0% to 72%. The results of herd prevalence are influenced from the low sensitivity of the test.

We calculated also the confidence interval (CI). Instead of estimating the parameter by a single value, an interval likely to include the parameter is given. Thus, confidence intervals are used to indicate the reliability of an estimate.

Minimum requirements (according to Nielsen and Toft, 2009)	Our data
Region and country	Lazio /Viterbo, Italy
Year of study	2005
Animal species	Sheep
Test used	ELISA Pourquier
Age	>1 year
Number of animals studied	1500
Number of herds studied	50
Number of animals positive	45
Number of herds positive	20
Sampling strategy	50 sheep flocks, randomly selected and proportionally allocated according to the 5 sanitary districts of the Viterbo province

Animal prevalence

District	Animals tested (n)	ELISA positive	Prevalence (AP)%	AP 95% konfidence interval	TP%
Vt1	300	13	4.3	2-6.5	7.3
Vt2	300	7	2.3	0.6-4	2.1
Vt3	300	10	3.8	1.6-6	6
Vt4	300	10	3.8	1.6-6	6
Vt5	300	5	1.6	0.19-3	0.26
Total	1500	45	3	2.1-3.8	3.9

Herd prevalence

District	Herds tested (N)	ELISA positive	HAP %	HTP %
Vt1	10	5	50	36.11
Vt2	10	3	30	-53.0
Vt3	10	6	60	72.5
Vt4	10	4	40	9.4
Vt5	10	2	20	-13.07
Total	50	20	40	13.1

The assessment of prevalence obtained should be comparable to other studies and might be important to plan prospective studies or to make rational health planning decision.

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#139 Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in adult Danish non-dairy cattle sampled at slaughter

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A voluntary, risk-based control program for paratuberculosis in dairy herds was started in 2006 in Denmark. The program does not include non-dairy herds, and the occurrence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in these herds is unknown. The objective of this study was to establish the prevalence of MAP infected adult non-dairy cattle in Denmark.

Serum samples were collected between October, 2008 and January, 2009 from every 6th animal over 24 months of age, sent to slaughter to Danish slaughterhouses from non-dairy herds. The sample included 2,368 cattle, the largest breed was crossbred (of unknown breeds) (30%) and the three dairy breeds (Holstein, Jersey and Danish Red Cattle) comprised 27% of the cattle. The serum samples were tested using an antibody ELISA (IDScreen® kit from ID-Vet) and positives were defined as the sample-to-positive ratio greater than 0.60. Information about test sensitivity (Se) and specificity (Sp) were based on literature data. Se was set for each year-age-group from 0.2 (95th percentile: 0.30) to 0.5 (95th percentile: 0.60) for 2-3 year-age-group and older than 5 year-age-group, respectively. Sp was set to 0.995 (5th percentile: 0.90) regardless of the year-age-group.

Using the test information, estimation of the true prevalence (TP) (stratified by breed) was done in a Bayesian analysis (with a random effect of breed) using WinBugs software. Overall, the estimated TP was 0.01 (95% CPI: 2.7E-6 - 0.06), with large differences between breeds. The dairy breeds Jersey, Holstein and Danish Red Cattle had highest ranked TP (mean: 0.13, 0.10, and 0.06, respectively). The former two were the only breeds significantly different from the population mean. The results needs to be further scrutinized, but the indication of dairy-breeds having a higher prevalence might provide a starting point for further analyses into the potential causes of this difference.

#144 The suitability of macroscopic preselection for prevalence estimation of bovine paratuberculosis at the slaughterhouse

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Objective: To examine the probability of detecting bovine paratuberculosis by sampling slaughtered cattle which are macroscopically suspicious and unsuspicious for granulomatous enteritis.

Materials and Methods: The survey was subdivided into two studies in german slaughterhouses. In study 1 sampling was done on 50 cattle with macroscopic lesions of granulomatous enteritis (thickening of the intestinal wall, gyrus form mucosa folds, granular appearance of the intestinal mucosa, dilated mesenteric lymphatics). In study 2 sampling was done on 150 cows which were not suspicious for granulomatous enteritis. Samples for bacteriological, histopathological and immunohistochemical examinations were taken from ileum, jejunum, Nll. mesenteriales and Nll. caecales.

Results: By sampling cattle with macroscopic lesions of granulomatous enteritis in study 1, 98% were MAP-positive. The prevalence of macroscopic suspicious, MAP-positive cattle was 1.35% of all slaughtered cattle. The lesions reflected mainly advanced stages of disease. Macroscopic alterations were most commonly seen in the ileum. Thickening of the intestinal wall and gyrus form mucosa folds were most frequent findings. In study 2, 28% of the 150 sampled cows were MAP-positive without any macroscopic lesion seen in the intestine.

Tissue culture turned out to be the most sensitive test. Histological examination and immunohistochemical or AFB staining identified fewer cattle to be infected. This was most obvious in study 2, in which sampled animals were in less advanced stages of disease. Immunohistochemical examination of the tissue was more sensitive then Ziehl-Neelsen staining. Ileum and Nll. mesenteriales can be especially recommended as sampling sites in cattle.

Conclusion: This study shows that a reliable detection of MAP-infected cattle is possible when macroscopic lesions of granulomatous enteritis are evident. In prevalence studies on the basis of slaughterhouse inspections it is necessary to examine cattle.

#151 Explaining persistence of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds

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In this work, we use a mathematical model to illustrate MAP transmission dynamics on dairy farms. Following the results of recent analysis of age-and-dose dependent shedding of MAP, we are able to build a model incorporating true values of exit from each infectious category. The model is evaluated with and without an environmental reservoir. This reservoir allows the infectious contribution from animals which have already exited the population to decay over time.

We show the influence of changing assumptions concerning environmental persistence and super-shedder adult animals as well as the relative importance of calf-to-calf transmission. We find that environmental loading could be a contributing factor allowing persistence of MAP on farms, and that calf-to-calf transmission is necessary to produce a spectrum of prevalence observed worldwide. The presence of age- and dose-dependent shedding of MAP among calves creates a bistable prevalence of infection following intervention.

#154 Effect of *Mycobacterium avium* subsp. *paratuberculosis* infection on time to calving and time to culling in dairy

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Among the costs attributed to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in dairy cattle, the impacts on reproduction and culling are the least documented. In order to properly estimate the cost of MAP infections in a dairy herd, the rates of conception and culling were calculated for cows in each stage of MAP infection relative to uninfected cows. Two well-defined databases, each consisting of culling and reproduction data from 3 commercial dairy herds, were used for analysis, consisting of 2,856 cows with 6,380 calvings and 1,696 cullings. Every cow in each study herd was tested regularly for MAP, and herds were followed for between 4 and 7 years. Infection status (uninfected/latent vs. low- or high-shedding) was defined as a time-dependent variable for all cows with at least one positive test result. Cox's proportional hazards model, stratified on herd and controlling for the time-dependent variable was used to analyze time to culling. Latent and uninfected animals were significantly less likely to be culled in comparison with animals in the low- and high-shedding categories. Time to conception was analyzed using a proportional means model, an analog to the Cox regression model suitable for recurrent event data, stratifying on herd and weighted to adjust for the dependent censoring caused by the culling effects describe above. Low- and high-shedding animals had longer calving intervals in comparison with latent and uninfected animals, but these results were non-significant using a robust variance estimate. These results can be incorporated into economic models for the cost of MAP in dairy herds.

#164 Diagnosis of paratuberculosis in slaughtered calves in the Northwest of Castilla y León (Spain) by pathological methods

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Objectives: To assess the prevalence of Map infection in calves from extensive herds in the slaughterhouse, using histopathological and PCR methods .

Materials and Methods: A total of 15,546 slaughtered calves between 8-12 months-old and different breeds, from 137 extensive herds in the Northwest of Castilla y León region (Spain) were examined between 2001 and 2008. Samples of ileocaecal lymph were examined microscopically for the presence of lesions characteristic of Map infection and the presence of acid-fast organisms by Ziehl-Neelsen (ZN) Selected sections were examined immunohistochemically using a polyclonal anti-Map antibody. Lesions were classified as focal, multifocal and diffuse according to the number of granulomas. Lesions with 1-3 granulomas formed by less than 20-25 macrophages, containing a yellow granular pigment were considered as doubtful. A PCR for detection of Map DNA was performed in 182 paraffin wax embedded tissue samples.

Results: Lesions associated with paratuberculosis infection, including doubtful lesions, were observed in 440 calves. Diffuse lesions appeared in 1 case, multifocal lesions in 8 calves, focal lesions in 137 and doubtful lesions in 294. Diffuse lesions were positive by ZN, immunohistochemistry and PCR. Seven multifocal lesions were PCR positive and one case only was positive by immunohistochemnical methods. PCR gave positive results in 60.8% and 24.7% of calves with focal and doubtful lesions respectively.

Conclusions: A remarkable percentage of the calves from extensive herds in this study (2.83%) had lesions associated with paratuberculosis. This prevalence was higher if considering herds with at least one infected animal (49.63%).

The sampling and technical methods used have demonstrated to be efficient in the detection of lesions in the early stages of paratuberculosis infection (subclinical cases).

#178 Age structure of *Mycobacterium avium* subsp. *paratuberculosis* infection in culled Friesian cattle

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ABSTRACT

The aim of this study was to determine the age structure of paratuberculosis infections in culled dairy cattle as a mean to improve the design and use of diagnostic tests and control strategies.

581 adult Friesian cattle slaughtered in the Basque Country from March 2007 to December 2008 were included in the study. Samples of blood, intestinal tissue and mesenteric lymph nodes were taken from each animal. Blood was used in an ELISA test whereas intestinal tissue was used for bacterial isolation, DNA detection by a commercial real time PCR (RTi-PCR) and histopathological examination. All methods showed a peak at 3-4 years of age that ranged from 42.3% (histopathology) to 16.5% (ELISA). Minimum prevalence was found at 7-8 years. Overall prevalence ranged from 8.3% (ELISA) to 20.0% (RTi-PCR). Sampling within the 3-5 years of age group could improve the chances of herd infection detection with a minimum number of samples. Focusing testing on this age group could also save resources and maximize efficiency in testing and culling programmes.

INTRODUCTION

It is generally accepted that paratuberculosis (PTB) is a slow infection that begins during the first weeks of life in a contaminated environment, but that do not develop into visible lesions and disease until the early adult life of cows in some animals.

Sensitivity of PTB diagnosis varies with the stage of the infection and lack of sensitivity of diagnostic tests is the main hurdle for the successful use of test and cull strategies.

In this slaughterhouse study, the main goal was to determine the age structure of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in Friesian cattle as well as to obtain new data on the efficacy of four diagnostic tests for detection of subclinically infected animals.

MATERIALS AND METHODS

Animal selection and sampling

Using a weekly systematic sampling, 581 Friesian animals were examined from March 2007 to December 2008, at two local slaughterhouses in the Basque Country. After stunning, blood samples from the jugular vein were collected into sterile tubes containing EDTA. Additionally, fresh and formalin-fixed samples of jejunal caudal lymph node (JCL) and distal ileon and ileocecal valve (ICV) were aseptically taken. Bovine identification documents (BID) were reviewed for age data collection.

ELISA

The Pourquier® ELISA Paratuberculosis Antibody Screening kit (Institut Pourquier, Montpellier, France) was used on plasma samples according to the manufacturer's instructions. Samples with positive and doubtful results for the screening ELISA were retested using Pourquier® ELISA Paratuberculosis Antibody Verification kit (Institut Pourquier, Montpellier, France). Positive results corresponded to sample to positive (S/P) ratios above 70%.

Culture

Gut tissue samples, consisting in 1 g of JCL and 1 g of mucosa from the ICV area and distal ileon, were decontaminated with hexa-decyl pyridinium chloride (HPC) (0.75%) and inoculated in duplicate mycobactin J supplemented Herrold's Egg Yolk (HEYM) and Löwestein-Jensen (L-J) media as previously described (Juste *et al.,* 1991). Cultures were first examined after 8 weeks of inoculation and subsequently every 4 weeks up to 20 weeks. No evidence of bacterial growth after this incubation period was considered as a negative result. Isolated colonies were confirmed by conventional PCR amplification of MAP specific IS900 insertion sequence (Moss *et al.,* 1992).

RTi-PCR

Sample preparation involved the homogenization of 2.5 g of JCL and mucosa from the ICV and distal ileum area (1:1) in 10 ml of sterile water for 1 min at medium speed in a Stomacher® 80 Biomaster (Seward, Worthing, UK). Afterwards, 300 μl of the homogenized sample were submitted to a modified protocol of Adiapure® MAP DNA extraction and purification kit (Adiagene, Saint Brieuc, France) for tissue samples. Purified DNA samples were eluted to a final volume of 100 μl. MAP DNA detection, based on the amplification of the specific IS900 insertion sequence, was carried out using the ADIAVET® PARATB Real Time commercial kit (Adiagene) and ABI Prism® 7000 Sequence Detection System instrument (Applied Biosystems, Foster City, CA). Samples were considered positive if the cycle threshold (Ct) value was <37.

Histopathological examination

Formalin-fixed tissue samples were processed using standard histological procedures. All sections were stained by haematoxylin and eosin (HE) and observed under the light microscope. In case of paratuberculosis compatible lesions Ziehl-Nielsen (ZN) staining was performed. Lesions were classified according to González *et al.*, (2005).

Test combinations: Prevalence analysis and sensitivity and agreement study

PTB prevalence was estimated by serial and parallel analysis. Only animals with all tests positive were scored positive in serial evaluation while animals with at least one positive result in any of the tests were scored positive in the parallel analysis.

Complementary sensitivity was calculated in order to evaluate test combinations and maximize sensitivity in PTB diagnosis. This value was defined as the additional number of samples testing positive to an evaluated test in relation to the reference one.

Agreement between tests was measured by Kappa (κ) value. Sensitivity estimations and Kappa values were calculated with WinEpi software (www.winepi.net).

Statistical analysis

Fisher's exact probability was calculated in order to detect significant differences in MAP diagnosis according to age. The level of significance was set at p-value <0.05.

RESULTS

Overall PTB prevalence ranged from 8.3% by ELISA (n=581) test to 20.0% by PCR (n=581). The prevalence of positive MAP tissue cultures was 18.7% (n=576). Characteristic paratuberculosis lesions were observed in 13.6% of the studied samples (n=213). Serial prevalence analysis estimated PTB prevalence at 9.5% whereas parallel analysis estimation was nearly 3 times higher (27.7%).

The average age at which MAP infection was detected by serology, culture, RTi-PCR and histopathology was 4.6, 5.3, 5.4 and 4.2 years; respectively. All tests evidenced a peak of paratuberculosis infection prevalence when animals were 3-5 years. At this age prevalence determined by a combination of tests read in parallel was 33.2%. Age distribution of PTB infection is shown in Fig. 1.

A statistically significant lower frequency in MAP infection was observed in animals older than 5 years compared to 3-5 years-old animals (p_{ELISA}=0.0002; $p_{CULTURE}$=0.0087; $p_{RTi-PCR}$=0.0078; p_{HP}< 0.0001).

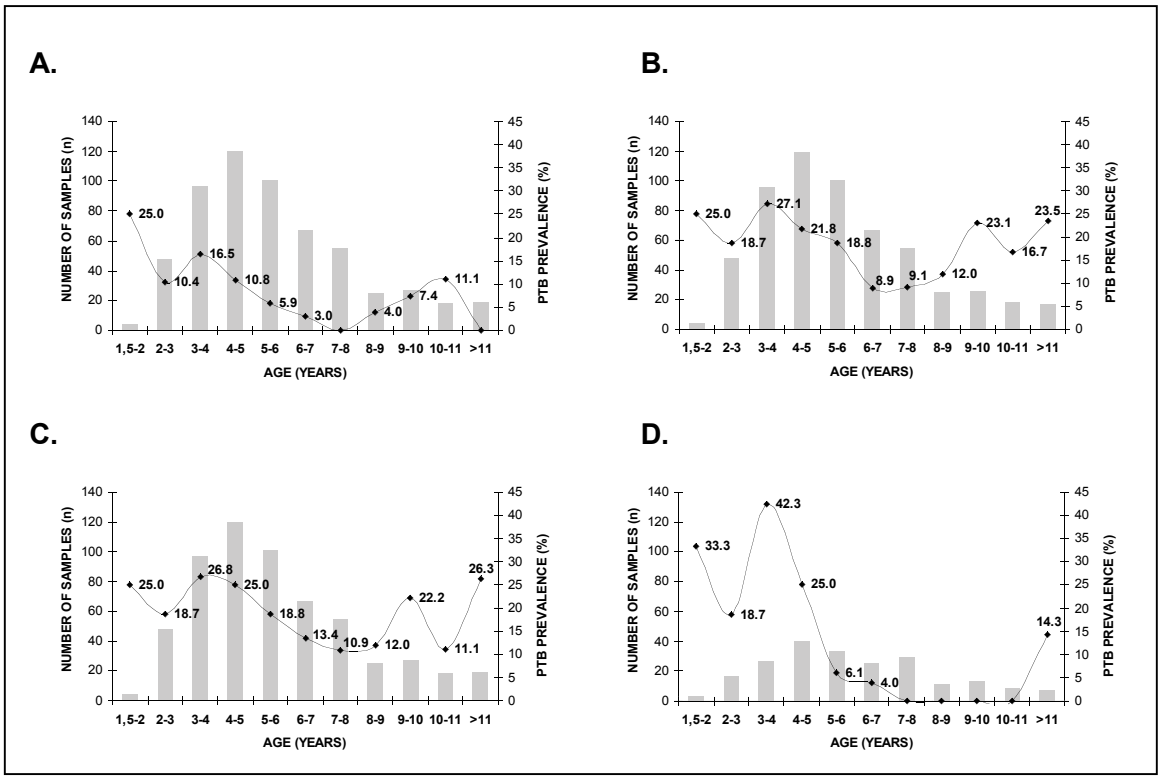

Fig. 1. Age structure of MAP infection in relation to diagnostic tests: ELISA (A), culture (B), RTi-PCR (C) and histopathological examination (D). Line: MAP prevalence and bars: number of samples tested.

Table 1. Sensitivity, complementary sensitivity and agreement (κ) values of diagnostic test combinations. Interpretation of agreement: poor (κ=0.00-0.20), fair (κ=0.21-0.40), moderate (κ=0.41-0.60), good (κ=0.61-0.80) and excellent (κ=0.81-1.00).

EVALUATED TEST	Sensitivity (%)				Complementary sensitivity (%)			
	REFERENCE TEST				REFERENCE TEST			
	ELISA	CULTURE	RTi-PCR	HP	ELISA	CULTURE	RTi-PCR	HP
ELISA	-	37.0 (κ=0.454)	37.1 (κ=0.461)	72.4 (κ=0.696)	-	6.5	4.3	24.1
CULTURE	85.1	-	63.4 (κ=0.562)	85.7 (κ=0.536)	144.7	-	33.0	92.9
RTi-PCR	89.6	65.7	-	86.2 (κ=0.525)	152.0	38.0	-	96.5
HP	75.0	48.0	47.2	-	28.6	8.0	7.5	-

Broadly, diagnostic test combinations resulted in moderate agreement (κ=0.41-0.60) excepting for the combination ELISA and histopathological examination that showed a good

agreement (κ=0.696). The highest relative and complementary sensitivity values were obtained with microbiological methods (RTi-PCR and Culture)(Table 1.).

DISCUSSION

Microbiological methods as well as histopathological examination corroborated that most PTB infected animals were between 3 and 5 years old as traditionally described. Our serological findings were in agreement with the assumption of the age of 2.5 to 4.5 years as the highest risk of detecting antibodies against MAP suggested by other authors (Nielsen Ersbøll, 2006). The fact of finding one seropositive 18 months-old animal with diffuse lesions and MAP isolation from gut tissue may support the implication of animals younger than 2 years in horizontal MAP spread suggested by other authors (Wells *et al.*, 2000).

With regard to diagnostic tests, RTi-PCR showed a similar sensitivity to MAP isolation. The good agreement between ELISA and HP indicates that ELISA could be a good predictor of intestinal lesion and therefore might anticipate clinical disease. However, the poor complementary sensitivity of ELISA and culture and RTi-PCR indicates that humoral immune response is a bad predictor of subclinical infection. Therefore, a combination of RTi-PCR and ELISA appears to give the best chances to maximize PTB diagnosis.

CONCLUSION

Based on the yearly infection rates obtained from this study it seems appropriate to intensify time testing at the age of 3-5 years in order to maximize the likelihood of detecting infected animals before they spent a too long time in the herd acting as a source of infection for the rest of the susceptible animals. On the other hand, we can conclude that no single test can detect all infected individuals and that, therefore, a combination of tests yields the maximum sensitivity.

ACKNOWLEGMENTS

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#183 Sero-prevalence of *Mycobacterium avium* subspecies *paratuberculosis* infection in low productive domestic ruminants with clinical and sub-clinical Johne's disease in India

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Objective: Status of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection was estimated in domestic ruminants' in India using 'indigenous ELISA kit'.

Methods: Indirect ELISA (un-absorbed) using protoplasmic antigen from 'Indian Bison Type' MAP of goat origin was used to screen all species of domestic ruminants. OD values were converted to S/P ratios to know status of Johne's disease (JD) as negatives, suspected, low positive, positive and strong positives. Animals in strong positives were considered positive for JD. Sero-prevalence was estimated on the basis of screening of serum samples submitted to Microbiology laboratory of Central Institute for Research on Goats, Makhdoom, over period of 4 years (2004 – 2008), in-view of lack of diagnostic kits against JD in the country. Total of 2478 serum (1289 cows, 515 buffaloes, 536 goats and 138 sheep) were screened in 4 years and samples were driven from Punjab, Hayana, Uttar Pradesh, Tamil Nadu and Rajasthan states of India..

Results and Conclusions: Of 2478 samples sero-prevalence of MAP was 22.5% in domestic ruminants in 5 states of India. Species-wise sero-prevalence was 23.3, 21.3, 23.5 and 17.4% in cattle, buffaloes, goats and sheep, respectively. Distribution of 2478 serum samples with respect to the status of JD was 20.4, 6.4, 8.5, 42.0 and 22.5% as negatives, suspected, low positives, positives and strong positivess, respectively. Comparison of ELISA kit with fecal culture, show maximum correlation with strong positive category, however, fecal culture detected positive animals from all categories of S/P ratio. Samples in the positive category also exhibited substantial correlation with culture, therefore if positive and strong positive were considered positive, then sero-prevalence of MAP infection in domestic ruminants in India was 64.5%. Low per animal productivity substantiated this high sero-prevalence of MAP infection in domestic ruminants of country. Still considering culture being positive in other categories also, these figures may still be under statement. Sero-prevalence of MAP infection in the domestic ruminants on the basis of screening of 2478 animals over period of 4 years (2004-08) from the 5 states of India was 22.5%.

#201 Prevalence and distribution of paratuberculosis (Johne's Disease) in cattle herds in Ireland

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A simple random survey was conducted in Ireland during 2005 to estimate the seroprevalence of paratuberculosis, commonly called Johne's disease (JD), in the cattle population. Serum samples were collected from all 20,322 females/breeding bulls over twelve months of age in 639 herds (0.7% of breeding herds). All samples were tested using a commercially available absorbed ELISA. The overall prevalence of seropositive herds was 21.4% (95% CI = 18.4%, 24.9%). Herd prevalence levels amongst dairy herds (mean 31.5%; CI 24.6%-39.3%) was higher than among beef herds (mean 17.9 %; CI 14.6%-21.8%). However, the animal level prevalence was similar. The majority of herds had only one seropositve animal. Only 6.4% (95% CI 4.7%-8.7%) of all herds had more than one seropositive animal; 13.3% (CI 8.7%-19.7%) of dairy herds ranging from two to eight seropositive animals and 3.9% beef herds (CI 2.4%-6.2%) ranging from two to five seropositive animals. The true prevalence of herds infected and shedding *Mycobacterium avium* subspecies *paratuberculosis* is estimated to be 9.51% for all herd types; 20.63% for dairy herds and 7.58% for beef herds based on a test specificity of 99.8% and test sensitivity (i.e. ability to detect culture-positive animals shedding at any level) of 27.8-28.9%.

#216 Calf shedding of MAP on two farms with high and low environmental exposure

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Two cohorts of calves were followed for 11 months by monthly fecal culture on two northeastern USA dairy farms with known MAP prevalence. Index calves were selected from dams classified as moderate or high shedders based on fecal culture. The remainder of the cohort was selected to be most likely to be raised in the same environment as the index animal.

On both farms, fecal samples were collected monthly and stored until completion of the study. Samples were batch processed to decrease variability between timepoints. Samples were evaluated at New Bolton Center by Tetracore's Vet-Alert™ real-time PCR and fecal culture of those samples with positive PCRs.

On farm A, no positive samples from animals were identified including the index animal. On farm B, 4 animals in the cohort had positive (Ct≤42) or suspicious (Ct> 42) PCRs (a total of 7 potential positive samples were identified out of 146). The index animal was negative for all cultures.

Calves in farm B were housed in the same barns as the milking herd for 6 months before transitioning to heifer barns. Environmental samples were gathered from within calf pens and the contiguous milking herd pens to evaluate the likelihood of post-birth exposure. Environmental samples were positive by RT-PCR at 8 of 11 sampling timepoints, and for 4 of the 6 timepoints in which calves were housed exclusively with other calves.

Although this analysis did not reveal positive cultures from the index animal, on farm B there were multiple potentially positive calves in the cohort, one of which cultured positive 3 times in the course of 4 months. While we cannot illustrate calf-to-calf transmission, we do see culture positive young animals, even when housed in an environment which should not involve substantial exposure from adult animals.

#218 Johne's disease in the New Zealand national dairy herd - an analysis of a decade of dairy cow culling records

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The LIC National Dairy Cow Database allows New Zealand (NZ) dairy farmers to record culling (including deaths) due to Johne's disease (JD). Analysis of these voluntary JD culling records over ten dairy seasons from 1998/99 to 2007/08 reveal that approximately 8% of New Zealand dairy herds cull 0.5% of their cows with signs of Johne's disease per annum. JD risks in the NZ dairy cow population have remained essentially static over that period with 0.05% reported annual incidence nationally.

In spite of typically high levels of dairy herd and cattle movements in NZ, the data reveals distinct regional and breed differences in JD culling rates. The Northland region records the lowest level of Johne's disease with 2% of dairy herds culling less than 0.2% per annum, while the West Coast is the only region where within-herd culling rates exceed 1% amongst herds recording JD culls. Jersey breed is associated with significantly higher risk than Friesian (RR = 4.26; $p < 0.001$), although it remains unclear whether this reflects differences in breed susceptibility.

Forty percent of all JD culls and deaths occur over three months in spring – coinciding with calving and onset of lactation. As expected JD risks are strongly correlated with increasing age and peak amongst eight-year-old cows, which are almost three times as likely to succumb to Johne's disease as three-year-old cows.

#223 A stochastic and age-structured compartmental model for MAP infection on a US dairy herd with test-based culling intervention

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Modeling of the transmission of MAP on dairy herds helps us understanding the dynamics and evaluating the effectiveness of control options. When herd size is small, stochastic effects such as fadeout become important and stochastic modeling is necessary. To study the phenomena of highly variable prevalence and fadeout of MAP infection, a stochastic compartmental model, with age structure including calves, heifers, and cows, was developed. We then investigated the impact of various testing and culling strategies on the prevalence over time and the cumulative distribution function of fadeout. To incorporate the model parameter uncertainty and find the most important parameters for the probability of fadeout, we performed global parameter uncertainty and sensitivity analyses. Our results show that: (1) the threshold property of the reproduction ratio, R, is generally not an accurate criterion to determine the invasion and persistence of MAP infection; (2) evaluation of the effectiveness of testing and culling strategies based on prevalence over time and the cumulative distribution function of fadeout is more appropriate; (3) for a farm of good management (low transmission rate), culling of only high shedders based on ELISA tests is effective to eradicate MAP infection within 20 ~ 30 years; however, for a farm of poor management (high transmission rate), culling of only high shedders is not enough to eradicate MAP infection within 50 years, although the prevalence still decreases; (4) higher test-based culling rates for low and high shedders increase the probability of fadeout which may be important for the eradication of MAP infection.

#225 Impact of Johne's disease vaccines on a dairy herd

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Aiming to reduce the prevalence of MAP infection, vaccination was applied as a control measure in some dairy herds. However, as vaccines of Johne's disease are imperfect and many effects are reported, including reduction in susceptibility, infectiousness, shedding level, infectious period, and clinical cases. Quantitative evaluation of the effectiveness of vaccines is, therefore, lacking. In this study, we developed a deterministic vaccination model, with age structure including calves, heifers, and cows, to investigate the impact of imperfect vaccines on the overall vaccine efficacy and the prevalence over time. Using the developed model, we derived an analytical expression for the overall vaccine efficacy which may be used as a measure for comparing the efficacy of various vaccines. In addition, we numerically compared the dynamics of prevalence in a 100% vaccinated herd with a control herd.

#227 Association between cow reproduction and calf preweaning growth traits and Elisa s/p ratio scores for paratuberculosis in a multi-breed herd of beef cattle

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MAP ELISA is used as a herd-screening tool that can detect approximately 50% of infected animals. Despite low sensitivity, evidence suggests association between ELISA s/p ratio and cow and calf traits. The objective of this study was to assess association between beef cow reproductive and weight traits, pre-weaned calf traits and MAP ELISA sample to positive (s/p) score (0 = negative, 1 = suspect, 2 = weak-positive, and 3 = positive) in a multi-breed herd of cows. Cows in the University research herd were tested by ELISA as part of the annual herd monitoring and control program. Cows and calves in the herd were monitored for reproductive and production traits according to the operating protocol of the unit. Records for the years 2002 - 2006 were collated and summarized to evaluate study objectives. Cow data included: 624 measures of gestation length (GL), 358 records of time to conception (TO), 605 calving intervals (CI), and 1240 measures of weight change (from November to weaning, WC), from 502 cows. Calf data consisted of 956 birth weights (BWT), 923 weaning weights (WWT), and 923 weaning weights adjusted to 205 d of age (WW205) from 956 calves. Traits were analyzed individually using multi-breed mixed models. Fixed effects were year, age of cow, sex of calf, year × age of cow interaction (except WC), age of cow × sex of calf interaction (only for WC), ELISA score, and covariates for breed fraction of sire and cow and heterosis of cow and calf. Random effects were sire (except for TO and CI), dam, and residual. Estimates of differences between cows with non-zero and zero ELISA scores were associated with lower cow fertility (longer TO), a lesser ability of cows to maintain weight (negative WC), lower calf BWT, and lower calf weaning weight (WWT and WW205).

#244 A survey to estimate the herd-level prevalence of paratuberculosis in the dairy herd of the United Kingdom using serum antibody

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A survey was conducted to estimate the prevalence of dairy herds in the United Kingdom that were infected with paratuberculosis. This report details the serological study carried out in support of this objective. Herds were selected at random from throughout the UK and 353 farmers were invited to participate. 136 (39%) of the contacted farmers were recruited. Farms with a previous history of clinical paratuberculosis were more likely to join the study. The herd size ranged from 57 to 413 milking cows. In each herd all animals of three years of age and above were sampled (mean sample size of 101). In total blood samples from 13,691 cows were tested using a commercially available enzyme-linked immunosorbent-assay (ELISA) to test for the presence of antibody to *Mycobacterium avium* subspecies *paratuberculosis* (Map). The results of the survey will be reported and related to the other parts of this study that involved faecal culture and examination of the bulk tank milk for the presence of Map.

#245 The validity of repeated serological and culture results to determine the true infection status for *Mycobacterium avium* subsp. *paratuberculosis*

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There is no real gold standard for detecting the infection status for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in live cattle. The likelihood of a (non-)infected status may be increased by repeated test results with different diagnostic tests. The objective of our study was to determine the added value of repeated test results to determine the real infection status for MAP.

Seventeen MAP infected herds were selected for the study. Twice a year all dairy cows in a herd were tested in parallel with a serum-ELISA (Pourquier) and fecal culture on solid LJ medium . Eight rounds of testing were carried out. The farmers only received the results for the first round of testing, the others were not disclosed. Hierarchical models were made in WinBUGS 14.0, in which the ELISA result was regressed against the fecal culture result, the age of the cow, the round of testing and a random cow and herd effect.

The multilevel model showed that only the random cow effect (3.87(SE 0.36)) was significant and that there was no significant random herd effect (0.016 (SE 0.0460)). Thus, repeated observations on a cow were correlated but cows within a herd were not correlated. The sensitivity of the ELISA in faecal culture negative cows is very low (1-2%). The sensitivity increased with age and with increasing amounts of bacterial shedding up to 89% for a heavy shedder older than 4 years. The specificity for non-shedding cows in infected herds slightly decreased from 99.2% in heifers to 97.9% in cows older than 4 years.

The correlations for the currently ELISA positives (S/P>90) with the previous results were fairly low (0.4-0.6) and only significant until two samples back (t-2). Therefore, infected herds should be tested annually.

Control Programs

Convenor: David J. Kennedy and Steve Hendrick

Results from the US JD Demonstration Herd Project: Key findings for disease control

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The National Johne's Disease Demonstration Herd Project (NJDDHP) in the United States was initiated to evaluate the long-term feasibility and effectiveness of management-related practices designed to control Johne's disease on dairy and beef cattle operations. The NJDDHP was started in 2003, but a few States had demonstration herds prior to the start of the National Project. The NJDDHP includes approximately 90 beef and dairy operations in 17 states. The project was designed for 5-7 years. Participating herds began with culture-confirmed *Mycobacterium avium* subspecies *paratuberculosis* (MAP) on the operation. Risk assessments and management plans were completed on an annual basis. Six different assessments of effective MAP control will be addressed: Attributes to a successful program as described by the investigators, changes in risk assessment scores by year and cattle class, changes in incidence and prevalence since the beginning of the project, evaluation of the assessment areas to determine practices associated with disease transmission, and evaluate the transmission of disease from dam to daughter. Ranked attributes from investigators addressed calving area/maternity management, producer dedication, motivation and education, and implementation of a feasible program. Although total beef operation risk assessment scores did not decrease over the 5 year period, decreases in risk were observed for the calving area and nursing calf area. Dairy operations scores decreased for calving area, bred heifer area and for operation total score. Fecal culture prevalence in dairy herds has decreased since the beginning of the project. The findings from these operations demonstrate that the disease can be managed.

#238

Passive MAP fecal shedding in dairy cattle

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Detectable MAP in fecal samples has been the long acknowledged Gold standard diagnostic test for Johne's disease in most species. With the recognition of MAP supershedder cows excreting up to 50 billion MAP organisms per day, some culture positive fecal samples have been attributed to ingestion of small amounts of feces from supershedders resulting in passive shedding.

Objective: to estimate the frequency of passive MAP shedding in dairy cattle.

Materials and Methods: Semi-annual whole herd fecal cultures were done in three dairy herds with more than 560 adult cows for four years. Passive shedders were defined as cows with low to moderate numbers of MAP on the surface of HEY culture tubes with at least two subsequent negative fecal cultures, ELISA negative or no MAP in tissues at slaughter. Fecal and tissue samples (ileum, IC valve, and 2 ileo-cecal lnn) from selected cows followed to slaughter were cultured to determine the extent of MAP infection in the tissues in passive shedders compared to active shedders.

Results: Data collected over four years (4,155 fecal samples) in three dairy herds found 35 (30%) of all positive fecal cultures (117 samples) as passive shedders. The extent of passive shedding was proportional to the presence of herd mates as MAP supershedders. Low shedders had orders of magnitude less MAP/gm of tissue compared to active shedders.

Conclusions: Based on these herd investigations, passive shedding occurs frequently in low to moderate MAP infected herds. Positive fecal cultures should not be considered as the Gold Standard test to determine infection in cattle. On occasion, more than 60% of positive fecal cultures in a whoe herd test may be due to passive shedding attributable to a MAP Supershedder in the herd.

#202 Milk quality assurance for paratuberculosis: effects of infectious young stock.

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INTRODUCTION

A bulk milk quality assurance program (BMQAP) for paratuberculosis in Dutch dairy herds was initiated in 2006, aiming at a reduction of the concentration of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) in milk delivered to the milk factories (Weber and van Schaik, 2007). The development of the program was supported by various modeling studies (van Roermund et al., 2005; Weber et al., 2005; Franken, 2005; Velthuis et al., 2006), in which it was assumed that cattle do not become infectious before adulthood. However, shedding of *Map* in young stock has been described in several studies (Rankin, 1959; Rankin, 1961; McDonald et al., 1999; Waters et al., 2003; Weber et al., 2005; van Roermund et al., 2007), and transmission of *Map* amongst young stock was found in an experimental study (van Roermund et al., 2007). Therefore, we evaluated the effect of transmission amongst young stock on key output parameters of the BMQAP in simulated dairy herds.

MATERIALS AND METHODS

Closed dairy herds participating in a BMQAP were simulated with a stochastic simulation model, JohneSSim. Transmission of *Map* amongst young stock was simulated, assuming various probability distributions for the age at onset of infectiousness and various contact rates between individuals.

RESULTS

Transmission amongst young stock resulted in an increased average within-herd prevalence, and a decrease of the beneficial effect of preventive management measures directed at a separation of adults and young stock on herd-level and within-herd prevalence's. When including transmission amongst young stock, infected herds were detected earlier within the simulated BMQAP. Consequently, the proportion of certified herds ('green' herds, i.e. status A in the Dutch BMQAP) decreased. Moreover, the beneficial effect of preventive measures on the proportion of certified herds was reduced. However, the effect of transmission of *Map* amongst young stock on the concentration of *Map* in milk from certified herds was small and became negligible beyond year 10 after the start of the program.

CONCLUSION

It is concluded that transmission of *Map* amongst young stock does not affect the quality assurance of milk from certified herds. However, the proportion of herds that are certified in a BMQAP is decreased by transmission of *Map* amongst young stock.

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#141

Control of paratuberculosis by vaccination - a systematic review and meta-analysis

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Objective: The effects of vaccination against paratuberculosis and its significance in the control of the disease were examined.

Materials and Methods: After thorough literature searches, vaccination studies in domestic ruminants published between 1961 and 2009 were selected by predefined criteria. Publications were separated in experimental (n=12) and field studies (n=14). Important criteria for the selection were direct verification of disease (pathological/histological examination, organ culture, fecal culture) and comparison of results for vaccinated and non-vaccinated groups. In experimental studies, all animals were infected orally with MAP after vaccination of one group. In field studies, young animals in infected herds were vaccinated, the infection status of the individual animal was unknown at the time of vaccination. Odds ratios (OR) were calculated to estimate the effects of vaccination on pathological/histological, organ culture and fecal culture results. In meta-analyses, results of primary analyses were integrated. Studies summarized in each meta-analysis were tested for homogeneity (Cochran´s Q-Test, I^2-statistic) and fixed or random effect models were chosen to estimate the pooled OR as an indicator of effect size.

Results: In experimental studies the chance for a positive pathological/histological result (OR=0.3535) or a positive organ culture result (OR=0.3496) was significantly reduced in animals vaccinated prior infection. In field studies, significantly decreased MAP detection rates were recorded in vaccinated animals regarding pathological/histological lesions (OR=0.1726), organ culture (OR=0.3992) and fecal culture (OR=0.2369).

Conclusions: No general protection against paratuberculosis infection can be achieved by vaccination. It is impossible to prevent infection in dairy cattle only by implementation of vaccination programs. Reduction of pathological findings and decrease of positive culture results indicate a lower risk of infection for vaccinated animals. The decrease of positive fecal culture results after vaccination can possibly reduce the environmental load in infected herds.

#235

Update on a Voluntary Johne's Disease Control Program in Ontario and Western Canada

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In 2005-07 a voluntary Johne's disease (JD) risk assessment (RA) control program was introduced to dairy producers in Ontario and Western Canada. The program included a RA and JD milk ELISA herd test. The aim of this study was to evaluate this RA based control program. Of the 499 contacted herds, 240 agreed to participate in this follow-up study and to re-test their entire milking herd with a JD milk ELISA test more than 2 years after the initial RA. The data collection is ongoing and so far 154 herds have retested their milking herd with a milk ELISA test.

The preliminary results indicate a decline in the average within-herd test-positive prevalence. Linking the current within-herd prevalence to management practices recorded with the RA from 2005-07 indicates that farm-level hygiene and the purchase of cows are important areas for JD spread. Herds that purchased cows, not bulls or heifers, in the 5 years prior to the RA showed higher prevalences today than closed herds. Interestingly, herds that asked about the JD history of the seller's herd tended to have lower prevalences than herds that did not inquire. The recorded hygiene levels on farm, in particular for the calving area, were also associated with a herd's current prevalence – the lower the farm scored two years ago (i.e., the cleaner), the lower the test-positive prevalence at present. Herds utilizing individual boxstalls had lower prevalences today than herds with group calving pens. But also other areas, like manure contamination of feeders and waterers for weaned heifers and cows, were associated with increased apparent prevalence in the herds current test. Culling of JD milk test-positive cows was not linked to greater decrease in JD within-herd prevalence. In conclusion, the program identifies risk areas correctly.

#198

Use of small experience groups to enhance knowledge and increase motivation of farmers in the process of controlling paratuberculosis

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The organic co-operative dairy Thise Dairy decided in 2008 to eradicate paratuberculosis from their member herds. Continued focus on transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for a period of 5-10 years will be needed to achieve eradication.

The objective of this project was therefore to enhance knowledge and increase motivation of farmers in the process of controlling paratuberculosis cost-effectively.

The 83 member herds were divided into 8 small experience groups, and a facilitator was associated with each group. The group meet every 6 month at one of the farms, and the owner describes how control of MAP is managed and the group give feed back. The role of the facilitator is to ensure that the group discuss the different risk areas and provide advice to the owner, and to summarise the advice given by the group.

The actual management related to factors of importance to transmission of MAP was recorded by May 2008 through a questionnaire. The results showed that approximately 75% of the farmers did not have management practices sufficient to control MAP. Subsequently, each group have had two herd visits and the farmers have been intensively involved in providing advice, which should result in reducing the number of herds with insufficient management practices, which can reduce MAP transmission. The outcomes of the project so far are: a) farmers do believe that experience groups are valuable and approximately 2/3 of the farmers participate in each group session; and b) the quality of the discussions and advices given have increased since the first meeting. The management practices will be recorded again in September 2008 to determine if the approach has resulted in improved management practices. However, many farmers have already improved their practices and appear to be highly motivated for continuing the control process.

#214

JDConsult: An example of immersion learning

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JDConsult is a novel approach to hands-on learning in a virtual environment. Our objective was to design a simulation that provides veterinarians the opportunity to assess and advise a variety of producers with different infection rates, business goals, and management styles. Using pilot funding from the University of Wisconsin Department of Informational Technology, and additional funding from JDIP, we created a game where learners take on the role of a new veterinarian in a national consulting practice. As such, the learner visits different farms across the country. The game was designed based on the Best Test Special Report paper published by M.T.Collins, et al. in the December 15, 2006 issue of JAVMA. Learners look around the farm, get information from the producer and then make recommendations regarding management changes, testing, and what actions to take based on testing. The owner of the practice gives immediate personalized feedback to the learner and provides expert modeling. Use of animated avatars provides a more realistic experience and adds to the gaming quality. By playing this game, learners visit different types of operations, are exposed to the myriad of issues affecting control of Johne's disease, and get first hand experience at advising producers. We will demonstrate the features of the game, share the technical aspects and considerations encountered in developing the game, explain the iterative nature of the development process, and discuss the pedagogical basis underlying the design of the game. We will conclude with a discussion with the audience of potential uses and applications for this educational game.

Control Programs

Poster Abstracts

#12

The FUIDI Herd Management Schema

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ABSTRACT

The FUIDI Herd Management Schema is an integrated set of tests and recommendations based upon Infectious Diseases Incorporated's (IDI) conceptualization of the natural history of infection due to *Mycobacterium avium* subspecies *paratuberculosis* and related pathogenic bacteria. The intent of the sequential use of a series of tests and resultant recommendations is designed to enhance retention of productive animals in the herd while reducing the introduction of pathogenic mycobacterium into the human food chain.

INTRODUCTION

The FUIDI Herd Management Schema is based upon an expanded understanding of the natural history of infection due to *Mycobacterium avium* subspecies *paratuberculosis* (Map). The pathogenic mycobacteria in the *M. avium* spectrum of mycobacterium appear to be deeply embedded in the natural food chain of herbivores, accounting for their prevalence in herbivore and rodent populations on a global level (Harris and Barletta, 2001; Turenne et al., 2007). The concentration of animals into herd groups makes animals that cannot attain immune governance over the pathogenic strains prime disseminators of infection, but they are not the ultimate reservoirs of infection. The congregation of animals makes it likely that over time every animal in a large herd will be infected by Map or a polymorphic variant of Map. When a herd is studied from, not the presence or absence of Map specific antibodies, but rather from the point of whether or not active Map replication is occurring, over 70% of animals will attain immune governance and cease to have evidence of continued Map replication (Monif et al., 2009). By so doing, these animals have achieved the prolonged cell-mediated immunity against the prevailing Map strain at the portal for infection. In these animals, relative immunity (auto-vaccination) to reasonable environmental re-expose at the portal of infection (the gastrointestinal tract) is theoretically in place. The probability of persistence and/or progression of infection to clinical Johne's disease are a partial function exposure dose and the strain's relative virulence is not effectively counterbalanced by the animal's cell-mediated immune system.

Animals exhibiting continued Map replication constitute a subgroup for which the risk of organism shedding into milk is heightened. Once substantial infection is established, the probability of subsequent immune capture of Map replication is usually remote. Animals identified as having had prior significant Map antigen processing, using the FUIDI #1 Map ELISA test, but no evidence of active infection, constitute a subgroup of animals that are potentially at risk of Map re-activation of replication at calving, particularly if environmentally and/or nutritionally stressed.

METHODOLOGY

The FUIDI Herd Management Schema is composed of two divergent types of Map ELISA tests: a direct milk Map PCR test and a nested Map PCR test or fecal culture and sets of directives that emanate from the resultant data.

The two FUIDI Map ELISA tests are used to dissect out subgroups at varying risk within the herd. The antigenic composition of the FUIDI #1 test identifies animals that have had significant antigenic exposure to Map at some time. The FUIDI #2 test assesses the probability of active mycobacterium replication.

RESULTS

Figure 1: FUIDI Map Test #1

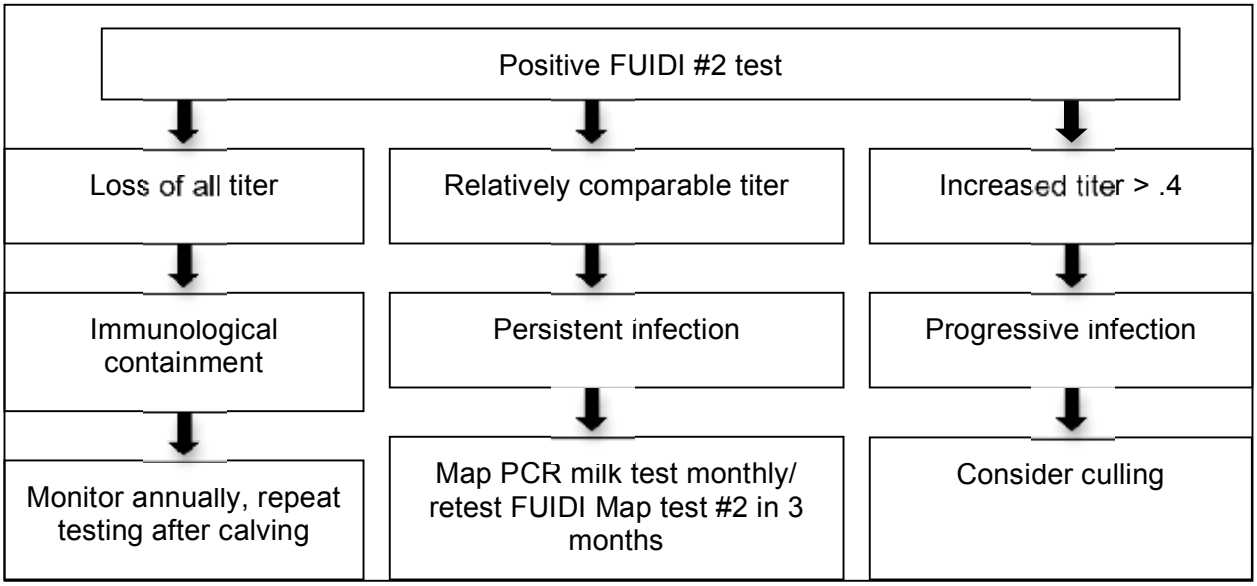

Figure 2: Repeat FUIDI Map Test #2

DISCUSSION

The majority of animals with low-level organism replication and subsequent documented immune capture constitute a subgroup for long-term retention in the herd. Theoretically, such naturally auto-vaccinated animals have now in place permanently enhanced cell-mediated immunity and are better able to handle continued environmental Map challenges. Potentially more important, the progeny from such animals in time will result in heifers better able to handle continued environmental Map challenges.

The animals with ongoing evidence of active antigen processing over time identify animals at augmented risk for intra-herd Map dissemination, shedding into the milk, and progression to clinical disease. Sequential use of the FUIDI tests in conjunction with selective use of Map PCR testing of milk and with resultant guidelines may allow retention of productive animals in the herd while reducing the introduction of pathogenic mycobacterium into the human food chain.

CONCLUSION

The FUIDI Herd Management Schema

1. gives milk producers on-going assessment of their herd's condition,
2. lessens the probability of having bulk milk rejected because of Map detection,
3. over time, allows producers to develop herds with greater inherent immunity to environmental Map challenge, and
4. reduces the introduction of Map into the nation's food supply and in so doing diminishes liability.

By being proactive, dairy producers, more likely than not, can partially shield themselves from the legal liability that will occur when, in a court of law, a jury finds cause for a relationship between Map in commercial milk and Crohn's disease.

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#37 Antimicrobial activity of gallium nitrate against *Mycobacterium avium* subsp. *paratuberculosis* (MAP) *in vitro*

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The objectives of this study were to evaluate the *in vitro* susceptibility of various virulent strains of MAP to gallium nitrate (GaN), and to investigate the potential use of GaN as a prophylactic agent for the control of MAP infections in cattle. Gallium, a trivalent semi-metal that shares many similarities with ferric iron and functions as an iron mimic, has been shown to have antimicrobial activity against various microorganisms, including *Rhodococcus equi*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*.

The *in vitro* susceptibility of several virulent strains of MAP to GaN was tested using broth culture with detection of MAP growth by a non-radiometric automated detection methodology, which employs fluorometric detection of oxygen consumption. Ten different strains of MAP were used, including 4 cattle strains, 2 bison strains, 1 alpaca strain, and 3 human strains. For each MAP strain, a series of 7 dilutions of GaN were tested, ranging from 200μM/L to 1000μM/L concentrations. GaN was determined to have caused 99% inhibition of the MAP growth at that GaN concentration where the time to detection of the MAP undiluted stock + GaN was lengthened to that of the 10-4 dilution of MAP without GaN.

All 10 MAP strains were susceptible to GaN. The susceptibility to GaN was variable between each isolate, and was dose-dependent for all isolates. Overall, the 99% inhibition ranged from < 200μM/L for the most susceptible strains to > 1000μM/L for the least susceptible strains. In general, the most susceptible strains were of alpaca or bison origins, whereas the least susceptible strains were of human and bovine origins.

Gallium nitrate demonstrated antimicrobial activity against all 10 strains of MAP tested *in vitro*, and could potentially be used as a prophylactic agent to aid in the control of MAP infections during the neonatal period.

#40 Managing Bovine Johnes Disease in South Australia using a Dairy Scoring System

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Bovine Johne's disease (BJD) is endemic in cattle in South Eastern Australia and mainly affects the dairy industry. In Australia dairying is primarily based on irrigated pasture and supplementary feeding of cows, rather than housed cattle.

Until February 2005, the management of BJD in the South Australian dairy industry involved mandatory reporting and quarantines to control the spread of disease from known infected herds plus within herd measures to control infection; usually a Test and Cull program and improved calf rearing systems. However, reluctance by producers to report or diagnose BJD in their herds meant the disease continued to spread.

The prevalence of BJD in Australian beef herds is very low and 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. In order to resolve the conflicting interests of the two cattle sectors, a new strategy was designed using assurance based trading principles to replace the regulatory approach.

By 2009 over 95% of SA dairy producers have enrolled in the Dairy ManaJD program and test results confirm the low prevalence of BJD (17%) in the SA dairy sector. Within infected herds the prevalence of BJD is typically 1-2% ELISA positives in animals four years and older, and significantly lower in herds undertaking control measures including Test and Cull programs. The widespread uptake of the voluntary program has enabled producers to trade cattle using the Dairy Score as a risk assessment tool. Higher scores (tested negative herds) have attracted higher prices and greater market access both within SA and nationally.

This paper describes the methodology and outcomes of implementing a voluntary assurance based trading scheme in the SA dairy industry.

#41 Paratuberculosis surveillance in Austria

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Introduction: In April 2006 paratuberculosis in cattle, sheep, goat and farmed deer became a notifiable disease in Austria. Clinically suspicious animals have to be reported to the local authorities.

The aim of the Austrian paratuberculosis surveillance program is the detection and elimination of clinical cases and the implementation of hygiene and management measures at the affected farms.

Material and methods: For laboratory verification of clinical infections blood and fecal samples or tissue samples from the intestine, the intestinal and hepatic lymph-nodes of slaughtered or perished animals, have to be sent to the national reference laboratory for paratuberculosis. Blood samples are tested by ELISA (Pourquier, Montpellier, France) for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) specific antibodies. For the detection of MAP in feces a commercially available real-time PCR kit (Adiagene, France) is used.

Results: Between the years 2006 and 2008 samples from 459 clinically suspicious cattle from 185 farms, from 26 sheep (4 farms), from 2 goats (2 farms) and from 4 farmed deer (2 farms) were examined according to the regulations. 120 cattle and one sheep were tested positive. Table 1 lists the positive results by laboratory method.

Table 1: Positive results in cattle by laboratory method. Number of positive samples: ELISA pos/ PCR (feces) pos: 81; ELISA pos/ PCR (feces) neg: 5; ELISA neg/ PCR (feces) pos: 11; ELISA pos: 7; PCR (feces) pos: 7; PCR (tissue) pos: 9

Discussion: Because of the difficulties in detecting the subclinically infected animals, it is unlikely to obtain paratuberculosis-free herds. This surveillance program is aiming at the detection and elimination of clinical cases in order to reduce the infection pressure in the affected farms. Due to intermittent shedding and pre-mortal antibody-decrease a combination of ELISA and real-time PCR is applied.

#43 A comparison of skin test, ELISA and mycobacteriologic examination in detection of Map-exposed heifer calves: a field study

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Johne's disease is present in a high percentage of dairy herds and causes major economic losses to the dairy industry. Diagnostic tests for detecting serum antibody are not of suitable sensitivity for monitoring *M. avium* ss *paratuberculosis* (Map) infection in young replacement cattle or for detecting subclinical infection in cattle. The specific objectives were to: 1. Obtain information on skin test responses in heifers from Map positive herds, and 2. Determine the specificity of skin tests using Map PPD in heifers 10 to 26 months of age.

The MAP-PPD used in this investigation was prepared by precipitation with trichloroacetic acid (TCA) from the culture filtrate of *Mycobacterium avium* ss *paratuberculosis* ATCC Neotype Strain 19698. The skin test was conducted in the mid-cervical region; skin thickness measured (mm) before injection of PPD and 72 hours following injection. Skin tests were conducted in 842 heifers were from 10 herds in which Johne's disease had been diagnosed by mycobacteriologic examination and in 468 heifers of the same age which were from 16 herds in which Johne's disease had not been diagnosed.

The results revealed that positive skin test responses were detected in heifers 10 to 26 months of age in each of the 10 Johne's positive herds; positive ELISA reactions were detected in heifers in only 3 of the 10 Johne's positive herds. The specificity of the skin test using Map was 98% in heifers 10 to 26 months of age, 95% in cows 26 to 38 months of age and 97% in cows 38 to 50 months of age.

#57 Expert-survey on basically management suggestions for MAP-positive farms

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Beside the ongoing discussions about the improvement of laboratory procedures, the genome of MAP and other important scientific topics a supraregional discussion about a basically on farm program against paratuberculosis should be started.

Many different countries have established voluntary programs to control paratuberculosis and prevent further spreading of the disease. In other countries compulsory registration for paratuberculosis is performed, or strict control and stamping out programs are in action.

To start the discussion and try to find agreement on cost effective and easy to perform steps to fight paratuberculosis, we would like to perform an expert-survey during the 10th ICP on basically management suggestions for MAP-positive farms. We suggest that the survey is introduced in a short presentation at the beginning of the congress and carried out during brakes and poster sessions.

The aim of this survey is to generate effective and simple measurements to reduce the incidence of paratuberculosis in affected herds. This should be the possible start of a trans-national basic program in the fight against paratuberculosis and the protection of free herds and areas which could be implemented immediately.

Beside all differences in actions and opinions this could be considered as a "minimum level" which could be achieved in reasonable time and would be accepted by many countries.

Results of the survey could be presented in the IAP Paratuberculosis Newsletter.

#74 Economic analysis of Johne’s disease control strategies for dairy herds

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Johne's Disease (JD) is a challenge to the dairy industry because of the pervasive economic loss it generates on dairy farms over extended periods of time. Effective control often requires years of diligent efforts. It is crucial for dairy producers to find economically optimal strategies for JD control that will reduce economic losses and thereby maximize profitability. The objective of this study was to determine the most economically optimal method for JD control. A discrete optimal control model was constructed to maximize Net Present Value (NPV) of a farm profit subject to economic and production constraints as well as equations of motion that model the dynamics of MAP transmission within the dairy herd. A GAMS/MINOS program was coded to empirically solve this model for optimal control strategies. Control strategies included test-based culling intervention with annual, semi-annual, and quarterly MAP testing, improved calf hygiene practices as well as off-farm calf-rearing, both for a closed farm and an open farm. Control strategies were compared to determine the most effective method for JD control over the extended planning duration of a dairy farm. Parameters related to JD such as the rates of transition between JD states, and animal turnover in the dynamic model of MAP transmission, were both obtained from previous literature and ongoing research at Cornell. The model allows evaluation of effective controls by farm characteristics. Results show that control or elimination of Johne's disease at the farm level requires at least one of the JD control strategies, otherwise the disease spreads unchecked through the herd. The most cost effective control depends upon economic parameters, which varies by farm and year. Control tactics often display interesting dynamics. Test and cull often produces a cyclical but damping pattern as calves become infected before diseased cows are identified and culled.

#79 Vaccination for Paratuberculosis in New Zealand farmed deer herds

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Aim: To evaluate Silirum® in farmed deer.

Method: Initial studies demonstrated 0.5, 1.0 and 2.0ml doses to three-month-old farmed red and red x wapiti deer (C. elaphus) were clinically safe, produced equivalent antibody and cellular immune responses, and caused few significant injection site lesions. Subsequently, six commercial deer farms previously reporting ≥5% annual incidence of clinical disease in young deer were selected for investigation of efficacy and cross-reactivity with bovine tuberculosis tests.

In March-April 2008, 180 - 1120 deer/farm were randomised to vaccination (0.5ml Silirum®, n=1671) and control (n=1664) groups at 3.5 – 4.5 months of age, and managed together on pasture. Clinical disease, mortality, weight and slaughter data were collected, and 25 vaccinated and control deer per farm faecal sampled for Map culture in November 2008. In January 2009, 180 vaccinated and 181 control deer from three farms underwent a comparative mid-cervical test (CCT) for bovine tuberculosis, also interpreted as a single mid-cervical test (MCT).

Results: By May 2009 when 95% of deer had been slaughtered, disease had been diagnosed in nine vaccinated and 22 control deer on four farms, a tentative overall efficacy of 59% against clinical disease (p=0.02). Some injection site residual lesions were evident but did not affect carcass processing or value. Fifty nine (47%) vaccinates and 68 (55%) controls were faecal culture positive (p=0.2). Tuberculosis testing yielded 44% vaccinates and 23% controls MCT positive, respectively, and correspondingly, two and three were CCT positive. Two controls remained positive to an ancillary Elisa test. Final data will be presented.

Conclusion: Despite the low incidence of clinical Ptb, these data suggest that Silirum® may aid control of clinical disease in young farmed deer. Further data are now needed to better quantify efficacy. Tuberculin testing suggests that the risk of indistinguishable cross-reactivity is low in herds without tuberculosis.

#83 Prevalence reducing factors in the Danish control programme on bovine paratuberculosis

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A risk-based control programme on paratuberculosis in Danish dairy cattle was initiated in 2006. In total, 1265 herds (~28% of all dairy herds and ~40% of all dairy cows) were enrolled in the programme by April 2009. Cows are categorised as High-Risk or Low-Risk animals based on 3-4 annual milk antibody ELISA tests. High-Risk animals require management practices reducing calves exposure to milk and faeces from these cows. Moreover, repeated test-positive cows are recommended to be slaughtered prior to next calving.

The objective of this study was to assess the development in within-herd test-prevalences for different management practices.

A questionnaire on management practices was distributed to all participating herds in January 2009. A total of 1075 (85%) herd-managers returned the questionnaire. Only the 679 herds that started in 2006 were included in the present analyses, because the effect of changes in management on prevalence would be expected at the earliest after 2 to 3 years.

Results of univariable analyses showed that: a) culling of repeated test-positive cows reduced the test-prevalence by 7%-points; b) cleaning of calving area subsequent to High-Risk cows' calvings led to a reduction of 3%-points; c) separation of High-Risk animals from Low-Risk animals resulted in a reduction of 2%-points; and d) use of colostrum from Low-Risk animals only, resulted in a reduction of 1.5%-points. Feeding practices using milk from High-Risk cows did not reduce the prevalence.

The control programme has been running for ~3 years in the herds included, and it is assumed that the full effect will only be observed after 4-8 years. However, already after 3 years, reduced prevalences were observed due to changes in specific management practices. The reductions are not additive due to possible confounding, which could not be assessed at this point in time because of the distribution of management factors.

#87 Evaluation of the surveillance system for MAP infection in Swedish cattle

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ABSTRACT

Previous investigations suggest that the prevalence of *M. avium* subsp. *Paratuberculosis* (MAP) in Swedish cattle is close to zero and all recent cases have been linked to imported animals (the two latest cases were detected in 2000 and 2005). According to Swedish legislation clinical suspicions are notifiable and prompt official investigations. Moreover, eradication measures are taken on detection.

The aim of this study was to evaluate the surveillance system for MAP infection in Swedish cattle and to estimate the probability of freedom from disease. Calculations of surveillance sensitivities and probability of freedom were made using scenario-tree modelling. This type of model allows inclusion of information from several different sources, e.g. random or non-random surveillance data as well as documentations of differences in risk.

The surveillance components identified and included in the present model were fallen stock necropsies, screening of importing herds, surveys of dairy herds, surveillance programme in beef herds and clinical surveillance. Import of animals and participation in the on-going surveillance programme was specified for each tested herd, in order to adjust for differences in risk. Calculations were made for each year from 2005 to 2008 and this formed the basis for a final estimate covering the whole period until 2009. Data including the identity of tested herds, the number of tested individuals etc. were collected from competent authorities and animal health organisations.

Final estimates of the surveillance system sensitivities and the probability of freedom from MAP infections at the end of 2008 are underway.

INTRODUCTION

Infection with *M. avium* subsp. *Paratuberculosis* (MAP) has been included in the Swedish Epizootic Act (SFS 1999:657) since 1952. According to this legislation, clinical suspicions of MAP are notifiable in all animal species and prompt official investigations and eradication measures upon detection.

In 1993, paratuberculosis was detected in an imported beef cow. This led to a thorough tracing and the discovery of a few more infected herds; all of beef breeds and linked to imported individuals (Engvall *et al.*, 1994; Viske *et al.*, 1996). Since then, several activities have been undertaken for the surveillance and control of MAP infections in Sweden (Sternberg Lewerin *et al.*, 2007; Anonymous, 2008). The purpose has been to eradicate MAP from the cattle population and the results indicate that implemented strategies have been successful. In order to evaluate the current probability of freedom, and to estimate the contribution of the different surveillance components to this, a complete investigation of the whole surveillance system regarding MAP infection in cattle has been requested. For MAP infection, which is a slowly progressing disease, it has also been considered important that this type of evaluation is performed while accounting for surveillance data from previous years. It is not reasonable that results from just one year of surveillance would be sufficient to significantly demonstrate freedom from MAP, as it takes several years for clinical disease to develop.

The aim of the present study was to evaluate the surveillance system regarding MAP in Swedish cattle, to quantify the contribution from different surveillance components and to estimate the probability that Swedish cattle are free from MAP.

MATERIALS AND METHODS

A stochastic scenario-tree model was used to estimate the probability of freedom from MAP infection. This method has previously been described by Martin and co-workers (Martin *et al.*, 2007a; Martin *et al.*, 2007b) and allows information from several different sources, e.g. random or non-random surveillance data as well as documentation of differences in risk, to contribute to the quantitative estimation of surveillance sensitivities and probability of disease. In this type of model, factors relating to the structure of the study population and to the probability of detection, or infection, are included as nodes for which input proportions or probabilities are given. One example of the tree-like structure of the model is given in Fig. 1 which shows the risk categories and relevant detection pathways relating to paratuberculosis investigations in fallen stock.

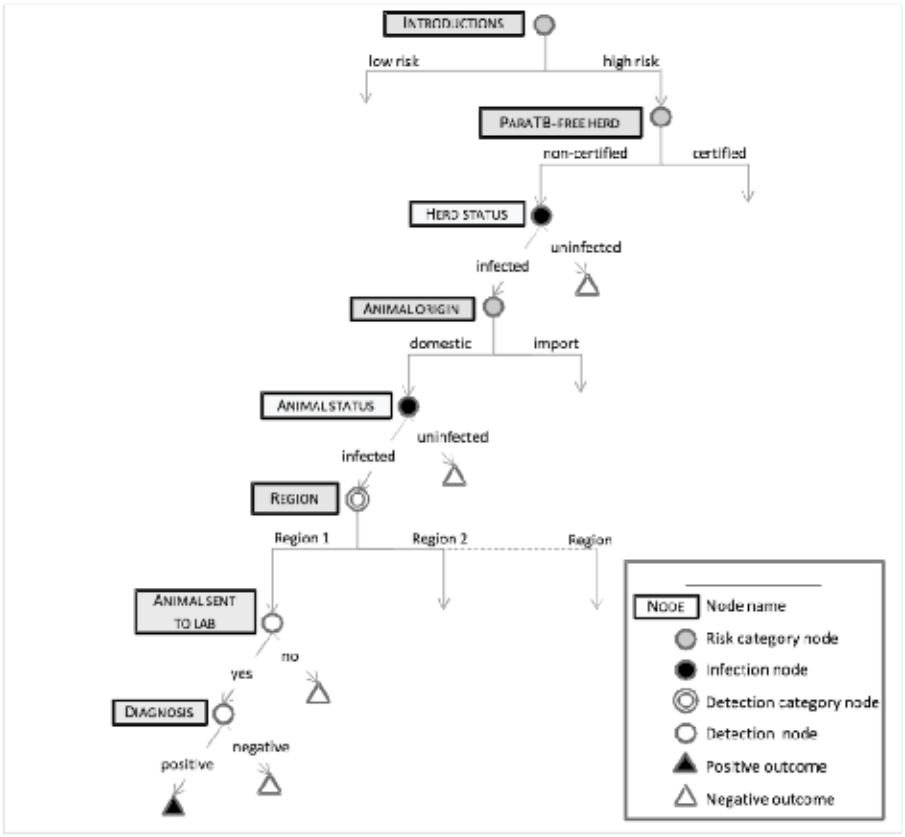

Figure 1. Structure of the scenario-tree model for evaluation of MAP surveillance in Sweden. This example illustrates the surveillance component covering investigations of fallen stock. Empty arrows indicate corresponding branches.

In brief, the scenario-tree model is used to calculate the probability of freedom based on the different probabilities of detecting each type of infected unit, in case infection is present in the population. By this approach more weight is given to investigations in herds and animal of known risk categories.

The probability of detection at the animal level, i.e. the animal-level sensitivity, is used to calculate the herd-level sensitivity. In our model, the herd sensitivity was calculated

separately for each existing herd of cattle in Sweden and was based on the type of herd and the actual number of individuals included in a specific surveillance activity. The numbers of herds and animals included for 2008 are presented in Table 1.

Table 1. Numbers of examined herds and animals within each surveillance component covering paratuberculosis in Swedish cattle in 2008.

Surveillance component	Number of herds	Number of individuals (>2 years)	
		imported	domestic
Fallen stock	8698	3	31371
Surveillance programme	233	42	3198
Clinical surveillance	23994	213	702495

The study includes the main surveillance activities as regards paratuberculosis in Swedish cattle (i.e. both dairy and beef). For the scenario-tree model, these were categorized into the following surveillance system components (SSCs): 1. Fallen stock necropsies, 2. Screening of herds with imported animals, 3. Surveys of dairy herds, 4. Voluntary surveillance programme in beef herds and 5. Clinical surveillance. The probability of freedom was estimated for each year from 2005 to 2008 and the SSCs for each year are presented in Fig. 2. In order to account for the value of previous data, the probability of freedom at the end of the study period was also estimated based on data from all years, 2005-2008. This time period was chosen because the most recent case (one imported animal) was sampled in 2004, and enough detailed data were available for the entire period. The calculations were made in one-year intervals, as illustrated in Fig. 2.

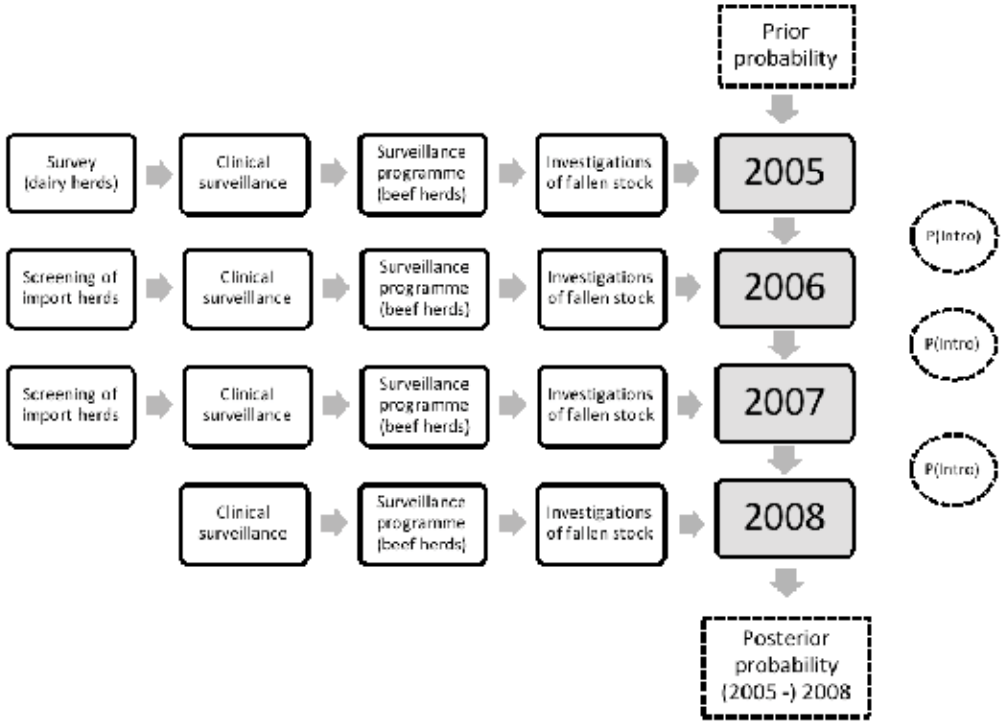

Figure 2. The surveillance components included for each year in the scenario-tree model of MAP surveillance in Swedish cattle, 2005-2008.

For each year, a prior probability of MAP infection in the population was based on the estimated probability from the previous year combined with the annual risk of introducing MAP infection in the population. For 2005, there were no previous estimate available and therefore a probability of 0.5 (i.e. a non-informative estimate between 0 = “not infected” and 1 = “infected”) was chosen. The risk of introduction was based on the number on new imports related to the historical relationship between number of cases and imports. The posterior probability of MAP infection from each year was then calculated based on the prior probability of infection and the value of the new surveillance activities.

RESULTS AND DISCUSSION

Calculations of the sensitivity of different surveillance components, the sensitivity of the surveillance system and the probability of freedom from MAP infections at the end of 2008 are underway. Additional data, such as results from a recently initiated sampling programme at abattoirs may also be included in the final model.

ACKNOWLEDGEMENTS

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#98 An inter-laboratory ring-trial evaluating molecular and culture detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy products: Outcome of an EU ParaTBTools workpackage

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There is a need for standardization and harmonization of culture and molecular methods for the detection/isolation/recovery of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from dairy matrices. The objective of this study was to perform a dairy products ring-trial of culture and molecular methods across European laboratories. The prescribed molecular method used was a Real Time IS*900*-based TaqMan assay in combination with the commercial Map DNA extraction kit (Adiapure®). The prescribed culture method used was HEYM agar supplemented with PANTA, nisin and penicillin.

The 'blind' ring-trial was completed according to the NordVal validation method: eleven participant laboratories, one Map strain, three contamination levels, duplicates for each level and five dairy matrices. The dairy matrices analysed were Map-infected faecally-contaminated milk, artificially-contaminated Gouda, Danish Blue and Munster cheeses and yogurt. The contamination levels of cheeses and yogurt were 5 x 10^3 cfu/g(high) and 50 cfu/g (low) and 1.8 x 10^3 cfu/g(high) and 18 cfu/g(low) for faecally-contaminated milk. In addition to the prescribed methods assessed, 12 alternate molecular and 14 culture 'own laboratory' detection protocols were employed on test samples.

For the prescribed molecular method, average sensitivities across all laboratories, for combined high and low contamination levels, were 95%, 96%, 100%, 84% and 96% for faecally-contaminated milk, yogurt, Gouda, Danish Blue and Munster cheese respectively. Specificities for all matrices were in the range 95-100%. Overall accordance (repeatability) and concordance (reproducibility) values were 0.975 and 91.7% respectively for the molecular method assessed. Culture methods employed showed lower sensitivities than molecular methods, Map was recovered more frequently at 'high' inoculum levels.

The reproducibility of a commercial Map DNA extraction kit used in combination with a Real Time TaqMan assay was demonstrated in this ring-trial. The extraction and assay protocol was applicable to a range of dairy matrices whereas the trial demonstrated the idiosyncrasies associated with Map culture methods.

#106 A gap in the bio security fence. Bought in beef bulls in dairy herds in Ireland (2004-2008)

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Beef breeds are often used as bulls in dairy herds once a sufficient number of cows have been bred to dairy AI sires to ensure adequate supply of replacement dairy stock. The introduction and use of such bulls curtails the potential genetic improvement of the herd but it also leads to an increased bio-security risk especially when source herds are not participating in a disease monitoring/control programme. Diseases such as Johne's disease may be introduced into dairy herds in this way.

A total of 942,000 calves were born to dairy cows in 2008. 51% of these had a dairy sire, 45% of which were by AI, and 49% a beef sire, 32% of these by a stock bull.

If a Johne's control programme in dairy herds is not extended to the producers of these beef bulls then these bulls may be the vehicle for the organism to enter the dairy herd. While a control programme would be much more difficult to implement in a beef herd due to the impossibility of separating calves from adult animals, a certified free programme in closed herds would be possible. A previous study of Irish beef herds had examined animal movements in to and out of case herds infected with Johne's Disease (faecal positive) compared with control herds that were not infected.(negative on a serum survey) [1]. In the current study a total of 478 bulls were purchased by dairy herds from beef case herds, 96 of these bulls subsequently moved to a second herd. The average number of bulls sold from case herds was eleven. A total of 931 bulls were purchased by dairy herds from 167 of the 277 beef control herds, 176 of these bulls subsequently moved onwards to other herds. The average number of bulls sold from control herds was seven.

[1] Mullowney,P.C., Barrett,D., Egan,J., Fallon,R., Blake,M., Good,M ;Cattle movements in to and out of Johne's infected beef suckler herds in Ireland. Proceedings 9th International Colloquium on Paratuberculosis, Tsukuba, Japan, 29 October – 2 November 2007.

#120 Paratuberculosis vaccine interference with bovine tuberculosis diagnosis in farmed red deer (Cervus elaphus)

Colin Grant Mackintosh, Geoff de Lisle, Frank Griffin, *AgResearch Invermay, Mosgiel, New Zealand; AgResearch Wallaceville, Upper Hutt, New Zealand; University of Otago, Dunedin, New Zealand*

Objective: To measure the accuracy of skin tests and ancillary blood tests for diagnosing bovine tuberculosis (Tb) in farmed red deer that have been vaccinated within the previous 12 months with Silirum™, a commercial oil-adjuvanted Johne's vaccine intended for use in cattle and deer.

Materials and Methods: Four groups of 15 three-month-old red deer received the following treatments: Group 1 vaccinated with SilirumTm and challenged with *M. bovis* 20 weeks later; Group 2 vaccinated with SilirumTm and not challenged; Group 3 not vaccinated but challenged with *M. bovis*: Group 4 not vaccinated and not challenged. A bovine skin test (MCT) was conducted 12 weeks after Tb challenge, and a comparative avian/bovine skin test (CCT) was conducted 24 weeks after challenge. The deer were slaughtered and sampled 27 weeks after Tb challenge.

Results: All the deer in Groups 1, 2 and 3 were MCT positive (≥ 2 mm increase), while all Group 4 deer were negative. In the CCT, only 36% of Group 1 were positive (ie increase at bovine site≥ avian site≥2 mm), while Group 3 animals were all CCT positive and Group 4 were all negative. One vaccinated animal in Group 2 had avian = bovine reaction and was classed as CCT positive.

Vaccination also interfered with an IgG1 antibody ELISA for Tb (ETB) conducted 2 weeks post skin testing, causing >80% false-positives in Group 2. Vaccination masked Tb infection in one Group 1 deer. At Week 46, all the deer that had only been challenged

Conclusion: This study showed that vaccination with Silirum™ has the potential to interfere with diagnostic tests for bovine Tb in deer. Vaccination resulted in false-negative Tb tests in Tb-infected deer and false-positive Tb tests in uninfected deer.

#126 The National Sheep Health Statement - a tool for assessing Johne's disease risk in the sheep industries

Rodney Watt [1], David Kennedy [2,] Lorna Citer [3]

[1.] Sheepmeat Council of Australia. [2.] Technical Adviser National Johne's Disease Control Program Animal Health Australia [3.] Manager Endemic Disease, Animal Health Australia

ABSTRACT

Ovine Johne's disease (OJD) has spread widely in Australia since its detection in 1980 and is now established in higher rainfall areas in south-eastern and south-western Australia. Initially the national program was based on traditional tools of zoning, quarantine and attempted eradication from individual farms, and was complemented by the Market Assurance Program to promote low risk flocks. However, without financial support for the owners of infected flocks, producers actively sought to avoid detection of the disease.

A mid-term review of the program provided the impetus to evaluate the risk factors in the spread of the disease and to undertake a quantitative risk assessment that could be form the basis of an assurance declaration form. This was developed into the Assurance Based Credit (ABC) Scheme which has been incorporated into the National Sheep Health Statement (SHS) to encourage prospective purchasers to obtain the NHS before purchasing sheep. The declaration provides producers with the ability to assess the risk associated with the flock of origin and activities undertaken to reduce the risk. The SHS also includes declarations for other important production diseases of sheep.

The current national program was developed and implemented through a partnership between industry and governments and is largely funded by the national sheep industries. It provides trading opportunities for both infected and not assessed flocks using a risk based trading system underpinned by surveillance information, a readily available vaccine and a uniform vendor declaration which is supported by government regulation.

BACKGROUND

Ovine Johne's disease has spread widely since its detection in central New South Wales in 1980 and is now established in higher rainfall production systems in south-eastern and south-western Australia. Sheep are regularly traded across state borders in Australia and, depending on local pastoral conditions, may move several hundred kilometres. This has the potential to seed disease to areas not previously infected with OJD.

Initially the national program was based on traditional tools of zoning, quarantine and attempted eradication from individual farms, and was complemented by the Market Assurance Program to promote low risk flocks. State authorities regulated the program which led to different requirements for disease control and certification between states, and an increasing reliance on state borders to control disease.

Producers were required to complete a state based OJD declaration form each time sheep moved or were traded, which further impacted the sheep trade. Producers were also required to complete additional documentation for movement requirements and for other disease conditions.

Since 2004 there has been a progressive de-regulation of OJD and the adoption of an industry initiated program that has been developed with the support of state animal health authorities. This change of direction in national policy is consistent with similar approaches developed for the management of bovine Johne's disease by other livestock industries.

The National Sheep Health Statement was developed by national industry groups, livestock agents and state governments to provide a standard for the voluntary declaration of sheep health risk across Australia. Prospective buyers of sheep are able to assess the risk a line of sheep represents to their own flock and in some states, is used for declaration of interstate movements.

The statement provides:

- A standardised animal health declaration form that would allow movement of sheep across state borders.
- Information on the health status of other to minimise paperwork.
- A tool for promoting and managing farm biosecurity.

THE NATIONAL SHEEP HEALTH STATEMENT

Industry organisations are very supportive of the move away from regulatory control and an increased emphasis on farm biosecurity. The Statement is a readily accessible tool to assist producers manage their risk of inadvertently sourcing sheep with OJD and also improves consistency and easier movement between states.
There are five sections to the National Sheep Health Statement.

Section 1 - Consignment information: Confirms the origin of the sheep and identification for traceability purposes.

Section 2 - ABC score: A key feature of the National Sheep Health Statement is a producer's declaration about the ABC score of the sheep in the consignment that indicates the level of risk of ovine Johne's disease.

Section 3 and Section 4 – Footrot and other health information: Endemic diseases which had previously required separate state based declaration forms.

Section 5 – Declaration: Although using the Statement is voluntary in most states, it is a legal declaration under the stock diseases legislation and producers may be prosecuted for false declarations. The statement is not valid unless it is signed.

How do producers work out their ABC score?

Under the ABC Scheme, a sheep producer can claim credit points for his or her sheep under four Categories:

A. Area and flock of origin - The known ovine Johne's disease status of the flock or the Prevalence Area from which the sheep originated. (Refer to Figure 1)

B. Testing history, including negative flock tests for the Sheep Market Assurance Program, as well as negative abattoir monitoring.

C. Vaccination history and whether the sheep are vaccinated themselves, as well as whether they come from a fully vaccinated flock and are the progeny of vaccinated sheep. The use of Gudair ™ vaccine is encouraged in all prevalence areas. Vaccinated sheep are eligible for ABC points for vaccination if they are identified by a standard NLIS (Sheep) ear tag incorporating a "V" identifier.

D. Individual veterinary risk assessment for a consignment of sheep undertaken by an Approved Veterinarian.

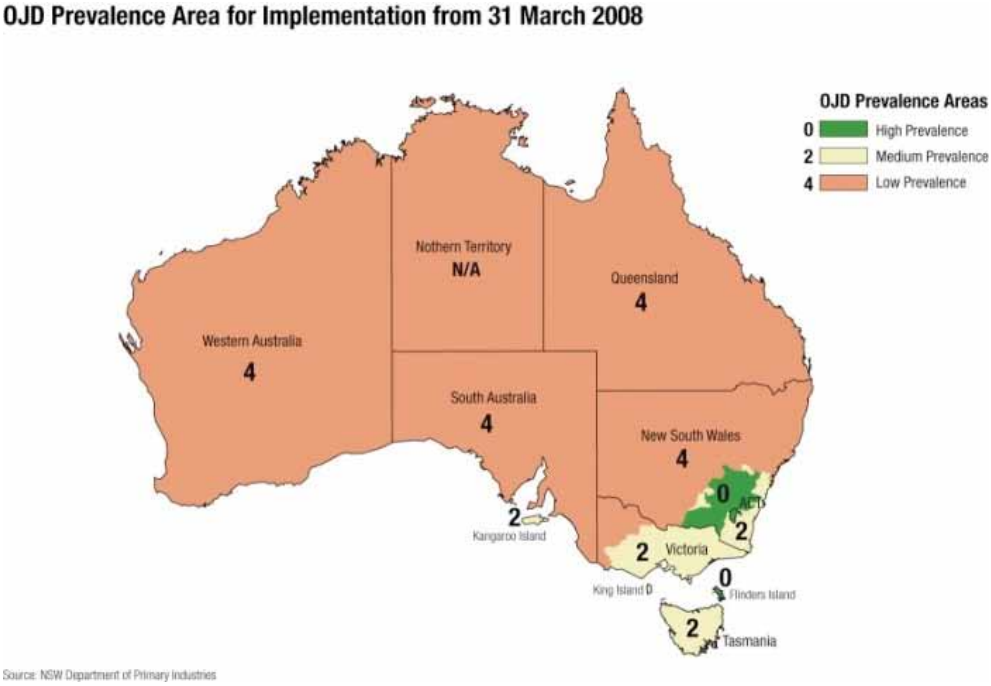

Figure 1. Prevalence Areas, based on flock prevalence estimated from abattoir surveillance of slaughtered sheep, and their respective ABC credits.

Sheep buyers are encouraged to obtain a declaration for any consignments they are interested in buying and to learn what the ABC information on it means for them. Buyers can then determine the risk that those sheep present to their own flock, by relating the score of the sheep offered for sale to their own flock score, and determining whether they want to take that risk. The clear message is that the higher the ABC score of introduced sheep, the lower the risk and the greater confidence in protecting a flock's ovine Johne's disease status.

Extensive promotion of the use of the National Sheep Health Statement was undertaken prior to its launch in March 2008. A simple one page flier was developed with the message *Don't be fleeced when next you buy sheep* and an easy to remember website was developed, www.ojd.com.au . The website is promoted as the central source of information, with links to and from each state website and key industry websites. In some states, animal health authorities have printed hard copies of the National Sheep Health Statement for distribution, but this is not encouraged as it often leads to producers using out of date statements. In the future, an E-form is planned for electronic lodgment and inclusion in pre-sale catalogues.

To reinforce the biosecurity component of this message the National Sheep Health Statement is also readily accessible from a high profile website used to promote general farm biosecurity, www.farmbiosecurity.com.au.

COMPLEMENTARY ASPECTS

On-Farm Disease Reduction Practices

Grazing management and biosecurity planning are also tools of choice for controlling disease on-farm and reducing the risk of spreading infection. Producers are encouraged to develop property disease management plans that

- most importantly, determine the level of risk associated with sheep prior to purchase and introduction by requesting a National Sheep Health Statement.
- use grazing management to reduce bacterial load on contaminated pastures and the development of 'safe' pastures (spelled or grazed with non susceptible stock for 12 months),
- implement biosecurity activities such as double fencing, and
- test purchased rams or other valuable individual animals by faecal culture before they are released into the flock.

Abattoir Monitoring

Abattoir monitoring involves examination of the intestines during the normal meat inspection process at several key sheep abattoirs. It generates data which is used to estimate flock prevalence in geographic regions of Australia and to provide feedback to individual producers on the occurrence of OJD in their sheep. This feedback enables them improve their Johne's disease control or, where monitoring is negative, to declare additional ABC credits under the testing section (C) on the National Sheep Health Statement.

#140 The therapeutic effect of the immunization against paratuberculosis with a heat-killed vaccine results in an increase of the productive life of vaccinated cattle

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The objective of this study was to evaluate the therapeutic efficacy of a heat-killed *Mycobacterium avium* subspecies *paratuberculosis* (Map) vaccine in six tuberculosis-free herds of dairy cattle. The prevalence of infection with paratuberculosis in these herds ranged from 2-10 %. Cattle from the 6 vaccinated farms (n=50) and from 2 unvaccinated farms (n=50) were slaughtered due to paratuberculosis or to other reasons, and feces and gastrointestinal tissues were taken and analyzed by bacteriological culture and histopathology. Clinical signs of paratuberculosis were observed in 28 and 30 % of the vaccinated and control animals, respectively. Immunization with the heat-killed vaccine reduced 23 % the overall mortality and 53 % the culling due to paratuberculosis of the 3-4.5 years old vaccinated animals when compared with the control group; p=0.012 and p=0.047, respectively. The peak of culling was between 4.5-5 years (21 %) in the vaccinated animals and between 3-4.5 years (60 %) in the control animals. Animals were culled at an average age of 4.7 and 3.7 years in vaccinated and control farms, respectively. Thus, vaccination resulted in 1 year of increase in the productive life of the vaccinated herds when compared with unvaccinated animals (p=0.038). In addition, vaccination diminished a 33 % the presence of paratuberculosis-associated lesions in gut tissues of the vaccinated animals (p=0.006) and 24 % the development of severe lesions (p=0.002), multibacillary forms, when compared with the control group. It also reduced a 38 % and 47 % the presence of bacteria in feces and in intestinal tissues of the vaccinated animals, respectively. Although vaccinated animals are not fully protected, the clear regression of the lesions and the substantially reduced Map load in their feces and gut tissues suggests that vaccination protects against the infection by decreasing Map load, which provides important benefits to the farmers.

#148 The externalities associated with the cross-reactivity of Johne's Disease vaccination with tuberculosis in U.S. dairy cattle

Jonathan Dressler, Rebecca Smith, Loren Tauer, Ynte Schukken, Yrjo Grohn

Cornell University, USA

We conceptualize and construct a dairy herd model to capture the externalities associated with the cross-reactivity of MAP vaccination within TB accredited regions given a TB outbreak occurs. We use partial budgeting methods to determine the net benefit of MAP vaccination within each TB accredited region in the U.S. by comparing the benefit of MAP vaccination (ex ante costs) to the extra costs associated with the cross-reactivity with TB test results (ex post costs). We also compare the net benefit of MAP vaccination to non-MAP vaccinated herds within each TB accredited region. Tentatively we find that the benefit of MAP vaccination exceeds the costs of cross-reactivity for a net gain of $16.33 per cow (range of $12.02 per cow to $27.49 per cow), lowering the losses attributed to TB. Overall the losses attributed to TB for MAP vaccinated herds were less than non-MAP vaccinated herds with an average net loss of $44.22 per cow (range $28.04 per cow to $54.19 per cow) compared to $60.55 per cow (range $55.52 per cow to $67.04 per cow).

#168 Paratuberculosis in Iran: past, now and future

Masoud Haghkhah,*Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Iran*

Iran in terms of area at 1.6 sq. km and with a population of over seventy million is a country in the Middle East and Southwest Asia. It has a wide range of climate; ranging more than +50°C in summer to -40°C in winter in some areas. There are two large deserts in the central region with nearly no rain, and vice versa more than 2000 mm raining per year in the north.

The first report of paratuberculosis in Iran comes back to 44 years ago by Talatchian (1965) which showed that the origin of infection was imported animals. From that time till now, there are several other reports from various parts of the country about the occurrence of disease and its undesirable consequences in cattle, sheep, and goats.

In recent years, several researches have been done on the disease which most of them are molecular aspects. Findings of these researches and some unpublished data show that the incidence of the disease has been increased in last decade due to unknown reasons.

There is a scientific committee on paratuberculosis in the Iranian Veterinary Organization (IVO) from 2007. The committee is responsible to make a national program for control and prevention of the disease. Unfortunately, there is no official data on the incidence of the disease in the country. However, the incidence of the disease may be completely different in different provinces on the basis of the climate and the dominancy of the animal species.

In this review, the last information on the disease in Iran and the IVO programs will be discussed.

#169 Therapeutic effect' of goat based 'Indigenous vaccine' in farm flocks of Bharat Merino and Magra sheep naturally infected with Johne's disease and located in two different agro-climatic regions

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Objective: 'Indigenous Johne's disease (JD) vaccine' with known effect (preventive and therapeutic) in infected and challenged goats, was evaluated in naturally infected sheep flocks.

Materials and Methods: Farm of Bharat Marino and Magra sheep located in hot and humid climate (Mananmanur, Tamil Nadu) and hot and arid region (Bikaner, Rajasthan), respectively were endemic for JD. Of 127 Bharat Marino sheep, 97 were vaccinated and 30 were controls. Of 40 positive adult Magra sheep, 30 were vaccinated and 10 were controls. Serum and fecal samples (vaccinated and controls) before and after vaccination were screened (Indigenous ELISA kit' and microscopy). Sheep were monitored for improvements in physical improvements, infection status, mortality, morbidity (diarrhea, etc.), production (growth rate, total wool yield, wool quality) and reproduction performances (twining and tupping percent), survivability, ELISA titers, fecal shedding of MAP. Inactivated JD vaccine' was prepared from native, 'Indian Bison Type' *Mycobacterium avium paratuberculosis* (MAP) strain of goat origin.

Results and Conclusions: Sero-conversion with long term increased titer was observed in vaccinated sheep. Rate and number of MAP shedders decreased with marked overall improvement in body conditions of vaccinated sheep. Average gain in body weights was more in vaccinated sheep. Percent survivability in different age groups and young lambs also increased after vaccination. Survivability of young lambs was much higher in vaccinated sheep. Tupping percent was 100.0% in Bharat Merino farm and problem of diarrhea was completely stopped after vaccination. Morbidity and mortality was also lower in vaccinated sheep as compared to controls. Lambing rate, birth weights and twinning was higher in vaccinated groups. At Bikaner vaccinated adult sheep were cured and mixed with general flock at the end of trial. Quality and quantity of wool was also superior.

Goat based 'indigenous vaccine' had similar 'Therapeutic effect' in sheep as seen in goatherds endemically infected with JD.

#170 Impact of Johne's outreach efforts

Kenneth Earl Olson, *JDIP, USA*

The Voluntary Bovine Johne's Disease Control Program (VBJDCP), operational in the U.S. since 2002, is available to producers in all states. Federal funding for the program has decreased from $21m in 2003 to $10.05m in FY08. Metrics used to evaluate effectiveness of the program include herds enrolled as well as "official" samples tested. In FY07 8,818 herds were enrolled in the program, including approximately 10% of the dairy herds and roughly 0.3% of the beef cow-calf operations raising concerns relative to the effectiveness of the investment. In contrast to these indicators:

i) 94.1% of participants in the NAHMS Dairy 2007 study indicated they were fairly knowledgeable or knew some basics about Johne's;

ii) 30% of the participants reported participation in a Johne's program;

iii) 30% of the dairy producer in a separate national survey indicated participation in a program;

iv) In addition, many DHIA organizations offer Johne's milk ELISA testing.

To document outreach activities and identify impacts not captured in current metrics, surveys were developed and distributed to the Designated Johne's Coordinator in each state as well as select extension, DHIA and industry representatives. Survey results found:

I) A cadre of over 2,000 trained Johne's certified veterinarians available across the nation to work with producer Johne's programs;

II) Meetings held with industry groups reach substantial numbers of producers;

III) Nearly 200,000 milk ELISA samples are run annually by DHIA with producers paying the cost;

IV) DHIA is seeking new ways to provide milk ELISA results with management information to producers.

Survey results demonstrate that outreach activities have had a substantial impact beyond program enrollment. They will be used to help set directions for implementation of the new Johne's strategic plan and provide information useful in maintaining financial support for the program.

#180 Behavioural change in owners of Johne's disease infected beef herds

Nick Keatinge[1], David Kennedy[2], Lorna Citer[3],

[1.] Cattle Council of Australia [2.] Technical Adviser National Johne's Disease Control Program, Animal Health Australia. [3.] Manager Endemic Disease, Animal Health Australia

ABSTRACT

Bovine Johne's disease (BJD) occurs most commonly in Australia in dairy cattle in the south-east of the country. The pure beef sector has very little BJD and trades largely independently of the dairy sector. Beef industry leaders are keen to protect this situation and are supporting owners of infected and suspect herds.

Beef herds that are diagnosed with BJD can suffer discrimination that can have a number of unintended consequences for them and for the national program. They can suffer financial loss through restricted trading options, a decline in land value and social isolation. Unless addressed, these can result in disengagement from the national animal health system, significant under-reporting and undetected spread of the disease.

The Cattle Council of Australia has initiated a program that funds assistance to beef producers whose herds are affected by BJD. The non financial assistance component is provided by two BJD Counsellors who offer an independent mediation service, provide general business advice and facilitate the development of plans to eradicate infection from the herd. Cattle producers who place their herd at risk by introducing dairy cattle or dairy cross cattle cannot access the financial component of the Package.

The BJD Counsellors have been crucial in engaging producers to address their suspicions of new infections or to eradicate long standing disease. For recently diagnosed herds, the support cushions the impact of market discrimination and regulatory control and has led to improved cooperation between producers and regulators and an increased awareness of on-farm biosecurity.

BOVINE JOHNE'S DISEASE IN AUSTRALIA

In Australia bovine Johne's disease is essentially a disease of dairy herds of south-eastern Australia. The dairy and beef industries operate as two distinct sectors with little crossover of stock between dairy and beef breeding herds. About two-thirds of Australia's beef herds are located in Free and Protected Zones areas where there is little or no evidence of Johne's disease. Surveillance of beef herds in south-eastern Australia in the past ten years has indicated a very low prevalence of BJD in the beef sector, with known infected herds accounting for less than 0.1% of the nation's 60,000 beef cattle herds. All these infected herds are located in south-eastern Australia. Control of bovine Johne's disease is based on nationally agreed Standard Definitions and Rules that cover zoning, movement controls and quarantine, testing and culling in infected herds.

THE NATIONAL BOVINE JOHNE'S DISEASE FINANCIAL AND NON FINANCIAL PACKAGE

The Cattle Council of Australia, representing the beef cattle sector, supports strong bovine Johne's disease control to ensure the disease remains contained and any perceived risk to product integrity is minimised. Animal Health Australia, Cattle Council and state governments, have put in place the National Financial and Non Financial (FNF) Assistance Package ('The Package') to assist producers whose cattle businesses are impacted by bovine Johne's disease. A key element of the Package is the contracting of counsellors who provide support to affected producers and facilitate the development of property disease management plans.

The two trained rural counsellors,

- facilitate a 'pathway forward' for producers who were previously unable to trade breeding stock because of their herds' infected status;
- offer support and general farm business advice;
- re-establish links between producers and the state regulatory authorities where communication had broken down;
- heighten awareness of the importance of farm biosecurity; and
- change purchasing decisions to reduce the risk of re-introducing disease.

How the package works

The Package is voluntary and confidential but all beef producers whose herds have been confirmed as 'infected' with BJD or are classified as 'suspect' are eligible.

The counsellors coordinate social and financial support for beef producers who voluntarily undertake to eliminate BJD from their herds. Government or private veterinarians, working with the counsellors, provide the technical advice upon which to base the disease eradication plan in each herd and to ensure that this complies with the Standard Definitions and Rules for BJD.

When a new infected beef herd is detected the owner is sent information about the Package. Those who take up the invitation are visited by the counsellor to help them decide how to proceed. Follow-up support involves the approved veterinarian and the producer's own financial adviser. The team assesses possible investigation and eradication options, whether they are likely to succeed and the financial impacts on the farm business.

Once an approach is agreed to eradicate the infection, an Enhanced Property Disease Management Plan (EPDMP) is developed with the producer. Producers receive funding to support its implementation, which typically involves identification of the high risk groups in the herd (based on history and testing) and culling these to slaughter. After the infected cattle are removed, a 12-month decontamination period starts. The funding assists in the re-stocking of the beef herd with cattle that must be sourced from a low risk *Beef Only* herds or beef herds in the Australian Johne's Disease Market Assurance program for Cattle (CattleMAP). *Beef Only* is a herd category to help assure cattle buyers about the very low risk of bovine Johne's disease in beef herds that have had no contact with dairy cattle

Results

Since the commencement of the Package, A$3.8 m has been committed to assisting 89 producers, 77% of whom have now progressed so that their herds' 'infected' or 'suspect' statuses have been successfully resolved.

Table 1. Progress in the National Financial and Non Financial Assistance Package since 2004.

Number of herds	Total
Invited to participate	149
Financial assistance accessed	89
Non Financial assistance accessed	21
Completed program	72

A recent independent review of the Package considered "that the role of the bovine Johne's disease Counsellors has a very high impact on the effectiveness of the Package. Both private and government veterinarians were highly appreciative of the Counsellors, especially

the fact that they had the time to sit down with the owners to discuss budgets and the various marketing options before a final decision on EPDMPs was made".

The review also noted that veterinarians involved in the program considered that the Counsellors had developed good technical knowledge of bovine Johne's disease control over the life of the Package and their "second opinions" provided reassurance to producers.

ACKNOWLEDGEMENT
Cattle Council of Australia and Animal Health Australia wish to acknowledge the support, enthusiasm and skills of Mr David Allan and Mr Campbell Trotter, the bovine Johne's disease counsellors working with producers as part of the National Financial and Non Financial Assistance Package.

FURTHER INFORMATION
For further information please refer to:

http://www.animalhealthaustralia.com.au/programs/jd/bjd.cfm

http://www.animalhealthaustralia.com.au/aahc/programs/jd/nbjdsp$/financial_assistance.cfm

http://www.cattlecouncil.com.au

#181 Continued shedding of MPtb from Australian sheep vaccinated with Gudair™

Jeff Eppleston, Jeff Eppleston, Peter A Windsor, Evan Sergeant, Navneet K Dhand, Richard J Whittington,
Tablelands LHPA, Australia; University of Sydney, Australia; AusVet Animal Health Services, Australia

Gudair™ was registered in Australia to control paratuberculosis in sheep after our previous research demonstrated its efficacy for reducing mortalities and shedding of *Mycobacterium avium* subsps. *paratuberculosis* in treated lambs. Subsequently a national risk-based trading scheme was developed that allowed trade in 'low-risk' vaccinated sheep. However our original study was conducted in high challenge environments while in practice vaccine has also been used in lower prevalence flocks. There has also been a recent trend to not vaccinate wether lambs because they are being sold at young ages. We report 2 further studies investigating the impact of vaccination on faecal shedding in flocks of variable prevalence, and the risk associated with leaving wethers unvaccinated.

In a longitudinal study we monitored the prevalence of shedders across 4 generations of vaccinated lambs in 11 flocks that varied in their initial disease prevalence. Cultures from 3, 4, 5 and 6 year-old sheep were collected every 2 years on 3 occasions. Vaccinates had significantly less shedding than non-vaccinates (0.63% versus 1.66%; $P < 0.001$) with unvaccinated sheep 7.9 times more likely to be pool positive compared to vaccinates. The flock prevalence declined from 2.1% at Sampling 1 when all age groups were unvaccinated, to 0.9% at Sampling 3 when all age groups were vaccinated. However at Sampling 3, 10 of the 11 flocks had detectable shedding (range 0.1% -1.3%) indicating that despite almost no mortalities in flocks vaccinating for the 6 years of the study, shedding persists at levels capable of spreading disease.

In Study 2 shedding in 1-2 year old ewes vaccinated as lambs and their unvaccinated wether cohorts was compared across 6 flocks. Shedding was detected in 5 of the 6 wether flocks but in only 2 of the 6 ewe flocks. The implications for flock and national control programs will be discussed.

#182 An integrated risk based approach to the management of Johne's disease in Australia

Lorna Citer[1], David Kennedy[2],

[1.] Manager Endemic Disease, Animal Health Australia [2.] Technical Adviser National Johne's Disease Control Program, Animal Health Australia.

ABSTRACT

Australia's National Johne's Disease Control Program was established in 1996 to provide effective coordination of Johne's disease programs across all states, territories and affected industries.

The program aims to protect the favourable status of Johne's disease in large parts of Australia and reduce the impact of the disease and control measures on industries by managing the risk across a number of affected species and the impacts that one industry's control strategy could have on another industry.

The initial approach, based on Market Assurance Programs (MAPs) at the farm level and a traditional model of regulatory animal disease control, was inadequate within endemic areas as Johne's disease continued to spread. Affected industries and governments sought better management strategies which resulted in the development of additional management tools. As a result, Australian industries have moved toward risk-based trading for endemically infected regions, with governments taking a more targeted regulatory approach in other regions.

Operational assurance systems, such as the Assurance Based Credit (ABC) scheme for sheep, *Beef Only* and *Q-Alpaca* have been developed for each industry sector. These are supported by nationally standardised written declarations which identify the level of assurance of the animals being sold or moved. The declarations are known as "national animal health statements" and are supported by state legislation to ensure the integrity of the statements that are made by producers.

BACKGROUND

The Australian National Johne's Disease Control Program (NJDCP) is a cooperative program involving Australian livestock industries, government and the veterinary profession and managed by Animal Health Australia (see Figure 1).

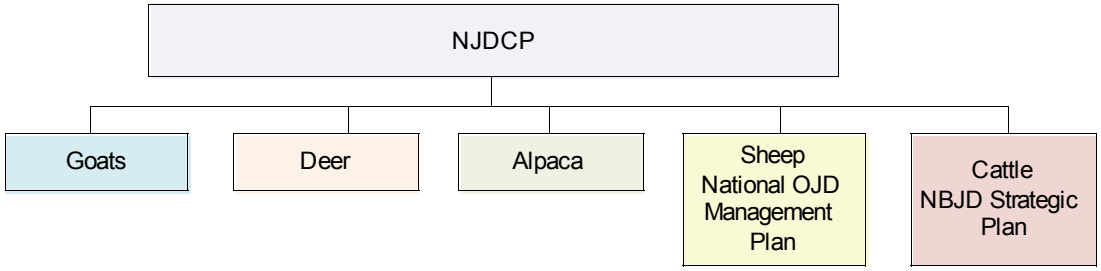

Figure 1. Structure of the Australian National Johne's Disease Control Program

The earliest national approaches comprised:

- Voluntary Australian Johne's Disease Market Assurance Programs (MAPs) at the farm level;
- Traditional state regulatory animal disease control programs based on quarantine and area movement restrictions conforming with a set of nationally agreed Standard Definitions and Rules; and.
- National standards for diagnostic tests.

Regulatory programs had successfully eradicated bovine tuberculosis and brucellosis from Australia and, in the 1990s, was the accepted methodology for the management of infectious disease generally, including Johne's disease. In northern regions of Australia, where the Johne's disease is rare or absent, risk assessment and management is still undertaken at a population level and governments manage regulatory programs that include quarantine and depopulation whenever the disease is identified. However, regulatory practices proved inadequate to control the spread of both bovine and ovine Johne's disease (called BJD and OJD respectively) in south-eastern Australia. In 2003, OJD was also detected in the previously free zone of Western Australia.

It was agreed that an alternative approach was needed to address some of the inequities that made producers reluctant to investigate suspect Johne's disease in their herds and flocks. A search for better risk assessment and management strategies redirected the Australian approach towards voluntary risk-based trading schemes. Over the past five years different flock or herd risk based trading schemes have been developed to assist producers or industries to manage the risk of Johne's disease. Each of the industry schemes is complemented by nationally agreed written declarations that producers can use to declare the level of assurance of the animals being sold or moved. The declarations are known as "national animal health statements" and are underpinned by state legislation to ensure the integrity of the statements that are made by producers.

The benefits of the change from regulation to an effective voluntary risk based approach include:

- The need to involve all producers is recognised.
- Producers' fear of having infected herds or flocks is reduced.
- Trading options are provided, whilst the risk of and rate of disease spread should be reduced.
- A fair, supportive environment encourages cooperation.
- Tools are developed to help producers of infected herds/flocks to trade.
- Awareness and understanding of Johne's disease risk increases.
- Low risk herds and flocks are promoted as sources of replacements.

ASSURANCE BASED CREDIT (ABC) SCHEME FOR SHEEP

The scheme was introduced in 2004, based on a quantitative OJD risk assessment model that was developed for the Australian wool industry. A sheep producer can claim credit points for his or her sheep under four categories up to 10 credits in total. The higher the ABC score, the greater is the assurance that the sheep are unlikely to be infected with ovine Johne's disease. The declaration is made by the owner in writing on the National Sheep Health Statement.

The four categories are:

A. Area and flock of origin - The known Johne's disease status of the flock or the prevalence of OJD (estimated from abattoir surveillance) in the area in which the flock is located.
B. Ovine Johne's Disease testing history, including flock tests for the Sheep Market Assurance Program and inspection of sheep at abattoirs.
C. Vaccination history of the sheep and the flock.
D. Risk Assessment of the particular consignment of sheep by a veterinarian.

NATIONAL DARIY BOVINE JOHNE'S DISEASE ASSURANCE SCORE

The dairy industry's Dairy Score aims to help dairy farmers assess the risk of bovine Johne's disease (BJD) when they are buying or selling stock, or seeking to improve the Score of cattle within their herd. It is not a quantitative risk assessment but ranks the existing regulatory and MAP herd statuses, based on scientific principles for bovine Johne's disease management and provides clear pathways to progress. A herd can declare a base score

between 0 to 10, depending on the herd's BJD history, testing history and prevalence, geographical location (zone) and calf rearing practices. The higher the Score the greater is the assurance. Herds that do not test or implement calf rearing are rated below infected herds at a Score of 0.

As hygienic calf rearing can significantly reduce the risk of calves becoming infected with bovine Johne's disease, calves reared under the Victorian Johne's Disease Calf Accreditation Program (JDCAP) are eligible for 3 extra points and those reared under an approved 3-Step Calf Rearing Plan are eligible for 1 extra point.

Q-ALPACA

After an initial outbreak in the early 1990s, Johne's disease has been virtually eradicated from alpaca in Australia. However, alpaca often graze with other livestock and to help assure its members' herds, the Australian Alpaca Association initiated this voluntary scheme with the endorsement of State and Federal animal health authorities in 2005. By requiring veterinary investigation of any death within a herd it not only monitors and assures for Johne's disease, but also monitors and assists management of other significant endemic diseases, such as severe worm infestation, liver disease, liver fluke infestation and coccidiosis.

GOAT RISK RATING

The most recent scheme aims to improve understanding of goat health and reduce risks associated with Johne's disease and a number of other important production diseases including footrot, caprine arthritis encephalitis (CAE) and lice, as well as drenching and vaccination history. Developed in 2008 by the Goat Industry Council of Australia (GICA) with state animal health authorities, it also promotes a simple message that higher the rating, the lower the risk. Like the sheep scheme, owners can voluntarily declare their herd's Goat Risk Rating on the National Goat Health Statement

CONCLUSION

The implementation of risk based trading has largely 'depoliticised' the management of Johne's disease and facilitated trade in south-eastern Australia where eradication of the disease is not considered possible in the foreseeable future.

ADDITIONAL INFORMATION

For additional information please refer to:

Animal Health Australia
http://www.animalhealthaustralia.com.au/programs/jd/jd_home.cfm

Australian Alpaca Association
http://www.alpaca.asn.au/pub/AAA/qa/docs/qa_faq2009.pdf

Dairy Australia
http://www.dairy.com.au/bjd

#185 Efficacy of 'Indigenous vaccine' in extended field trials conducted on the farm goatherds naturally infected with Johne's disease

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Objective: Extended field trials were conducted to know the efficacy of 'Indigenous Johne's disease (JD) vaccine' in goatherds naturally infected with MAP (therapeutic effect).

Materials and Methods: 'Indigenous vaccine' (extended field trials) was evaluated in two herds of goats naturally infected with Johne's disease, consisting of tall breeds from south- western India located at CIRG, Makhdoom. Goats were vaccinated with 'indigenous vaccine', after failing to contain MAP infection by traditional test and cull method for past 25 years. One herd consisted of 219 existing goats of Sirohi, Jamunapari, Marwari, Barbari and local non-descript breed and newly purchased 66 goats of Sirhoi and Barbari breeds were vaccinated. Another herd consisting of 228 goats was of Jakharan breed of northern Rajasthan and was endemic for JD. Existing parameters in-terms of health status and productivity were control. Serum and fecal samples of vaccinated goats were collected before and after vaccination and screened by Indigenous ELISA kit' and microscopy. Goats were monitored for improvements in physical conditions, infection status, mortality, morbidity (diarrhea, etc.), production (growth rate, milk yield) and reproduction performances (twining percent), kid survivability, ELISA titers, fecal shedding of MAP. Inactivated JD vaccine' was prepared from native, 'Indian Bison Type' *Mycobacterium avium paratuberculosis* (MAP) strain of goat origin.

Results and Conclusions: There was visible improvement in the physical condition of vaccinated goats. Goats regained luster and shine of body coat, were alert, active, glistening eyes etc. Sero-conversion with long term increased titer was observed in vaccinated goats. Number of MAP shedders decreased with marked overall improvement in body conditions of vaccinated goats. Average gain in body weights recorded constant improvement. Percent survivability, birth weight and growth rate was higher especially in young kids. There was significant improvement in twining percent (60.0%) and milk yield (90 day, 120 days and total milk yield). Morbidity and mortality was reduced significantly. Improvement was more in newly purchased goats as compared to existing goats at the farm.

'Indigenous vaccine' showed 'Therapeutic effect' in goatherds endemically infected with JD.

#203 Milk quality assurance for paratuberculosis in the Netherlands

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INTRODUCTION

A bulk milk quality assurance program (BMQAP) for paratuberculosis in Dutch dairy herds was initiated in 2006. The aim of the BMQAP is to reduce the concentration of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) in milk delivered to the milk factories. The BMQAP is run alongside the pre-existing 'Intensive Paratuberculosis Program' (IPP; aiming at elimination of *Map* and low-risk trade of cattle).

PROGRAM

The BMQAP starts with an initial assessment consisting of a single herd examination. Test-negative herds enter a surveillance procedure consisting of biennial herd examinations and are assigned 'Status A' (formerly 'green'). Test-positive herds enter a control procedure consisting of annual herd examinations and are assigned 'Status B' if test-positives are culled and 'Status C' otherwise (formerly 'red'). Herd examinations consist of a milk-ELISA of all lactating cattle. Milk samples regularly collected for Milk Production Registration are used for testing. ELISA results may be confirmed by individual fecal culture.
The program promotes preventive management measures to reduce the risk of introduction and spread of Map in participating herds.

RESULTS

Results of the program achieved in 2006 and 2007 were presented at the 9ICP (Weber and van Schaik, 2007). The results of the program are in line with our expectations based on modeling studies, presented at 8ICP (van Roermund et al., 2005; Weber et al., 2005). The program turned out to be farmer friendly, easy to communicate, and easy to manage.
Based on the results achieved in 2006 and 2007, the Dutch dairy processing industries have taken the lead to increase the uptake of the BMQAP. In 2008, the majority of costs for participating farmers were covered by the dairy processing industries. This resulted in a major increase of the uptake of the BMQAP. By June 1st, 2009, approximately 17,000 (85%) of the 20,000 Dutch dairy herds participated in the BMQAP, alongside approximately 450 (2%) herds in the IPP (Fig. 1).

FUTURE DEVELOPMENTS

The Dutch dairy industries have decided that by 2010 all Dutch dairy herds delivering milk to their factories are required to participate in either the BMQAP or the IPP. Moreover, by 2011 all herds are required to have at least status 'A' or 'B'.

CONCLUSION

It is concluded that the bulk milk quality assurance program attracts increasing numbers of participants, is appreciated by the Dutch dairy industries, and is a practical and attractive way to assign a herd-status in relation to the aim of the program.

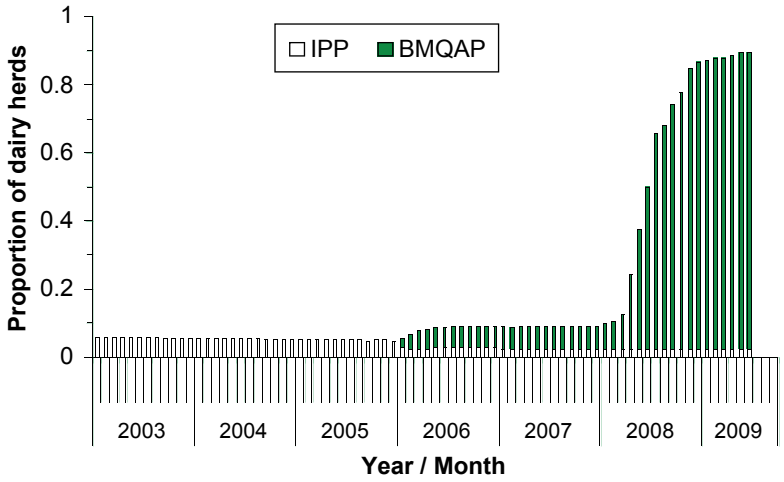

Fig. 1. Proportion of participating dairy herds in the bulk milk quality assurance program for paratuberculosis (BMQAP) and the pre-existing 'Intensive Paratuberculosis Program' (IPP) in the Netherlands.

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#234 Johne's disease vaccine: a cohort study measuring long-term effectiveness of the whole cell killed bacterin

Barbara Knust, Darlene Konkle, Jeffery Bohn, Scott Wells, Elisabeth Patton, *University of Minnesota, USA; Wisconsin Department of Agriculture, Trade and Consumer Protection, USA*

In this prospective cohort study, three commercial dairy herds vaccinated every other heifer calf using a whole cell killed Johne's vaccine until two cohorts were obtained. Other Johne's disease management efforts on the farms were used, including annual risk assessments, but the management of vaccinates and controls was the same in the herd. Fecal samples from heifers from the cohort groups were collected at first calving and at 90 days of pregnancy at each subsequent lactation and tested using bacterial culture with liquid media. Fecal samples from the rest of the herd were also collected and cultured annually at 90 days of pregnancy. Herd production data was collected on a semiannual basis, and data regarding culling and reasons for culling was recorded. Baseline prevalence estimates indicated the herds were moderately to heavily infected with Johne's disease at the start of the study. Five years after the initial cohort animals were vaccinated, significantly fewer vaccinates were culled due to clinical Johne's disease, and vaccinates had a significantly lower level of fecal shedding. Survival analysis found vaccinates survived a significantly longer time until being culled for clinical disease, and had a longer time to first positive test result than controls. Vaccination however did not have an effect on the overall survival in the herd if culling for any reason was measured. Whole herd fecal shedding prevalence decreased on all three farms from the beginning of the study. The results of this study demonstrate that use of the commercially available Johne's disease vaccine serves as a valuable tool in reduction of clinical signs and eventual disease eradication.

#236 Producer's perception of a voluntary Johne's disease control program in Ontario and Western Canada

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Producer's compliance with recommended management practices is crucial for the success of Risk Assessment (RA) based Johne's disease (JD) control programs. The objective of this study was to assess dairy producers' perception of JD and a JD RA based control program.

A voluntary RA based control program for JD was initiated in Ontario and Western Canada in 2005-07. A subset of 238 producers were contacted through a telephone survey in 2008 asking about their perception of the importance of JD, the program and costs for implementation of farm-specific recommendations as well as their reasons against implementing suggestions.

In 2005-07 most farms were test-negative and in 2008 most producers did not see JD as a problem for their farm. Producers indicated that they were participating in the program, because they saw the economic impact of the disease, wanted to be proactive or because they were concerned that the consumer's perception of an association between MAP and Crohn's disease could affect the dairy industry. The program and the farm-specific recommendations were generally well received. The majority of producers had implemented at least one recommendation. However, on average only 2 out of 6 suggestions were implemented. The recommendation that had the highest compliance was culling of test-positive cows. This was also the main reason why producers decided against further changes. After they culled the test-positive cow, they did not see the necessity for additional changes. Most producers were unable to quantify the costs associated with management changes, but felt that some changes actually saved time and money. In addition, many producers reported better herd health or less calf disease after implementation of at least one recommendation. In conclusion, these results indicate that these recommendations can lead to improved herd health which may be used to convince producers to implement recommended management changes.

#240 A survey of dairy producers in the United States concerning Johne's program participation

Ernest Hovingh, Kenneth Olson

Penn State University, USA; KEO Consulting, USA

The United States Department of Agriculture has promoted a voluntary Bovine Johne's Disease control program with funds being made available to states to support program implementation. The number of enrolled herds showed evidence of plateauing and information was desired about the reasons that producers did or did not participate in the program, and the perceived value of the program.

A survey was developed and mailed to a systematic random sample (N=8,013) of all USA dairy herds. Additional mailings were used to increase the return rate, and 2601 surveys were returned.

Herd size distribution of respondents was consistent with other national data. Herds participating in their state's JD program (27%) were slightly over-represented in the dataset. 48% of the respondents "agreed" or "strongly agreed" that JD was currently a concern in their herd, and 55% indicated they expected it to be a future concern.

Logistic regression modeling was used to determine the factors associated with the probability of participation in the state Johne's program. Regional, herd size, disease knowledge and motivational factors were among those evaluated. 24% of responders that were not participating in the state program felt that they were already doing everything they could to manage JD, such that program participation was perceived to provide no additional benefit. The respondents appeared to have a reasonable level of knowledge of the transmission of JD, but misunderstanding about the interpretation and use of diagnostic test results was evident in the results.

Questions were asked to determine the economic importance of JD. 74% of the respondents indicated that they would be willing to pay a premium for replacement animals that had at least a 95% probability of being free of JD.

The results of this study will be useful to guide Johne's education and control program implementation efforts.

Pathogenomics and MAP Biology

Convenors: Adel M. Talaat and Luiz E. Bermudez

Genomic Perspectives on the Pathogenesis of *M. avium* subsp. *paratuberculosis.*

Adel M. Talaat

University of Wisconsin-Madison, USA

The way we approach the study of molecular pathogenesis of *M. paratuberculosis*, the causative agent of Johne's disease in cattle, has changed dramatically with the arrival of the genomics era by sequencing the genome of *M. paratuberculosis*. My group among other groups led the way in developing novel tools to dissect the genetic basis of *M. ap* infections on a genome-wide scale. One of the employed approaches was the construction of mutant library of *M. ap* that was screened to identify genes important for virulence as well as for lipid biosynthesis. Another approach involved the development of DNA microarrays to analyze the transcriptional profile of both the bovine host and the infecting *M. ap* growing under variable conditions. Finally, sequence-based approaches were introduced to investigate genomic composition and genomic topology of *M. ap*. In this presentation, I will discuss the latest on the pathogenesis of *M. ap* infection following the application of key genomic approaches. Our goal here is to shed lights on the current and future efforts that will shape the field of Johne's disease to better understand the pathogenesis of this disease and to develop novel intervention strategies against Johne's disease.

#45

Identification of genes expressed by *Mycobacterium avium* subsp. *paratuberculosis* in subclinical Johne's disease and *in vitro* infected macrophages

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Veterinary Population Medicine, University of Minnesota, USA; Minnesota Supercomputing Institute, University of Minnesota, USA; Veterinary Diagnostic Laboratory, University of Minnesota, USA

Mycobacterium avium subsp. *paratuberculosis* (MAP) uses a diverse set of genes in the cellular environment for its survival and pathogenesis. We hypothesized that genes expressed exclusively in the in tissues or in macrophages would contribute to MAP virulence. We studied the MAP gene regulation in ileum and mesenteric lymph nodes of two subclinical Johne's disease (JD) animals or in an *in vitro* macrophage infection model. Total RNA was extracted from tissues or *in vitro* infected monocyte derived macrophages. Mycobacterial mRNA was enriched and amplified, reverse transcribed to cDNA and competitively hybridized to a MAP K10 microarrays. We evaluated only those genes differentially expressed in tissues distinct from those expressed in a nutrient rich broth environment. Genes related to cell envelope biogenesis, carbohydrate metabolism (glucose-6-phosphates, glucokinases), fatty acid metabolism (acyl-coA dehydrogenases, glycosyl transferases), virulence (MCE family proteins, PPE family proteins, serine/threonine protein kinases), transcriptional regulators, ABC transporters and oxidoreductases were differentially expressed by MAP in tissues or macrophages. Gene expression patterns were similar between the infected tissues and macrophage infection at three time points and shared common functions as determined by pathway analysis. Our analysis establishes a repertoire of genes used by MAP in the host, which is expected to aid in the development of successful vaccination strategies for the effective control of JD in herd level.

#89

Mycobacterium avium subspecies *paratuberculosis* isolates recovered from infected goats in the central Spain contain deletions of mce genes and an increased capacity for persistence in macrophages

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This study used the ParaTBtools MAPAC microarray to perform comparative genomic hybridization (CGH) on MAP isolates recovered from a goat herd in Spain. These animals presented unusual features on histopathological investigation including moderate to severe diffuse lymphocytic infiltration with a heavy macrophage load but a very low cytoplasmic bacterial load as determined by Ziehl-Neelsen staining.

CGH analysis revealed a novel deletion, consisting of 19 consecutive ORFs which including a complete *mce* gene operon, *PE*/*PPE* family genes, a transcriptional regulator and several other genes with either putative or unknown function. Findings were confirmed by a PCR-based approach and sequencing. A panel of 97 Spanish MAP isolates was also screened using this PCR-based approach revealing 53 isolates containing the same deleted region. Epidemiological analysis correlated *mce* negative MAP isolates with herds having a history of animal transfer.

Parallel infections of a representative mce negative isolate (MAP 464) and a reference isolate (MAP K-10) into a macrophage cell line showed a significant increase in intracellular persistence associated with MAP 464. Further studies of differential *mce* expression between MAP 464 and MAP K-10 will also be presented.

#117

Adsorption of MAP organisms to soil particles

Navneet Kumar Dhand, Jenny-Ann LML Toribio, Richard Whittington

University of Sydney, Australia

Attachment of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to soil particles could increase their availability to farm animals which typically consume hundreds of grams of soil per day during grazing, as well as facilitate transportation of MAP to water sources. To investigate the possibility of such attachment, we passed a known quantity of MAP through chromatography columns packed with various substrates (clay soil, sandy soil, pure silica, clay-silica mixture and clay-silica complexes) and quantified the organisms recovered in the eluent using culture or quantitative PCR. Experiments were repeated using buffer at a range of pH levels with pure silica to investigate the effect of pH on attachment. Linear mixed model analyses were conducted to compare the proportional recovery of MAP in the eluent between different substrates and pH levels.

Of the organisms added to the columns, 54 to 99% were estimated to be retained in the columns after adjustment for those retained in blank columns. The proportions recovered were significantly different across different substrates with the retention being significantly greater ($p < 0.05$) in pure substrates (silica and clay-silica complexes) than soil substrates (clay soil and sandy soil). However, there were no significant differences in the retention of MAP between silica and clay-silica complexes or between clay soil and sandy soil. The proportion retained decreased with increasing pH indicative of greater adsorption of MAP to soil particles at an acidic pH ($p < 0.05$). The results suggest that under experimental conditions MAP adsorbs to a range of soil particles and this attachment is influenced by soil pH.

#160

Hidden gems in the *Mycobacterium avium* subsp. *paratuberculosis* genome

John P Bannantine[1], Adel M Talaat[2], Michael L Paustian[1], Srinand Sreevatsan[3]

[2]National Animal Disease Center, USDA-ARS, Ames, Iowa, USA; University of Wisconsin, USA; University of Minnesota, USA

If 4,350 genes annotated in the *M. avium* subsp. *paratuberculosis* strain K-10 genome wasn't already enough to study, more genes have recently been uncovered, hidden deep within this genome sequence. Genomic and proteomic studies, both published and unpublished, have revealed a handful of new genes missed by annotation of the K-10 genome. BLAST searches of the intergenic regions present in the annotation shows several new genes. Furthermore, an automated annotation of the K-10 genome by the Venter Institute (JCVI) has revealed an additional 188 genes. Most of the genes missed in the original annotation are located between two annotated genes (intergenic region) while a few others are either discovered as part of a misalignment or exist on the opposite DNA coding strand. A catalog of these genes will be presented with a focus on three selected genes of special interest in diagnostics and pathogenesis. One of these encodes a protein that is detected by a monoclonal antibody that uniquely binds to *M. avium* subsp. *paratuberculosis*.

#222

Atypical structural features of two MAP P60 family members

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[1]University of Missouri Kansas City, USA; [2]National Animal Disease Center, USDA-ARS, Ames, Iowa, USA

The majority of Map gene products have no known function. In order to better understand the pathobiology of this mycobacterium, we have begun to study the structure-function relationship of a subset of Map proteins. We have selected a number of gene products unique to Map which are either predicted to be secreted or expressed on the surface of the mycobacterium. Here we report the atomic resolution crystal structures of two Map proteins, 1272c and 1204. Both proteins are members of the NlpC/P60 superfamily of peptidylglycan hydrolases and are predicted to be expressed on the surface of the bacteria. Surprisingly, neither protein appears to have a functional catalytic core. It is clear from the structure of 1272c that the residues required for hydrolysis are absent, strongly suggesting a role as a binding protein or receptor for this protein. While the Cys-His-Glu catalytic triad is present in 1204, other residues occlude access to the catalytic site. Based on these two structures, and a thorough search of the Map genome, we can conclude that the canonical role of a peptidylglycan hydrolase is not fulfilled by either 1204 or 1272c. As both these proteins are strongly recognized by antibodies from infected animals, we propose that they function in an as yet undetermined role during pathogenesis.

#150

Structural characterization of lipid 550/760 from *Mycobacterium avium* subspecies *paratuberculosis*

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease which is a chronic inflammatory bowel disease found in cattle and other ruminants. Intriguingly, mycobacteria's cell envelope is composed of more than 40% lipids, most of which are found in the outer layer of the cell wall. This led to a recent research focus to determine the role of cell wall lipids and their interactions with host cells. Recently, it was demonstrated that MAP undergoes a striking change in its lipidome when grown intracellularly in bovine macrophages. Several lipids of MAP that were not seen in-vitro could now be detected which suggested that they are either newly synthesized or over-expressed. It is hypothesized that MAP changes its lipidome to enhance the pathogen's survival by increasing virulence or by camouflaging the surface of MAP from host immune surveillance. One of these lipids that is over-expressed under intracellular conditions is lipid spot 550/760. This lipid was also found in a recombinant MAP strain over-expressing MAP0486, a luxR homolog transcriptional regulator that seems to be responsible for the intracellular lipidomic changes. The goal of this research project was to structurally characterize the lipid(s) within spot 550/760. Mass spectrometry, 1H-NMR, 1H{13C}-NMR, and 1H1H-TOCSY demonstrated clearly that the spot consists of two lipid components: mycolic acids (a-MA, keto/methoxy-MA) and a lipopeptide. The lipopeptide seems to consist of a b-OH fatty acyl chain linked to either a single lysine that forms an internal ring through an amide bond with its carboxyl group, or to two amino acids. However, the non-polar character of the lipopeptide would rather suggest the single lysine. Further NMR and MS/MS analysis will be performed to confirm the final structure of the lipopeptide.

MAP Biology Perspectives.

Luiz E Bermudez.

College of Veterinary Medicine, Oregon State University, Corvallis, OR.

Mycobacterium avium subsp paratuberculosis (MAP) causes Johne's disease. It is encountered in the environment as well infecting ruminants. The organism is acquired at the calf stage, and for several years the infection evolves, triggering clinical signs of the disease. We know very little about sources of infection, ability to survive in the ruminant's rumen, about crossing of the intestinal barrier and interaction with the host immune system, both when immature and mature. It is assumed that MAP lives and replicates in macrophages but the possible role of other host cells is not well understood. What kind of nutrients the pathogen needs when inside phagocytic cells, and how it influence the intracellular lifestyle is unknown. The importance of the innate and adaptive immunity in host defense and how MAP subvert them are key questions for which we have few answers. So, how do the papers presented at the meeting add to our knowledge? The concept of biofilm is an obvious one, but the role of biofilm in transmission has not been investigated. The studies of the MAP transcriptome in macrophages. Encouraging first steps to understand how the pathogen manage to live inside of a phagocyte. The comparison with better studied Mycobacteria can help significantly in the understanding of MAP pathogenesis. The role of MAP lipids in the pathogenesis of the infection. Very difficult topic to address, but of remarkable importance. Novel findings are beginning to add important pieces to out knowledge gap. Role of iron and gene regulation in MAP. Here too, a significant topic that would benefit from past findings using different mycobacteria. Shedding is an enigmatic topic. Host-mediated or pathogen-triggered? Much work needs to be done, but the value of the finding can have significant impact on transmission. Proteomic analysis, as well as cell wall protein analysis can unveil crucial understanding on how MAP interacts with the intracellular environment. Many of these studies are bringing some new excitement to the field, but we need to start asking specific questions related to the biology of the organism as well the pathogenesis of the infection. A move from the general information to the specific observation that might impact prevention or treatment of the disease. We all have heard that these are golden times for biology, but we need to be creative and use the techniques that are available to us to ask relevant questions, create animal models that would allow us to test important hypothesis and provide precise answers without the complexity of the natural host. In summary, the field is positioned for a dynamic "beginning", but major challenges are ahead of us.

Pathogenomics and MAP Biology

Poster Abstracts

#35 Evaluation of subspecies-specific proteins for the diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infections

Valerie Hughes[1], John P Bannantine[2], Susan Denham, Jim McNair, Samuel Strain, Mike L Paustian[2], Karen Stevenson[1]

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Mycobacterium avium subspecies *paratuberculosis* (Map)-specific proteins (35) were identified by comparing the proteomes of Map isolates with those of the genetically similar subspecies IS*901*$^{+}$ *Mycobacterium avium* subspecies avium or silvaticum. This approach identified subspecies-specific proteins including the products of differential expression that would not have been detected by a comparative genomics approach. The genes encoding the proteins of interest were cloned into pMAL-c2X and expressed in Escherichia coli and the immunogenicity of the recombinant proteins determined to assess their potential as specific immunological reagents for the diagnosis of paratuberculosis and epidemiological studies. Immunogenicity was evaluated using the interferon-gamma enzyme-linked immunosorbent assay (IFN-γ ELISA), serum ELISA and immunoblotting. Responses to the recombinant Map-specific proteins were compared to those of PPD from M.avium subspecies avium (PPD-A) and Map (PPD-J). Immune responses were evaluated in naturally Map-infected sheep with subclinical and clinical disease and in calves experimentally infected with either Map or IS*901*$^{+}$ M.avium. Three proteins were found to have diagnostic potential when incorporated in the IFN-γ ELISA. Seventeen proteins were detected in at least one of the immunoassays and eleven proteins were detected by serum ELISA with an optical density in excess of the cutoff of 0.1 in four of six sheep sera tested. The immunoreactivity of these proteins indicates their potential as unique diagnostic antigens for the specific immunodiagnosis of paratuberculosis.

#52 Is 'Indian Bison Type' *Mycobacterium avium* subspecies *paratuberculosis* represents' a new MAP Biotype'?

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Indian Council of Agricultural Research, India; Central Institute for Research on Goats, India

Objective: In India, first case of Johne's disease was identified in 1913, but active research was started only after 1984. Indian MAP has been found to be highly pathogenic and endemic in domestic animals. First time culture of Indian MAP isolates in 1988 by our group, helped in initial characterization using radio-active probes, protein and lipid profiles. Genotypic analysis with help of Spanish and Australian labs, first time indicated that 'Indian MAP strains' were similar to 'Bison type' MAP, but possibly represents a new biotype. Present study aimed to identify the genetic variations of 'Indian MAP strains' and their evolution compared with other MAP strains

Materials and Methods: Different molecular markers (IS elements, LSPs and SSRs) of native MAP were analyzed in order to study above objectives.

Results and Conclusion: Sequencing of different markers identified SNPs/indels unique to 'Indian Bison Type' MAP with potential to be used as 'Molecular Signatures' for native strains of MAP. Positional polymorphism was also identified in few IS elements. Studies on 'Phylogenetic Analysis' indicated that the 'Indian Bison Type' MAP may be most recent to evolve in the evolutionary ladder, when compared with MAP strains reported outside India. Molecular phylogeny studies also indicated that the other strains of MAP were perhaps evolutionary intermediates during its' journey from *M. avium* to 'Indian Bison Type' MAP through 'Sheep type', 'Cattle type' and 'US Bison type' strains. Study also identified genetic rearrangements in the genome of 'Indian Bison Type' MAP that resulted in duplications of LSP regions in it's genome. These 'duplicated regions' encode for important proteins including virulence factors, antigenic proteins and enzymes. The diagnostic kit (ELISA based) and 'indigenous vaccine' developed using 'Indian Bison Type' strain have been reported to be highly efficacious. Increased gene dosages due to duplicated regions may be responsible for over-production of virulence factors and antigens that may have bearing on over all virulence and efficacy of vaccine prepared using 'Indian Bison Type' MAP strains Further studies at proteome and transcriptome level and complete sequencing of 'Indian Bison Type' MAP, will impart novel insights into the biology of native MAP. Documentation of new 'Indian Bison Type' MAP strain, (reported to be highly pathogenic) will help initiating efforts towards the control of paratuberculosis in the country.

#53 Novel IS*900* sequence polymorphisms in Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis* of human and bovine origin

Ajay Vir Singh, Shoor Vir Singh, Pravin Kumar Singh, Jagdeep Singh Sohal, M C Sharma

Indian Council of Agricultural Research, India; Central Institute for Research on Goats, India

Objective: Study aimed to characterize clinical isolates of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) of humans and bovine origin by sequencing two regions of IS*900* elements.

Materials and Methods: DNA was extracted from representative 11 native isolates of MAP of human and bovine origin from North India. Stool samples from human beings (N=2) exhibiting clinical symptoms indistinguishable to Crohn's disease (CD) and were employed in goatherds endemic for Johne's disease (JD). Two DNA belonged to Crohn's disease patients. Seven DNA were recovered from commercially pasteurized bovine milk samples. All the DNA samples were characterized by IS*900* PCR at two regions (A and B) and PCR products were subjected for sequencing using big dye terminator chemistry (courtesy Tim Bull, UK). Using online and offline alignment tools, sequences were analyzed for the global and local sequence comparisons. **Results and Conclusions**: Sequencing confirmed that region B of these native MAP isolates of human and bovine origin were identical to MAP K10 and other sequences deposited in GenBank database. However, region A of these isolates carried a stretch of nucleotides (from region 62 to 121) having number of sequence variations compared to MAP K10 and other sequences deposited in GenBank. Within native isolates there were no variations (homologous) and had identical sequences. However, earlier studies reported highly conserved nature of IS*900* in MAP isolates recovered from different species and geographical locations outside India. Previous studies by this group also reported presence of new 'Indian Bison type' genotype from different species of animals (domestic and wild) and human beings. This study also exhibited that 'Indian MAP' isolates may probably represent a new group of genotypes. Variations in the A region of IS*900* sequences of 'Indian MAP' isolates may be used for designing one step assays to discriminate Indian and non-Indian MAP isolates. However, more extensive validation of the above observations is needed.

#69 *In vitro* susceptibility of *Mycobacterium avium paratuberculosis* to antibacterial drugs individually and in combination with the immunosuppressive drug 6-mercaptopurine

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Objectives: To determine 1) the *in vitro* antibiotic susceptibility of *M. avium* ss. *paratuberculosis* (MAP) and compare to those of M. a. hominisuiss (MAH) and M. a. avium (MAA) and 2) the effect of the immunosuppressive drug 6-mercaptopurine–antibiotic interactions on *in vitro* growth of MAP.

Materials and Methods: Clinical isolates of MAP (n=18, human and animal origin), MAH (n=10) and MAA (n=5) were used. The MICs of 11 antibiotics were determined by the proportion method adapted for the BACTEC™ MGIT™ 960 culture system. Drug interactions were tested by the chequer board method adapted for the MGIT™ 960 system and interpreted based on fractional inhibitory concentration (FIC) indices.

Results: In the absence of interpretative criteria specific to MAP, those for *M. avium* complex were used to define susceptibility. Azithromycin and clarithromycin were the most effective (100% isolates) against MAP, followed by amikacin (83.3%), ciprofloxacin (55.5%), levofloxacin (50%), rifampicin (44.4%) and rifabutin (38.8%). Twenty two percent of the isolates were either susceptible or moderately susceptible (≤0.3 µg/mL) to clofazimine. Clofazimine MICs for the resistant isolates ranged between 0.6 or 1.2 µg/mL, which may indicate *in vivo* susceptibility, since the drug is known to concentrate in tissues. Ethambutol was effective against 28% of the isolates, but isoniazid and dapsone were completely ineffective. MAP was more susceptible to amikacin than MAH and MAA, less susceptible to rifamycins than MAH, and more susceptible to ethambutol than MAA. Synergism was observed when 6-mercaptopurine was combined with azithromycin/clarithromycin/rifamycin / ethambutol for multiple MAP human isolates. No antagonism was observed with any of the drugs tested.

Conclusions:

- MAP strains are susceptible to macrolides>amikacin>fluroquinolones=rifamycins> clofazimine=ethambutol;
- MAP strains are more susceptible to amikacin than are MAH and MAA, but relatively less susceptible to rifamycins than MAH;
- 6-mercaptopurine may be synergistic with macrolides and rifamycins against MAP.

#71 In-Silico identification of peptides for the diagnostics of paratuberculosis

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Identification of bovine MHC class II reactive peptides that are specific/unique to paratuberculosis and conserved across pathogenic variations of the paratuberculosis proteome will be of high value for development of new vaccines and immune based diagnostics. Here, we present an in silico screening procedure that can identify such peptides.

In short, the procedure identifies 20mer peptides that fulfill the following criteria:
a) Conserved in a set of positive (i.e. pathogenic) genomes;
b) Absent in a set of negative genomes (i.e. non-pathogenic or related pathogen);
c) High density of Bovine MHC-II epitopes.

As positive genomes were used full genome sequences of two *Mycobacterium avium* subsp. *paratuberculosis* strains, strain K-10 and the newly sequenced strain Ejlskov2007. As negative genomes were used five complete genomes of *Mycobacterium tuberculosis*, bovis and avium.

All reading frames were virtually translated and converted into overlapping sub-peptide of length 20. Peptides fully conserved in the positive strains were selected as potential positive hits. All positive hits were compared to the set of 20mers peptides from the negative strains and discarded if an overlap of 8 consecutive amino acids was found. This resulted in approximately 80000 20mers that were 100% conserved between the two positive strains, sharing no 8mer overlap to any negative genome.

Residual 20mers were next in-silico checked for binding to each of five prevalent bovine class-II MHC molecules using the NetMHCII-pan method [2]. Studies covering Bovine ligands from public data-resources revealed that this method, even though trained so

References: 1. Larsen, T.S. and A. Krogh, EasyGene-a prokaryotic gene finder that ranks ORFs by statistical significance. BMC Bioinformatics, 2003. 4: p. 21. 2. Nielsen, M., et al., Quantitative predictions of peptide binding to any HLA-DR molecule of known sequence: NetMHCIIpan. PLoS Comput Biol, 2008. 4(7): p. e1000107.

#90 Differential proteome analysis (*in vitro* vs. *in vivo*) of *Mycobacterium avium* ssp. *paratuberculosis*

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In order to identify novel proteins of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) expressed *in vivo*, MAP was isolated from the mucosa of four cows with clinical Johne's disease, fractionated into cytoplasma- and membrane fractions and compared to the respective isolates cultured on Middlebrook Medium 7H9 supplemented with OADC and Mycobactin J. The yield of MAP isolated from mucosa was calculated in relation to the mucosa mass and varied from 1.4 % to 4.4 % in three different cows; in one cow the yield was below 0.5 % and, therefore, not sufficient to study the proteome expressed *in vivo*.

The MAP fractions were subjected to an in-gel trypsin digest and subsequently investigated by UPLC-Q-ToF-MS/MS analysis using a nano Acquity (Waters) and ESI Q-TOF MS (Q-TOF Ultima, Waters). For the membrane fraction the approach resulted in the detection of more than hundred MAP-proteins expressed *in vivo*, and a similar number was detected *in vitro*. A total of 32 different proteins were found to be expressed *in vivo* only; five of these were expressed *in vivo* in two cows, and one protein (FO synthase FbiC) was expressed *in vivo* only in all three cows. Only one of the proteins (30S ribosomal protein S2 RpsB) had been identified in a previous *in vivo-in vitro* proteome comparison (Stevenson et al., Microbiology 2007; 153, 196-205). Among the other proteins identified were a number of hypothetical proteins of unknown function, a hypothetical fatty acyl dehydrogenase (FadE3_2), and 3-hydroxyacyl-CoA dehydrogenase, all of which are possibly relevant for *in vivo*-metabolism.

#118 Cloning, expression and purification of *Mycobacterium avium* subsp. *paratuberculosis* stress-associated genes

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It is believed that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) can survive in an unfavourable environment for a long time in a dormant state. In a previous study, we developed an *in vitro* model of stressed/dormant MAP under three different environmental stresses of temperature flux, nutrient starvation and oxygen limitation, and identified proteins expressed under these conditions by proteomics analysis. It was hypothesised that these stress response proteins might also be expressed *in vivo*, and could be useful targets for early diagnosis of JD. In this study, 28 MAP stress-associated proteins were cloned, expressed and purified, and the immunogenicity of these recombinant proteins was evaluated by ELISA.

Twenty eight MAP genes previously identified as differentially regulated under conditions of physiological stress were randomly selected for cloning and expression. 15 of 28 were cloned using Invitrogen Gateway® Technology, while the other 13 clones were obtained using a traditional restriction enzyme-based cloning method. His-tagged proteins were expressed in BL21 *Escherichia coli* and purified using affinity chromatography. Soluble proteins confirmed by Western-blotting analysis were purified under native conditions, while insoluble proteins were solubilised with denaturing buffer and then purified.

The immunogenicity of purified recombinant proteins was evaluated for detection of MAP infection by ELISA using serum samples from 50 infected and 50 uninfected sheep. Some antigens showed significantly higher OD values in the infected group compared to the uninfected group. When an arbitrary cut off line was set, these antigens had similar sensitivity to a commercial Pourquier ELISA, but detected more infected sheep that had no detectable histological lesions and fewer that had multibacillary lesions. The results suggest that these recombinant MAP stress-associated proteins might be expressed in MAP infected sheep at an early stage of infection. This is consistent with the hypothesis of dormancy.

#121 Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) using culture from blood during early, subclinical and clinical stages of disease

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MAP organisms have been found in infected animals at sites that are distant from the gut including culture of MAP from milk, liver, mammary tissue, spleen, foetal tissues, reproductive tissue, and extra intestinal lymph nodes. These studies, in association with detection of MAP DNA in the blood, indicate that at some stage of the disease a bacteraemia may occur.

Demonstration of the organism in blood from infected sheep and cattle by PCR has led to the question of the temporal pattern of the bacteraemia during the course of paratuberculosis infection. This study aims to determine when bacteraemia is detectable by culture during the time course of paratuberculosis infection, using an optimised method of culturing S-strain of MAP from blood samples.

One hundred and eleven Merino or Merino-cross lambs aged 3-4 months were used in four experimental infection trials. The lambs were shown to be free from detectable MAP infection prior to the study. Lambs were inoculated with either a pure culture of MAP strain Telford 9.2 or a gut homogenate from a clinically infected sheep.

Blood was collected from all animals prior to inoculation with MAP, every 3-4 months throughout the trial and then prior to necropsy. Additionally, in one trial, blood was sampled monthly during the first 6 months. Naturally infected animals were also studied, with blood collected from animals from heavily infected flocks prior to necropsy. Infection status of all animals was assessed by culture of gut and faeces, histology and antibody ELISA.

Results from both naturally and experimentally infected animals indicate that MAP can be isolated from the blood of a low proportion of sheep at late stages of the disease. Though preliminary, these studies provide some indications of the nature of the bacteraemia and identify the difficulties associated with culture from blood.

#132 Full genome sequence of a Danish isolate of *Mycobacterium avium* subspecies *paratuberculosis*, strain Ejlskov2007

Mamuna Afzal, Soad Abidi, Heidi Mikkelsen, Sheila Tuyet Tang, Ole Lund, Morten Nielsen, Claus Lundegaard, Gregers Jungersen, Dave Ussery

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We have sequenced a Danish isolate of *Mycobacterium avium* subspecies *paratuberculosis*, strain Ejlskov2007. The strain was isolated from faecal material of a 48 month old second parity Danish Holstein cow, with clinical symptoms of chronic diarrhoea and emaciation. The cultures were grown on Löwenstein-Jensen media by standard procedures and passed once to new tubes before DNA extraction and being sequenced on a 454 FLX machine. Currently, the genome has been assembled into 70 contiguous pieces, for a total of around 5.0 Mbp, with a 63% GC content. We have predicted a total of 4687 proteins, consisting of 4317 unique gene families. Comparison with *M. avium paratuberculosis* strain K10 revealed only 3436 genes in common (~70%). We have used GenomeAtlases to show conserved (and unique) regions along the Ejlskov2007 chromosome, compared to 2 other *Mycobacterium avium* sequenced genomes. Pan-genome analyses of the sequenced Mycobacterium genomes reveal a surprisingly open and diverse set of genes for this bacterial genera.

#176 A reference proteomic profile of the cell wall of the *Mycobacterium avium* complex

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All four *M. avium* subspecies (*avium*, *silvaticum*, *hominissuis*, *paratuberculosis*) and *M. intracellulare* are collectively known as the *Mycobacterium avium* complex (MAC) and possess a high degree of genetic similarity but can inhabit diverse environments and infect a diverse range of host species. Despite their divergent phenotypes and the diseases they cause, the genomes of *M. avium* subspecies share greater than 97% nucleotide identity and 91% with *M. intracellulare*. Therefore, a better understanding of the host specificity and differences in pathogenicity within the MAC could come from a thorough proteomic comparison. The cell wall proteins are particular interesting in this respect. The aim of this study was to construct a reference cell wall protein map for the MAC and identify differences between the MAC members. Method: A proteomic analysis approach, based on one dimensional polyacrylamide gel electrophoresis and LC-MS/MS, was used to identify and characterize the cell wall associated proteins of all 5 members of the MAC. An enzymatic cell surface shaving method was used to determine the surface-exposed proteins. **Results**: Between 300 and 400 cell wall proteins and more than 40 surface-exposed proteins were identified for each MAC member. The overlap of the different cell wall proteomes was determined and mapped against a consensus reference MAC cell wall protein profile. Detailed information about functional classification, signal peptides and number of transmembrane domains were added to the reference profile. **Conclusion**: We have generated a comprehensive profile of the MAC cell wall subproteome. This work will help us focus in on proteins in the cell wall that might be involved in Map specific virulence mechanisms and those of the other MAC members.

#179 Rhodanine agents active against non-replicating intracellular *Mycobacterium avium* subspecies *paratuberculosis*

Tim J Bull, Richard Linedale, John Hermon-Taylor, Jason Hinds,*St George's University of London, United Kingdom; King's College London, United Kingdom*

Introduction and Objective: The capacity for long term persistence inside macrophages and other cell lineages combined with the ability to enter a non-replicating viable (NRV), often non-culturable state, allows MAP to develop paucimicrobial chronic infection states leading to disease. Therapy is difficult because the lack of bacterial division, diminution of cell wall antigens and low transcriptomic turnover in NRV drastically reduces the effectiveness of conventional antibiotics. In addition, MAP can manipulate host intracellular killing mechanisms and disrupt pathogen antigen presentation leading to dysregulation of host cell mediated clearance. In this study we evaluate a novel anti-MAP agent Rhodanine(D157070) that aims to re-assert the normal surveillance and activation of host cell defense mechanisms by inhibiting MAP from releasing anti-reactive nitrogen intermediates.

Materials and Methods: Culturable viability (CV) and viable persistence (VP) of MAP K10 in a variety macrophage models and culture alone as a control, with and without Rhodanine(D157070) treatment was monitored over a 7 day period. CV was measured using conventional colony counting. VP was determined as the degree of ribosomal turnover represented by the qPCR DNA:RNA ratio of a 102bp pre16SrRNA leader sequence present as single copy number (DNA) but cleaved and degraded during ribosomal assembly (RNA). Full MAP genome transcriptomic profiles were also obtained during treatment using the ParaTBtools MAPAC microarray.

Results: Rhodanine(D157070) did not kill MAP in conventional extracellular culture but transcriptome profiles were altered. Rhodanine(D157070) was highly active against intracellular MAP in macrophage infection models increasing MAP killing as measured by both CV and VP assays. Rhodanine(D157070) alone showed no toxicity to cell lines.

Conclusion: Rhodanine(D157070) does not kill MAP directly but can enter host cells and induce inhibition of MAP derived processes that normally decrease effectiveness of intracellular killing mechanisms. This agent thus shows good promise as a therapy directed at non-replicating intracellular MAP infection.

#206 The role of TcrXY, a two component system in *Mycobacterium avium* subspecies *paratuberculosis*

Michelle Pinto, Lucy Mutharia, *University of Guelph, Canada*

Bacteria are able to survive in environments that experience continuous fluctuation in both physical and chemical aspects by adapting to these changes using sensory-response mechanisms such as Two Component Systems. These systems involve autophosphorylation of membrane histidine protein kinase receptors on detection of certain stimuli and phosphoryl transfer to a cytoplasmic response regulator, which generally acts as a transcription regulator. *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the etiological agent involved in Johne's disease in ruminants, has ten putative two component systems in addition to one orphan histidine protein kinase and five orphan response reglulators. The TcrXY system has been studied in M. tuberculosis and infection assays using a partial deletion construct resulted in hypervirulence in SCID mice. The *tcrXY* promoter in MAP_K10 was used to generate promoter: *lacZ* fusions and used in *in vitro* β-galactosidase assays in *Mycobacterium avium* subsp. hominisuis 104 (MAA104). The *tcrXY* promoter was induced in acidic conditions (pH 4.5 to 5.5). It is possible that TcrXY may be responsive to changes in pH in the environment in addition to what it faces in the gut and feces of the host as well as in phagosomes. A MAP tcrXY gene knockout will be characterized by investigating the survival of the mutants in macrophages and when exposed to acidic gradients with the goal of identifying the role that TcrXY plays in virulence and pathogenesis. The recently described conditionally replicating shuttle phasmid pHAE87 is used to generate the TcrXY mutants.

#213 Construction the auxotrophic *leuD* mutant of *Mycobacterium avium* subspecies *paratuberculosis* K10 as a live attenuated vaccine candidate

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Background: *Mycobacterium avium* subspecies *paratuberculosis* is an obligate pathogenic bacterium in the genus Mycobacteria. It is intracellular pathogens and reside inside host cells primarily macrophage. MAP causes Johne's disease in cattle and other ruminants, and is responsible for an economy loss to dairy industry. It has long been suspected as a causative agent in Crohn's disease in humans, although this connection is controversial. It is urgently needed to develop a vaccine with a better protection and fewer side effects. We have constructed a live attenuated MAP *leuD* mutant as a live vaccine candidate.

Methods: A phage-mediated allelic exchange technique was used to delete *leuD* gene. The MAP *leuD* gene deletion was confirmed by both PCR and DNA sequence. The complementation of leuD by pVV16-leuD vector abolished its leucine requirement for growth. Mice were used to perform a vaccine trial.

Result: C57BL/6 mice were used to determine the pathogenicity of MAP *LeuD* mutant. We found that mice infected with wild type of MAP with a larger liver and spleen than that of *leuD* mutant. Histopathological examination also showed the *leuD* mutant strain infected mice have a mild inflammation in spleen and liver than that of wild type. Preliminary data also indicated that the *leuD* mutant could induce protection against challenge in a mouse model.

Conclusion: Management of Johne's disease has been a challenge to farmers. We constructed auxotrophic *leuD* mutant of MAP K10 as a live attenuated vaccine candidate which could induce a better protection against MAP infection. Based on our preliminary mouse study, this new vaccine candidate may be used as a live attenuated vaccine against Johne's disease.

#219 *Mycobacterium avium* subsp. *paratuberculosis* PPE protein MAP1152 and conserved protein MAP1156 are antigenic in experimentally and naturally infected cattle

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Mycobacterium avium subsp. *paratuberculosis* causes Johne's Disease (JD) in ruminants. A transposon insertion upstream from the MAP1152-MAP1156 genomic region causes a change in colony morphotype and results in an attenuated phenotype in bovine macrophages. Bioinformatic analysis indicates that MAP1152 encodes a PPE protein, while MAP1156 is a member of the uncharacterized protein family UPF0089. Transcriptional analysis suggests that the corresponding genes are organized in two overlapping operons. Maltose-binding protein tagged recombinant proteins were overproduced and purified from *E. coli*. The antigenicity of MAP1152 and MAP1156 was examined against sera of experimentally infected mice, rabbits; and experimentally and naturally infected cattle. MAP1156 yielded a stronger positive Western blot signal than MAP1152 against sera from cattle with JD. Four positive and four negative sera, previously classified by culture and the ELISA Idexx^{R} assay, were re-tested in triplicate by ELISA using the Idexx^{R} reference antigen and the recombinant proteins. MAP1152 displayed 2- to 3-fold greater reactivity (optical density values) against positive sera as compared to negative controls ($p < 0.001$). Likewise, the reactivity of MAP1156 was 2 to 4-fold greater for positive sera ($p < 0.05$.). In contrast, the Idexx^{R} antigen reacted 5 to 12-fold greater with positive than negative sera ($p < 0.0001$). Classification of positive and negative status was determined by cutoff value determined as the average OD of the negative sera ± 3 SD. Positive/negative classification of sera with MAP1152 was in exact agreement with the IdexxR antigen (k= 0.96). However, reaction with MAP1156 resulted in a disagreement with 1 of 4 presumed negative sera testing positive (k= 0.68). The data presented suggest that MAP1152 and MAP1156 are antigenic and hold strong potential as vaccine candidates or differential diagnostic antigens if used in conjunction with the corresponding attenuated mutants.

#230 Iron concentration dependent stressome of *Mycobacterium avium* subsp. *paratuberculosis*

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Mycobacterium avium subsp. *paratuberculosis* (MAP) residing inside host macrophages regulates expression of iron acquisition, iron transport and oxidative stress response genes. Since MAP is mycobactin dependent for its growth in laboratory media functional analysis of MAP in response to iron limitation in-vitro would increase our understanding of its iron physiology in the host as well as aid in the development of improved culture methods. Therefore, we undertook a high-resolution proteomic and transcriptomic analysis of MAP using iTRAQ (isobaric tag for relative and absolute quantification) and microarrays. MAP was grown to mid-logarithmic phase in MB7H9 medium containing mycobactin J (MJ) and washed several times in PBS and inoculated separately into minimal essential medium (MEM) (nutrient starvation) (20%glycerol, K_2HPO_4 and $MgSO_4$) containing (i) no iron or (ii) iron at 50µM. Three hours following inoculations, total cellular protein extracted from bacterial pellets was analyzed using iTRAQ labeling for protein identification and total RNA extracted was used in MAP K-10 microarrays for genome wide transcriptome analysis. Results show that under iron deplete conditions, MAP downregulated expression of iron utilizing proteins and upregulated stress response proteins similar to nutrient deprived conditions (estimated iron concentration 1µM). Further, we identified two novel proteins belonging to the ESAT-6 family and a conserved hypothetical membrane protein, which were not previously identified by genome annotation. Identification of these proteins is significant as it opens new areas of functional characterization to understand MAP physiology during infection. The data also show that MAP may be unsuccessful in accumulating sufficient quantities iron deplete conditions and allows us to interrogate this stress induced metabalome to device better culture methods.

#232 PrrA is important in *Mycobacterium avium* subspecies *paratuberculosis* virulence

Chia-wei Wu, Chung-Yi Hsu, Adel M. Talaat,*University of Wisconsin-Madison, USA*

Two-component systems are widely found in bacteria and involved in sensing environmental changes and subsequently altering gene expression patterns to adapt to various survival challenges. One of such systems, PrrA-PrrB, was shown to be required for early intracellular replication of *Mycobacterium tuberculosis* (*M. tb*) in a macrophage infection model. The response regulator, PrrA, was also shown to be auto-regulated through promoter binding.

BLAST analysis indicated that the prrAB operon (MAP0833c and MAP0834c) of *Mycobacterium avium* subspecies *paratuberculosis* (*M. ap*) is homologous to that of M. tb (82% identical), and those in other mycobacteria. Additional sequence analysis showed that *M. ap prrA* has a signal receiver domain and a DNA binding domain, suggesting the signal transduction and gene regulation roles of PrrA. We have screened a *prrA*::Tn5367 mutant from our transposon mutant library with the genetic background of *M. ap* ATCC 19698. In a mouse infection model of paratuberculosis, the *prrA*::Tn5367 mutant showed significant reduced levels of colonization in the liver and intestine compared to the wild type. Furthermore, the mutant also showed less-defined granulomatous structure in pathological examinations, suggesting a reduced virulence phenotype of the mutant. In addition, microarray analysis of M. ap culture in an acidic condition (pH 5.5) showed a 3.5-fold induction of the *prrA* gene. Currently we are testing the intracellular survival of the mutant in a mouse macrophage infection model and identifying genes under the control of *prrA* with microarray analysis. In summary, we showed that disruption of the *prrA* gene results in reduced virulence of M. ap and we believe the PrrAB two-component system is important in *M. ap* pathogenesis.

#239 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in cull dairy cows

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Objective: To correlate fecal shedding to MAP tissue bio-burden and to determine what proportion of fecal culture negative cows would have culture positive fecals and tissues at slaughter.

Materials and Methods: Tissues and fecal samples were harvested from 230 cows culled from 3 dairy herds over four years. Five samples were collected from each animal; 2 intestinal lnn, ileum, IC valve and a fecal sample. Samples were cultured on HEYM.

Results: Of the 230 cattle with harvested tissues, 17 (9.8%) were previously fecal culture positive for MAP. Fourteen of 17 animals were positive at slaughter (82%) and 3/17 (18%) cattle had all 5 samples culture negative. Of the 20 fecal culture positive cattle at the time of slaughter, 9 were heavy shedders each massively infected in the four tissues examined (>300 cfu MAP/tube). No other fecal culture positive or negative cows had such massive tissue infection with MAP. Interestingly 5/20 (25%) fecal culture positive cows at slaughter had negative cultures on all four tissues. Three positive fecal culture cows (very low shedders) had only one colony on the four tissues cultured, suggesting infections as adults.

Of the 156 cattle with all negative fecal cultures prior to culling, 58/156 (37%) had at least one positive sample at slaughter. The lnn was positive most frequently, followed closely by ileum and IC valve. 26/58 (45%) cattle had less than 10 total cfu of MAP on 20 tubes of HEYM for the five samples suggests a more recent infection.

Conclusions: 35% of fecal culture negative cattle had positive tissues at slaughter. Cattle shedding more than a few colonies of MAP or with multiple positive fecal cultures had multiple tissues positive at high MAP concentrations.

Public Health

Convenors: Marcel A. Behr and Carol Nacy

Public Health Keynote Lectures (Sunday Evening)

Paratuberculosis and Crohn's Disease: Already Beyond Reasonable Doubt.

Tim Bull

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Crohn's Disease (CD) is an important chronic disabilitating inflammatory disease that is increasing in incidence. There is good scientific evidence that CD has both an underlying genetic susceptibility and an infectious aetiology. That the infectious agent may be MAP has been a subject of much speculation, argument, controversy and misinformation over the past decade. Despite detailed analysis, and partly because of the complexities and technical difficulties in studying CD and MAP, the position has not been clarified further than suggestions of 'a possible link' or 'insufficient and inconclusive scientific evidence'. This ambiguity has left many clinicians sceptical and allowed a conservative and cautious attitude to persist. Of particular relevance is that any successful resolution of this issue will not be without wide reaching consequences. A general acceptance of MAP as a true zoonotic agent will have ramifications in many fields including domestic and wild animal welfare, sustainable agriculture, economic planning of infection control policies, food and environmental safety, clinical approaches to CD therapy and possible legal complications. Thus any conclusions or truths that can be derived from past or present studies should not be undertaken lightly, should be founded on good scientific practice and devoid of conjecture.

A common perception has been that this is an insolvable dilemma, particularly since ethical restrictions forbid human challenge studies. However, recent advances in technology combined with a more complete understanding of immunological mechanisms in CD and mycobacterial mechanisms of pathogenesis have enabled studies designed to confront this dogma. This presentation will summarise the crucial developments and insights that are being revealed in this field including good scientific evidence for MAP presence, virulence, infectivity, pathogenesis and response to treatment. It will show that consistent with the increased exposure of humans to MAP from animals and the environment, MAP is present, unequivocally viable and persistent in both normal human controls and with a significantly increased strength of association in CD patients. It will show that persistence of MAP in humans, as in other pathogenic mycobacteria is associated with a viable non-replicating intracellular phenotype and that MAP from humans are virulent in animals. It will show that the degree of MAP infection and colonization in the human gut mucosa is patient specific but is able to stimulate innate inflammatory responses predominant in CD. It will show that CD patients have MAP specific immunological reactivities that are fully consistent with our knowledge of immune dysregulations underlying inflammatory bowel disease. It will show that JD and CD have related genetic susceptibilities that are indicative of defective resistance to mycobacteria and that these defects can preferentially favour MAP pathogenesis. It will additionally show that therapy which includes anti-MAP activity can lead to improvement of CD. In conclusion it will suggest that we now have more than enough good scientific evidence to show that MAP has sufficient motive, means, opportunity and association with the scene of the crime to convince a jury to deliver a verdict.

Where are the Weapons of Mass Destruction --- the MPTB?

Herb Van Kruiningen

University of Conneticut, Stors, CT, USA

Abstract not available at press time.

#59

CD4 T cells from intestinal biopsies of Crohn's disease patients react to *Mycobacterium avium* subspecies *paratuberculosis*.

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The role of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease (CD) remains controversial. One issue that has been raised is the lack of data showing a cellular immune response to MAP. Earlier studies have mostly focused on responses in peripheral blood which have several limitations. We thus wanted to characterize the response in the affected tissue and consequently made T-cell lines from intestinal biopsies of patients with CD (n=11), ulcerative colitis (UC) (n=13) and controls (n=10). The T cells from CD patients showed higher proliferation in response to MAP compared to UC patients ($p<0.025$), while no differences were detected in response to commensal bacteria. T-cell clones (n = 28) were made from four CD patients and tested for responses to various mycobacterial species. One T-cell clone responded only to MAP and the very closely related *M. avium* subspecies avium (MAA) while another responded to MAP, MAA and Mycobacterium intracellulare. A more broadly reactive T-cell clone reacted to MAP1508 which belongs to esx family. The T-cell clones produced IFN-γ and/or IL-17 but minimal amounts of IL-4. The presence of MAP responsive T cells producing pro-inflammatory cytokines suggests that MAP may contribute to the inflammation in CD.

#174

Sharing of 'Indian Bison Type' *Mycobacterium avium* subspecies *paratuberculosis* between goatherds endemic for Johne's disease and their animal attendants

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Objective: Sharing of 'Indian Bison Type' *Mycobacterium avium* subspecies *paratuberculosis* (MAP) between goatherds endemic for Johne's disease (JD) and their attendants with Crohn's disease (CD) was investigated.

Methods: Stool from 40 persons with clinical profiles indistinguishable to CD (Infrequent bowel movement, tendency to tire easily, frequent abdomen pain, diarrheal episodes, weight loss, low grade fever) and history of consumption of raw goat milk and had variable duration (1-5, 6-10, 11-15 and >15 years) of contact with goatherds endemic for JD. Forty stool samples from healthy human beings with no history of contact with animals also screened. The 26 (65.0%), 20 (50.0%), 27 (67.5%), 20 (50.0%) and 22 (55.0%) attendants had clinical profiles; history of abdominal pain, diarrhoeal episodes, weight loss, fever and consumption of raw goat milk, respectively. Stools were screened by microscopy and culture. IS*900* PCR and IS*1311* PCR-RE, used to characterized colonies as MAP and 'Indian Bison Type'

Results and Conclusions: Of 80 stool samples, MAP was isolated from 35.0% (28) human subjects and 15.0% (12) were acid fast. Of 40 healthy human beings, nil and 12.5% (5) were positive for MAP by microscopy and culture, respectively. Of 40 animal attendants, 30.0% (12) and 23 (57.5%) were positive in microscopy and culture, respectively.. MAP were recovered from 65.3, 75.0, 70.3, 45.0 and 54.5% attendants with clinical profiles of abdomen pain, diarrhea, weight loss, and fever and milk consumption, respectively. MAP was isolated from a person suffering from rectal bleeding. Of 23 attendants' positive for MAP, 39.1, 30.4, 26.0 and 4.3% had worked >15, 11-15, 6-10 and 1-5 years, respectively in JD infected goatherds.

Risk of contracting MAP infection and colitis with clinical symptoms indistinguishable to CD were higher in attendants working with goatherds' endemic for JD as compared with human beings with no history of contact with animals and risk was directly correlated with duration of association with endemic goatherds.

#135

Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from muscle tissue of naturally infected cattle

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Although dissemination of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from the gastrointestinal tract to liver, spleen, reproductive organs, and kidney has been reported; no previous studies have detected dissemination of MAP to muscle tissue of infected cattle. The purpose of this study was to assess whether MAP may also be present in muscle tissue of naturally infected cattle. Forty-seven cows, originating from farms with a prevalence of MAP infection ranged between 3-10 %, were slaughtered due to clinical signs associated to paratuberculosis (27 %) or for other reasons (73 %). Samples of gastrointestinal tissues, lymph nodes, blood and diaphragm muscle were taken and analyzed by histopathology and bacteriological culture. Our results revealed the presence of MAP in gastrointestinal tissues of thirty-one of the slaughtered animals (66 %). MAP was also detected by bacteriological culture, PCR and Real-Time PCR in the diaphragm muscle of six infected animals (19 %). The six animals showing evidence of MAP in muscle showed a diffuse type of paratuberculosis with heavy bacterial load in gut tissues and four of them showed severe clinical signs of paratuberculosis including diarrhea, weight loss and low milk production. The other two positive animals did not show clinical signs of paratuberculosis but had heavy bacterial load in gut tissues. This result suggests that the presence of Map in diaphragm muscle may also occurs at an early stage of infection with no identifiable clinical signs of paratuberculosis. MAP was also found in feces of three of the six animals showing evidence of MAP in diaphragm muscle and in blood of only one of them. In conclusion, we provide the first evidence that Map can be detected and cultured from muscle of MAP-infected cattle destined for human consumption and suggest a possible route of exposure of humans to MAP via contaminated meat.

#177

Associations between CARD15 polymorphisms, MAP DNA in blood and lactase persistence in a Crohn's disease case-control study in North-Spain

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Polymorphisms in CARD15 gene have been associated with Crohn's disease (CD). Also an epidemiologic association between national CD incidence and lactase persistence frequencies has been reported. CARD15 polymorphisms and presence of MAP DNA in blood results are controversial. Here we test the association of R702W, G908R and L1007fs polymorphisms in CARD15, as well as CT-13910 polymorphism in the lactase gene with the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) DNA in blood and the diagnosis of CD.

Blood samples from 150 CD patients and 65 blood donors from Asturias (Spain) were genotyped by RTi-PCR for CARD15 and by PCR and restriction enzyme digestion for lactase. MAP was detected by nested PCR. A marginally significant association between CD and MAP in blood was found (controls: 37.7%, CD: 25.6%, p=0.0598). NOD2 R709W variant was associated with higher rate of MAP detection in blood (60.0% versus 27.8%, p=0.0048). Associations between CD and genotypes were weak, but were partially confirmed by comparing allelic frequencies. The T allele in lactase gene was more frequent in CD patients (61.9%) than in controls (47.1%)(p=0.0275), while only marginal differences were observed for CARD15 at R709W (5.0% vs. 6.4%, p=0.3641) and L1007fs (3.9% vs. 0.8%, p=0.0792). Logistic regression analysis yielded odds ratios of over 4 between both genes and MAP in blood.

These results confirm an association of genetic factors to CD and to MAP infection in humans. The observation of higher frequencies of MAP in controls than in patients is controversial, but has been explained as the result of bacterial reduction by the antibiotic effect of standard CD therapy. Alternatively, it could represent evidence of a more continuous and efficient boosting of protective immune mechanisms in slow infections. This hypothesis matches better with observation of more frequent MAP-haemia in herds without paratuberculosis clinical cases and in vaccinated animals.

#207

Nicotinic and Salicylic acids and α & β nicotinamide adenine dinucleotide (NAD) cause dose dependant enhancement, and iso-nicotinic acid (INH) and para-amino-salicylic acid (PAS) cause dose dependant inhibition of *M. avium* subspecies *paratuberculosis* (MAP)

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Background: There is increasing concern that MAP may be zoonotic, responsible for, at a minimum, Crohn's disease. Tobacco usage may exacerbate Crohn's disease and tobacco cessation results in clinical improvement. We hypothesized that nicotine, its structural analogs or possible metabolites, may influence the growth of MAP.

Methods: Nicotine, nicotinic acid, α & β NAD, INH, and as experimental controls salicylic acid and PAS were evaluated in $14CO_2$ radiometric culture (Bactec 460®.) Strains studied included MAP isolated from cattle (ATCC 19698 & 303), or humans with Crohn's disease (Dominic & UCF-4), *M. avium* subspecies avium (ATCC 25291 & 101) and BCG (a level II Biosafety surrogate for M. tb.) Growth inhibition or enhancement is indicated by "percent decrease (-) or increase (+) in cumulative Growth Index" (%-/+ΔcGI) from control. All data in this Abstract are at 64μg/ml.)

Results: Responses vary amongst sub-species and strains. Salicylic and nicotinic acids and α & β-NAD may cause dose dependant enhancement. Nicotinic acid: Dominic; +82%: UCF-4; +58%: MAP 303; +62%: Salicylic Acid: 303; +66%: 25291; +104%: BCG; +95%: α-NAD; Dominic +120%. β-NAD: Dominic +101% Both PAS and INH may cause dose dependant inhibition. INH: Dominic -74%: UCF-4; -89%: 25291; -99%: PAS: 19698 -74%: 303; -80%: 25291; -98%: BCG; -98%. In contrast, Nicotine has no demonstrable effect on any strain tested.

Conclusions: We show dose dependant mycobacterial growth enhancement by nicotinic and salicylic acids and α & β NAD. Additionally we show anticipated dose dependant inhibition by their spatially modified analogs, the antibiotics INH and PAS. In contrast, we find no effect of pure nicotine. Our data indicate that, at the doses tested, pure nicotine has no growth enhancement effect on MAP but that its structural analogs profoundly influence MAP's growth kinetics.

Perspectives Talk: Public Health

Mycobacterium avium paratuberculosis: Infrequent Human Pathogen or Public Health Threat?
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More than 800,000 people in North America suffer from Crohn's Disease (CD), a debilitating chronic gastrointestinal disorder in search of a cause and a cure. Researchers and clinicians agree that CD onset requires inherited genetic traits, an environmental stimulus, and an overzealous inflammatory response. Long considered an autoimmune inflammatory disorder, current CD therapies treat symptoms of overactive inflammation in the gut. Chronic inflammation, however, does not generally induce itself. Inflammation is normally caused by a "foreign body," an inanimate object or rogue tissue cells or microorganisms. Until the cause of inflammation is eliminated, the body continues to send in its clean-up crew, the white blood cells of inflammation whose job it is to expel the tissue invader. Inflammation subsides when the causative agent is finally banished. Evidence suggests that CD may have a currently unrecognized infectious origin, and one suspect is *M. avium paratuberculosis* (MAP). People with CD have 7:1 odds of having a documented presence of MAP in blood or gut tissues than those without CD, thus the association of MAP and CD is no longer in question. The critical issue today is not whether MAP is associated with CD, but whether MAP causes CD or is only incidentally present, not an inciter or participant in the disease. If MAP is involved in the disease process of CD or other gastrointestinal disorders, then we need to determine how people are exposed to this microorganism, how to prevent that exposure, and how to treat the infection. In this session, investigators report the isolation of MAP from meat of Johne's diseased cattle, transmission of MAP from infected goats to goatherd attendants who develop CD-like gastrointestinal symptoms, correlation of human genetic factors with CD, identification of MAP-reactive CD4 T cells from CD patients, and the absence of nicotine effects on MAP growth.

Public Health
Poster Abstracts

#30 Genetic analysis of MAP in bovine milk samples in Brazil

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ABSTRACT

Paratuberculosis studies has increased considerably in Brazil, however the economic impact of the disease in the country has not yet been measured. Data on the prevalence of the disease do not exist in the state of Minas Gerais, although some cases have been reported. The aim of this study was to confirm the genetic identity of MAP (*Mycobacterium avium* subsp. *paratuberculosis*) detected in raw milk samples in the region of Viçosa, MG, Brazil. A total of 220 individual bovine milk samples were analyzed by PCR using the primers BN1/BN2 derived from the insertion sequence IS*900*. Eight samples (3.6%) amplified fragments of the expected size, 626bp. In order to confirm the genetic identity, the eight samples were cloned, sequenced and compared with the insertion sequence IS*900* deposited in GenBank (X16293.1). Out of the cloned samples, three (37.5%) and the positive control were successfully sequenced, but it was not possible to sequence the others. The genetic analysis showed 99% of identity between the sequences of this study and the sequence X16293.1, 90% with *M. avium* (AF527957.1), 91-92% with *Mycobacterium* sp. (AF455252), not-yet-identified, and 74% with *Streptomyces turgidiscabies* (AY707080.1) Among all the compared sequences, 11 were randomly selected to carry out a genetic sequence alignment among the 626 nucleotides, together with the four sequences of this study. From this alignment, a gene cluster map among the 15 gene sequences was built with high levels of similarity, since sequences were pooled together within the same tree branch. Only one sequence showed lower similarity and is located in another branch, probably for being a not-yet-identified *Mycobacterium* species. The sequencing proved that the fragments amplified in PCR reactions were MAP fragments. The results of this study allow us to affirm that MAP is present in bovine milk samples in Brazil, and it is reasonable to consider them as a first survey on the disease in the Minas Gerais State, Brazil

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a Gram-positive, acid-fast and facultative anaerobic, intracellular bacterium. It is a fastidious microorganism that requires the growth factor mycobactin *J* for *in vitro* growth. MAP belongs to Mycobacteriacea Family and under microscopy, usually forms small clusters (Collins, 2003). Some researchers indicate a possible role of MAP in Crohn's disease (Hermon-Taylor, 2002; Feller, et al., 2007), while others express doubt on this association (Abubakar et al., 2007; Waddell et al., 2008). Studies show that the etiology of Crohn's disease can involve a variety of viral and bacterial agents, including MAP, or an immunological origin. Evidence points to an interaction between a persistent environmental stimulus, such as a microbial antigen, and genetic factors that regulate an immunological response or a mucosal intestinal function (Shanahan and O'Mahony, 2005). The vehicle for transmission of MAP from animals to humans would be milk, but the causal association between MAP and Crohn's disease remains unclear. In the near future, the demand for milk MAP free will be a reality.

OBJECTIVES

Considering the disease socio-economical and public health significance, the significance of milk quality and presence of MAP in Brazilian milk (Carvalho et al., 2009), the aim of this study was to confirm the genetic identity of MAP detected in raw milk samples in the region of Viçosa, MG, Brazil.

MATERIALS AND METHODS

A total of 206 quarter milk samples were aseptically collected, and bulk tank milk samples from each of the 16 dairy herds in the region of Viçosa, MG, totaling 16 bulk tank milk samples, were collected. All the samples were processed and PCR was performed according to Sivakumar et al. (2005). A wild certified MAP strain was used as positive control; milli-Q water was used as negative control and φX174/HaeIII as molecular marker. In order to confirm the identity of the amplified fragments (626bp), they were cloned in the pGEM vector using the pGem T-Easy™ Vector System. Plasmid DNA was purified with Wizard® Plus SV Minipreps DNA Purification System. DNA sequencing, adapted by Sanger et al. (1977), was performed to confirm the sequences, by using the M13 forward and reverse primers. The obtained sequences were edited by the DNA MAN software and compared to IS*900* (Green et al., 1989) deposited in Genbank under the accession number X16293.1, using the Basic Local Alignment Search Tool (BLAST) software, available at National Center for Biotechnology Information – NCBI website (http://www.ncbi.nlm.nih.gov). Multiple alignments were obtained using the software ClustalW and from these, genetic groups were performed using the software MEGA version 4.0 Windows, by the UPGMA method. Statistical analysis was performed using 1000 replications of bootstrap.

RESULTS AND DISCUSSION

Eight quarter milk samples (3.6%) and none of the bulk tank milk samples amplified fragments of the expected size, 626bp (Figure 1). Out of the cloned samples, three (37.5%) and the positive control were successfully sequenced, but it was not possible to sequence the others.

The genetic analysis showed 99% of identity between the sequences of this study and the sequence X16293.1; 90% with *M. avium* (AF527957.1); 91-92% with *Mycobacterium* sp. (AF455252) not-yet-identified; and 74% with *Streptomyces turgidiscabies* (AY707080.1). Among the compared sequences, 11 were randomly selected to carry out a genetic sequence alignment among the 626 nucleotides, together with the four sequences of this study (Figure 2). From this alignment, a gene cluster map among the 15 gene sequences was built, showing high levels of similarity, since the sequences were pooled together within the same tree branch (Figure 3). Only one sequence showed lower similarity and is located in another branch, probably for being a not-yet-identified *Mycobacterium* species.

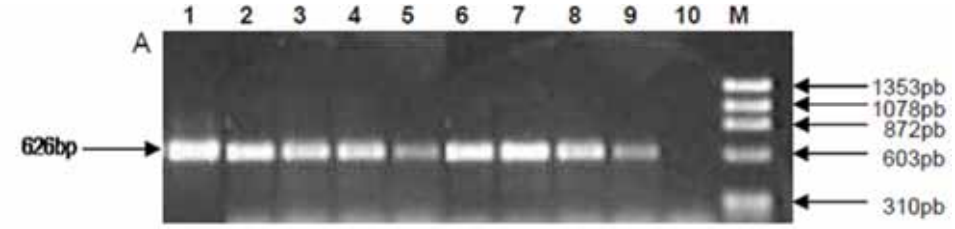

Figure 1: PCR products visualized in 1% agarose gel electrophoresis, using primer pairs BN1/BN2. 1, 2, 3, 4, 5, 6, 7 and 8) amplified samples; 9) positive control; 10) negative control: milli-Q water; M) molecular marker: ϕX174/*Hae*III.

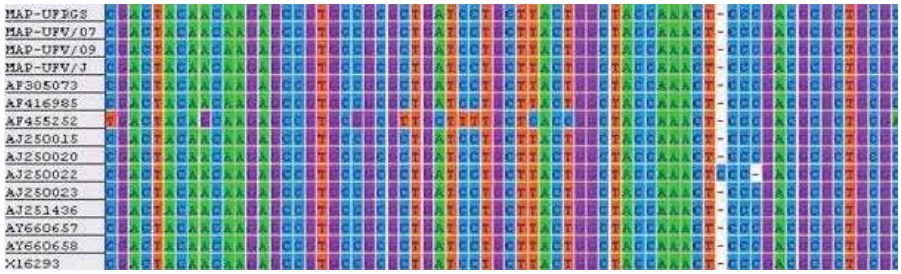

Figure 2: Part of the genetic alignment between DNA fragments sequenced and sequences existing in GenBank.

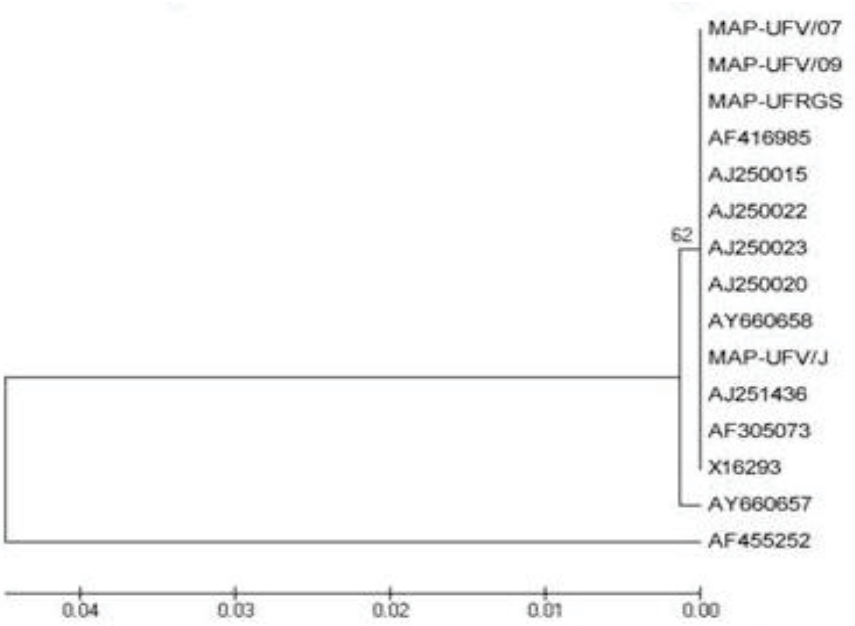

Figure 3: Gene cluster map between genetic sequences of MAP described in this study (MAP-UFRGS; MAP-UFV/07; MAP-UFV/09 and MAP-UFV/J) and sequences available on GenBank (X16293, AF416985, AJ250015, AJ250022, AJ250023; AJ250020, AY660658, AJ251436, AF305073, AY660657 and AF455252). Method: UPGMA; Statistical Support: 1000 replications of bootstrap.

CONCLUSION

Results confirmed presence of MAP in the analyzed samples that showed identity with the insertion sequence IS*900*. This study confirmed MAP in the tested milk samples, providing key information about presence of paratuberculosis in dairy herds in the region of Viçosa, Minas Gerais State, Brazil. Since there is a lack of official paratuberculosis surveys and control programs in Brazil, further studies will be needed to support the adoption of national paratuberculosis control measures. The dilution that occurs in bulk tank samples makes them non-ideal for PCR analysis, despite being the type of sample chosen to perform a herd screening. This is the first report of MAP detected in raw milk samples from dairy cattle in Brazil and it is reasonable to consider its as a first survey on the disease in the state of Minas Gerais, Brazil.

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#34 Viability of *Mycobacterium avium* subsp. *paratuberculosis* during traditional elaboration and storage of yogurt

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Objective: In order to evaluate the viability of Map during traditional elaboration and storage of yogurt, an inoculation experiment was conducted.

Materials and Methods: Typified strain of Map, previously isolated form commercial milk, was cultivated onto HEYM. The bacterial suspension was adjusted to McFarland tube 0.5 using PBS (pH 7). A traditional yogurt was prepared using 360mL of UAT milk with 125gr of commercial yogurt. Five mL of the bacterial suspension was added. The mixture was incubated at 43ºC during 3 hours in shaker (New Brunswick Scientific Exella 24). Once fermentation was complete it was kept at 4ºC for 20 days. Samples were taken out before (T0) and throughout the elaboration process, every 45 minutes (T1, T2, T3 and T4), and afterwards, during storage (T5: 4.5 Hs. T6: 24 Hs, T7: 48 Hs, T8: 4 days, T9: 10 days, T10: 15 days, and T11: 20 days). To each sample pH and Map counts, after decontamination with 5% Oxalic Acid (Tacquet et al, 1980), were recorded.

Results: pH measurement: During the elaboration pH dropped from 6.1 at T0 to 4.7 at T7, while during storage pH was kept stable at 4.4 (T8 – T12). Map viable count: Map viable counts were 127 CFU/mL at T0. Map was not re-isolated from samples taken at T1 – T4. Map was detected at T5 (2.5 CFU/mL), gradually increased up to 62.7 CFU/mL (T8) followed by a decrease to 0.33 CFU/mL (T11).

Discussion and Conclusion: Map may resist low pH levels and high temperatures during the traditional elaboration of yogurt and its storage. During this process no viable Map were detected. Nevertheless, Map was detected during storage showing adaptation to the new conditions of pH and temperature. Map counts are low due to the decontamination process.

Map may be present in raw material, in low concentrations, and might survive elaboration process and reach the final product.

#49

Two Worlds on a Collision Course

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ABSTRACT
The veterinary and medical perspectives as to the zoonic potential of *Mycobacterium avium* subspecies *paratuberculosis* are on a collision course.

INTRODUCTION
There has been a long simmering controversy between the worlds of medicine and veterinary sciences as to whether *Mycobacterium avium* subspecies *paratuberculosis* (Map) and related genomic variants constitute a threat to the public welfare.

The theoretical bridge linking the two worlds is the failure of pasteurization to completely destroy Map and thus enter the human food chain and evidence that Map has the potential to function as a zoonic pathogen.

HISTORICAL PERSPECTIVE
In 1999, the National Institute of Allergy and Infectious Diseases (NIAID) published its research agenda in which it targeted an infectious cause of Crohn's disease. The U.S. Congress elected not to fund this initiative.

In 2000, the Centers for Disease Control and Prevention (CDC) issued a working document related to identifying risk factors for human zoonic infections including Crohn's disease. The project was not funded.

In 2000, the United States Department of Agriculture introduced and advocated the implementation of the U.S. Voluntary Johne's Disease Herd Program Herd Status Program (Anon., 2000b:2000c; 2000d; 2005). The U.S. Voluntary Johne's Disease Herd Program Herd Status Program provided voluntary guidelines for herd risk assessment and identified management practices designed to reduce the prevalence of infection. In the same timeframe, revisions to parts 71 and 80 of the Code of Federal Regulation (CFR) were made that restricted the interstate transportation of Map-infected animals except to recognized slaughter establishments (USDA 2000).

In June 2001, the United Kingdom Food Standard Agency issued its report for food standards. The conclusion statement states "*There is undoubtedly sufficient cause for concern (relative to Map as being the cause of Crohn's disease) for further action to be taken urgently to determine what the available data means …… This question can be divided into two areas: What action should be taken to reduce exposure to Map even though the causal link is not established; and what action can be taken to increase the knowledge base so that future decisions may be based upon more information* (1)."

In April 2002, USDA-APHIS published the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program and instituted a 5 year Johne's Disease Prevention Dairy Herd Demonstration Program to develop voluntary guidelines designed to reduce Map herd prevalence.

In 2003, The National Academy of Press published the national Academy of Sciences' analysis of the Crohn's disease/Map controversy *"There remains insufficient evidence to prove or disprove that Mycobacterium avium subsp. paratuberculosis is a cause of some or all cases of Crohn's disease. … A causal link between Map and Crohn's disease remains a plausible hypothesis…."*

In 2008, the American Academy of Microbiologists published its report *on Mycobacterium avium paratuberculosis*: Infrequent human pathogen or public health threat (2). The executive summary states, "*the association of MAP and CD is no longer in question. The critical issue today is not whether MAP is associated with CD, but whether MAP causes CD or is only incidentally present."*

By 2008, the majority of Koch's postulates for causation had been effectively met from data engendered by the medical side of the controversy (3-9).

In 2009, three independent diagnostic laboratories (Michael T. Collins, Saleh A. Naser, and that of the Centers for Disease Control and Prevention) recovered Map from the blood of individuals with Crohn's disease (10). Naser's previous recovery of Map from the blood of Crohn's patients and the breast milk of two postpartum CD females without corresponding recovery from non-Crohn's diseased individuals had been largely under appreciated.

CONCLUSION

From a medical infectious disease point of view, the validation of Naser's original findings cuts short the argument as to causality. If an individual has certain retroviruses in his or her white blood cells, he or she has HIV infection. If the individual has hepatitis B or C virus in his or her white blood cells, he or she has hepatitis infection. If an individual has Map in his or her white blood cells, he or she has infection with map. If the vast preponderance of individuals with map in their white blood cells has Crohn's disease or irritable bowel syndrome, the critical element of Koch's postulates for causation has been met.

The Rio Declaration Relative to Food Safety states, "*Where there are threats of irreversible damage, lack of full scientific certainty shall not be used as reason to postpone cost-effective measures to prevent environmental degradation."*

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#56 *Mycobacterium paratuberculosis* – The trigger for Type 1 Diabetes and cardiovascular disease?

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Unfolding appreciation of shared genetic susceptibility to mycobacterial infection and autoimmune disease has created a link between *Mycobacterium avium* ss. *paratuberculosis* (MAP) and Type 1 Diabetes Mellitus (T1DM). Animal and clinical studies implicate mycobacteria in the immune genesis of atheromatous lesions. Beyond classic risk factors for atheromatous cardiovascular disease, cellular and humoral immunity against heat shock protein 60 (HSP60) is increasingly identified in the genesis of atheromatous disease. HSP60 is an immunodominant antigen and there is marked homology between human and mycobacterial HSP60. MAP is pervasive in the food chain, causes Johne's disease of ruminant animals and is the putative cause of Crohn's disease in humans. This article describes the link between MAP and T1DM and postulates that MAP is the mycobacterium responsible for HSP60: the immune trigger for atheromatous disease.

#115 Crohn's disease and ruminant farming. Got lactase?

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Crohn's disease (CD) is a well known chronic pathological condition whose aetiology has remained unrecognized for nearly a century. Complex immune mechanisms in a specific genetic background causing an abnormal local inflammatory response are thought to be directly responsible for the clinical picture, but no external factor triggering such host responses has been identified. Humans lose the capability of breaking down milk lactose early in life and, afterwards, ingestion of large amounts of lactose causes a transient digestive illness known as lactose intolerance. Since some populations have developed a lactose tolerance mutation, we submit the hypothesis that this adaptation to dairy farming could be related to CD as a collateral effect of exposure to a intestinal inflammation-causing ruminant parasite to an epidemiological analysis of association throughout the world.

Data from published sources regarding by country CD and type I diabetes incidence, lactose tolerance, livestock population, food production, Gross National Income and human population were submitted to correlation, multiple regression and principal components analyses. Multiple regression was also applied to a published 20-year time series for CD incidence in Japan. These analyses showed a strong association between country incidence of CD and frequency of lactase persistence as well as other ruminant production and consumption variables that further supports the meaning of those observations.

The evolutionarily plausible framework provided by this association with the species suffering a similar inflammatory bowel disease (IBD), its coincidence with the expanse of the Friesian cattle lineages, that could act as a Trojan horse, in addition to recent microbiological, immunological and therapeutical observations consistent with a slow infection type of pathogenesis, supports a mycobacterial aetiology of human IBD. Further research challenging the hypothesis of a shared aetiology by *Mycobacterium avium* subsp. *paratuberculosis* of human and ruminant IBD is needed for confirmation or rejection of this hypothesis.

#247 Effective reduction of *Mycobacterium avium* subspecies *paratuberculosis* in raw milk by industrial centrifugation

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Heat treatment is the most applied process to ensure the microbial quality and safety of milk and milk products. With the application of centrifugation or bactofugation an additional reduction of the number of microbes in milk can be realized. To determine its contribution to the quality of milk, the reduction of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in raw milk by centrifugation prior to pasteurisation was determined.

In the framework of the EUParaTBtools project two rounds of experiments were conducted. The reduction of raw milk contaminated with MAP by centrifugation was quantified. Raw bovine milk was artificially contaminated with MAP by addition of the collection strains, ATCC 19851=NCTC8578 from a bovine clinical sample; strain Niebuell from bovine milk; strain NIZO2962 from bovine faeces. Additional MAP infected feces of cows with clinical symptoms of Johne's disease was used to contaminate the raw milk mimicking possible natural contamination. MAP contaminated raw milk was submitted to centrifugation, using a pilot scale bactofuge mimicking industrial scale at different temperatures. Part of the samples was additionally submitted to pasteurization using different time-temperature combinations. MAP levels were monitored by real time PCR and survival was determined by growth in MGIT media. Preliminary results indicate that an MAP reduction of at least 1 log is obtained by centrifugation.

International Johne's Disease Initiatives

Convenor: Douwe Bakker

#76

Towards improved reporting standards for test evaluation studies for paratuberculosis

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A 2008 review of test evaluation studies for paratuberculosis concluded “there is a profound lack of reliable test evaluations, and future assessments should be conducted more stringently to allow appropriate interpretation and comparison across populations”. One approach to improve quality is to develop consensus-based reporting standards. Development of standards for paratuberculosis has several potential benefits. First, standards could help avoid tests of poor utility that don’t improve management decisions by livestock producers or reduce potential public health risks. Second, provision of design and analysis guidelines for researchers, grant proposal reviewers and test developers interested in licensure of their products would help improve consistency of the review and assessment process. Finally, standards could help guide choices of sample types to be included in repositories or sample banks developed for use in test validation studies.

In human medicine, the STARD (**St**andards for **R**eporting of **D**iagnostic **A**ccuracy) initiative was started to improve the accuracy, completeness and transparency of studies reporting on diagnostic accuracy. More than 200 biomedical journals (including 2 veterinary journals, Acta Veterinaria Scandinavica and BMC Veterinary Research) endorse the STARD statement (available at www.stard-statement.org/) and most recommend use of the STARD checklist of 25 items in their instructions to authors. The STARD checklist was primarily developed for evaluation of tests in individual human patients with clinical disease and hence, may not be directly extrapolated to testing scenarios in animal health, which often focus on subclinical infection in individuals, herd tests, and pooled tests. Also, unlike human medicine we often do not have the financial or technical resources to truly define the infection status of study units and hence, latent class modeling approaches may be needed for statistical analysis.

A consensus meeting to discuss reporting standards will occur prior to the ICP meeting and this presentation will update progress on the initiative.

#78

National surveillance for Paratuberculosis in New Zealand Farmed Deer Herds: Development of a Database

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Aim: Develop a national database for paratubercluosis surveillance in New Zealand farmed deer.

Method: Meat inspectors characterise and electronically record lymph nodes (LN) as “normal” or “enlarged” based on investigation of circumference distribution, or resembling tuberculosis (*M. bovis*) lesions. During set-up, LN and carcass data from 1,309,566 deer from 3380 farms were recorded from January 2007 – 2009. Data is now recorded from almost every animal slaughtered, expected to be ~0.5 million in 2009, from every deer slaughterhouse. Data are forwarded regularly to the central database for checking and collation. Validation involved physical and bacteriological investigation.

Results: Evaluation of four meat inspectors recording 1287 mesenteric LN (MLN) showed 99% specificity and 25% sensitivity for categorisation of MLN enlargement. Ongoing supervision is improving inspector diagnosis. Initial screening showed 95% of130 “enlarged”, and 69% of 154 randomly selected “normal” MLN from 25 farms were *Mycobacterium avium* subsp. *paratuberculosis* (MAP) culture and/or histology positive. Data from subsequent MLN screening of four deer per farm from 57 randomly selected farms from four regional slaughterhouses has been collected for culture and histology for modelling of MLN characteristics and frequencies as a tool for predicting farm infection and possibly clinical disease status. Results will be presented.

Conclusion: The database allows spatial and temporal tracking to quantify and monitor the disease and control measures, reported to industry. Data is being used to heighten awareness of paratuberculosis, target high prevalence herds for intensive control programmes and cost-effectively identify herds of various disease and infection strata for case, case-control, longitudinal and intervention studies to better understand the disease. The database is supported by the deer industry and is now owned and managed by an independent processor-funded agency. Routine analysis and report presentation is by Massey University (see accompanying abstract), and data is available for approved research purposes.

#97

The EU ParaTBTools Project – Thematic Area 3: The inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk and dairy products

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The objectives of Theme Area 3 of the EU ParaTBTools project were to develop improved processing and manufacturing parameters for milk and dairy products to achieve improved inactivation of Map and to standardize culture and molecular methods for the detection/isolation/recovery of Map from dairy matrices. The Work-packages (WP's 7, 8 & 9) involved research institutes from Ireland, UK, Germany, France, Netherlands and Belgium.

In WP7 molecular and culture methods were developed for the detection/enumeration of Map from yoghurt, Gouda, Danish Blue and Munster cheeses. Real-Time PCR using an IS*900*-based TaqMan assay and a commercial Map DNA extraction kit was the most effective molecular method. Culture methods using HEYM supplemented with the antibiotic supplements PANTA, nisin (125 U) and penicillin (12 IU) was most suitable for the recovery of Map from Yoghurt, Danish Blue and Gouda cheeses. However, this medium was less suited for the recovery of Map from a raw milk cheese.

In WP 8 eleven laboratories participated in a dairy products ring trial using the developed methodologies according to the NordVal validation method. Dairy samples contaminated with 'medium' and 'low' levels of Map were prepared and distributed to participant laboratories. Average sensitivities across all laboratories at medium and low levels of inocula were 95%, 98%, 100%, 84% and 96% for faecally-contaminated milk, yogurt, Gouda, Danish Blue and Munster cheese respectively. Calculated specificities for all matrices tested were in the range 95-100%. The sensitivity and specificity of the culture methods were lower than those recorded for molecular methods.

In WP 9 the inactivation of Map during the manufacture of Gouda, Danish Blue, a smear ripened raw milk soft cheese and yogurt has been assessed. The inactivation of Map during high temperature short time pasteurisation of milk in combination with homogenization (up-stream and down-stream) and bactofugation are under investigation.

#65

A deer model for heritable resistance to MAP infection and Johne's disease

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Objective: To identify breeds of deer with polarized phenotypes for susceptibility or resistance to MAP Infection.

Materials and Methods: An ELISA test was used to screen naturally infected deer stud herds to identify the relative prevalence of infection from eight uniquely different deer breeds (genotypes). Seropositive animals were necropsied and detailed microbiological and histopathological examinations carried out to confirm their infection status. Cross-bred hybrids were also examined. Followup studies were carried out using embryos from the distinct genotypes studied in the herd screen.

Results: The different deer breeds had MAP seroprevalence ranging from 0 - 80%. Animals with a given genotype displayed similar infection rates when farmed in different herds. The influence of genotype was explored further using embryos derived from different breeds as purebred or hybrid lines. The advantage using embryos is that exposure risk from the dam was controlled as all embryos were transferred randomly into test negative recipient hinds. Hybridising parental breeds that displayed low infection prevalence (0%) with breeds showing high prevalence (58%) produced offspring with intermediate prevalence (24%) suggesting Mendelian heritability of resistance traits.

Conclusion: High levels of line breeding are carried out within the New Zealand deer industry where some of the top performing stags are overrepresented in the gene pool. Selection criteria have been largely focused solely on superior production (venison and antler) and temperament. This selective breeding strategy within deer studs appears inadvertently to have selected animals with genotypes that display extremes of susceptibility or resistance to mycobacterial infections (tuberculosis and paratuberculosis). The purebred lines of deer that display these extreme phenotypes are being studied to identify the genes that contribute to susceptibility/resistance using immunoassays for innate and adaptive immunity. Polymorphisms in Immune response genes (SNPs) and the mononuclear cell transcriptome of these animals is being studied using 454 sequencing.

#241

US Vaccine Initiative through the JDIP Program

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Investigators across the United States and some abroad have submitted their best candidate vaccine formulations for standardized testing in preselected labs. Vaccine candidates include mostly attenuated *M. avium* subsp *paratuberculosis* mutants and some recombinant proteins. There will be three phases of testing with the final phase using the goat model. The logistics of this coordinated effort and early progress will be described in detail.

#116

Associations between clinical paratuberculosis, production performance and species interaction in co-grazing pastoral ruminant species in New Zealand

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Aim: The objective of this study was to evaluate farmer awareness and observations of clinical paratuberculosis (Ptb), and the impact of observed Ptb on productivity in mixed species, pastoral farming systems in New Zealand.

Methods: Twenty-six veterinary practices were contracted to mail out questionnaires to 8,314 commercial livestock farming clients in five of 16 regions of New Zealand. The requested information included herd characteristics and denominator data, observed clinical Ptb and records, as well as estimates of mortality and reproductive performance in sheep, deer, beef and dairy cattle. This paper presents farmer awareness about Ptb and annual rates of observed clinical Ptb in these species, adjusted for region, farm type and herd/flock sizes. For the analysis of production effects, reproductive performance and mortality were classified as low or average/high for each species. For each species enterprise, a multivariable logistic regression model assessed the relationship between production performance and clinical Ptb, using co-grazing species, region, farm type and farm size as co-variables. A second model evaluated the association between clinical Ptb for each species and other species present or absent on farm.

Results: This study will be completed by end of May 2009. A total of 1,713 (20.6%) commercial farmers returned the questionnaire with valid data. Results of the analysis will be available and presented at the conference.

Conclusions: Expected inferences include the perceived importance that farmers attribute to Ptb, population based estimates of the impact of clinical Ptb on productivity, and associations between clinical Ptb and on-farm species composition.

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