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9th International Colloquium on Paratuberculosis

October 28 – November 2, 2007
Tsukuba, Japan

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International Association for Paratuberculosis, Inc.

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Kei Nishimori
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9th International Colloquium on Paratuberculosis

Tsukuba, Japan

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58 **Characterization of *Mycobacterium avium* subsp. *paratuberculosis* isolates in Tamil Nadu, India.**

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59 **Strain typing based on sequence polymorphisms in a surface exposed PPE protein of *Mycobacterium avium* subspecies *paratuberculosis***

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Title: Modulation of cytokine expression and lymphocyte subsets during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s): Karcher EL, Beitz DC, Stabel JR.

Institution: 1 Department of Animal Science, Iowa State University, Ames, IA 50010, 2 USDA-ARS, National Animal Disease Center, Ames, IA 50010.

Presentation: Keynote, winner of JDIP Award

Abstract: On-farm observations suggest that dairy cows infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) may demonstrate increased signs of clinical disease during the weeks following parturition. To date, limited research is available characterizing host immunity in periparturient dairy cows infected with MAP or the potential impact of periparturient immunosuppression. Therefore, the objective of this study was to characterize cytokine gene expression and secretion in dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows. Twenty-three Holstein cows were placed into 3 groups consisting of 5 noninfected healthy cows, 14 subclinical cows, and 4 clinical cows. Blood was collected from the jugular vein at -21, -14, -7, +1, +7, +14, +21, and +28 days relative to calving. Peripheral blood mononuclear cells (PBMCs) were isolated and cultured for 24 h with and without concanavalin A (ConA) throughout the sampling period. Real-time PCR was performed on each sample to evaluate the expression of IFN-gamma, IL-12, IL-10, TGF-beta, IL-4, and beta-actin. RT-PCR data was analyzed using 2-ddCt values calibrated to dCt value at +1 d for each animal. Additional PBMCs were incubated for 24 h with and without ConA or MAP whole cell sonicate (MPS). Cell culture supernatants were collected and ELISA assays performed to evaluate secretion of IFN-gamma, IL-10, TGF-beta, and nitric oxide (NO). All of the animals displayed concentrations of progesterone, 17beta-estradiol, and IGF-1 that are consistent with values reported in the literature for periparturient dairy cows (parturition effect, \( P < 0.001 \)). Regardless of infection group, expression of IFN-gamma from non-stimulated (NS) PBMCs declined as parturition approached and did not recover during the postpartum period (\( P < 0.03 \)). IL-12 and TGF-beta expression for all groups remained relatively stable during the 3 wks before calving and the 4 wks after calving. Expression of IL-4 by ConA-stimulated PBMCs isolated from infected cows, declined between -21 d and +1 d (\( P < 0.05 \)). IL-10 expression by NS PBMCs, for both infection groups, declined as parturition approached (\( P < 0.05 \)). However, during the postpartum period, there was an increase in expression of IL-10 by control cows (\( P < 0.05 \)). Secretion of IFN-gamma by ConA-stimulated PBMCs, tended to be greater in infected cows compared with the controls (\( P < 0.12 \)). Parturition did not have an effect on TGF-beta secretion by either NS or MPS-stimulated PBMCs and effects due to infection status were minimal. When subclinical and clinical cows were analyzed together, secretion of IL-10 tended to be greater for infected cows compared with control cows at +1 d and during the postpartum period (\( P < 0.10 \)). Stimulating PBMCs with MPS resulted in a 7.7, 9.7, and 12.0-fold increase in IL-10 secretion for control, subclinical, and clinical cows, respectively, compared with secretion from NS PBMCs. MPS-stimulated PBMCs from clinical cows tended to have greater NO production compared with the control (\( P < 0.09 \)) and subclinical cows (\( P < 0.15 \)). In addition to characterizing cytokines, peripheral blood CD4+, CD8+, and gamma-delta T-cells and B-cells percentages were analyzed with flow cytometry. Cell populations were further delineated by staining for CD5, a marker for T and B-cell activation. Compared to the prepartum period, the percent of CD4+ T-cells increased at parturition for clinically infected cows (\( P < 0.08 \)), whereas healthy control cows showed a gradual decline in CD4+ T-cells from -21 to -7 d. Subclinical cows expressed an overall greater percentage of both CD8+ and gamma-delta T-cells (\( P < 0.05 \)) throughout the periparturient period compared with controls. Clinical cows expressed a lower percentage of CD4+/CD5bright and CD8+/CD5bright compared with control cows, but greater percentages of CD5dim cells for all lymphocyte subsets (\( P < 0.05 \)). Results suggest that cytokine gene expression and secretion, and the percentages of lymphocyte subsets are modulated by both infection status of the
animal and the periparturient period.

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<td>The University of Edinburgh</td>
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<td>Three forms of Johne's disease have been described in sheep - multibacillary, paucibacillary and asymptomatic. We used real-time RT-PCR to compare the expression of thirteen cytokine and cytokine-related genes in ileal tissue from sheep with these three forms of the disease to try to understand the immune responses underpinning these three pathologies. Three pathological forms of sheep paratuberculosis were defined on the basis of histopathology, cytochemistry (Zeihl-Neelsen) and IS900 PCR. Paucibacillary lesions have lymphocytic infiltration and are ZN negative; multibacillary lesions have macrophage infiltration and large numbers of acid-fast bacteria. The pauci- and multibacillary forms are linked to the differential expression of IFN-gamma and IL-10 respectively. In addition the increased levels of the proinflammatory cytokines (IL-1beta and TNF-alpha), IL-8, IL-18 and TRAF-1 in both diseased forms is indicative of persistent inflammatory lesions. No changes were seen in IL-1alpha in any sheep ileum tissues. Asymptomatic animals are IS900+ with normal histology but have significantly decreased levels of IL-18 and increased levels of TNF-alpha and thus can be distinguished, in terms of cytokine expression profile, from uninfected controls.</td>
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<tr>
<td>Institution</td>
<td>Department of Microbiology and Immunology, University of Otago, New Zealand</td>
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<td>There are two possible disease outcomes in ovine Johne's Disease. Paucibacillary (PB) disease is defined by lymphocyte infiltration, a Type 1 immune response and few acid fast organisms present within lesions. Multibacillary (MB) disease is defined by macrophage infiltration, a Type 2 immune response and many acid fast organisms. Sheep with severe Johne's Disease were identified from within several small herds, necropsied, and blood and mesenteric lymph node samples harvested. Culture and histology results were used to define two groups; sheep with varying severity PB disease, and sheep with severe MB disease. Samples were further analysed by IFN-gamma ELISA, IgG1 antibody ELISA, flow cytometry and real-time PCR. This data was correlated with both disease status and disease severity to determine whether these naturally infected sheep have classical PB or MB immune profiles. This data revealed a strong johnin specific proliferative response in the PB diseased group and a less responsive antibody-based response in the MB diseased group. This was demonstrated by high levels of IFN-gamma protein and mRNA accompanied by increases in all cell populations monitored after stimulation with johnin in PB diseased animals. There were also higher levels of inherent IFN-gamma mRNA in the blood but not the posterior jejunal lymph node. These observations were in contrast to MB diseased animals which exhibited low levels of IFN-gamma protein and mRNA, no change in the cell populations monitored after stimulation with johnin, but high IgG1 antibody levels, high circulating levels of BCR+ cells, and high levels of inherent IL-10 mRNA in the posterior jejunal lymph node but not the blood. Interestingly both diseased groups had lower levels of inherent IL-4 mRNA in the</td>
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blood and posterior jejunal lymph node than the control non-diseased animals. This data will also be compared to data from a current vaccine trial to investigate the effects of vaccination on disease development.

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

Author(s) Allen AJ¹, Hamilton MJ², Barrington G¹, Lahmers K², Park KT², Davies C², Davis W².

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Presentation Poster

Abstract Crohn's disease (CD) is a clinically defined syndrome of unknown etiology. Although the etiology of CD is not fully understood, genetic and epidemiological studies indicate individuals with one or more allelic variants of genes involved in regulation of the immune response may be at higher risk for developing CD. The factors that induce persistent immune mediated inflammation of the bowel in these individuals are thought to include exposure to specific pathogens, bacteria present in normal microflora of the intestine, or other undefined factors. The mechanisms of induction and persistence of inflammation appear to include modulation of the immune response by regulatory T cells and activation of the IL-23-IL-17 pathway that promotes chronic inflammation. *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the pathogen most frequently implicated in playing a role in the pathogenesis of CD. The implications are due to similarities in pathogenesis between CD and Johne's disease in cattle and the increased frequency of finding *Map* in CD patients over patients of other inflammatory bowel diseases. These findings could indicate *Map* plays a role in pathogenesis in a subset of genetically susceptible individuals with CD or that, individuals with CD are serving as sentinels that reveal the prevalence of *Map* in the environment. Elucidation of the mechanisms of pathogenesis mediated by *Map* in its natural host could provide an opportunity to clarify the role of *Map* in CD pathogenesis and also insight into the sequence of events leading to erosion of protective immunity and development of clinical disease. To explore this possibility, we developed a bovine cannulated ileum model to analyze the immune response to bovine and human isolates of *Map* and determine how *Map* modulates the immune response during the early and late stages of disease. Comparative studies have revealed no significant differences in the capacity of bovine and human isolates of *Map* to infect calves. Both types of isolates elicit a prominent CD4 memory T cell response to PPD and soluble *Map* antigens detectable by flow cytometry 3 months post infection (PI). Morphologic changes in the ileum consistent with disease progression are detectable by 8 - 10 months PI. Although difficult to culture from tissue biopsies, presence of *Map* is detectable by PCR. Quantitative RT-PCR has revealed a complex pattern of expression of genes encoding IFN-gamma, IL-17, and granulysin in experimentally infected animals 10 - 12 months PI indicating the presence of CD4 memory T cells associated with a Type I immune response and Th17 CD4 T cells associated with development of a proinflammatory response. The increase in expression of granulysin suggests the presence of effector memory T cells with bactericidal activity. The findings indicate a detailed analysis of immunopathogenesis of JD will facilitate understanding the role of *Map* in the pathogenesis of CD.

Title Proliferation of lymphocyte subsets in experimental ovine Johne's disease

Author(s) de Silva K, Begg D, Taylor D, Di Fiore L, Whittington R.

Institution University of Sydney, Australia

Presentation Poster

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Abstract
Protection against mycobacterial disease relies on T-cell dependent immune responses. In Johne's disease, cell-mediated immune responses predominate in subclinical disease. As disease progresses these responses diminish and can be undetectable while there is a concurrent strong humoral response, demonstrated by elevated serum anti-Mptb (\textit{Mycobacterium avium} subsp. \textit{paratuberculosis}) antibodies. If the infection is not eliminated by the initial T-cell response then Mptb are able to persist and proliferate resulting in lesion formation and chronic infection. Much work has been published on cellular responses throughout the course of bovine Johne's disease but knowledge of similar responses in sheep is lacking. The aim of this study was to identify changes in antigen-driven proliferation of lymphocyte subsets during the course of early ovine Johne's disease (OJD). Merino lambs aged 4 months were randomly assigned into three groups, \( n = 20 \) per group. Two groups were orally challenged with either a clonal inoculum of Mptb (Group 1) or a gut homogenate prepared from an animal with clinical OJD (Group 2) while the third group was left unchallenged (Group 3). Blood samples were collected at 4 monthly intervals and prior to necropsy at 13 months post-challenge. Lymphocytes isolated from blood and lymph nodes (ileal, posterior jejunal and prescapular) were labelled with CFDA-SE and cultured in the presence or absence of Mptb antigen for 5 days. Cells were labelled for phenotype - CD5, CD4, CD8\(\alpha\beta\), CD8D (gamma-delta T cells) and WC4 (B cells) - prior to data collection by flow cytometry. Faecal shedding and presence of Mptb in tissue samples were determined by culture in a radiometric system (Bactec). Diseased sheep were categorised based on histological lesion type. Distinct differences were seen in the antigen-induced proliferative response in both blood and lymph node cells from sheep challenged with Mptb compared to the unchallenged controls. While 30\% of Group 1 and 50\% of Group 2 animals responded to Mptb antigen stimulation in the proliferation assay as early as 4 months post-challenge, none in Group 3 responded. The number of responders in Groups 1 and 2 continued to be higher than in Group 3 throughout the study. Differences in the phenotype of proliferating subsets in relation to infection status will be discussed.

Title
Association between Two Polymorphisms in the Bovine CARD15/NOD2 Gene and Paratuberculosis Infection in Florida Dairy and Beef Cattle.

Author(s)
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Institution
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Presentation
Winner of Richard Merkal Award

Abstract
Paratuberculosis has been suspected to have a genetic component and estimations of heritability of about 0.15 have been reported. Caspase recruitment domain 15 (CARD15/NOD2) is a gene codifying for a cytosolic protein implicated in bacterial recognition by cells involved in innate immunity. This protein modifies inflammatory responses to bacterial triggers through activation of the nuclear factor-kB. Crohn's disease (CD) is an idiopathic inflammatory bowel disease in humans similar in many features to bovine paratuberculosis and involves an aberrant mucosal immune response in genetically susceptible individuals. The association between mutations in the CARD15/NOD2 gene and increased risk of CD has been described. The objective of this candidate gene case-control study was to characterize the distribution of two polymorphisms in the bovine CARD15/NOD2 gene and test their association with paratuberculosis infection in Florida dairy and beef cattle. The study population consisted of 432 adult cows composed of Holstein, Jersey and Brahman-Angus crosses distributed in four herds. The infection status for cases and controls was determined using five diagnostic tests (serum ELISA, milk/blood/fecal nested PCR, and fecal culture). Parallel interpretation of the results was used to compensate for limitations in sensitivity of available diagnostic tools. Two previously reported single nucleotide polymorphisms (SNP1; C733R and SNP2; Q1007L) in the bovine CARD15/NOD2 gene, responsible for two amino acid substitutions, were established.
for the study population by the TaqMan® genotyping assay. The statistical analysis was based on Chi-square and Fisher's Exact Test and different models were proposed for the logistic regression analysis. It was our central hypothesis that a combination of particular alleles in our candidate gene would be present in higher frequency in controls compared to cases, suggesting a role in resistance to infection. The resulting ratio of cases to controls was 1:2.5. Frequencies for the major allele in SNP1 and SNP2 two were 0.957 and 0.543, respectively. The population was in Hardy-Weinberg equilibrium for SNP2 but not for SNP1. Values for coefficient of linkage disequilibrium (LD), the normalized LD, and the correlation between the two SNPs were -0.027, 1.0, and -0.23. Chi square test indicated that SNP1 and SNP2 are in linkage disequilibrium. The statistical analysis resulted in significant differences in allelic frequencies between cases and controls for SNP1 (p<0.001) indicating a significant association between infection and mutant allele. In the analysis of genotypes a significant association was found between SNP1 and infection status (P<0.0001). A significant association between allele combinations and infection status was found (P<0.0001) when both SNPs were considered in the genotype. The low representation of the variant allele for SNP1 in Holstein and Jersey breeds raises the prospect of a potential confounding role of breed for its connection with infection. However, a significant association between SNP1 and infection was confirmed when tested within the Brahman-Angus sub-population (P=0.02). Preliminary results suggest a role for CARD15/NOD2 gene in the susceptibility of cattle to paratuberculosis infection. Amino acid substitution C733R (SNP1) appears to be associated to paratuberculosis infection in Florida cattle. These results could be the basis for further research to create a rapid method to select for more resistant individuals, genetically contributing to the control of Johnne’s disease.

Title Innate resistance of mice to *M. avium* subsp. *paratuberculosis* is controlled by SLC11A1.

Author(s) Rosseels V, Roupie V, Piersoël V, Zinniel D, Barletta RG, Huygen K.

Institution WIV-Pasteur Institute, Belgium, WIV-Pasteur Institute, Belgium, WIV-Pasteur Institute, Belgium, University of Nebraska, USA, University of Nebraska, USA, WIV-Pasteur Institute, Belgium

Presentation Poster

Abstract We have recently described an enumeration method using luminescent *M. paratuberculosis* strain expressing the *luxAB* genes of *Vibrio harveyi* which replaces fastidious and costly CFU counting by plating. Here we have re-evaluated the effect of *Slc11a1* (formerly *Nramp1*) polymorphism on susceptibility against *M. paratuberculosis*, using this luminometric method. A series of inbred mouse strains were infected intravenously with luminescent *M. paratuberculosis* S-23 and monitored for bacterial replication in spleen, liver and lungs for 12 weeks. Results clearly indicate that - as for *M. avium* subsp. *avium-* innate resistance to *M. paratuberculosis* infection is genetically controlled by *Slc11a1*. In BALB/c, congenic BALB.B10 (BALB/c background, H-2b), C57BL/6 and mutant C57BL/6bg/bg mice (all *Slc11a1s*) bacterial numbers in spleen and liver remained stable during the first 4 weeks of infection, whereas in DBA/2, (C57BL/6xDBA/2)F1 and congenic C.D2 mice (all *Slc11a1t*) bacterial number decreased more than 30 fold during the first month after infection. Both male and female mice displayed the genetic difference. At later time points, additional differences in bacterial replication were observed between the susceptible mouse strains, particularly in the liver. Whereas bacterial numbers gradually decreased more than one-hundred fold in C57BL/6 mice between week 4 and week 12, bacterial numbers remained more or less constant in BALB/c and mutant C57BL/6bg/bg mice. Mycobacteria-specific IFN-gamma responses developed earlier and to a higher magnitude in C57BL/6 mice than in BALB/c mice and were lowest in resistant C.D2 mice.
Title | Susceptibility to paratuberculosis is associated with functionally relevant single nucleotide polymorphisms in bovine Toll-Like Receptor 2
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Author(s) | Koets A.*, Mertens H.*, Oostenrijk D.*, Keestra M., Overdijk M.*, Franken P. †, Frijters A., Rutten V.*
Institution | † Department of Infectious Diseases and Immunology, Immunology Division; † Department of Infectious Diseases and Immunology, Infection biology Division; ¹ Department of Farm Animal Health, Epidemiology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; ³ GD Animal Health Service, Deventer, The Netherlands; ⁴ HG B.V., Arnhem, The Netherlands.
Presentation | Oral
Abstract | Paratuberculosis is a chronic intestinal infection in ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). To study the role of host genetics in disease susceptibility 9 candidate genes (Toll-like receptors 2 and 6, Interleukin-10, Interleukin-12p35, Interleukin-12p40, Interleukin-12 receptor beta1, Interferon-gamma, Interferon-gamma receptor 1, and NOD2/CARD15) selected for their potential role in immunity to mycobacterial infections were analysed for single nucleotide polymorphisms (SNP) and disease association. For SNP discovery and disease association, a case-control study including 24 cows from farms with paratuberculosis was conducted. Sequence analysis of the 9 candidate genes from 12 paratuberculosis infected animals, and 12 age-matched healthy herd-mates, revealed 35 different SNP. The TLR2-1903T/C SNP was significantly associated with resistance to *Map*. This and 11 additional SNP were studied in a subsequent cohort study with 553 cows from farms with paratuberculosis. The allelic distribution of the TLR2-1903T/C SNP was confirmed, and the TLR2-385T/G SNP was also found to be significantly different between the infected and non-infected animals. In *in vitro* functional assays, ligand binding by the TLR2 of the resistant haplotype induced higher *in vitro* NFkB production as compared to the TLR2 of the susceptible haplotype. These findings suggest that higher activity may contribute to enhanced cell activation and a lower susceptibility to paratuberculosis. In conclusion these data support previous work indicating a role for host genetics in susceptibility to bovine paratuberculosis, and the current study specifically identified the diversity in the TLR2 gene in the cattle population to be involved in resistance to bovine paratuberculosis.

Title | Experimental paratuberculosis in sheep with a caprine isolate of *Mycobacterium avium* subsp. *paratuberculosis*
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Author(s) | Kumar AA, Tripathi BN.
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Presentation | Poster
Abstract | The objective of the study was to investigate pathogenesis of paratuberculosis in sheep experimentally infected with a caprine isolate of *M. paratuberculosis* (MAP) and also the transmission potential of the infected sheep to the in-contact sheep over a period of 390 days post-infection (DPI). Cross-bred lambs, 8-12 weeks old, negative to faecal culture/PCR and ELISA to MAPinfection were divided into infected (15) and in-contact (8) groups. Lambs were orally infected with 4x10⁹ MAP ten times within a period of one month and the in-contact animals similarly received sterile phosphate buffered saline. A new classification system based on the results of a set of diagnostic tests (faecal smear examination, culture and PCR, tissue PCR, and ELISA, immunoperoxidase and histopathology) were used to categorise infected and in-contact animals. Amongst 15 infected sheep, 2 (13.3%) each were found to be in the negative and the suspected, 5 (33.3%) in the mild and 3 (20%) each were in the...
moderate and the severe infection categories. Four (50%) sheep of the in-contact group had mild infection. Gross lesions consisting of mild to severe thickening of intestinal mucosa, enlarged mesenteric lymph nodes and gelatinisation of fat could be observed at 150 DPI. Up to 90 DPI, histological lesions were not detected, which was a major point of difference from earlier experiments. The mild infection lesions were characterised by the presence of focal granulomas, mostly in the Peyer's patch area of the ileum and ileocaecal valve (ICV) and occasionally multinucleated giant cells without demonstration of acid-fast bacilli. The moderate infection lesions consisted of multiple focal granulomas containing few to numerous AFB from proximal jejunum to the ICV, besides diffused infiltration of lymphoid cells and macrophages. The lesions in Peyer's patch areas were more severe. The severe lesions varying from large multifocal epithelioid granulomas to formation of epithelioid cell sheets containing abundant AFB were observed in 3 sheep and were similar to the classical lesions of paratuberculosis. It was concluded that caprine strain could produce characteristic lesions in sheep after 150 days of infection and in-contact sheep could develop only mild infection. Pathogenicity of caprine isolate of MAP to sheep and its implication in relation to India where sheep and goat husbandry goes side by side has been discussed.

Title Screening of tissues to estimate comparative distribution of Mycobacterium avium subspecies paratuberculosis in target and non-target organs of goats and sheep in India, using culture and PCR

Author(s) Vohra J, Singh SV, Singh AV, Singh PK, Sohal JS.

Institution Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - FARAH, District - MATHURA (UP), INDIA.

Presentation Poster

Abstract Target and non-target tissues of 41 goats and 20 sheep (farm and farmer's stocks were screened by culture and PCR, to know the distribution of MAP in different tissues and organs. Species-wise, prevalence of MAP was 42.5 and 40.0% in goats and sheep, respectively and was 48.1 and 39.3% in animals (goats and sheep) from farm and farmer's stocks, respectively. Of the 215 tissues screened by culture, 47.9% were positive for MAP. Prevalence was 50.8 and 40.0% in tissues of goats and sheep from farm stock, respectively and 60.0 and 44.2% in tissues of young and adult animals, respectively. Tissues-wise, 42.5 and 53.8% farm animals were positive in culture of mesenteric lymph nodes (MLN) and intestine tissues, respectively, whereas, 37.5 and 41.1%, respectively in farmer's animals. Screening of supra-mammary lymph nodes (SMLN), 52.5 and 43.1% were positive from farm and farmer's animals, respectively. Inflamed SMLN from farm stocks showed higher infectivity. MAP was recovered from 73.6, 64.2, and 54.5% tissues of uterus, udder and testes, respectively in farm stocks. Of the MAP cultured from goats, 82.0% were pauci and 17.9% multi-bacillary and from sheep, all the cultures were pauci bacillary. Majority of colonies appeared between 45 and 120 days post inoculation. Decontaminated pellets from tissues and MAP cultures were processed for DNA isolation and screened by IS900 PCR. Positive DNA samples on amplification yielded specific229 bp band from pellets of intestine, MLN, SMLN, udder, uterus and testes. From MAP cultures, 4 of 13 DNA were amplified. Protoplasmic (PPA) antigen from MAP 'Bison type' strain cultured from a terminal case of JD in goat was used in ELISA. Study revealed that MAP was distributed widely in target and non-target tissues of goats and sheep. Of the 3 tests culture of tissues was most sensitive. This is the first report of recovery of MAP from non-target tissues of goats and sheep in India.

Title Presence and characterization of Mycobacterium avium subspecies paratuberculosis from vaginal secretions of post-parturient farm goats, using culture, IS900 PCR and ELISA kit
### Abstract
Vaginal secretions and serum from 29 post-parturient farm goats were screened by culture, IS900 PCR and indigenous ELISA kit to know the presence of MAP in reproductive tract discharges after parturition. Decontaminated pellets from vaginal secretions and MAP cultures were processed for DNA isolation and screened by IS900 PCR. Positive DNA samples on amplification yielded specific 229 bp band from pellets of vaginal secretions (VS). Of 10 DNA obtained from 29 pellets, 4 were amplified. Soluble protoplasmic (PPA) antigen from MAP 'Bison type' strain cultured from terminal case of JD in goat was used in ELISA kit. The 37.9%, vaginal secretions each were positive in culture and ELISA from 29 goats of farm herds. Of the 3 tests culture was most sensitive. There was poor correlation (37.5%) between culture of VS and ELISA kit (18.1% in strong positives and ELISA and very good correlation between positives (80.0%), low positive and suspected (50.0%) and culture. These results clearly established the immuno-suppression in pregnant goats and damage to immune system by Johne's disease. The study is maiden attempt and reports high presence and recovery of MAP in vaginal secretions of post parturient farm goats in India by culture and ELISA kit. Positive IS900 PCR in DNA pellets confirmed the presence of MAP. Vaginal secretion was potential source of contamination to newborn kids and animal handlers and was a good clinical material to screen post-parturient female goats against MAP infection.
CD40L-stimulated MDM cells is only transient, we observed a sustained p38 activity in MAP-infected MDM cells upon CD40L stimulation, suggesting p38 plays a role in MAP interference with CD40 signaling. Additionally, our data revealed the possibility of a third potential mechanism. We observed a dramatic increase in IL-10 gene expression in MAP-infected MDM upon CD40L stimulation relative to uninfected cells. IL-10 has been shown to negatively regulate IL-12p40 gene expression. Therefore, we hypothesize that MAP-induced IL-10 expression at early times following CD40 signaling interferes with subsequent IL-12p40 and iNOS expression. Continuing studies are underway to uncover the role of sustained p38 activity and enhanced IL-10 expression for MAP interference with CD40 signaling in infected macrophages.

Title Apoptosis of mononuclear cells in experimental and natural ovine Johne's disease
Author(s) Browne S, de Silva K, Begg D, Whittington R, Emery D.
Institution University of Sydney, Australia.
Presentation Poster
Abstract Cell death by apoptosis is a part of normal development. Apoptosis also controls the immune response and is involved in the cytotoxic killing of infected cells. Cell-mediated immune responses play an important role in mycobacterial disease. Increasing our current knowledge of the immunological mechanisms involved in disease progression, including apoptotic responses, may allow advancements in the area of early diagnosis, identification of resistant animals and disease control. For experimental infection, Merino lambs (randomly drafted into groups of 20) were challenged with either 3.36x10^8 CFU of a clonal inoculum (Telford) or 3.76x10^8 CFU of a gut homogenate from an animal with clinical ovine Johne's disease (OJD). One group received no treatment. Sequential blood samples were taken over a period of 12 months. Faecal shedding and presence of Mycobacterium avium subsp. paratuberculosis (Mptb) in tissue samples were determined by culture in a radiometric system (Bactec). Diseased sheep were categorised based on histological lesion type. In addition, samples were taken from sheep sourced from OJD-infected and disease-free farms. Mononuclear cells were isolated from peripheral blood using density gradient centrifugation. Isolated cells were incubated with medium alone, Mptb antigen (10 µg/ml) or Con A (10 µg/ml) for up to 6 days. Caspase activity, a marker of apoptosis, was determined by flow cytometry on day 6 of culture. Phenotype markers were also used to identify specific apoptotic lymphocyte subpopulations. Apoptosis in intestinal tissue was studied by TUNEL assay and by the expression of apoptosis-related genes by Q-PCR. Mptb antigen-driven apoptosis of mononuclear cells in experimentally challenged animals varies as disease progresses. There were also significant differences in the presence of apoptotic cells, as well as expression of apoptosis-related genes, in intestinal tissues from disease-free sheep and those with histological lesions.

Title DNA Cocktail Vaccination Induces Th1 Response and Protects Mice against Mycobacterium avium subsp. paratuberculosis Challenge
Author(s) Chang YF¹, Park SU¹, Kathaperumal KU¹, McDonough S², Stehman S¹, Akey B¹, Huntley J³.
Institution ¹ Animal Health Diagnostic Center, Department of Population Medicine and diagnostic Sciences; ² Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY-14853; ³ New York State Department Agriculture and markets, Albany, NY-12201, USA.
Presentation Poster
Abstract Several novel antigens of Mycobacterium avium subsp. paratuberculosis (MAP), have
been studied as vaccine components and their immunogenicity has been evaluated. 85 antigen complex (85A, 85B, and 85C), Superoxide dismutase (SOD), and 35kDa protein of MAP has been found to induce significant lymphocyte proliferation as well as Th1-associated cytokine response. Based on these results, we cloned and expressed 85A, 85B, and 85C, SOD, and 35kDa protein genes into eukaryotic expression plasmid pVR1020. C57BL/6 mice were immunized three times intramuscularly with the recombinants as a DNA cocktail and pVR1020 vector DNA alone as control. A significant reduction has been detected in the bacterial burden of the spleen and liver of mice immunized with DNA cocktail in contrast to the control group. Also, the relative liver and spleen histopathology data paralleled with the MAP culture results and showed more multifocal granuloma and acid-fast bacilli in the control animals. Moreover, mice immunized with DNA cocktail developed both CD4+ and CD8+ T cell responses to the recombinant antigens and showed significant lymphocyte proliferation. The Th1 response related cytokine (IL-12, IFN-gamma, TNF-alpha) gene expression levels increased in immunized animals. The results of ELISPOT assay also revealed an increase in the number of IFN-gamma secreting cells in the immunized group than the control group, indicating a Th1 type of response. These results indicate that the use of recombinant DNA vaccine cocktail can induce protective immune response against MAP infection with a predominant Th1 response.

Title
Immunization with a Novel Map74F Fusion Protein Protects Mice against Mycobacterium avium subsp. paratuberculosis Challenge

Author(s)
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Presentation
Oral

Abstract
Paratuberculosis, also called as Johne's Disease (JD) is a chronic infectious disease of ruminants caused by Mycobacterium avium subsp. paratuberculosis (MAP), and development of improved vaccines is urgently required considering its economic importance. Here, we report the cloning and expression of a 74kDa recombinant fusion protein (Map 74F) and evaluation of its protective efficacy against MAP challenge in mice. Map74F was generated by the sequential linkage in tandem of the ORFs of the ~17.6-kDa C-terminal fragment of Map3527 to the full-length ORF of Map1519, followed at the C terminus with ~14.6-kDa N-terminal portion of Map3527. C57BL/6 mice immunized with Map74F along with MPL+TDM emulsion had a significant IgG1 response but not IgG2a. In vaccinated animals, the IgG1/IgG2a ratio which increased until 4 wk after MAP challenge, decreased gradually from 8 wk indicating a possible shift to a Th1 response. Antigen specific IFN-gamma responses measured by IFN-gamma ELISA and ELISPOT assay were significantly higher in mice immunized with Map 74F than the control animals. The results revealed that IL-4 and IL-10 mRNA expression by spleen cells from immunized animals were higher than that of control animals, whereas no significant differences were detected in the expression levels of other cytokine genes including IL-2, IL-12, TNF-alpha and IFN-gamma. Antigen specific CD3+ and CD4+ T cell populations increased significantly in mice immunized with Map 74F, whereas, no significant differences were detected in the CD8+ T cell populations between the immunized and control animals. Following challenge, MAP load was significantly lower in spleen, liver and mesenteric lymph nodes of immunized animals compared to the control animals indicating protection against MAP infection. This was further evident by the improved spleen and liver pathology of the immunized animals which had fewer granulomas and lesser numbers of acid-fast bacilli. Results of our study indicated that
immunization of mice with Map74F protected mice against MAP infection.

Title New proposed immunopathological model for paratuberculosis in ruminants
Author(s) Geijo MV, Molina ME, Sevilla I, Bastida F, Garrido JM, Juste RA.
Institution NEIKER-Tecnalia, NEIKER-Tecnalia, NEIKER-Tecnalia, VACUNEK S.L., NEIKER-Tecnalia, NEIKER-Tecnalia, Spain.
Presentation Oral
Abstract In a field study designed to improve the knowledge of the immunopathogenic mechanisms of paratuberculosis, cohorts of vaccinated and unvaccinated animals from cattle herds with and without clinical history of paratuberculosis were followed up for 18 months with samplings every 6 months. Determinations of humoral immunity and specific and non-specific cellular immunity in response to a PPA-3, avian PPD and PBS were carried out. In addition, a blood PCR technique was used to detect bacteraemia. Animals were classified into four stages or immunopathological forms based on the results of immunity and bacteraemia: animals showing basal levels of cellular and humoral immunity and, therefore, considered as uninfected defined an Apathogenic/Non-bacteraemic Form. Animals showing a moderately intense but apparently efficient non-specific innate immune response and both specific cellular and humoral adaptive immune responses at moderate values, apparently tolerated the presence of Mycobacterium avium subsp. paratuberculosis(Map), were classified as in an Apathogenic/Bacteraemic Form. These could correspond with the focal lesional forms with little presence of acid alcohol resistant bacilli that do not alter the histological structure of the intestine or lymph nodes as described by Gonzalez et al. (2005). A Pathogenic/Non-Bacteraemic Form was defined for animals with an intense and uncontrolled innate immune response, which would likely be responsible for the tissue damage, and an equally increased specific cellular response that could be containing the diffusion of Map, and which would be translated into negative bacteraemia. This form could be assimilated to the previously defined as diffuse limphocytic paucibacillar lesional forms. The Pathogenic/Bacteraemic Form would represent an intense innate immune response that would be producing tissue damage. The specific cellular response was higher than that of apathogenic forms, but lower than those of the non-bacteraemic group. This suggests an inability to control the spread of the infection that would allow the circulation of mycobacteria. These, in turn, could act as an stimulus for humoral immunity which causes this group to have high levels of antibodies. The diffuse multibacillary lesional forms according to Gonzalez et al. (2005) would be consistent with this form. In conclusion, the model presented here, although very broad and panoramic and with few specific details, points out the relevance of innate immune responses that probably are common to other slow infections caused by low virulence pathogens.

Title Comparison of the cell-mediated immune responses to reduced doses of Mycobacterium avium ssp paratuberculosis vaccine in cattle
Author(s) Platt R¹, Roth J¹, Stalberger R², Thoen C¹, Chiang YW³, Chu HJ³.
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Presentation Poster
Abstract The effectiveness of Mycobacterium avium ssp paratuberculosis (MAP) killed-cell in oil vaccine at full, half, and quarter doses for inducing T cell mediated immune responses was evaluated using three groups of 10 age-matched calves (1-35 days of
Age). Another group of 10 calves were mock vaccinated and served as a control group. At 2 and 4 months after vaccination, blood samples were collected for cell-mediated immunity assays. MAP-PPD was used as recall antigen in the whole blood (WB) IFN-gamma assay to detect the extracellular interferon gamma (IFN-gamma) expression and in the multi-parameter flow cytometry assay to detect the expression of high affinity IL-2 receptor (CD25), intracellular IFN-gamma and intracellular interleukin-4 (IL-4) expression by CD4+, CD8+, and gamma-delta TCR+ T cells. At 2 months after vaccination, the WB IFN-gamma assay and the multi-parameter flow cytometry assay showed significant (P<0.05) increases in CD25, IFN-gamma, and IL-4 expression in all T cell subsets of the full dose vaccinated group compared to the control group (except that the gamma-delta TCR+ T cells did not express IL-4).

Among the 3 vaccinated groups, the responses were not significantly different. At 4 months after vaccination, the WB IFN-gamma assay responses of the full and quarter dose vaccinated groups were still significantly higher than the control group (P<0.05). By 4 months after vaccination, at least 2 of the 3 vaccinated groups had significant increases in CD4+ cell expression of CD25, IFN-gamma, and IL-4. The CD8+ T cells did not have detectable increases in expression of either CD25, IFN-gamma, or IL-4, and the gamma-delta TCR+ T cells had increased expression of CD25 and IFN-gamma but not IL-4. There were no significant differences between the vaccinated groups. The results demonstrate that the WB IFN-gamma and the multi-parameter flow cytometry assays could detect significant T cell specific responses to MAP-PPD at 2 months after vaccination but the responses decreased when detected at 4 months after vaccination. All three doses of vaccine were able to induce similar significant responses to MAP-PPD with both assays.

Title
Identification of bovine and caprine B cell epitopes of M. avium subspecies paratuberculosis 70 kD heat shock protein

Author(s)
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Presentation
Poster

Abstract
Bovine paratuberculosis is caused by infection of young calves with Mycobacterium avium ssp. paratuberculosis (MAP), and results in chronic granulomatous infection of the ileum. A recent vaccination-challenge study (Koets et al. Vaccine 2006) identified the recombinant 70 kD heat shock protein (Hsp70) of MAP as a promising subunit vaccine candidate. Contrary to expectations the major post vaccination immune response associated with protection appeared to be an antibody response. The aim of the present study was to define antibody epitopes of the 70 kD heat shock protein of MAP through the generation of monoclonal antibodies. Mouse monoclonal antibodies were generated against recombinant MAP 70 kD heat shock protein (Hsp70) using conventional hybridoma technology. Subsequent epitope mapping was performed using synthetic peptides representing different parts of the Hsp70. Monoclonal antibodies recognizing linear epitopes were tested for use in western blot, immunohistochemistry and electronmicroscopy. Sera from cattle and goats infected with MAP and/or vaccinated with Hsp70 were used to determine if linear epitopes were recognized. In total, 8 hybridomas which recognized MAP Hsp70 were generated. Five hybridomas produced antibodies recognizing conformational epitopes on the Hsp70. Three hybridomas produced monoclonal antibodies recognizing 2 different linear epitopes of Hsp70. These 3 antibodies could successfully be used in peptide specific ELISA, western blots, immunohistochemistry and electronmicroscopy of tissues of animals infected with paratuberculosis. One epitope was conserved in multiple mycobacterial species, with the other epitope differentiation between MAP and M. tuberculosis / M. bovis was possible, however this epitope appeared to be present in at least some of the M. avium ssp. avium species tested. The conserved N-term linear epitope was also recognized by cattle and goats.
vaccinated with Hsp70. In the less conserved C-term of the protein different epitopes were recognized by different species. In conclusion, monoclonal antibodies recognizing linear epitopes of MAP Hsp70 were generated, which may prove useful in commonly used techniques to study the presence of MAP in tissue and various substrates. This study also showed that antibodies are induced against multiple linear B cell epitopes of MAP Hsp70 following vaccination of cattle and goat with Hsp70.

Title
Early local immune responses to Mycobacterial 70 kD heat-shock protein vaccination

Author(s)
Broere F¹, van Eden W¹, Rutten V¹, Koets A¹².

Institution
¹ Department of Infectious Diseases and Immunology, Immunology Division; ² Department of Farm Animal Health, Epidemiology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

Presentation
Poster

Abstract
Paratuberculosis is a chronic granulomatous inflammation of the small intestine of cattle and other ruminants, caused by infection with Mycobacterium avium ssp. paratuberculosis (MAP). The disease can be found in ruminant herds worldwide, causing substantial economic losses at farm level due to premature culling and production losses. We have documented previously that mycobacterial heat-shock proteins (Hsp) are dominant antigens in various stages of bovine paratuberculosis. Especially the 70 kD Hsp (Hsp70) induces cell mediated responses in natural infection. Furthermore, recombinant MAP Hsp70 has been shown to be a successful subunit vaccine against bovine paratuberculosis. Surprisingly the main hallmark of vaccination induced immunity was antibody production rather than T cell immunity. To explore the immunological mechanisms of induction of early cellular responses at the local draining lymphnodes within days after vaccination we adopted a murine model. Balb/c mice were vaccinated with Hsp70 and DDA adjuvant (Hsp70/DDA), comparable to the cattle vaccine. OVA/DDA was used as a control treatment. BrdU incorporation was measured by flowcytometry 4 and 7 days after vaccination with either vaccine. In addition lymph node cells and splenocytes were restimulated in vitro to address the functional differentiation of the immune response as measured by in vitro restimulation and antibody production. Enhanced BrdU incorporation was observed in draining lymphnodes of mice that were immunized with Hsp70 compared to OVA treated mice 7 days after immunization. No differences in BrdU incorporation were observed in non-draining lymphnodes or at day 4 after immunization. Cellular proliferation following in vitro restimulation at 7 days after vaccination indicated equal responses against OVA and Hsp70. In addition, in vitro B cell restimulation showed an enhanced antigen specific B cells response in the draining lymph nodes only after Hsp70 vaccination at day 7, whereas B cells isolated from OVA treated mice did not produce significant amounts of antibodies in an antigen specific fashion. Similar to the immunization outcome in cattle in the murine model there is a preferential activation of B cell activity following subcutaneous Hsp70/DDA vaccination. Therefore, the murine model presented in this study offers a convenient means to study the mechanism leading to this immuneresponse bias which is opposite to Hsp70 immune responses in natural infection and yet confers protective immunity to paratuberculosis.

Title
Development and characterization of attenuated mutant candidate vaccines for control of paratuberculosis.

Author(s)
Park KT¹, Dahl JL², Bannantine JP³, Barletta RG⁴, Allen AJ¹, Hamilton MJ¹, Park MK¹, Davis WC¹.

Institution
¹ Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine; ² School of Molecular Biosciences, Washington State University, Pullman, WA; ³ National Animal Disease Center, USDA-ARS, Ames, Iowa; ⁴ Department of
Abstract

*Mycobacterium avium* subsp. *paratuberculosis* (Map) is the causative pathogen of Johne’s disease, a chronic inflammatory wasting disease in ruminants. The disease has been difficult to control because of the lack of an effective vaccine. To develop a live attenuated vaccine for Map, as well for the study of specific gene function in Map, an efficient method for generating targeted gene mutation is urgently needed. Here, we report an efficient allelic exchange mutagenesis system in Map using an in-vitro generated specialized transducing mycobacteriophage (phAE87). Three genes were selected for this study based on their known function in other mycobacteria: *pknG* and *relA*, genes known to be important virulence factors in pathogenic mycobacteria and *lsr2*, a gene regulating several important pathways related to lipid biosynthesis and multi-drug tolerance in mycobacteria. All three genes were successfully disrupted in a virulent strain, Map K10, as well as in a recombinant strain expressing the green fluorescent protein gene, *gfp*. The GFP tagged mutants will prove useful for intracellular studies as well as distinguishing vaccinated animals from naturally infected animals. With the optimized conditions we developed, we obtained allelic exchange frequencies of 78 - 100 % with a transduction frequency of 9.5 x 10-8 - 1.6 x 10-7. As predicted by its role in other mycobacteria, Delta-*lsr2* showed strikingly different morphology on agar medium compared to wild type. In addition, it failed to form a pellicle in standing broth culture without shaking. To investigate whether the disrupted genes affect the capacity of Map to survive following phagocytosis, an in-vitro infection assay was conducted. Peripheral blood mononuclear cell derived macrophages were infected with each mutant or wild type at MOI of 10. Colony forming units (CFUs) were measured at each time point. On day 1 after infection, while CFU of K10 was increased about 38 % compared to baseline CFU (Time 0), CFU of three mutants was similar or slightly decreased compared to baseline CFU. On day 3, all mutants showed a significant decrease in survival compared to wild type (Percentage of CFU on day 3 compared to baseline CFU: K10, 84.4 %; Delta-*lsr2*, 38.9 %; Delta-*relA*, 37.8 %; Delta-*pknG*, 49.5 %). Further studies on characterization of the mutants are now in progress. The improved method of selectively disrupting genes provides an opportunity to gain insight into specific gene function, mechanisms of pathogenesis and development of an effective vaccine for Map.
eliminated immunological differences between the low and high-grade histopathology
groups and affected Johne's disease diagnosis. These profiles provide information on
the different immune processes that affect Johne's disease progression in red deer.

Title Mycobacterium paratuberculosis Under Stress: What Can the Bacteria Do?
Author(s) Talaat AM.
Institution University of Wisconsin-Madison, Madison, WI USA and Cairo University, Egypt.
Presentation Oral
Abstract Mycobacterium avium subspecies paratuberculosis(M. ap) causes an enteric infection
in cattle, with a great impact on the dairy industry in the United States and worldwide.
Characterizing the gene expression profile of M. ap exposed to different stress
conditions could improve our understanding of the pathogenesis of M. ap. Recently,
we profiled the stress responses of M. ap on a genome-wide level (stressome) using
oligonucleotides DNA microarrays. Expression data analysis revealed unique gene
groups of M. ap that were regulated under in vitro stressors or in biofilm cultures
while additional groups were regulated in fecal samples collected from clinically
infected cows. Interestingly, acidic pH induced the regulation of a large number of
genesis (N=597) suggesting the high sensitivity of M. ap to acidic environments.
Generally, responses to heat shock, acidity and oxidative stress were similar in M. ap
and M. tuberculosis suggesting common pathways for mycobacterial defense against
stressors. Additionally, we analyzed the virulence of 7 M. ap mutants with
inactivation of differentially-regulated genes using a murine model of
paratuberculosis. Both bacterial and histopathological examinations indicated the
attenuation of all gene mutants, especially those selected based on their expression in
the cow samples (e.g., lipN). This analysis also indicated the key role played by genes
encoding lipases that are induced in clinically infected cows. Overall, the employed
approach profiled mycobacterial genes responsive to variable stress conditions
including those activated in fecal samples. Also a list of potential virulence genes was
characterized. In this communication, we will further analyze the contribution of our
findings to the understanding of the molecular pathogenesis of Johne's disease.

Title Proteomic evaluation of sera and milk from healthy and paratuberculosis infected
cow.
Author(s) Roncada P¹, Deriu F³, Soggiu A¹, Arrigoni N², Bonizzi L³.
Institution ¹ Istituto Sperimentale Italiano "Lazzaro Spallanzani", Laboratorio di proteomica
ISILS-UNIMI, Facoltà di medicina veterinaria - Università degli Studi di Milano; ²
Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Centro
di Referenza Nazionale per la Paratubercolosi - Piacenza, Italy; ³ Dipartimento di
Patologia Veterinaria, Igiene e Sanità Pubblica, Università degli Studi di Milano.
Presentation Poster
Abstract Johne's disease or paratuberculosis is a chronic enteritis of ruminants caused by
Mycobacterium avium subspecies paratuberculosis. It is endemic in Europe and the
U.S.A and responsible for significant economic losses to the livestock industries
through premature culling, lost productivity, infertility, susceptibility to disease, lost
export markets and direct cost on diagnosis and control. Current control measures are
generally inefficient because they depend on culling or removing animals that show
positivity in sub-optimal diagnostic tests that lack specificity and have poor
sensitivity. In this study we investigated proteins expression changes associated to
Johne's disease using a proteomic approach. 2D-PAGE coupled to mass spectrometry
was used as a tool to investigate up or down regulated milk and serum proteins during
natural infection. Healthy and affected bovine sera and milk were harvested,
quantitated and prepared for 2-DE by dilution in isoelectric focusing (IEF) rehydration solution on 18 cm pH 3-10 IPG strips and focused on the IPGphor III apparatus. Second dimension electrophoresis was performed on large format SDS-polyacrylamide gels. Silver stained and Coomassie stained proteins maps were analysed using ImageMaster 2D Platinum software. Some different proteins expression were detected in both healthy and affected sera and milk. Image analysis of serum proteome from healthy and paratuberculosis infected bovine showed an increased expression of immunoglobulin IgG1, IgM, IgG light chain, IgG heavy chain, and a transthyretin decrease. In both gels about 435 spot proteins were detected and only 35 did not match each other. Image analysis of milk proteome have shown that caseins are most abundant in milk from infected bovines in control milk. Their potential role in biomarker discovery and disease progression will be discussed.

Title Decreased expression of MMP-9 and increased expression of TIMP-1 in peripheral blood of paratuberculosis-infected cattle in the ELISA-negative subclinical stage

Author(s) Momotani E, Wang X, Wang H, Aodongeril, Shu Y, Momotani Y, Mori Y.

Institution Research Team for Paratuberculosis, National Institute of Animal Health, 3-1-5 Kan-nondai, Tsukuba 305-0856, Japan

Presentation Poster

Abstract We investigated the gene expression of matrix metalloproteinases-9 (MMP-9) and tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) in peripheral blood cells from infected cattle with Mycobacterium avium subsp. paratuberculosis (Map) in the ELISA-negative subclinical stage compared with uninfected control cattle. MMP-9 and TIMP-1 gene expression analysis was carried out using real-time RT-PCR. The activity of MMP-9 was analyzed by gelatin zymography. Significant decreased MMP-9 expression and increased TIMP-1 expression were found in peripheral blood cells from Map-infected cattle after stimulation with Map lysate and Map purified protein derivative (PPD) than in control cattle by real-time RT-PCR analysis. In contrast to the uninfected controls, the activity of MMP-9 was also decreased in peripheral blood cell culture supernatants from Map-infected cattle at 24h after Map lysate and Map PPD stimulation by gelatin zymography analysis. So the MMP-9 may play an important role in the development of Mycobacterium avium subsp. paratuberculosis disease.

Title CRH and urocortin expression in peripheral blood from experimentally infected cattle with Mycobacterium avium subsp. paratuberculosis

Author(s) Momotani E, Wang H, Aodongeril, Shu Y, Momotani Y, Wang X, Mori Y.

Institution Research Team for Paratuberculosis, National Institute of Animal Health, 3-1-5 Kan-nondai, Tsukuba 305-0856, Japan.

Presentation Poster

Abstract Urocortin (UCN) is a new neuropeptide of the corticotrophin-releasing hormone (CRH) family which plays an important role in immune responses. Mycobacterium avium subsp. paratuberculosis (Map) is the etiological agent of paratuberculosis (Johne's disease). The role of UCN or CRH in the pathogenesis of Map-infection is unknown. In the present study, we first cloned the bovine UCN gene and demonstrated the profile of UCN or CRH expression in peripheral blood cells from Map-infected cattle and uninfected controls by real-time reverse transcription-polymerase chain reaction (RT-PCR) and ELISA analysis. These data are the first observations of the characteristic kinetics of these neuropeptides in Map-infection. UCN or CRH expression in non-stimulated blood samples from infected cattle was higher than that in similarly treated samples from uninfected...
controls; however, exposure to *Map* lysate and live *Map* resulted in down-regulated expression of UCN in infected cattle compared to their counterparts from uninfected controls. These results have provided a direction in understanding the pathogenesis of paratuberculosis and improving diagnostic methods for *Map*-infection.

**Title**
Association between milk antibody and interferon-gamma (IFN-gamma) responses in cattle from *Mycobacterium avium* subsp. *paratuberculosis* infected herds

**Author(s)**
Mikkelsen H1,2, Nielsen SS2, Jungersen G1.

**Institution**
1 National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark; 2 Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark.

**Presentation**
Poster

**Abstract**
Paratuberculosis is a chronic, granulomatous enteric infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in ruminants. Eradication of MAP in cattle herds is complicated by lack of diagnostic tests for early diagnosis of infected animals. Available diagnostic methods include detection of MAP by cultivation, cell-mediated immune responses by IFN-gamma assays on blood samples or antibodies (Ab) in milk and blood by ELISA. The objective of the present study was to evaluate the association between IFN-gamma test results in calves and antibody ELISA status in the adult cows. During a three year study period, blood was repeatedly (3 to 4 times) sampled from 15-24 months old heifers in 18 Danish dairy cattle herds and analysed the following day by a whole blood IFN-gamma test supplemented with IL-12. After calving, milk samples were analysed for MAP Ab three times per year per animal. For the present analysis, the result of the latest available ELISA test was used. Faecal samples were cultured once per year from adult cattle. Animals were retrospectively grouped by their faecal culture (FC) status. Animals were considered FC-negative if negative in all samples. The IFN-gamma test was considered positive if IFN-gamma >= 1000 pg/ml in PPDj stimulated and IL-12 potentiated blood samples. The ELISA test result was considered positive if ODCorrected>0.3. Preliminary analyses of the results were carried out by FC-stratified Fishers'exact tests of 2x2 contingency tables of ELISA results cross-tabulated by IFN-gamma results. No associations were found between early IFN-gamma results and later ELISA results in animals positive (n=77) or negative (n=1180) by FC. Of the 77 FC positive animals, 13 animals were tested both IFN-gamma and ELISA positive. However, 31 heifers that had been tested IFN-gamma positive were not ELISA positive later on. Conversely, 11 cows that were ELISA positive had been tested negative as heifers by the IFN-gamma test. A large part of the tested animals were FC negative. Of 1180 FC negative animals, 17 were both IFN-gamma and ELISA positive. Close to half of the FC negative animals, or 593 heifers, were tested positive only by IFN-gamma. A smaller part of the FC negative animals, that is 50 cows, were only ELISA positive. In fact, 57% of the FC positive and 52% of the FC negative animals had been tested positive by the early IFN-gamma test. The lack of association between tests documented here may partly be related to concerns regarding the low specificity of the present IFN-gamma test for individual diagnosis of paratuberculosis in young calves. In addition, it may partly be ascribed to the lack of a true gold standard replacing FC. At present, all tests have limitations at certain points during progression of MAP infection. Further evaluation and optimisation of the IFN-gamma test using new and more specific antigens is necessary for diagnosis in young animals. Consequently, an association between milk antibody and IFN-gamma may not be expected until a specific and sensitive IFN-gamma test has been developed, but the results still suggest that a cell-mediated immune response is infrequently followed by a humoral immune response.
The objective of this study was to investigate suitability of BALB/c mice as laboratory animal model and appropriateness of various routes of inoculation for the study of pathogenesis of paratuberculosis. Fifty-four mice aged 2-4 weeks were divided into 3 groups viz. oral, intraperitoneal and intravenous with 12 animals each and their respective control groups. The bovine isolate was grown in Middlebrook 7H9 medium with ADC and mycobactin J, harvested and final concentrations of 10^7 cfu were suspended in pasteurised milk for oral (10 times) and 10^6 and 10^5 cfu in sterile saline for I/P and I/V inoculations (3 times), respectively. The infection was monitored for a period of 9 months by serological, immunological, bacteriological, molecular and sequential pathological methods at 3 time points (3, 6 and 9 months post infection, MPI). Clinical symptoms such as depletion of perineal and mesenteric fat reserve, muscle wasting, emaciation and unthriftiness were observed more frequently in IP & IV groups than the oral group mice. Gross lesions suggestive of paratuberculosis infection were not observed in any group. Histologically, the small intestine particularly ileum showed broad, flat and fused villi with increased infiltration with lymphocytes and macrophages without formation of distinct granuloma in lamina propria from 3 to 9 MPI in all the 3 groups. Distinct granulomas were observed mostly in the lymphoid tissues. The mesenteric lymph nodes revealed multifocal granulomas at 9 MPI in the oral group, whereas animals from I/P and I/V groups had multiple granuloma at 6 and 9 MPI. The liver in the oral group showed only a few small aggregates of lymphoid cells, in the I/P group 1-2 focal granulomas, whereas in the I/V group, it had focal to multifocal granulomas from 3-9 MPI. The acid-fast bacilli were detected in the mesenteric lymph nodes of only one mouse from I/V group at 9 MPI. Serologically, there was rising titres of antibodies to MAP most prominently in the oral than in the I/P and I/V groups in the ELISA. None of the mice of three groups reacted positively to intradermal johnin skin test. Faecal excretion of the bacteria was not detected in culture. Only one mouse was found positive in tissue culture at 9 MPI from I/V group. One mouse each in I/P and I/V groups at 3 MPI in tissue PCR, and one mouse each in oral and I/V groups at 6 MPI were found to be positive in the faecal PCR. The results of this study suggested that BALB/c mouse was a suitable model and I/V route of inoculation was better than the other two routes for experimental reproduction of the disease.
respectively. The infection was sequentially monitored for a period of 9 months after last inoculation by serological, immunological, bacteriological, PCR and histopathological methods. Most rabbits in all the 3 groups did not exhibit any clinical signs except rough hair coat, depletion of perineal and mesenteric fat reserve, and emaciation and decrease in the body weight at 9 MPI in I/V group. No gross lesion suggestive of paratuberculosis infection was observed in any group. Histologically, the I/V group had more pronounced lesions in the organs such as ileum, sacculus rotundus, vermiform appendix and mesenteric lymph nodes. Focal to multifocal granulomas were observed in the lymphoid follicles of these organs. The oral and I/P groups did not have significant differences in terms of type and severity of the lesions which were produced in the ileum, sacculus rotundus, mesenteric lymph nodes and vermiform appendix but lesions were less pronounced than the I/V group. The acid-fast bacilli were observed in the sacculus rotundus and mesenteric lymph nodes of 2 rabbits from I/V group. There was rising antibody titres in all the groups from 3 MPI onwards but was most prominently observed in the I/V group. MAP was isolated from tissues of one rabbit each at 3 and 6 MPI from I/V group. Faecal and tissue PCR results were positive at 6 MPI for all the 3 groups. The results of this study suggested that young rabbits could be used as laboratory animal model for pathogenesis of paratuberculosis infection and I/V route of inoculation was better than oral and I/P routes in terms of severity of lesions

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<th>Title</th>
<th>Immunologic responses to Mycobacterium avium subsp. paratuberculosis in neonatal calves after oral or intraperitoneal experimental infection.</th>
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<tr>
<td>Author(s)</td>
<td>Stabel JR, Robbe-Austerman S, Davis WC.</td>
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<tr>
<td>Institution</td>
<td>USDA-ARS-NADC, Ames, IA; USDA-APHIS-VS, Ames, IA; Washington State University, Pullman, WA.</td>
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<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>Infection models are useful for studying host responses to infection to aid in the development of diagnostic tools and vaccines. The majority of experimental models for ruminants have utilized an oral inoculation of live Mycobacterium avium subsp. paratuberculosis (MAP) in order to establish infection, thereby mimicking the fecal-oral route of transmission generally observed in the field. The current study was designed to compare the effectiveness of oral and intraperitoneal inoculation on the host immune response to MAP infection. Twenty neonatal Holstein calves were obtained from status level 4 herds and randomly assigned to 5 treatment groups: 1) control noninfected (C), 2) oral (Oral), 3) oral with dexamethasone pretreatment (Oral/DXM), 4) intraperitoneal (IP), and 5) oral/mucosal (Oral/M). The oral group was fed milk replacer containing 1010 cfu of live MAP, strain K-10, 2x per day for 14 consecutive days. The Oral/DXM group were inoculated in the same manner as the Oral group but the calves were administered 0.25 mg/kg BW dexamethasone IV for 3 consecutive days prior to bacterial challenge, and again on days 28 and 56 post-challenge. Intraperitoneal inoculation of calves with 1010 cfu MAP, strain K-10, was performed on days 0, 7, 14, and 21 of the study. The Oral/M calves were inoculated by feeding milk replacer containing live MAP obtained by scraping the ileal mucosa from a clinically infected cow on days 0, 7, and 14. All calves were housed in AAALAC-accredited BSL-2 facilities during the study. Throughout the study, blood and fecal samples were obtained from calves on days -5 and -4 prior to the first inoculation of MAP, and then on days 7, 14, 21, 28, and monthly thereafter for the 12 month term of the study. Blood samples were processed for isolation of peripheral blood mononuclear cells (PBMC) followed by incubation with medium only (nonstimulated), concanavalin A (ConA), a whole cell sonicate of MAP (MpS), and johnin purified protein derivative (JPPD) for 24 and 48 hr for determination of cytokine secretion, lymphocyte proliferation, and flow cytometric analyses. Results demonstrated that oral inoculation of calves significantly increased lymphocyte proliferative responses to K-10 MpS at 12 months. Secretion of antigen-stimulated iNOS by PBMC was higher for oral infection groups at both 6 and 12 months.</td>
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post-infection compared to control calves. IP calves had the earliest antigen-specific IFN-γ responses at 7 d post-infection, preceding responses noted for other infection groups that followed between 90 and 120 d. Average IL-10 responses to ConA and MPS were higher at 1 and 6 months and declined significantly by 12 months post-infection. At 1 month, Oral and Oral/M calves had higher MPS-stimulated IL-10 than other treatment groups. By 12 months only the Oral/M calves had higher IL-10 secretion than control calves. Intracellular IFN-γ and IL-10 levels were measured for CD4+, CD8+, and gd T cell subpopulations. At 3 months post-infection, there was significantly higher IFN-γ in CD4+ cells stimulated with MPS in the Oral treatment. Intracellular IL-10 was higher in CD4+ and CD8+ T cells in Oral and IP calves compared to the other treatments. These results demonstrate that exposure and infection to MAP will invoke early immunologic responses characterized by IFN-γ, IL-10, and iNOS secretion.

Title Pathogenesis of *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves after oral or intraperitoneal experimental infection.

Author(s) Stabel JR¹, Palmer MV¹, Robbe-Austerman S², Harris B².

Institution ¹ USDA-ARS-NADC, Ames, IA; ² USDA-APHIS-VS, Ames, IA.

Presentation Poster

Abstract The current study was designed to compare the effectiveness of different methods of experimental inoculation on the pathogenesis of MAP infection. Twenty neonatal Holstein calves were obtained from status level 4 herds and randomly assigned to 5 treatment groups: 1) control noninfected (C), 2) oral (Oral), 3) oral with dexamethasone pretreatment (Oral/DXM), 4) intraperitoneal (IP), and 5) oral/mucosal (Oral/M). The oral group was fed milk replacer containing 10¹⁰ cfu of live MAP, strain K-10, 2x per day for 14 consecutive days. The Oral/DXM group were inoculated as the Oral group but calves were administered 0.25 mg/kg BW dexamethasone IV for 3 consecutive days prior to challenge, and on d 28 and 56 post-challenge. IP inoculation of calves with 10¹⁰ cfu MAP, strain K-10, was performed on d 0, 7, 14, and 21 of the study. The Oral/M calves were inoculated with milk replacer containing live MAP obtained from ileal mucosa from a clinically infected cow on d 0, 7, and 14. Throughout the study, blood and fecal samples were obtained from calves on d -5 and -4 prior to the first inoculation of MAP, and on d 7, 14, 21, 28, and monthly thereafter for the 12 month term of the study. Fecal culture and PCR data demonstrated that calves in the oral inoculation groups experienced shedding on d 7, 14, 21, and 28, indicative of "pass-through" shedding that is typically observed after large oral boluses of bacteria are administered. Shedding was minimal and infrequent over the course of the study for calves in the Oral, Oral/DXM, and IP treatment groups. Calves in the Oral/M treatment group shed high numbers of bacteria up to 4 months post-inoculation. By 4 months post-infection, shedding was significant only in 1 of the 3 calves (79 cfu/slant), followed by sporadic shedding of few organisms thereafter. Fecal PCR results mirrored the culture results with infrequent positive reactions after the first 4 weeks of infection, regardless of infection group. Colonization was present in a number of intestinal tissues and lymph nodes with the lowest number of affected tissues in the IP calves and the highest for calves in the Oral/M group. Recovery of viable MAP was low in tissues regardless of treatment group with the exception of one calf in the Oral/M. Histopathologic lesions were predominantly found in the ileal and jejunal sections and their associated lymph nodes, as well as the ileocecal valve and node. Lesions were characterized by multifocal small infiltrates in the submucosa of intestinal tissues and small aggregates of macrophages with and without granuloma formation within the lymph nodes. Lesions were most predominant within the tissues from Oral/DXM calves and secondarily for the Oral group. Few lesions were found in the tissues of IP and Oral/M calves. Four SSR loci in the MAP genome (Locus 1, Locus 2, Locus 8 and Locus 9) with the highest indices of diversity were compared across the 2 strains of MAP utilized (K-10, clinical cow isolate) and between the original inoculum of each
strain and the output from fecal shedding of infected calves. Analysis of the MAP isolates recovered from the infected calves indicated that there was no variation in the MLSSR types as compared to the original inoculum. However, strain K-10 demonstrated the genotype of >14-10-5-5, consistent with previous reports, but the clinical cow isolate yielded two similar MLSSR types; 7-9-4-3 and 7-10-4-3, suggesting that 2 strains of MAP were present in the ileum of the infected cow. These data suggest that oral inoculation remains the most effective method of experimental infection for MAP and that inoculation with a low passage strain of MAP may induce more clinical signs.

**Title**  
Experimental infection of sheep with *M. avium* subspecies *paratuberculosis*: a brief review and introduction to an Australian ovine challenge model

**Author(s)**  
Begg DJ, Taylor D, de Silva K, Di Fiore L, Whittington R.

**Institution**  
Faculty of Veterinary Science, The University of Sydney, Australia.

**Presentation**  
Poster

**Abstract**  
Experimental animal infection models are crucial tools in the continuing fight to control and eradicate Johne's disease. The animal model selected and how it is utilised will depend on the outcomes required, such as immunological testing, pathogenesis and vaccine trials. The factors that appear to influence the outcome of experimental infections with *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) are the species, breed and age of subject used for the infection, the route of infection, and the strain, dose and number of doses of *Mptb* used to inoculate the subjects. Studies to date have been lacking in the use of a defined type strain of *Mptb* in pure culture prepared from an archived seed stock that can be used at the same passage level in later trials. An ovine experimental oral infection model has been developed for Australian conditions using a pure culture of *Mptb* (Telford) retained as a freeze dried seed stock. This has been directly compared to oral infection with infectious gut tissue homogenate. While both experimental infections created disease closely resembling natural infection, not surprisingly the gut tissue homogenate challenged animals developed clinical disease earlier than animals given Telford strain *Mptb*. The results with the pure culture were repeatable over 3 trials.

**Title**  
Signal Transduction in *Mycobacterium avium* spp. *paratuberculosis*

**Author(s)**  
Bach H, Wong D, Sun J, Av-Gay Y.

**Institution**  
Department of Medicine, Division of Infectious Diseases, University of British Columbia, Vancouver, Canada.

**Presentation**  
Poster

**Abstract**  
Signal transduction is a ubiquitous mechanism responsible for cell adaptation to environmental changes in both prokaryotes and eukaryotes. Cellular responses to the dynamic changes in the environment are mediated by a cascade of events involving protein kinases, which activate protein substrates by ATP-dependent phosphorylation on specific residues such as histidine and aspartate in prokaryotes (two-component systems) and serine/threonine, or tyrosine in eukaryotic or eukaryotic-like protein kinases. The reverse regulation of kinases (dephosphorylation) is mediated by protein phosphatases. Protein phosphatases participate in modulating a variety of cellular events such as metabolism, gene transcription, cell cycle control, immune response, and cell growth, etc. In addition, protein phosphatases have also been associated with virulence contributing to the intracellular survival of pathogens. For example, the tyrosine phosphatase YopH of *Yersinia pseudotuberculosis* dephosphorylates host proteins, the tyrosine phosphatase SptP from *Salmonella typhimurium*, which is translocated into the host, causes a disorganization of the actin cytoskeleton, while
Stp, a serine/threonine phosphatase from *Listeria monocytogenes* dephosphorylates the host elongation factor EF-Tu. Signal transduction in *Mycobacterium avium* spp. *paratuberculosis* (Map) is regulated according to the annotated genome by twelve two-component systems based on signal-transducing histidine kinases, nine serine/threonine protein kinases, five proteins containing serine/threonine kinase catalytic domains, and two tyrosine phosphatases. Interestingly, the annotated genome does not possess a defined tyrosine kinase, suggesting that both proteins might act in the host upon infection. Recently, we have reported that map1985 is a functional low-molecular tyrosine phosphatase, which is secreted intracellularly upon macrophage infection. Then, interfering with the host signal transduction could contribute to the pathogen survival in macrophages.

**Title**

Biofilm and Virulence of *Mycobacterium avium* subspecies *paratuberculosis*

**Author(s)**

Wu CW, Schmoller SK, Talaat AM.

**Institution**

Laboratory of Bacterial Genomics, Department of Pathobiological Sciences, University of Wisconsin-Madison, 1656 Linden Drive, Madison, WI 53706.

**Presentation**

Oral

**Abstract**

Formation of biofilms by pathogenic bacteria plays a key role in their pathogenesis, especially when the bacteria establish an infection in adverse environments. We examined the genetic basis of biofilm formation in *Mycobacterium avium* subspecies *paratuberculosis* (M. ap), the causative agent of Johne's disease in cattle and a potential risk factor associated with Crohn's disease in humans. A transposon mutant of *M. ap* with an inactivation of the *pstA* gene was shown to have reduced abilities to form biofilms on PVC plates, and to colonize mouse organs in a murine model of paratuberculosis. Similar results were obtained when a surgical model of intestinal invasion in cattle was utilized to assay the invasion of the *pstA* mutant, suggesting a role for this gene in biofilm formation and virulence of *M. ap*. Finally, genome-wide transcriptional analysis of biofilm and planktonic cultures of *M. ap* profiled *M. ap* biofilms as stress-responsive structures, especially against oxidation and hypoxia. Overall, the analysis of *M. ap* biofilm reveals the importance of the *pstA* gene in biofilm formation and the pathogenesis of *M. ap*. The knowledge generated in this study will facilitate the analysis of other mycobacterial species that infect humans and animals and can provide a model for the analysis of other biofilm-forming pathogens.

**Title**

*Mycobacterium avium* subsp. *paratuberculosis* enters intestinal mucosa through M cells and there is difference in the uptake across the ileal and jejunal mucosal epithelial cells in lambs

**Author(s)**

Duraisamy P, Tripathi BN, Periasamy S, Pal A.

**Institution**

Division of Pathology ¹ and Surgery ² Indian Veterinary Research Institute, Izatnagar-243 122 (ÚP), India.

**Presentation**

Oral

**Abstract**

The entry of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to the intestinal mucosa is critical to the pathogenesis and control of paratuberculosis in animals, which is poorly understood. An *in-vivo* multiple-intestinal loop model involving Peyer's patch (PP) and non-Peyer's patch (non-PP) areas was developed in lambs to examine (i) the attachment and uptake of MAP across the intestinal mucosa, (ii) the ability of various strains of MAP to invade the mucosa. By using a number of methods including polymerase chain reaction, in-situ hybridization (ISH), histology and transmission electron microscopy, it was observed that MAP entered the intestinal mucosa through follicular M cells (fM cells) in PP as
well as possibly through villous M cells (vM cells) in non-PP areas. The fM cells are the specifically and functionally modified cells lining the follicular associated epithelium (FAE) over Peyer's patch areas, whereas the vM cells are originally the transformed enterocytes present throughout the intestinal mucosa. The observation of more number of bacteria, bacterial antigen or bacterial genome in the ileal mucosa lined with continuous PP in comparison to the jejunal mucosa with and without discrete PP suggested that translocation of MAP across the fM cells were more efficient than the vM cells. The field strains of MAP isolated from cattle and goat showed greater ability (P<0.05) for invasion into the small intestinal mucosa of the lambs than that of the vaccine strain. The demonstration of MAP genome by ISH and its antigen by ICH in the intestinal mucosa, and the inability to isolate the bacteria from the mucosal homogenate of infected loop tissues suggested that the bacteria could transform into the cell-wall deficient forms after the invasion. This could be significant from early pathogenesis point of view.

Title  
Mycobacterium avium subsp. paratuberculosis induced apoptosis: a complex mechanism in bovine macrophage.

Author(s)  
Periasamy S, Singh N.

Institution  
Department of Pathology, Indian Veterinary Research Institute, Izatnagar-243122, India.

Presentation  
Poster

Abstract  
The interaction between macrophage and Mycobacterium avium subsp. paratuberculosis (MAP) was found to be complex processes involving survival or death of macrophage with bacterial persistence or clearance depending upon the number of bacteria infected per macrophage (multiplicity of infection, MOI). The bovine strain of MAP (C-123/IVRI) isolated from clinically infected cattle was found to be ingested by bovine blood monocyte-derived macrophages, multiplied within them, and resulted in differential expression of a number of pro-inflammatory cytokine genes and induction of macrophage apoptosis. At MOI>1, MAP was found to be not harmful for macrophages and down-regulated most of the pro-inflammatory genes. The maximum apoptotic index was found to be 2-3% at 24 h post-infection, which was as low as baseline apoptosis observed in uninfected control cells. MAP at MOI>10 induced apoptosis in 5% of macrophages at 6 h, 8 % at 12 h, and 13% at 24 h post-infection. A number of pro-inflammatory genes were down-regulated during this condition with activation of caspase-dependent and mitochondrial pathways of apoptosis. On the other hand, MAP at MOI>50 or 100 was found to be potentially cytotoxic for macrophages. MAP at MOI>50 induced apoptosis in 20, 29 and 34% of macrophages at 6, 12 and 24 h, respectively. Down-regulation of pro-inflammatory cytokines as well as activation of caspase-independent and NO-dependent pathways of apoptosis was observed in these conditions. The results of the present study suggest that interaction of MAP with macrophage is a complex process with activation of caspase-pathway dichotomously depending upon the multiplicity of infection.
A cultivation ring trial for *Mycobacterium avium* subsp. *paratuberculosis* in three certified laboratories.

**Author(s)**
Giese SB.

**Institution**
Technical University of Denmark.

**Abstract**
Faecal samples from Danish cattle were investigated for the presence of *Mycobacterium avium* subsp. *paratuberculosis* by 3 laboratories by their routine cultivation procedures. Out of 30 samples of which 14 previously had been found culture positive, the 3 laboratories (named A, B and C) found 12, 6 and 0 positive respectively by their direct standard culture methods. One laboratory (A) also performed direct PCR on the samples and found 13 positive. Two laboratories (A and C) also performed culture combined with PCR and found 14 and 10 positive respectively. These findings show that laboratories certified to perform cultivation for *Mycobacterium avium* subsp. *paratuberculosis* need to evaluate and renew their diagnostic methods regularly to ensure the best results.

Comparison of Liquid Culture to Solid Media for Quantification of Mycobacterium in Tissues Following Experimental Infection

**Author(s)**
Sweeney RW¹, Whitlock RH¹, Bowersock TL², Pruitt GW².

**Institution**
¹ University of Pennsylvania School of Veterinary Medicine, Kennett Square PA USA; ² Pfizer Animal Health, Kalamazoo MI USA.

**Abstract**
When experimental models of MAP infection are employed to investigate the efficacy of vaccination or other therapeutic strategies, quantification of tissue concentrations of MAP is desirable. This has most commonly been achieved through processing tissue samples for culture, and plate counts of MAP colonies grown on solid media such as Herrold's Egg Yolk Media (HEYM). This procedure requires up to 16 weeks of incubation, whereas growth in liquid media can be achieved in a much shorter time period. The time required for the mycobacterial growth to trigger detection in an automated liquid culture system (Time To Positive, TTP) is correlated to the amount of MAP in the original inoculum. The purpose of this study was to compare the use of automated liquid culture of MAP, using the MGIT system (Becton-Dickinson) to conventional quantification of MAP using HEYM, using tissues obtained from calves experimentally infected with MAP. Twelve male Holstein calves were given an oral challenge of 10⁹ CFU of MAP on days 21 and 22 of life. Calves were euthanized at 100 days of age, and 33 tissue samples (intestine, mesenteric lymph nodes) were processed for MAP culture. Tissue samples were disrupted with a stomacher, decontaminated with HPC, and plated on 4 tubes of HEYM (200 ul inoculum per tube) or in one tube of liquid MGIT media (100 ul/tube). The number of colonies per tube of HEYM were counted after 16 weeks of incubation. The MGIT tubes were incubated until signalled positive by the Bactec unit. Time to positive was recorded, and converted to a CFU value by use of a standard curve constructed by plotting time to positive vs. CFU/ml for inocula created from known concentration MAP suspension. Of 396 samples, 3 were lost to contamination and excluded from analysis. Of 393 remaining samples, 269 (68%) were positive on both culture systems, 83 were negative on both systems (21%), for a total of 89% agreement between methods. Of the discordant samples, 36 were positive on HEYM only, and 5 were positive on MGIT only. The CFU as determined by HEYM was significantly (but inversely) correlated with the TTP in MGIT (r = -0.96). On the basis of these results, automated liquid culture detection of MAP in tissue samples provides a more rapid method to quantify tissue concentration of MAP, compared with HEYM. Comparable results are obtained with both methods. In this experiment, in which half of the calves received
MAP vaccination prior to challenge, differences in MAP tissue colonization of experimental vs. control groups was detected at a similar level of statistical significance for the two culture methods.

Title Radiometric culture of farm slurry - inexpensive tool for the detection of paratuberculosis in dairy cattle herds
Author(s) Gwozdz JM¹, Carajias M¹, Mohammad I¹, Ridge S¹, Condron R².
Institution ¹ Department of Primary Industries, Victoria, Australia; ² Dairy Australia.
Presentation Poster
Abstract Objectives: To evaluate the usefulness of the radiometric culture of faecal slurry for rapid and inexpensive screening of dairy herds for paratuberculosis. Experimental design: Samples of faecal slurry were collected from yards immediately after milking from 70 herds in which paratuberculosis had previously been diagnosed. Results of the last blood ELISA test that was performed as part of the Victorian Bovine Johnes Disease Test and Control Program were also obtained from each herd. Faecal material was pushed together across the full length and breadth of the yard and mixed using a shovel or a shed scraper and triplicate samples were collected from each farm. After initial processing, two sub-samples were derived from each replicate. Subsequently, 6 sub-samples from each herd were submitted for culture and decontaminated using the double incubation method. Three of the 6 sub-samples were decontaminated at 37°C and the other 3 were decontaminated at 42°C. After decontamination, each sub-sample was inoculated into a BACTEC 12B bottle supplemented with egg yolk, mycobactin J and PANTA, and incubated for at least 12 weeks at 37°C. Cultures showing growth were subcultured to demonstrate mycobactin dependency and tested by the polymerase chain reaction for the presence of IS900. Results: M. paratuberculosis, or its DNA, was identified in faecal slurry from 50 of the 70 (71.4%) herds with paratuberculosis. The radiometric culture of slurry detected 53% of herds with low (0 to 1.5%) seroprevalence, 75% of herds with moderate (1.6 to 3%) seroprevalence and 100% of herds with high (>3%) seroprevalence. The growth of M. paratuberculosis in cultures of slurry from herds with high seroprevalence was detected significantly earlier than that observed in cultures from herds with low and moderate seroprevalences. These results show that this test is a good indicator of the prevalence of infection. Of the 210 paired sub-samples of slurry that were decontaminated at 37°C and 42°C, 175 produced concordant results. Among the 35 pairs of sub-samples with discordant results, M. paratuberculosis was detected in 23 (65.7%) sub-samples decontaminated at 42°C and in 12 (34.3%) sub-samples decontaminated at 37°C. This indicates that the decontamination at 42°C offers a sensitivity advantage. From these preliminary findings using replicate samples it has been estimated that about 50% of infected herds would have been identified with the culture of a single sample. Conclusions: The radiometric culture of farm slurry has the potential to be a useful and inexpensive tool to screen dairy herds for paratuberculosis.

Title Cross-pooled faecal culture and individual faecal PCR for paratuberculosis herd level monitoring
Author(s) Molina E, Sevilla I, Geijo MV, Garrido JM, Plazaola JM, Juste RA.
Institution NEIKER-Tecnalia, NEIKER-Tecnalia, NEIKER-Tecnalia, Animal Production and Health Department, NEIKER-Tecnalia, Diputacion Foral de Gipuzkoa, NEIKER-Tecnalia, Spain.
Presentation Poster
Abstract Isolation of Mycobacterium avium subsp. paratuberculosis (Map) by faecal culture is the gold standard tests to in vivo diagnose paratuberculosis in infected animals.
Culture is supposed to be able of detecting around 50-100 cells/g of faeces. But Map shedding varies according to the immuno-pathological status of animals. The presence of sub-clinically infected animals and other factors including infection with strains difficult to grow reduce considerably the sensitivity of this method. In addition, Map culture is expensive, slow and laborious. In this study preliminary results of a strategy based on cross-pooled faecal culture and individual faecal PCR for herd follow up are presented. The cross-pooled culture method lies in crossing pools of 5 samples in 5x5 squares of samples. This allows direct identification of shedder animals in the pool without subsequent individual culture for confirmation. During the setting up of the method results of pooled and individual cultures were compared. A reduction of 43% in the number of cultures needed to detect all positive animals was observed in a herd with a prevalence of 10.2%. Once the cross-pooled culture method was adopted 699 bovine faecal samples were used to inoculate 2 Herrold’s egg yolk (HEY) and 2 Lowenstein-Jensen (LJ) slants. Individual PCR (conventional PCR in the beginning and real-time PCR later: Adiavet kits) was used for confirmation of inconclusive results of cross-pooled cultures. Later all individual faecal samples were tested by PCR and compared with the previous culture results. Six-hundred and ten samples were directly diagnosed by cross-pooled culture, 21 classified as positive and 589 as negative. Eighty-nine samples needed individual PCR to be correctly identified, 19 were finally classified as positive and 70 as negative. Agreement between definitive culture qualifications and individual PCR general results was observed in 639 samples. Thirty-three out of those 639 were identified as positive, while 606 were identified as negative. Surprisingly, the individual PCR detected 53 positive samples not identified by pooled culture. The reduction in culture number using cross-pooled culture compared to individual culture was of 59.9%. But it should be considered that PCR was needed to confirm the final culture result in 89 samples. Our results also indicate that individual PCR alone can perform better than the cross-pooled culture in herd monitoring strategies. Nevertheless, this pooled culture method is still interesting when culture is the selected test for paratuberculosis screening of herds.

Title Culture of M. paratuberculosis from Blood
Author(s) Bower K, Begg D, Di Fiore L, Taylor D, Whittington R.
Institution University of Sydney, Camden, Australia.
Presentation Oral
Abstract Between exposure to Mycobacterium avium subsp paratuberculosis (M. ptb) and expression of clinical disease, there is a long subclinical phase in which shedding of the organism in faeces occurs intermittently. Current diagnostic tests are unable to provide sensitive and specific diagnosis during the early stages of the disease. Detection of M. ptb DNA in blood, and culture of M.ptb from milk, liver, mammary tissue, spleen, foetal tissues, reproductive tissue, and extra intestinal lymph nodes, indicate that at some stage in the disease, bacteraemia occurs. Demonstration of the organism in blood from infected sheep and cattle by PCR has sparked recent interest in developing a diagnostic test based on culture or molecular detection of the organism from blood. Various methods of processing blood prior to culturing in Bactec 12B culture media were compared using whole blood spiked with a known quantity of a sheep strain of M.ptb. The culture results and factors influencing the ease of processing were used to choose a method to be applied to samples from naturally and experimentally infected sheep. The chosen method utilises the intracellular location of the organism, concentrating the M.ptb by collecting the white blood cells. This allows efficient removal of the red blood cells which can be inhibitory to the growth of M.ptb in liquid media. Preliminary findings indicate that M.ptb can be isolated from blood of a low proportion of animals following exposure, and before development of clinical signs. Results will be presented from two trials with 152 sheep.
Title: In vitro effect of antibiotics on *Mycobacterium avium* subspecies *paratuberculosis* in Herrold's medium

Author(s): Traveria GE, Pinedo MFA, Peralta LM, Bartoletti LC, Quinteros M.

Institution: CEDIVE, Salta y Alvear, 7130 Chascomús, La Plata University, Argentina

Presentation: Poster

Abstract: The culture of *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is considered as the "Gold Standard" diagnostic test for paratuberculosis. On account of the high contamination rates with an average of 30% observed after conventional fecal culture from beef cattle herds, we address this problem by identification of *Pseudomona aeruginosa* as the most common contaminant. After antibiotics susceptibility test the following antibiotics were efficient controlling the grow of pseudomona: enrofloxacin, florfenicol and gentamicin. To check the in vitro breakpoint inhibition effect of these antimicrobial agents to *Map*, clinical isolates of paratuberculosis agent were tested to examine their growth responses in Herrold’s medium supplemented with these antibiotics at serial dilutions. Inhibition effect on *Map* was observed in enrofloxacin and gentamicin at low concentration. Florfenicol combined the best in vitro breakpoint inhibition effect on contaminant grow with minimal susceptibility to clinical isolates of *Map*.

Title: Can the detection rate of faecal shedders of MAP be increased by optimising the time point of faecal sampling?

Author(s): Gierke F¹, Ziller M², Köhler HU¹.


Presentation: Oral

Abstract: Paratuberculosis is endemic in the dairy cattle population of Germany. However, the actual prevalence on the individual animal as well as on the herd level is not known. A national paratuberculosis guideline came into force in February 2005 giving recommendations for paratuberculosis control on a voluntary basis. In Germany; because of the intensive cattle husbandry, paratuberculosis control in dairy cattle can only be successful combining strict hygienic measures in the herds with immediate removal of faecal shedders. Up to now, faecal culture is still the most sensitive method for the identification of faecal shedders, although it is expensive and time consuming. Improvement of the effectiveness of the identification of shedders is urgently needed. It was the objective of the present study to clarify, whether the detection rate of faecal MAP shedders can be increased by optimising the time point of faecal sampling. Therefore, the influence of individual host factors on faecal shedding was investigated. In two paratuberculosis positive dairy herds with an average of 245 (herd A) and 390 (herd B) lactating cattle, respectively, faecal samples were collected four times every 5 to 7 months from all lactating cows and heifers of more than 18 months of age. Bacteriological culture was performed for the detection of MAP. In herd A MAP positive animals were not removed systematically while in herd B, culling of shedders was performed regularly. Individual data of each animal included in the study were obtained from the herd records, i.e. age, lactation state, milk yield and others. For all animals with a culture positive faecal sample, individual factors at the respective time point were analysed for their contribution to the risk of faecal shedding. In herd A, a higher proportion of faecal shedders were detected in older animals and in animals in the third trimester of lactation. In herd B, however, the influence of age and lactation state on faecal shedding was not obvious. In both herds, there was no clear relation between milk yield and the risk of faecal shedding.
Analysing repeated sampling every 5 to 7 months, about 13.8% of the shedders which underwent at least three faecal examinations would have been detected by one sampling, additional 16.6% by two samplings and further 49.0% by three samplings. 20.7% of the shedders would not have been detected by three subsequent samplings. In conclusion, individual factors that influence faecal shedding of MAP seem to be herd specific. In the present study, no general, preferential time point for sample collection could be identified. Although older animals may have a higher risk of MAP excretion, faecal culture is also successful in young dairy cattle and these animals should be examined too. One way to increase the detection rate of faecal shedders is repeated sampling.

Title  Evaluation of Different Organism Based Methods for the Detection and Identification of Mycobacterium avium subspecies paratuberculosis from Bovine Feces

Author(s)  Payeur JB, Capsel RT.

Institution  USDA,APHIS,VS,National Veterinary Services Laboratories, Ames, IA USA

Presentation  Oral

Abstract  United States Department of Agriculture (USDA) regulations state that an organism-based test (culture/PCR) is the official test for determining the infective status of an animal for Johne's disease. Recent method evaluation tests performed for laboratory approval for the Voluntary Bovine Johne's Disease Control Program (VBJDCP) indicate multiple culture methods are being used in the United States (US). The annual evaluations indicate a wide range of sensitivities associated with the different culture methods. The National Veterinary Services Laboratories (NVSL) have been requested to establish a standardized protocol for detecting Mycobacterium avium subspecies paratuberculosis(Map) in fecal samples which is reproducible and has a known sensitivity. The NVSL have also been requested to establish the criteria for well-characterized bovine fecal panels for use in organism-based detection procedures and methods evaluation. These panels will be used to validate different diagnostic procedures, including serological assays and USDA licensed diagnostic kits used for Johne's disease detection. Based on the results of the last 11 years of proficiency tests for detecting Map, several methods were chosen for further evaluation. These methods included different decontamination techniques involving sedimentation or centrifugation and different media including solid and liquid which have been used by multiple laboratories. Preliminary evaluation based on proficiency test results indicate that centrifugation methods are more sensitive than sedimentation decontamination methods, and liquid media methods are faster than methods using tubes of Herrold's Egg Yolk (HEY) media with mycobactin J. More PCR methods have been introduced and evaluated by different laboratories each year. Currently the Tetracore VetAlertTM Johne's Real-Time PCR is the only USDA licensed PCR Kit available in the United States. Varied growth performances in the solid media used with different culture methods were also noted during the last 10 check tests. Two commercial sources of HEY media available in the US were evaluated along with in-house media for growth performance. Tissue culture flasks containing the same volume of HEY media and inoculums were evaluated and shown to have isolated more Map colonies earlier in an 8 week time period than tubes containing an equal volume of HEY media.

Title  Isolation and molecular confirmation of Mycobacterium avium subsp paratuberculosis in guanacos (Lama guanicoe) in Tierra del Fuego, Chile, by fecal culture and Real-Time PCR.

Author(s)  Salgado MÁ1,2, Herthnek D3, Bölske G1, Kruze JD1.

Institution  1 Microbiology Department, Faculty of Sciences, Universidad Austral de Chile, Campus Isla Teja, P.O.Box 167, Valdivia, Chile; 2 Postgraduate School, PhD
Mycobacterium avium subsp. paratuberculosis (MAP) is the causative agent of paratuberculosis. The primarily affected hosts are domestic ruminants, but paratuberculosis has also been reported in wild animal hosts. In Chile, the infection has been confirmed in cattle, sheep and goats, but there is no information about paratuberculosis in wildlife animals. In Chile, the infection has been confirmed in cattle, sheep and goats, but there is no information about paratuberculosis in wildlife animals. The broad range of hosts affected by MAP implies a possible intraspecies transmission, as well as wildlife reservoir. Guanaco is the only wild ungulate species widely distributed across the Patagonian steppe, sharing grazing land with domestic sheep. The aim of this study was to detect MAP infection in a free ranging wildlife animal species in Chile, using conventional diagnostic tools, as well as new molecular confirmation technology. Faecal samples were obtained from 501 guanacos populating the Rusfin area, Tierra del Fuego Island in August 2006. The sampling was synchronized with a controlled hunting activity carried out by a private company under the Ministry of Agricultural (SAG) supervision. Faecal samples were collected post mortem right after hunting and cultured on a homemade HEY medium with and without mycobactin J following the procedure recommended by the Cornell University. Colonies resembling MAP and showing mycobactin-dependence were confirmed by Real-Time PCR based on IS900 and F57. Twenty one out of 501 (4.2%) animals sampled were positive for Map all of which were confirmed by Real-Time PCR IS900 and F57. This represents the first isolation of Map from a free-ranging wildlife animal in Chile. These findings support an increasing body of evidence that indicates that a wide diversity of wildlife species as well as domestic ruminants can become infected with Map. In a control or eradication program of this disease, it is of special importance to know how to control the transmission. The presence of a wildlife reservoir of the disease has to be considered for the potential transmission to livestock. However, for free-ranging wildlife, the most likely initial source of infection is the shared range with domestic species, given the higher prevalence of Johne's disease in the latter species, an issue to be determined in the Chilean situation.
paratuberculosis could not be readily detected in the bulk milk from herds with infected cattle by either PCR or culture. This indicates that the hygiene measures in milk collection are effective and that in Australian conditions testing of bulk milk may not be a useful method to identify herds with paratuberculosis.

Title A review of diagnostic accuracies of ELISA and faecal culture in cattle
Author(s) Nielsen SS¹, Toft N².
Institution ¹ Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark; ² Danish Meat Association, Kjellerup, Denmark.
Presentation Keynote
Abstract Infections with Mycobacterium avium subsp. paratuberculosis (MAP) can be latent for years without affecting the animal, but the infection may result in the animal becoming infectious and developing clinical disease. Diagnosis can be a challenge primarily in latent stages of the infection, and because different decision makers have different target conditions for a diagnosis. The objective of this study was to provide a critical review of reported accuracies of ELISA and faecal culture (FC) tests used for diagnosis of three defined target conditions in cattle: MAP infected, MAP infectious and MAP affected animals. For each target condition and test, sensitivities (Se) and specificities (Sp) were summarised. The diagnostic test information varied substantially for tests of the same type and make, particularly ELISA. For affected and infectious animals, the Sp of FC was set to 1.0 by definition. Se reported for FC in infectious and affected cattle were 0.74 and 0.70, respectively, whereas Se for infected cattle were 0.23 to 0.29. Se for ELISA were in the ranges 0.50 to 0.87 for affected, 0.24 to 0.94 for infectious and 0.07 to 0.39 for infected cattle, but Se of ELISA should always be interpreted with Sp, which also varied considerably. The variation in reported Se and Sp may primarily be a reflection of the choices of the test-evaluators regarding weighing of either Se or Sp, study design and population. Comparison of the various tests accuracies was generally not possible, but stratification of test-evaluations by target condition improved the interpretation of the test accuracies. Infectious and affected animals can often be detected, but Se for infected cattle is generally low. A main conclusion of the review was that the quality of design, implementation and reporting of evaluations of tests for paratuberculosis was generally poor. Particularly, there is a need for better correspondence between the study population and target population, i.e. the subjects chosen for test evaluation should reflect the distribution of animals in the population, where the test is intended to be used.

Title Sensitivity and specificity of unique 'Multi-species indigenous ELISA kit' with respect to fecal, milk and tissues culture for the diagnosis of Johne's and Crohn's disease in India
Author(s) Singh SV, Singh AV, Singh PK, Sohal JS.
Institution Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - Farah, District - Mathura (UP), India.
Presentation Poster
Abstract Three ELISA kits were compared for screening of animals and human beings in India. Kit 1: Indigenous ELISA kit had protoplasmic antigen (PA) from MAP 'Bison type' of goat origin. Kit 2: Antigen (PA) of Kit 1 was replaced with commercial purified protoplasmic antigen (PPA) of MAP 'bovine' origin (Allied Monitor Inc., USA). Kit 3: Commercial ELISA kit for bovines (Pouquier, France). Kit 1 was used as. Serum ELISA kit 1 (s-Kit 1) and milk-ELISA kit 1 (m-Kit 1). Overall sensitivity and
specificity of s-Kit 1 with culture (Feces/milk/tissues) was between 28.5-95.6% and 50.0-90.6%, respectively. M-Kit 1 with respect to culture (milk and feces), sensitivity and specificity ranged between 28.5-91.9% and 50.0-75.0%, respectively. Using s-Kit 1 in homologous host, the sensitivity and specificity was 55.5 and 86.3% with respect to fecal culture and were 66.6 and 75.0% with respect to tissues culture, respectively. In advance stages of Johne's disease in a private farm (35 goats and kids with 100% morbidity due JD) with respect to fecal culture, the sensitivity and specificity was, 37.0 and 50.0%, respectively. However, in sheep, the sensitivity and specificity of s-Kit 1 with respect to fecal and tissues culture was variable between 40.0-68.7% and 75.0-90.0%, respectively. In cattle, s-Kit 1, had 50.0 and 90.6%, sensitivity and specificity with respect to fecal culture With respect to milk culture it had sensitivity of 95.6% and specificity could not be determined due to lack of negative samples. Using m-Kit 1, in goats, sensitivity and specificity was, 56.7 and 50.0%, respectively, with respect to milk culture. In cattle, with respect to milk culture, the sensitivity and specificity was 28.5 and 75.0%, respectively. Low correlation with ELISA and culture was due to low conversion of serum globulins to lacto-globulins. In another studies, sensitivities were 90.0 and 90.9%, respectively in milk and fecal culture. However, in human beings, using s-Kit 1, sensitivity and specificity were, 100.0 and 33.3%, respectively. PA in s-Kit 1, detected 10.5 and 46.7% kids positives in farmer's herds, whereas Kit 2 detected, nil and 2.7% kids, respectively. However, in adult farm goats and sheep using s-Kit 1, 25.0 and 43.7% animals were positives, as compared to 17.8 and 25.0% by Kit 2, respectively. In farmer's buffaloes s-Kit 1 detected 58.6% buffaloes positive, whereas, none was positive in Kit 2. In human beings, s-Kit 1 and Kit 2 had 34.0- 42.1 and 30.0-40.7%, positives, respectively, Comparative evaluation of 3 kits on 72 serum samples of farm goats and sheep showed that sensitivity and specificity were, 55.5 and 86.3 and 18.5 and 86.5 and 3.7 and 91.7% in Kit 1, 2 and 3, respectively. S/P ratios showed that Kit 1 in comparison to culture (fecal, tissues, milk) was never over sensitive. Indigenous ELISA kit (Kit 1), was a useful 'Multi-Species kit' for screening of MAP infection in animals and human beings in India.

Title Variability of Repeated Johne's Disease Milk ELISA Test Results in Canadian Dairy Herds
Author(s) Sorge US, Kelton DF, Sears W.
Institution Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.
Presentation Poster
Abstract Commercially available milk ELISA tests for Johne's disease (JD) are vary in sensitivity and specificity depending on the disease status of the cow. The objective of this study was to explore and describe subsequent test results from cows previously positive on the AntelBio Johne's Milk ELISA test. The cut point for a positive milk ELISA test was set at an optical density (OD) of 0.1. The study was conducted retrospectively utilizing the CanWest DHI milk ELISA records from 2,398 cows in 128 herds in the Canadian provinces of Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia. The study included all cows in these herds that had been tested at least twice between March 2005 and April 2007. The cows were in lactations one to eleven. Of all cows tested, 87 cows had a positive test result and were tested on at least one subsequent occasion. Of these, 36 (41%) tested negative and 51 (58.6%) tested positive for JD at a second milk ELISA test (27 to 632 days after the first test). Nine cows were tested three times, with five of these testing positive at all three tests. Of the four remaining cows, two tested negative on test two and three, while one cow changed from positive to negative and one from suspicious to positive between test two and three. One cow had four and another cow five subsequent positive tests within the period of one year. The OD of the test score and subsequent changes in test status were independent (p=0.1852). Neither the lactation number nor the breed of the cows (Guernsey, Jersey, Holstein) were associated with the occurrence of changes in
the test status (p > 0.1), nor were the differences in OD between tests of the 87 cows associated with interval between the tests (p > 0.6). The variability in the milk ELISA test scores and their test interpretations, even in cows with initially high OD test scores, underscore the limitations of this test as a diagnostic tool for individual cows.

Title An alternative for the preadsorption step in the paratuberculosis serodiagnosis: Mycobacterium fortuitum

Author(s) Marassi CD¹, Oelemann WM¹, Fonseca LS¹, Ristow P¹, Lilenbaum W².

Institution ¹ Universidade Federal do Rio de Janeiro; ² Universidade Federal Fluminense, Brazil.

Presentation Poster

Abstract Currently available ELISAs for paratuberculosis employ a preadsorption step with Mycobacterium phlei. As M. fortuitum is the most frequently isolated environmental mycobacteria in Brazil, we considered the hypothesis that the use of local strains of environmental mycobacteria might be useful. Ten negative sera and four positive sera of our collection, confirmed by results of bacteriological culture, plus one positive and one negative control serum were used. Adsorption of bovine sera was performed in three distinct ways: using M. phlei only, M. fortuitum only or a combination of M. phlei + M. fortuitum. In spite of the overall reduction on ODs values observed at the M. fortuitum-ELISA, three positive sera remained presenting much higher values (mean = 0.650) than negative sera (mean= 0.150), as expected. Only one positive serum became negative with an OD value of 0.261 (cut-off = 0.35). Two of the positive sera and four of the negative sera preadsorbed with the M. fortuitum + M. phlei solution presented higher ODs than with the standard assay. Nevertheless, with this preadsorption step, no serum changed its final status and correlation between both tests was also high for those samples (k> 0.8). In spite of one serum having its final result altered, the assays using different preadsorptions were demonstrated to be comparable (p<0.01) and no difference on efficacy could be detected between them (k>0.8). Besides of this, variation on ODs values observed among the three preadsorption assays was not significant (p< 0.01). Our results suggest that M. fortuitum, alone or combined with M. phlei, may be considered as an alternative for the preadsorption step of ELISAs for paratuberculosis.

Title Diagnosis of bovine paratuberculosis: sensitivity of a commercial ELISA test on bovine bulk milk

Author(s) Arrigoni N¹, Cammi G¹, Losini I¹, Taddei R¹, Tamba M², Belletti GL¹.

Institution ¹ Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna, Centro di Referenza Nazionale per la Paratubercolosi - Piacenza, Italy; ² Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna, Centro Emiliano-Romagnolo Epidemiologia Veterinaria- Bologna, Italy.

Presentation Poster

Abstract 52 dairy herds, infected with paratuberculosis, were submitted to repeated bulk milk sampling. 183 bulk milk samples (an average of 3.5 samples/herd) were tested by a commercial ELISA test validated on milk (Institut Pourquier); 30.1% of samples and 40.4% of herds resulted reactive (positive or doubtful) to paratuberculosis. The sensitivity of this ELISA test applied on bulk milk appears strongly correlated to the herd prevalence. The repeated sampling enhances the sensitivity of this diagnostic tool.

Title Evaluation of four commercial bovine-ELISA kits for the diagnosis of paratuberculosis in dairy goats
Goat paratuberculosis is a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), characterized by enteritis, progressive loss of body weight, and decrease in production, especially in dairy goats. The disease is worldwide distributed and in Chile the prevalence is suspected to be high in dairy herds with intensive management systems and specialized breeds for milk production. There is no single test able to detect all infected animals and the faecal culture and the ELISA test are the most widely used tests to diagnose the disease. However, faecal culture is laborious, expensive and takes a long time to give a result and, therefore, the ELISA test, despite its low sensitivity, is the best alternative to be used as a diagnostic tool in Chilean dairy goat herds. The main objective of this study was to evaluate the sensitivity and specificity of four commercial bovine-ELISA kits for the diagnosis of paratuberculosis in dairy goats. A total of 379 serum samples from dairy goats >2 years old with known infection status belonging to the bank of sera of the Paratuberculosis Laboratory, Microbiology Department, Universidad Austral de Chile, were analyzed. All sera were previously collected from animals in four infected and four non-infected dairy goat herds from different regions of the country. Serum samples were simultaneously assayed for anti-MAP antibody using four different commercial bovine-ELISA kits (A, B, C, and D), and performed after manufacturers' recommendations. Sensitivity and specificity of each test were calculated by means of 2x2 tables and for comparison between tests the Z test was used. Association and agreement between two kits were determined by means of the McNemar Chi square and the Kappa tests, respectively. Positive results were obtained in 49 (12.9%) samples assayed with kits A, 33 (8.7%) for kit B, 46 (12.1%) for kit C, and 42 (11.1%) for kit D. Test sensitivity varied between 69.4% (B) and 77.7% (A, C, and D). Test specificity was 100% for all four kits. The McNemar P value showed statistic differences between kits A and B, B and C, and B and D but no difference between kits A, C, and D. The highest kappa value was 0.806 for kits A and D, a high agreement between these two kits. These results suggest that ELISA test developed for diagnosis of paratuberculosis in cattle can be equally used for diagnosis in goats though differences in sensitivity and specificity exist between kits, in particular when applied to low shedder animals, being kit D, the only licensed ELISA kit for goats, the most accurate test for detecting low shedder animals. Consequently, the ELISA test can be recommended for the diagnosis of paratuberculosis in dairy goat herds as a more inexpensive and confident alternative diagnostic test.
paratuberculosis-free herds by two consecutive 100% negative fecal cultures one year apart. Fecal samples were collected via rectum using individual polyethylene sleeves, transported to the lab, and cultured within 24h on home-made Herrold's Egg Yolk Medium (HEYM) with mycobactin J (3 tubes) and HEYM without mycobactin J (1 tube), using the centrifugation method. Prior to culture, 2g of each fecal sample was decontaminated with HPC and an antibiotic solution containing nalidixic acid, vancomycin, and amphotericin B. A 0.15 ml aliquot of each suspension was used to inoculate all HEYM tubes which were incubated at 37°C for 16 weeks. Colonies resembling M. paratuberculosis and showing mycobactin-dependence were tested and confirmed by IS900 PCR technology. The following cutoff values recommended by the manufacturers were used for sensitivity and specificity analysis of each kit: S/P >= 0.25 (kit A), S/P% >= 70 (kit B), (OD-neg) >= 0.100 (kit C), and S/P% >= 70 (kit D). The sensitivity of the four ELISAs were 99.73% (kit A), 99.18% (kit B), 99.46% (kit C), and 98.76% (kit D). The sensitivity of the four kits for detecting fecal culture-positive cows were: 32.08% (kit A), 37.74% (kit B), 41.51% (kit C), and 37.74% (kit D). Receiver Operating Characteristic (ROC) analysis showed that kit C performed much better than the other three kits as the AUC values for the four kits assayed were 0.899 (kit A), 0.818 (kit B), 0.945 (kit C), and 0.854 (kit D). Assay agreement between kits was high (kappa 0.842 to 0.908) for categorical interpretations (positive or negative); the following kappa values were calculated for all four kits: A vs B = 0.843; A vs C = 0.843; A vs D = 0.842; B vs C = 0.908; B vs D = 0.907; and C vs D = 0.952. According to these results all four paratuberculosis ELISA kits evaluated were similar in sensitivity and specificity but kit C was most accurate.

<table>
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<tr>
<th>Title</th>
<th>Reproducibility of results in batches of three ELISA kits for the diagnosis of paratuberculosis in cattle: recommendations for kit evaluation criteria</th>
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<tr>
<td>Author(s)</td>
<td>Gwozdz JM, Carajias M.</td>
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<tr>
<td>Institution</td>
<td>1 Department of Primary Industries, Victoria, Australia.</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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</table>

**Abstract**

*Introduction:* In Australia, the ELISA technology is used in the National Johne's Disease Market Assurance Program for Cattle to assess herds for paratuberculosis. Currently, there are three commercially available ELISA kits that are approved for testing cattle for this disease in Australia. New batches of the ELISA kits are subjected to independent evaluation to assess reproducibility of the assay performance prior to release of a kit for diagnostic purposes. Objectives: To validate criteria for the evaluation of new batches of three ELISA kits. Experimental design: Three batches of each of the three ELISA kits were evaluated over a period of three years. Specificity sera from 180 cattle from a region considered as free of paratuberculosis and sensitivity sera from 40 cattle with paratuberculosis were tested following the kit manufacturer's recommendations. Results: The average CVs of OD values within a plate (among wells), between plates and between batches of the three kits were 6.8% (range 3.73 to 9.12%), 9.3% (5.35 to 14.9%) and 13% (8.8 to 16.98%), respectively. The overall average agreement of diagnostic classification for all kits and batches was 99% (98 to 100%). The overall average specificity and sensitivity for all kits and batches were 99.75% (98.53 to 100%) and 78.6% (70.6 to 90.9%), respectively. Conclusions: Data derived from this study was used to formulate acceptance criteria for evaluation of new batches of the ELISA kits. The high reproducibility of results warrants the use of these tests in market assurance programs to consistently assess level of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle herds.

<table>
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<th>Title</th>
<th>Application of three ELISA kits to bulk milk for detection of paratuberculosis in dairy cattle herds</th>
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<tr>
<td>Author(s)</td>
<td>Gwozdz JM, Carajias M, Mohammad L, Ridge S, Condron R</td>
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</table>

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Objective: To evaluate the usefulness of testing bulk milk samples using 3 commercially available ELISA kits for rapid detection of paratuberculosis in dairy cattle herds. Experimental design: Duplicate samples of bulk milk from 70 herds with paratuberculosis and 15 herds considered as free of infection were tested using 3 commercially available ELISA kits supplied by Prionics, Institut Pourquier and Svanova. Results: The 3 ELISA kits showed limited ability to discriminate between herds with paratuberculosis and herds considered as free of the disease. Of the 70 herds with paratuberculosis, 9 (13%) tested positive by the Pourquier ELISA and 12 (17%) tested positive by the Prionics ELISA when results were interpreted using a cut-off value that theoretically offers 95% specificity (2 SD + mean). However, at this cut-off value the Porquier ELISA gave a positive reaction in one of the 15 herds considered as free of paratuberculosis. Consequently, the results were also interpreted using a cut-off value of 3 SD + mean, which offers a test of approximately 99% specificity. Both the Porquier ELISA and the Prionics ELISA at the 3 SD cut-off detected 6 of the 70 (8.6%) herds with paratuberculosis. The Svanova ELISA consistently produced a high non-specific reaction in one sample of milk from the 15 herds considered to be free of paratuberculosis and was subsequently excluded from further analysis. Conclusions: ELISA technology applied to bulk milk samples is not a sensitive method for the identification of dairy herds affected by paratuberculosis. With further evaluation and refinement the technology may be suitable for the identification of herds with a high prevalence of infected cows.
highly sensitive and may improve the effectiveness of JD control measures.

Title: Development of ELISA method using purified protein derived from Mycobacterium avium subspecies Paratuberculosis isolate from Japan

Author(s): Wang X, Kojima H, Kato T, Ishigami S, Ishiguro S, Mori Y1, Yanaka T.

Institution: Advanced Technology Development Center, Kyoritsu Seiyaku Corporation, Japan; 1 Research Team for Paratuberculosis, National Institute of Animal Health, Japan.

Presentation: Poster

Abstract: The immunological diagnosis of Johne's disease is performed by an ELISA test and Johnin test. For the production of the diagnostic antigen for the tests, P-18 strain, classified as Mycobacterium avium subsp. avium(Maa) on the basis of genetic properties, has been used. Hence, in the present study, we used Kag-1 strain of Mycobacterium avium subsp. Paratuberculosis(Map), an isolate from Japan, to produce an ELISA antigen and examined its sensitivity and specificity in antibody detection. Sera from 141 fecal culture-positive and 103 fecal culture-negative cattle were used to examine the sensitivity and precision of the partially purified ELISA antigen from Kag-1 strain of Map. The sensitivity was 80.1% in ELISA and an agreement of 88.1% was found between the ELISA method and fecal culture method (Kappa value: 0.765, P<0.01). In addition, in the fecal culture-positive cattle, the ELISA-positive ratio was 71.8% for light shedders, 80.9% for intermediate shedders, and 92.9% for heavy shedders. The average ratio was 79.4%. In examination of cross reactions with bovine Mycobacterium antibodies, the purified ELISA antigen revealed weak cross reactions with antisera immunized by BCG Tokyo strain of M. bovis and Kumamoto-8 strain of Maa, and a strong cross reaction with an antiserum immunized by S-7 strain of M. intracellulare. A western blotting that uses infection serum of ATCC 19698 strain and Kag-1 strain of Map showed colored bands mainly at 56 kDa and 29 kDa. The above results indicate that the purified protein from Kag-1 strain of Map can be used as an antigen for ELISA.
variation coefficient, then standard deviation of the reproducibility results and Z-scores were calculated using the result of Immunology Laboratory of IDAH, as a reference value. Grubbs test was used for identifying aberrant results and for elimination of laboratories with unsuitable results. The results of Z-scores were presented in diagrams. In the end it was made a correlation between the obtained results of Z-scores and those of qualitative interpretation. Thus the correlation for negative samples E5 and E6 was 100%, for positive samples E4 of 95%, and E3 of 97.5% and for doubtful samples was as follows: E1 77.5% and E2-52.5%. The sum of Z scores was used for the classification of laboratories which had the same result at the quality interpretation test. Following the results obtained, 31 laboratories obtained satisfactory result, 6 questionable and 2 unsatisfactory. The statistical analyses together with the results of the test permitted a better evaluation of laboratories. Key words: paratuberculosis, ELISA, score Z, proficiency testing

<table>
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<th>Title</th>
<th>Determination of optimal conditions to test bovine milk for antibodies against <em>Mycobacterium paratuberculosis</em> using an ELISA test</th>
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<tr>
<td>Author(s)</td>
<td>Gwozdz JM, Carajias M, Mohammad I.</td>
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<td>Presentation</td>
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<tr>
<td>Abstract</td>
<td>Objectives: To determine optimal volume and type of milk samples for the Paracheck TM ELISA, a test that was developed to assay serum or plasma for antibodies against <em>Mycobacterium paratuberculosis</em>. Experimental design: Samples of milk and blood were collected from 12 cows that previously tested positive by the ELISA. Of the 12 cows, 11 tested positive by faecal culture and/or histopathology and one had no bacteriological or histopathological evidence of infection. In the first experiment, duplicate samples of serum, whole milk and skim milk the 12 cows were assayed for antibodies using a commercial bovine ELISA. Each replicate was diluted 1:20 in absorbing buffer and tested as recommended by the manufacturer. In the second experiment, 3 sets of duplicate samples of whole milk from 5 cows with paratuberculosis and one cow with no evidence of infection were assayed for antibodies. One set was diluted 1:20 in absorbing buffer (25 micro-L sample/475 micro-L absorbing buffer), the second set was diluted 1:10 (50 micro-L sample/450 micro-L absorbing buffer) and the third set was diluted 1:5 (100 micro-L sample/400 micro-L absorbing buffer). Results: In the first experiment, there was no significant difference (P&lt;0.05) between the mean OD values in whole and skim milk. The average coefficient of variation (CV) between OD values in whole and skim milk was 4.8%. Although there was strong positive correlation between OD values in samples of serum and that measured in corresponding samples of whole milk and skim milk, only 7 of the 11 cows with paratuberculosis gave positive reactions in both the whole and skim milk. The OD values in samples of serum were significantly higher than that measured in corresponding samples of whole milk and skim milk, only 7 of the 11 cows with paratuberculosis gave positive reactions in both the whole and skim milk. The OD values in samples of serum were significantly higher than that measured in corresponding samples of whole milk and skim milk. In the second experiment, the mean OD values in whole milk samples diluted 1:20, 1:10 and 1:5 were 0.217, 0.340 and 0.549, respectively. The latter was similar to the mean OD in corresponding samples of serum. Of the 5 cows with paratuberculosis, all tested positive when the test was applied to serum and samples of milk diluted 1:5. In comparison, only one of the 5 cows gave a positive reaction when samples of milk diluted 1:20 were tested. The negative control showed a slight, negligible increase in OD values when larger volumes were tested. The one cow with no evidence of infection gave consistent negative results throughout the testing. Conclusions: The likelihood of detecting infected animals increases when the ELISA is applied to larger volume samples of milk. The whole milk is a suitable sample as the differences between the OD values in samples of whole and skim milk are negligible and similar to normally expected well-to-well variation.</td>
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Title | Diagnostic performance of PARACHEK® for the detection of antibodies against |

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**Mycobacterium avium** subsp. *paratuberculosis* in milk and serum of Dutch dairy cattle

**Author(s)** Weniger P\(^1\), Schacher P\(^1\), Senn K\(^1\), Wisselink HJ\(^2\), Harders-Westerveen J\(^1\), Marg-Haufe B\(^1\), Meissner K\(^1\).

**Institution**  
\(^1\) Prionics AG, Schlieren, Switzerland  
\(^2\) Animal Sciences Group, Lelystad, The Netherlands.

**Presentation** Poster

**Abstract**  
Efficient control of Johne's disease is dependent on early detection and removal of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infected cows to prevent spreading of the disease and associated economic losses to the cattle industry. Testing schemes optimized for cost and ease of implementation are often based on screening of milk samples and ELISA methodology rather than the use of blood serum samples with ELISA. The goal of this study was to determine the diagnostic performance of the PARACHEK® *Mycobacterium paratuberculosis* antibody test kit (Prionics AG, Schlieren, Switzerland) using milk and blood serum samples compared to the culture results of feces for MAP bacteria. For this purpose milk, blood serum and fecal samples were collected from 531 dairy cows in the Netherlands. These cows derived from 10 dairy herds. Eight herds had a recent history of paratuberculosis and two farms had a confirmed status of freedom of paratuberculosis infection based on their participation in a Dutch herd-certification program (1). Serum samples were collected from all 531 cows and stored at -20°C until analyzed. Milk samples were obtained from lactating cows (n=483) and stored at -20°C until further use. Fecal samples (n=531) were obtained directly from the rectum of the cows. The feces samples were decontaminated (3) and subsequently cultured in the TREK ESP para-JEM Culture System II (TREK Diagnostic Systems, Cleveland, Ohio, USA). All samples detected by the system and all samples not yet detected by the system at the conclusion of the experiment (49 days) were further investigated via Ziehl Neelsen staining and PCR methodologies (2). Milk and serum samples were analyzed using the indirect enzyme immunoassay PARACHEK® to detect antibodies against MAP. The manufacturer has two different protocols for milk and blood serum samples using the same kit. The samples were analyzed according to the manufacturer’s instructions. The results showed that in total 4.0% of the cows (21 out of 531) were culture positive. The sensitivity of the PARACHEK® with regard to culture positive cows was 52.4% (11/21) in serum and 57.9% (11/19) in milk samples. The specificity was 98.6% (503/510) and 98.9% (445/450), respectively. None of the cows from certified MAP-negative herds were determined as positive with the PARACHEK® either with serum or with milk samples. This suggests that false positive determinations from herds with a recent history of paratuberculosis may in fact indicate truly infected cows. These cows are possibly only negative in culture analysis due to intermittent or low mycobacterial shedding in feces, i.e. levels which are below the detection limit of the culture method. The agreement between the results from milk and serum samples was excellent with a proportion of agreement of 0.98 and a weighted kappa value of 0.843. In conclusion, the results of this study indicate that surveillance of cattle herds with the PARACHEK® using either serum or milk is an efficient, cost effective and reliable method to detect paratuberculosis in infected herds. In combination with good farm management, this test can contribute substantially to the reduction in prevalence of Johne's disease in cattle herds.

Title  Antigenicity study of secreted proteins of *Mycobacterium avium* subsp *paratuberculosis* (*Map*) isolated from dairy herds in southern Chile.

Author(s) Pradenas MV\(^1\), Jara MC\(^1\), Zambrano ÁH\(^1\), Kruze DJ\(^1\), Collins MT\(^3\).

Institution \(^1\) Microbiology Department, Faculty of Sciences, Universidad Austral de Chile, Campus Isla Teja, P.O.Box 167, Valdivia, Chile; \(^2\) Postgraduate School, PhD Program, Faculty of Veterinary Sciences, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile; \(^3\) Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, USA.

Presentation Poster

Abstract Liquid culture filtrates (CF) of *Mycobacterium avium* subsp *paratuberculosis* (*Map*) contain more antigenic proteins that react with sera from infected cattle. The aim of this study was to identify proteins with potential diagnostic value. CF MAP proteins were separated by SDS-PAGE one-dimensional electrophoresis technology. The CF proteins were harvested from supernatants of liquid cultures in stationary-phase and concentrated by size exclusion filtration. Analysis of SDS-PAGE gels showed that the majority of CF proteins had low molecular masses (<50 kDa). The antigenicity of CF proteins was determined by 1-DE immunoblotting with sera of four different cows naturally infected with MAP. The sera reacted strongly with proteins in the range of 20 - 40 kDa. When sera from different infected cattle were tested by immunoblotting with CF proteins, a high degree of variability in protein binding patterns was observed. Additionally, when bovine sera were absorbed with environmental mycobacteria, namely *M. avium*, *M. phlei*, *M. terrae*, *M. scrofulaceum* and *M. smegmatis*, and tested by immunoblotting, there were no major differences in the antigenic patterns between absorbed and non-absorbed sera. These results indicate that serological tests like ELISA for bovine paratuberculosis may be improved by using CF proteins, and the serum preabsorption step using environmental mycobacteria could potentially be eliminated from the ELISA procedure.

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Title Three-year serological follow up of an ovine herd, naturally-infected by *Mycobacterium avium* subsp *paratuberculosis* (*Map*)

Author(s) Pourquier P\(^1\), Lesceu \(^1\), Foucras G\(^2\).

Institution \(^1\) ID VET, Montpellier France, www.id-vet.com; \(^2\) Ecole Nationale Vétérinaire de Toulouse, France.

Presentation Poster

Abstract Objective: The aim of this study was to follow within-herd Map infection using an absorbed ELISA and other diagnostic methods. Methods: An ovine herd (n=1000), endemically infected by Map, was bleeded twice a year for 3 years. Animals were killed at 5 years of age, and different tissue samples and a final serum sample were taken. Serum samples were tested by ELISA (ID Screen® Paratuberculosis Indirect, ID VET, France), and tissue samples were examined with other diagnostic techniques. Results: The poster will present and analyse the results in detail, allowing a better understanding of Map serology in ovine herds.

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Title Diagnostics for Resistance and Susceptibility to Johne's disease in deer

Author(s) Griffin F, Liggett S, Rodgers C.

Institution Disease Research laboratory, University of Otago, Dunedin, New Zealand

Presentation Poster
### Abstract

Johne's disease, caused by infection with *Mycobacterium paratuberculosis* (*Map*), is an important bacterial disease affecting productivity in New Zealand farmed deer herds. In addition to clinical losses, subclinical infection results in reduced growth and reproduction. Deer differ from other species of farmed ruminants in that they can present with clinical disease at a young age (8-12 months). Microbial culture of gut tissue obtained at necropsy has been the 'gold standard' for diagnosing *Map* infection. Diagnosis of infection in live animals continues to be a challenge. The Disease Research Laboratory's recently developed ParalisaTM test has good sensitivity (>90%) and specificity (>98%) for the detection of animals with Johne's disease. It has a lower sensitivity (75%) in diagnosing *Map* infected deer without lesions. The use of this test in severely infected deer herds can reduce the prevalence of reactors from high (>40%) to low levels (<5%) within 2-3 years and results in the elimination of clinical disease. As clinically detectable Johne's disease represents a minor proportion of the total number of *Map* infected deer within a herd, it is also important to have diagnostic tests that detect subclinically infected animals. Using the ParalisaTM, it is now possible to implement management systems to electively remove *Map* infected deer resulting in reduced environmental contamination and improved production and reproductive performances. While this approach can significantly reduce Johne's disease levels within a deer herd we do not claim that it can be used to eradicate *Map* infection. Diagnostics have also been used to identify susceptibility traits linked to genotype (breeds), phenotype (male vs female) and production.

### Title

New cases of JD and Map infection in two dairy herds from Pernambuco and Rio de Janeiro States - Brazil.

### Author(s)

Gomes MJP¹, Mota RA², Tokarnia CH¹, Galvão A¹, Brito MF³, Seixas JN³, Bercht BS¹, Snel GGM¹, Juffo GD¹, Chies JAB¹.

### Institution

¹ Universidade Federal do Rio Grande do Sul; ² Universidade Federal Rural de Pernambuco; ³ Universidade Federal Rural do Rio de Janeiro.

### Presentation

Poster

### Abstract

Two cases of bovine JD were detected by post-mortem examination in 4-6 years old Holstein cows of a 170 head dairy herd in the State of Pernambuco, Northeast-Brazil. During 2006, three more cases were diagnosed by post-mortem examination in a 130 head Girolanda dairy herd in the State of Rio de Janeiro. The histopathological findings were granulomatous enteritis, lymphadenitis and lymphangitis. The inflammatory infiltrate was composed of macrophages, epithelioid and Langhans cells. Samples of intestine tissue and feces were inoculated in HEYM with/without mycobactin for *Map* isolation. Cultures on HEYM supplemented with mycobactin yielded colonies identified as *Map* according to their phenotypic properties and PCR reaction. From intestinal tissues and feces from cows with JD and from asymptomatic cows of the Pernambuco herd, five *Map* strains, and of the Rio de Janeiro herd, two strains were isolated in HEYM. The prevalence of *Map* infection was estimated by whole herd testing in the two dairy herds by absorbed ELISA using PPA-3 commercial antigen. The ELISA test identified 55 positive cows (32.3%) among the 170 animals from Pernambuco and 57 positive cows (43.8%) among the 130 from Rio de Janeiro. The *Map* infection was disseminated in bovine dairy herds in Pernambuco and Rio de Janeiro States showing the necessity for adoption of control measures for the protection of Brazilian dairy herds.

### Title

Diagnostic-test characteristics of microscopic examination of ZN-stained faecal smears and ELISA in cattle suspected of clinical paratuberculosis

### Author(s)

Weber MF, Verhoeff J.

### Institution

GD Animal Health Service, PO Box 9, 7400 AA Deventer, The Netherlands
Testing cattle with clinical signs of paratuberculosis is an important element of surveillance for paratuberculosis. In many herds, control of paratuberculosis infection is only initiated after detecting clinical paratuberculosis cases. Therefore, the aim of this study was to evaluate the diagnostic-test characteristics of microscopic examination of Ziehl-Neelsen-stained faecal smears for acid-fast Mycobacteria (ZN-test) and serum-ELISA in cattle suspected of clinical paratuberculosis. Results of all samples submitted for ZN-test and serum-ELISA between April 2003 and April 2006 to our laboratory were retrieved. Results of cattle for which both tests were performed were analysed using three Bayesian models for evaluation of diagnostic tests in two populations without a gold standard, assuming conditional independence of tests (model 1), conditional dependence of tests in both infected and non-infected cattle (model 2) and conditional dependence of tests in infected cattle only (model 3). Sampled cattle were divided into two populations in two different ways using known risk factors for clinical paratuberculosis: region and age. Priors for sensitivity and specificity of tests were based on the literature; uninformative priors were used for prevalence's in the various populations. For 892 cattle suspected of clinical paratuberculosis, both ZN-test and ELISA results were retrieved: 250 ZN-positive and ELISA-positive, 12 ZN-positive and ELISA-negative, 260 ZN-negative and ELISA-positive, and 370 ZN-negative and ELISA-negative cattle. Posterior estimates of sensitivity, specificity, and positive and negative predictive values of the ELISA were always higher than those of the ZN-test, irrespective of the population and choice of model. Lower limits of the 95% credibility intervals of the posterior positive predictive values of the ELISA were always >=99.7%, and of negative predictive values of the ELISA >=57.0%. Upper limits of the of the 95% credibility intervals of the posterior positive predictive values of the ZN-test in the various models were always <=99.4%, and of negative predictive values of the ZN-test were always <=59.8%. It is concluded that the ELISA is preferred to the ZN-test to confirm the presumptive diagnosis of clinical paratuberculosis. Little diagnostic information can be gained by performing the ZN-test in addition to the ELISA.

Title
The use of MPB70 and MPB83 to distinguish between bovine tuberculosis (TB) and paratuberculosis (PTB)

Author(s)
Marassi C¹, McNair J¹, Pollock J¹, Ristow P¹, Fonseca L¹, Oelemann W¹, Lilienbaum W².

Institution
¹ Universidade Federal do Rio de Janeiro, Brazil; ² Universidade Federal Fluminense, Brazil; ¹ Department of Agriculture, Northern Ireland.

Abstract
Paratuberculosis (PTB) is characterized by chronic enteritis as a consequence of Mycobacterium avium paratuberculosis (Map) infection in cattle. Map shares several antigens with other Mycobacteria, including M. bovis. Some attempts with purified antigens have been described in order to differentiate between those two infections in cattle. The purpose of this study was to demonstrate the potential of two M. bovis-specific recombinant proteins MPB70 or MPB83 to distinguish PTB from bovine tuberculosis infection (TB). Two TB-free and six herds where TB occurs were studied. An ELISA using the recombinant proteins MPB70 and MPB83 was standardized. Two distinct populations of animals were selected, Group A (n = 23) with animals coming from TB-free herds with PTB and Group B (n = 48) composed by PTB-free animals (confirmed by culture) from where M. bovis infection was cultured. In Group A, 10 animals reacted to MPB70 (43.47%) and nine to MPB83 (39.13%). In Group B, 37 animals reacted to both MPB70 and MPB83 (77.08%). Our results indicate that both antigens presented very similar results, with a concordance (kappa index) of 0.91. The difference between the mean OD value for Group A and Group B measured against the same antigen was highly significant (p<0.01). MPB70 and MPB83 clearly detect M. bovis specific antibodies more often in tuberculous cows than in paratuberculous cattle, and therefore can be considered as valuable tools
to differentiate between these two infections at serology. The use of recombinant antigens derived from each microorganism, or a combination of them, would increase the specificity of serological assays by diminishing antigenic cross-reactivity between bovine PTB and TB.

<table>
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<th>Title</th>
<th>Recombinant enoyl-CoA hydratase (echA) antigen of <em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em> expressed in <em>Escherichia coli</em> can be used for serological diagnosis of Johne's disease.</th>
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<td>Author(s)</td>
<td>Nagata R¹, Yoshihara K¹, Wang X², Yanaka T², Mori Y¹.</td>
</tr>
<tr>
<td>Institution</td>
<td>¹ Research Team for Paratuberculosis, National Institute of Animal Health, Japan; ² Kyoritsu Seiyaku Corporation, Japan.</td>
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<td>Presentation</td>
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<td>Abstract</td>
<td>The phage library of <em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em> (Map) strain ATCC19698, which had been constructed using the Zap Express cloning vector (Infect. Immun. 73:3778-82, 2005), was screened with sera from calves infected with Map in order to detect the Map antigens with eliciting humoral immunity. The serum used for screening of the phage library was absorbed with plaques from non-recombinant phages, <em>Escherichia coli</em> and <em>Mycobacterium phlei</em> organisms to remove cross-reacting antibodies. After the screening of about 4 x 10⁵ plaques, we have finally cloned one recombinant phage expressing Map antigen which strongly react with serum antibodies from infected calves. The Map DNA insert cloned into the phage vector was excised out of the phage into the form of the phagemid vector with <em>E. coli</em> strain XLOLR, and a part of 5' end of the insert DNA was sequenced using T3 universal primer. The sequence results indicated that the insert Map DNA contained Map gene encoding &quot;echA12_2&quot;, an enoyl-CoA hydratase protein (echA), which plays a role for effective ATP synthesis in stationary phase survival. Therefore, the coding sequence of Map echA gene was amplified from DNA of Map strain ATCC19698 by PCR, and cloned into pQE plasmid vectors to obtain the recombinant Map-echA protein (rMap-echA). Sera from experimentally infected calves strongly reacted with the rMap-echA with immunoblotting, whereas sera from uninfected cattle did not. Although the homologous gene of <em>M. avium</em> subspecies <em>avium</em> (Maa) showed 99% nucleotide sequence homology with echA12_2 gene of Map, ELISA using the rMap-echA indicated high specificity without any cross-reaction to bovine hyperimmune sera against Maa and other related <em>Mycobacterium</em> species. These results suggest that the ELISA using the rMap-echA antigen may be useful for the diagnosis of Johne's disease.</td>
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<th>Title</th>
<th>Comparative proteomic analysis of mycobacterial tuberculins and identification of <em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em> antigens with diagnostic potential</th>
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<td>Author(s)</td>
<td>Santema W, Overdijk M, Barends J, Krijgsveld J, Rutten V, Koets A.</td>
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<td>Presentation</td>
<td>Oral</td>
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<tr>
<td>Abstract</td>
<td>Accurate immunodiagnosis of bovine paratuberculosis is among others hampered by the lack of specific antigens. One of the most frequently used antigen preparations is purified protein derivative, also known as tuberculin, produced from heat processed culture filtrates. This crude extract has limitations when used in diagnostic assays due to the presence of cross reactive antigens. The aim of the current study was to analyse the qualitative composition of tuberculins of the major mycobacterial pathogens and subsequently identify novel paratuberculosis specific antigens. Using one dimensional</td>
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gel electrophoresis followed by tandem mass spectrometry analysis of purified protein
derivatives from *Mycobacterium avium* subspecies *paratuberculosis* (MAP),
*Mycobacterium avium* subspecies *avium* (MAA) and *Mycobacterium bovis* we
identified 156, 95 and 132 proteins respectively. Subsequently, comparative sequence
analysis led to the selection of a MAP specific protein (MAP1718c). This protein as
well as MAP3515c (4 AA difference with the homologous protein in MAA) and
MAP1138c (LprG, the homologue of an interesting *M. tuberculosis* antigen) were
expressed as recombinant proteins in *E. coli* for use in lymphocyte proliferation
assays and serum antibody ELISA. While lymphocyte proliferation responses did not
indicate substantial diagnostic potential of the antigens tested, the antibody titers
measured by ELISA specific for MAP1138c, but not MAP1718c and MAP3515c, in
serum from paratuberculosis infected cows (N=20) were significantly higher (p<0.05)
than those in serum from control animals (N=20), despite the conserved nature of this
protein. In conclusion this study showed that a combination of proteomics and
genomics starting from complex protein mixtures can reveal novel antigens
supporting the development of more accurate diagnostics in mycobacterial diseases.

**Title**  
Interference of intradermal tuberculin tests on the serodiagnosis of paratuberculosis in
cattle.

**Author(s)**  
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**Institution**  
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**Presentation**  
Oral

**Abstract**  
Bovine paratuberculosis (PTB) is widely diagnosed by ELISAs. Nevertheless, in spite
of the pre-adsorption step with *M. phlei*, which intends to minimize cross-reactions
with environmental mycobacteria, lack of specificity may happen, due to the shared
antigens of its agent, *Mycobacterium avium paratuberculosis*, with *M. bovis*. A herd
that was proved to be TB and PTB free by serology, intradermal testing and bacterial
culturing three months before the study was selected. For this experiment, 63 animals
were divided in four groups. Group A was tested with PPDbov only, group B with
PPDbov and PPDav, group C only with PPDav and group D with PPD diluents, as
control. Blood samples of each animal were collected moments before PPD
inoculation and after 3, 15, 30, 60 and 90 days. All sera were tested for PTB by an
accredited "in-house" ELISA-PPA and 36 also selected to be confirmed by a
commercial ELISA (Pourquier). Three (4.76%) animals were reactive to ELISA-PPA,
one (1.58%) from group A and two (3.17%) from group B. Considering only animals
tested by recommended tests, i.e. single or comparative intradermal testing, reactive
animals represented 8.82% of the herd. Most samples became reactive between the
30th and 60th days and two animals remained reactive until the 90th day after ITT.
Although not reactive, an evident increase in S/P values along the experiment was
observed in other 29 cows, 7/17 from Group A (41.2%), 10/17 from Group B (58.8%)
and 12/17 (70.5%) from Group C. In the commercial ELISA, the three reactive
animals confirmed to be reactive. From the other 29 animals, two were reactive and
22 confirmed the phenomena. The two used PTB-ELISAs were highly correlated
(κ=0.78). We demonstrate that intradermal tuberculin tests may temporarily (up to 90
days) interfere in the immune status of the animal and determine false-positive
reactions in ELISA as used for the serodiagnosis of paratuberculosis. Therefore, in
order to avoid such occurrence, cattle should not be bled for PTB serodiagnostic for a
period of at least 90 days after tuberculin testing.

**Title**  
Detection of *Mycobacterium avium paratuberculosis* antibodies in bovine serum
using a conductometric biosensor

**Author(s)**  
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Institution 1 Department of Large Animal and Clinical Sciences; 2 Department of Biosystems and Agricultural Engineering, Michigan State University, East Lansing MI 48824 U.S.A.

Presentation Oral

Abstract Johne's disease (JD) in ruminants is caused by the bacterium Mycobacterium avium subsp. paratuberculosis (MAP). JD has caused significant economic loss in affected farms. The worldwide distribution of MAP and its potential link to Crohn's disease in humans raises a public health concern. To facilitate more widespread acceptance of JD control programs, inexpensive diagnostic method that requires less time, inexpensive equipment and that are user friendly must continue to be developed. Diagnosis of JD is done by either detecting MAP in feces or infected tissues or detecting an immune response. Common assays used to detect MAP antibodies include: compliment fixation (CF), agar gel immunodiffusion (AGID), and enzyme linked immunosorbent (ELISA) assays. In this study, a polyaniline-based conductometric biosensor was developed for the rapid detection of antibodies to MAP in cattle serum. The conductometric biosensor consists of three components; the biological sensing element, the transducer and the detector element. MAP antigen served as the biological sensing element, polyaniline (Pani) was the transducer and an ohmmeter was used as the detector element. The biological element and the transducer are immobilized in a membrane with four regions; sample application, conjugate, capture and absorption regions. The detection technique is based on the antigen-antibody coupling reaction with Pani as the conductive transducer. Pani is conjugated to the heavy chains of the monoclonal anti-bovine IgG (AB/IgG) forming Pani-AB/IgG, which is positioned in the conjugate region. MAP purified proteins, serving as capture antigens; are immobilized on the nitrocellulose capture region. Subsequently, silver electrodes are fabricated on both sides of the capture region. 0.1 ml of the sample is added to the application region and allowed to flow through the other regions through capillary movement. Theoretically, Pani-AB/IgG in the conjugate region binds to the bovine IgG present in the sample. The conjugated bovine IgG-Pani-AB/IgG flow through the capture zone where MAP specific antibodies are captured by immobilized MAP purified proteins. As MAP specific antibodies are captured, the attached Pani structures form a bridge across the electrodes flanking the capture region. This bridge closes an electrical circuit that can be measured as a decrease in electrical resistance as more antibodies are captured. In this proof of concept study, JD positive cattle sera, as determined by standardized ELISA technique, were compared to serum samples that were negative for JD. The average resistance for JD negative samples was 92.875 Kilo ohms (range 101.3-72.1 Kilo ohms) while the average resistance for JD positive samples was 53.338 Kilo ohms (range 66.4-37.0 Kilo ohms). These initial studies demonstrate that a conductometric biosensor can be fabricated to detect antibodies to MAP. Further testing to optimize the biosensor performance and test with large numbers of samples is underway.
verification was performed on serum and milk of the positive individual. We collected faeces from positive sheep and carried out PCR on the specific insertion sequence IS900. From our results the seroprevalence among flocks were from 0.7% to 11%. The 54% of the seropositive animals were also positive in milk ELISA. Individual showing an higher value of S/P are more likely to be positive in both tests. So we confirm that the ELISA S/P value is an useful tool in the diagnosis and control of paratuberculosis, particularly in subclinical cases. PCR analysis has confirmed as positive only the 11% of the samples, difficulties are experienced in recovering DNA from small number of organisms in clinical specimens, especially in complex samples such as faeces. The low sensitivity could be attributed to the presence of a intermittent shedding of the MAP in faeces or PCR inhibition by faecal constituents. Mycobacteria are commonly found in soil and water. Since this widespread distribution the present study was performed also to determinate if genetically identifiable individuals may have different tendency to develop paratuberculosis when exposed to the same infectious agent. Two genes that seem to have a major influence on the outcome of infection with Mycobacteria in many species, including mice and humans are the NRAMP1 gene and the CARD15/NOD2 gene. Genetic analysis was carried out on 31 sheep (18 infected and 13 healthy) We searched for polymorphisms linked to susceptibility or genetic predisposition to paratuberculosis in sheep. We sequenced exon 1 and exon 2 of NRAMP1 and we didn't find any SNPs. Concerning CARD 15 we sequenced exon 1 (270bp), exon 2 (500 bp), exon 3 (235 bp), exon 4 (2000 bp), exon 11(120 bp) and intron 1-2 (1008 bp) and the promoter region (541 bp). The exons 1, 2 and 11 were monomorphic as well as the intron 1-2. The exon 4 and the intron 5-6 were polymorphic. The sequences of exon 4 and exon 2 are published in the database Gene Bank (Number of accession EF141018). We found 4 haplotypes with different frequency estimates. We analysed the data with chi-squared test, and fisher's exact test. Up till now wee found no significant associations between variation in CARD15 and disease status. Further step of the study is to investigate on a large cohort of infected animal and controls.
experimental paratuberculosis in sheep

Author(s) Kumar AA, Tripathi BN.

Institution Division of Pathology, Indian Veterinary Research Institute, Izatnagar-243 122 (UP); 
: Department of Pathology, College of Veterinary Science, Hyderabad, India.

Presentation Poster

Abstract The lack of appropriate methods for subclinical diagnosis hampers the control and eradication of Johne's disease in sheep. As infection is insidious with prolonged incubation, it is usually difficult to assess performance of diagnostic methods during progressive stages of natural infection. In the present study, the performance of various tests were therefore evaluated on 15 experimentally infected sheep and 8 in-contact sheep. These animals had various grades of infection (negative, suspected, mild, moderate and severe) categorised on the basis of a set of diagnostic methods (faecal smear, culture and PCR, tissue PCR, ELISA immunoperoxidase and histopathology) over a period of 390 days. Four animals each from the infected and incontact groups were either in the negative or the suspected infection categories, and hence were not included in the assessment. Thus, the performance of the diagnostic methods was assessed on 15 sheep (11 from infected and 4 from incontact groups). Among 15 infected sheep, 9 (67.7%) were in the mild and 3 (33.3%) each were in the moderate and the severe infection categories. The sensitivities of the clinical diagnostic methods were 80% for I/D johnin test, over 73% for faecal smear and faecal PCR, 60% ELISA, over 53% for faecal culture and AGID, and 40% for blood PCR. Among post-mortem diagnostic methods, the highest sensitivity was obtained for immunoperoxidase test (80%), followed by tissue PCR (~67%) and acid-fast bacilli demonstration in tissues (40%). The study revealed that among antemortem tests, johnin test followed by fecal PCR, and among postmortem tests, immunoperoxidase were the sensitive test for the diagnosis of paratuberculosis. The performance of these tests in relation to severity of infection and its implication in the diagnosis and control of paratuberculosis has been discussed.

Title Dairy herd prevalence of Mycobacterium avium subsp. paratuberculosis in bulk-tank milk samples obtained from three regions in Fars province, Iran by nested PCR

Author(s) Masoud Haghkhah¹, Maryam Ansari Lari², Amir Mansour Novin³, Ayatollah Bahrami³.

Institution ¹Department of Pathobiology; ²Department of Food Hygiene and Public Health; ³Graduated, School of Veterinary Medicine, Shiraz University, Shiraz, 71345-1731, Iran.

Presentation Poster

Abstract Paratuberculosis, which is also known as Johne's disease, is a chronic, progressive enteric disease of ruminants and other species of animals including primates. Cattle become infected with Mycobacterium avium subsp. paratuberculosis (MAP) as calves but often do not develop clinical signs until 2 to 5 years of age. Therefore, it causes several economic losses in the cattle industry in all over world per year. Moreover, it has been evidenced by some researchers that MAP is also as the causal agent of Crohn's disease in the human population. To evaluate the prevalence of paratuberculosis in dairy herds, and to determine the association between herd infection status and herd management practices, this research was conducted. A nested-PCR method based on insertion sequence 900(IS900) was adapted for testing bulk-tank milk for the presence of the MAP in Shiraz, Marvdasht and Sepidan regions of Fars province dairy herds. Moreover, an extensive questionnaire based on the main risk factors of the disease was developed and used to collect data from the herds. Twelve of the 110 examined bulk-tank milk samples (11%, 95%CI: 5-17%) tested IS900-PCR positive. The herd prevalence of the MAP in the Shiraz, Marvdasht and Sepidan regions were 8.6, 8.5 and 23.5 percent respectively. Statistical analysis using
multivariable logistic regression showed that low sanitation of periparturient cows as measured by contamination of udders with manure (OR = 6.4, P = 0.02) and history of suspected Johne’s disease in the herd (OR = 6.7, P = 0.04) were significantly associated with herd infection status. No relationship between breed, herd size and other management practices was found in this study. As the bacterium is a slow-growing mycobacterium, the detection of MAP directly from bulk tank milk by IS900 nested PCR could become a valuable diagnostic or screening test for herds with Johne's disease.

Title Comparison of the isolation of Mycobacterium paratuberculosis from milk on the HEYM substrate and direct DNA isolation

Author(s) Szteyn J, Wiszniewska A, Ruszczyfk A.

Institution Warmia and Mazury University, Poland.

Presentation Poster

Abstract Mycobacterium paratuberculosis (MAP) is an aetiology factor in paratuberculosis in ruminants, both domestic and wild. Animals are the reservoir of MAP in the environment and the disease is mainly transmitted to the environment in faeces and milk of sick animals and of those which are infected without symptoms. The presence of mycobacteria in milk may be a source of infection for calves, but also for other animal species and for humans. Mycobacteria are introduced into milk in two ways: with macrophages when animals are infected and by contamination with faeces. In practical terms, faeces contamination is more likely and it increases the MAP count in milk to a greater extent. It has been proven that the HTST (high-temperature short-time) pasteurisation, commonly applied in dairy industry, fails to deactivate mycobacteria completely, which confirms their considerable resistance to high temperatures. The threat related to the mycobacteria presence in milk requires that studies should be conducted in its occurrence. MAP isolation from milk meets with a number of obstacles related to complex three-phase structure of milk on the one hand and the diversity of microorganisms on the other. MAP can be detected by either of two methods: direct isolation of DNA-MAP with the use of QIAamp DNA Mini Kit manufactured by Qiagen or culturing on HEYM substrate. 87 samples of udder milk were examined, taken from milk cows over three years old, belonging to a stock in which cases of subclinical type of paratuberculosis had been found earlier. DNA isolation was conducted according to the manufacturer's recommendations and the obtained solution was subjected to the PCR. MAP genetic material was found in 21 (24.1%) of the udder milk samples. When isolating MAP in the culturing method on the HEYM substrate, having first decontaminated and made milk samples uniform, 43 strains were cultured with the morphological features typical of genus Mycobacterium; however, the presence of an insertion fragment IS-900 was confirmed only in two cases. 18 samples of milk gave a positive result of direct isolation of DNA-MAP and an increase in the number of colonies typical of genus Mycobacterium on the HEYM substrate; in one case, no growth was observed despite the presence of DNA-MAP. Analysing udder milk for the DNA-MAP presence may be used as complement of diagnostic tests.

Title Real-time PCR testing of pooled (1:5) fecal samples comparison to HEYM culture


Institution 1 University of Pennsylvania, Kennett Square, PA 19348; 2 Tetracore Inc, 9901 Belward Campus Drive, Rockville, MD 20850; 3 Cornell University, Ithaca, NY 14853; 4 University of Vermont, Burlington, VT 05405; 5 USDA, ARS, Beltsville, MD 20705; 6 Penn State University, University Park, PA 16802; 7 Veterinary Services, USDA, Burlington, VT 05405.
Introduction. Increased sample submission for laboratory testing for the detection of *Mycobacterium avium* subspp. *paratuberculosis* (MAP) in bovine fecal has necessitated utilization of techniques to enhance efficiency of MAP detection. Pooling of fecal samples offers a method to enhance diagnostic efficiency when coupled with real-time PCR (RT-PCR) with minimal loss of test sensitivity. Most importantly, the action cut-point for the identification of individually infected cattle can be adjusted to account for herd prevalence for owner management decision making. Materials and methods. Individual fecal samples from 736 cows in four dairy herds were processed by standard techniques for the detection of MAP and 1:5 pools were created concurrently for both culture and for RT-PCR with the Tetracore Vet AlertTM Johne's Real-Time PCR assay. For the purposes of this investigation all individual and pooled fecal samples were cultured using the standard three day culture protocol with four tubes of HEYM. The 1:5 pools were created by transfer of five ml of each standard fecal water tube-step to a 50 ml conical tube. From the 25 ml of pooled fecal water tube, 5 ml was transferred to 25 ml of BHI and incubated overnight; centrifuged at 900 X G for 30 minutes and the pellet was re-suspended in 1 ml of antibiotic brew on day 2 and incubated overnight. On the third day the sample was vortexed and approximately 200 ul inoculated on the surface of each of four tubes of HEYM with mycobactin J. The remaining 20 ml of the pooled fecal water tube was centrifuged at 900 X G for 30 minutes, decanted and the pellet re-suspended in water and processed according to manufacturer's recommendations for RT-PCR. Samples tested by RT-PCR were assayed in duplicate wells. Results: Of the 148 pooled fecal samples representing 736 individual cows, 34 pools were RT-PCR positive on both wells. Of the 34 RT-PCR positive pools, 19 had all individual samples within the pools tested where 18/19 (95%) contained at least one RT-PCR positive individual sample. Culture identified MAP in 14/34 (41%) of the RT-PCR positive pools and in 26/170 (15.2%) of the individual samples from those 34 pools. Only one pool was RT-PCR positive where all individual samples were both RT-PCR negative and culture negative. Of the 31 culture positive fecal samples among the 736 tested, only one individual fecal sample (1, 0, 0, 0) was not detected by either RT-PCR or by culture in the 1:5 pool. An additional 24 pools had one of two wells positive on RT-PCR. These represented lower concentrations of MAP with Ct values between 37 and 42, the cut-off value for a negative sample. Of the 9 positive pools with one positive well and all individual samples tested by RT-PCR, 8/9 (89%) had at least one positive individual sample. Only four (3.5%) individual fecal samples within the 115 fecal samples represented by the 24 pooled samples were culture positive, all were low shedders and ¾ in one pooled sample. Conclusion. The use of a commercially available RT-PCR with pooled fecal samples offers a very economical, flexible, rapid and exquisitely sensitive method to identify those MAP infected cattle at the greatest risk to spread MAP infection to herd-mates. Only individual samples in pools with the highest concentration of MAP (lowest Ct values) need to be tested for MAP, thus significantly reducing the testing cost for the herd. Acknowledgements: Financial support for this work was provided by the USDA Agricultural Research Service Cooperative Grant (58-1265-3-115) and by USDA-APHIS-VS field studies funding.

**Title**

Nested Polymerase Chain Reaction for the Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Bovine Allantoic Fluid and Fetuses.

**Author(s)**

Buergelt CD, Williams JE, Decker JH, Monif G.

**Institution**

Dept. of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida, USA.

**Presentation**

Oral

**Abstract**

*Mycobacterium avium* subspp. *paratuberculosis* (Map), the etiologic agent of Johne’s disease, mainly is transmitted to susceptible calves via the oral-fecal route. Map also can be transmitted transplacentally, thus detection of Map in such infected fetuses...
would allow for more efficient culling of all infected animals in a herd, the preferred method of disease control. In this study, a percutaneous technique for the sterile collection of allantoic fluid during late gestational pregnancy on the locally sedated standing animal was employed for the analysis of Map DNA via nested PCR (nPCR). A total of 12 infected pregnant Holstein cows with signs of clinical Johne’s disease were studied using IS900 and primers P90,P91 and J1,J2 for the nPCR. Antemortem samples studied were blood and allantoic fluid from the dam and after necropsy intestinal and mesenteric lymph node tissues from the dam, placental fluid, and fetal tissues such as liver, spleen, brain, and cotyledon. Nested PCR performed on the allantoic fluid collected multiple times antemortem from three cows greater than 7 months into pregnancy were negative each time. Allantoic fluid collected at necropsy was positive on nPCR for Map DNA in 2 additional cows at mid-gestation. The spleens and liver of 5 fetuses (including one set of twins) and the lung, liver and brain of another fetus amplified on nPCR. One of the 12 cows had microscopically demonstrable Map bacilli in the placenta were verified by nPCR to be Map DNA. A total of 6 fetuses (50%) were PCR positive at least on one tissue. These results demonstrate that Map can be transmitted in-utero, even though bacterial DNA may not appear in the allantoic fluid very often. This observation may have implications to consider infected dams and their offspring for the test and cull program in the effort to control Johne’ disease.

Title  'Single Colony PCR' using physical method of DNA recovery for the characterization of tiny colonies of *Mycobacterium avium* subspecies *paratuberculosis*

Author(s)  Singh SV¹, Singh PK¹, Singh AV¹, Sohal JS¹, Subodh S², Narayanasamy K².

Institution ¹ Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - Farah, District - Mathura (UP), India; ² R & D Facility, Institute of Molecular Medicine, 254, Okhla Industrial Estate-III, New Delhi, 110020.

Presentation  Poster

Abstract  Presently amplification of specific loci (IS900) by PCR is popular for confirming the *Mycobacterium avium* subspecies *paratuberculosis* cultures but it requires loopful of growth, which is rarely available for sheep and human isolates. In case of other animals also MAP colonies are minute due to use of old batches of certain critical chemicals, (personal observation). Two methods of DNA isolation; a non-chemical and non-enzyme physical method referred from Challans et al., (1994) (Method 1) has been compared with standard DNA isolation method described by van Soolingen et al., (1991) (Method 2). Both methods were used to recover DNA from 1 (‘Single Colony PCR’) to few (1-3) extremely minute and tiny MAP colonies. Using standard protocol DNA (method 2) was only recovered from 33.3% of cultures processed and only 14.6% samples gave positive amplification in PCR. Using method 1 (physical method), DNA was recovered from all the cultures processed and 90.0% of cultures processed yielded positive amplification in IS900 PCR. The new ‘physical method’ recovered DNA of PCR quality and helped to characterized the minute colonies cultured first time from Crohn’s disease patients, dairy cattle, raw milk and pasteurized commercial milk supplies, in India.

Title  Comparison of four methods of DNA isolation from intestinal tissues of goats infected with *Mycobacterium avium* subspecies *paratuberculosis* and evaluation of the sensitivity of PCR with respect to tissues culture

Author(s)  Singh PK, Singh AV, Sohal JS, Singh SV.

Institution  Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - Farah, District - Mathura (UP), India.
Low sensitivity of the PCR reaction for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in tissue, fecal, milk and blood samples is attributed to the false negative results primarily due to unsuccessful isolation of DNA and presence of PCR amplification inhibitors in the samples. Therefore, selection of a suitable protocol for the isolation of DNA is crucial for high sensitivity in PCR based detection tests. Study aimed to compare 4 methods of DNA isolation and determined most suitable procedure for isolation of good quality MAP DNA from intestinal tissue samples that would be suitable for IS900 based PCR detection of MAP infection. A total of 25 intestinal tissues (near ileocaecal junction) were collected from farm goatherds endemic for Johne's disease (multi-bacillary colonies on fecal culture and with typical intestinal corrugation). Tissues were homogenized, suspended in sterilized distilled water and supernatant was processed for culture on HEY medium as well as DNA isolation by four different methods. Method 1: DNA was isolated from tissues by method of van Soolingen *et al.* (1991). Method 2: Supernatant of grounded tissues was treated with 0.9% HPC (Hexadecylpyridium chloride) over night and then DNA was extracted using method of van Soolingen *et al.* (1991). Method 3: Supernatant of grounded tissues was treated with tissue lysis buffer (2% Triton-X, 1% SDS, 100mM NaCl, 10mM Tris HCl) [pH 8.0] and proteinase K (20 mg/ml), for proper digestion and afterwards DNA was extracted using phenol and chloroform-isomyl (24:1) process. Method 4: Samples were first treated with 0.9% HPC as in method 2 and then DNA was isolated as per the method described in Method 3. Isolated genomic DNA was further purified by commercial kit and subjected to IS 900 PCR. For the further confirmation of amplicons, these were subjected to sequencing and BLAST. Using the first method of DNA isolation, 15 (60%) samples were positive. Using method 2, 18 (72.0%) samples were positive, as compared to 13 (52%) samples positive in each of the method 3 and 4, respectively. Amplification of DNA extracted from 25 intestinal tissues by four different methods indicated that Method 2, (treatment of the samples first with 0.9% HPC overnight and subsequent DNA isolation by method of van Soolingen *et al.*, 1992), was more efficient in extracting good quality DNA as compared to other 3 methods. Therefore method 2, as standard method for isolation of DNA from MAP culture may also be adopted for necropsy tissues for high sensitive detection of MAP using PCR based tests. However, results of culture on Herrold's egg yolk medium using same fraction of tissues samples showed 100.0% sensitivity in detecting MAP bacilli.
bp sequence of Map insertion sequence IS900. A second set of primers, J1J2 which overlapped and spanned a 333 bp region within the insertion sequence was then used as nested pcr.. Sections were stained with hematoxylin-eosin stain. At least one representative section was stained with acid-fast stain to determine the density of bacilli present. The J1J2 primers tested positive in 11 instances in which P90P91 primers had tested negative, and P90P91 primers tested positive in one instance not identifiedby the J1J2 primers. The addition of the J1J2 set of primers appears to extend the sensitivity of PCR analysis for Map DNA in bovine tissue. Simple PCR using primers P90P91 identified Map in 64% of the ileocecal lymph nodes, 69% of the mesenteric lymph nodes and 57% of the ileal tissue samples from cows with documented Map enteritis. The addition of the J1J2 set of primers identified 100% of ileocecal lymph nodes, 100% of the mesenteric lymph nodes and 86% the ileal tissue. The blotted tissue impression method offers an additional means of rapid identification of Map within diseased tissue.

Title Real-time PCR for detection of Mycobacterium avium subsp. avium in milk and comparison to culture of environmental samples for herd testing

Author(s) Herthnek D¹, Nielsen SS², Bölske G¹.

Institution ¹ National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden; ² Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark.

Presentation Poster

Abstract A possible mode of transmission for the ruminant pathogen Mycobacterium avium subsp. paratuberculosis (MAP) from cattle to humans is via milk and dairy products. Although controversially, MAP has been suggested as the causative agent of Crohn's disease and its presence in consumers milk might be of concern. Isolation of MAP has been reported from milk of infected cows, bulk tank milk and from pasteurized milk. For screening of farm bulk tank milk, a method to detect MAP in milk with real-time PCR was developed. With this method, both pellet and cream fraction of the milk were harvested for analysis. The bacteria were lysed enzymatically and by mechanical disruption and the DNA was extracted by robotized magnetic bead separation. The analytical sensitivity was determined to 100 organisms per ml milk (corresponding to less than 10 CFU per ml, as CFU measurement usually underestimates the actual numbers) for samples of 10 ml, although as few as 10 organisms per ml milk was detected in three of four replicates in spiked milk samples. The method was applied in a study of 55 dairy herds to compare PCR of farm bulk tank milk to culture of environmental samples for detection of MAP in the herds. In this study, 17 herds (31%) were negative with both methods, 21 herds (38%) were positive with environmental culture but negative with milk PCR, one herd (2%) was positive with milk PCR but negative with environmental culture and 16 herds (29%) were positive with both methods. Hence the sensitivity for detection of MAP in a herd was considerably higher for the environmental culture method than PCR testing of farm bulk tank milk. From the 37 herds that were proven positive by culture of environmental samples, 89 tank milk samples were tested. Eighteen of these milk samples (20%) tested PCR positive and altogether 16 of these 37 herds (43%) had at least one positive tank milk sample. By comparison with spiked milk samples, it was concluded that the positive milk samples contained low numbers of MAP, usually less than 100 organisms per ml and never more than a few hundred organisms per ml. The results indicate that although MAP may be shed into milk or transferred to milk by faecal contamination, it will only occur in low numbers in the farm bulk tank milk due to the dilution and it can be assumed to often fall below the detection limit. Thus, PCR detection of MAP in milk would be less suitable for herd prevalence testing, but useful for control of MAP presence in milk, in order to avoid transfer to humans. The results also suggest that the level of MAP in the bulk tank milk of Danish dairy herds with paratuberculosis is low.
Detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* in ovine and bovine faeces by direct quantitative PCR

Author(s) Kawaji S¹, Taylor DL¹, Mori Y², Whittington RJ¹.

Institution ¹ Faculty of Veterinary Science, University of Sydney, Australia; ² Research Team for Paratuberculosis, National Institute of Animal Health, Japan.

Abstract In this study, a new test based on a novel faecal nucleic acid extraction method and an IS900-based real-time quantitative PCR (QPCR) method was developed and evaluated for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) DNA in ovine and bovine faecal samples. Both the Cattle (C) and Sheep (S) strains of MAP were detected by the QPCR assay, and no cross reactions were detected with 51 other species of mycobacteria including 10 which contained IS900-like sequences. One copy of IS900 fragment cloned into plasmid pCR2.1 and 1 fg of MAP genomic DNA were consistently detected, while in spiked faecal samples the detection limit was 10 viable MAP (S strain) per one gram of negative ovine faeces. A total of 506 individual ovine faecal samples with known culture (BACTEC) results and histological status were tested in Australia, and a total of 666 bovine faecal samples cultured by Herrold’s egg yolk medium (HEYM) were tested in Japan. In ovine samples, the QPCR assay detected 68 of 69 (98.6%) BACTEC culture positive faeces and there was a strong relation between time to detection in culture and DNA quantity measured by QPCR (r=−0.70). Furthermore, when DNA quantities detected by the QPCR were analysed on the basis of histological classification, faecal samples representing sheep with multibacillary lesions showed significantly higher levels of MAP DNA than samples from sheep with paucibacillary lesions or no lesions, suggesting that the QPCR test could be used for estimation of the risk of transmission. In bovine samples, 54 of 60 (90.0%) HEYM culture positive faeces were detected by the QPCR. MAP DNA was also detected from some culture negative faecal samples from sheep and cattle exposed to MAP, suggesting that the QPCR has very high analytical sensitivity for MAP in faecal samples and detects non-viable MAP in faeces. None of the faecal samples from 176 sheep and 508 cattle that were not exposed to MAP were positive in QPCR.

Evaluation of three methods of DNA extraction for the detection of *Mycobacterium paratuberculosis* by polymerase chain reaction in milk

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Institution ¹ Department of Primary Industries, Victoria, Australia

Abstract Objectives: To evaluate three methods of DNA extraction from milk for the IS900 polymerase chain reaction (PCR) and compare analytical sensitivities of the PCR and modified double incubation radiometric mycobacterial culture (RMC) method. Experimental design: The comparative evaluation of the three DNA extraction methods (Beadbeater, InstaGene and Qiagene) and determination of the detection limits of the RMC and PCR were carried out on triplicate samples of milk inoculated with serial ten-fold dilutions of *Mycobacterium paratuberculosis*. Results: Among the three protocols of DNA extraction from milk, the Beadbeater method was the most efficient procedure for the preparation of *M. paratuberculosis* DNA template for the IS900 PCR. The average detection limit of the Beadbeater PCR system was about 70 viable *M. paratuberculosis* cells/50 ml sample. The InstaGene and Qiagene (QIAamp DNA Stool Kit) methods produced average detection limits by PCR of 600 and 700 cells/50 ml sample, respectively. The analytical sensitivity of the RMC was about 700 viable cells/50 ml sample. Conclusions: The analytical sensitivity of the Beadbeater PCR system is sufficient for this test to be used for the detection of low levels of *M. paratuberculosis*.
paratuberculosis contamination in milk. Further evaluation of this test on diagnostic samples is warranted.

Title A simple internal PCR control for *Mycobacterium avium* subsp. *paratuberculosis* constructed by PCR techniques

Author(s) Marsh I, McLoon M, Austin S, Fell S, Saunders V, Reddacliff L.

Institution

Presentation Poster

Abstract 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. 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. 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. We present a new 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.

Title Results from interferon gamma testing, ELISA testing, bacteriological and pathological examination in a Norwegian goat herd with naturally acquired paratuberculosis

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Institution ¹ Department of Animal Health, National Veterinary Institute, Oslo, Norway; ² Norwegian Goat Health Service, Norway.

Presentation Oral

Abstract Paratuberculosis was diagnosed in a herd with 160 goats that had performed sanitation for paratuberculosis. Sanitation was carried out by establishing a new herd from goat kids snatched from their dams, culling of all the adult goats, exclusion of the goat kids from potentially contaminated pastures and cleaning and disinfection of pens and outdoor areas. The goats were tested when approximately 10 and 23 months old, using an interferon gamma (IFN-gamma) test from Biosource, but no IFN-gamma positive goats were identified. Three years following sanitation some two- and three-year-old goats began loosing weight and had a reduced milk production just after kidding. One goat died, and paratuberculosis was diagnosed based on histopathology, presence of large amounts of acid fast rods, detection of *Mycobacterium avium* subsp. *paratuberculosis* by culture and IS900 by PCR. The herd was then tested with two months intervals using the IFN-gamma test and the Pourquier ELISA. Post mortem examination of selected goats revealed significantly enlarged intestinal lymph nodes with necrotic cortical areas and severe intestinal lesions compatible with paratuberculosis, located particularly in the proximal jejunum. Culturing of feces from two- and three-year-old goats identified *M. a. paratuberculosis* in more than one-third
of the animals. The ELISA and the IFN-gamma test had the same ability to identify culture positive animals (about 80%), while more culture negative goats were positive on IFN-gamma testing than on ELISA. Furthermore, some culture positive animals were negative on both ELISA and IFN-gamma testing, indicating that shedding can occur without any detectable immune response. IFN-gamma and ELISA testing of goats less than 18 months old rarely gave positive results. Some goats that were negative on the IFN-gamma test at 23 months of age, tested positive on the ELISA one year later. As expected the IFN-gamma test was the most sensitive in detecting sub-clinically infected animals. However, the ELISA also performed quite well, and the time from positive IFN-gamma results are seen, until goats become ELISA positive, appears to be short. In addition some culture positive goats had only antibody responses and no detectable IFN-gamma response. Results from testing in this herd suggest that the Pourquier ELISA can be well suited for screening goat herds for paratuberculosis. However, the disease progress might have been unusually fast in this herd, compared to herds with a well-established infection, and this could explain why antibody responses were seen earlier than expected.

Title
Seeing spots, developing an IFN-gamma ELISPOT assay to detect ovine *M. avium* subspecies *paratuberculosis* infection

Author(s)
Begg D, de Silva K, Di Fiore L, Taylor D, Whittington R.

Institution
Faculty of Veterinary Science, The University of Sydney, Australia

Presentation
Poster

Abstract
While current diagnostic tests can accurately identify sheep with late subclinical to clinical Johne's disease, tests need to be aimed at earlier stages in the disease process to limit the spread of infection within the flock. Diagnosis of Johne's disease in ruminants with subclinical disease is difficult as the available assays generally have low sensitivities. Among the common immunological diagnostic assays used are antibody and interferon gamma (IFN-gamma) ELISAs. The IFN-gamma ELISA will detect approximately 45-65% of the subclinically infected animals in a flock. However, the ELISPOT assay has been shown to be 10-200 times more sensitive in the detection of cytokines than the conventional ELISA assays. The ELISPOT assay will detect a different subset of IFN-gamma reactive animals as it detects the number of IFN-gamma producing cells compared to conventional ELISAs which detect the total amount of IFN-gamma. For these reasons an ELISPOT assay to detect ovine IFN-gamma has been developed and is being assessed for use in sheep infected with *M. paratuberculosis*. Results show that the assay has the potential to detect both naturally and experimentally infected animals, although the background response of unexposed animals increases over time. While the assay may be more sensitive than conventional ELISAs for detecting IFN-gamma it is limited by the antigens used for blood cell stimulation.

Title
Novel diagnostic criteria of the Interferon-gamma test for bovine Paratuberculosis

Author(s)
Mori Y, Nagata R, Yoshihara K.

Institution
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Presentation
Poster

Abstract
Interferon-gamma (IFN-gamma) test based on cell-mediated immunity against *Mycobacterium avium* subspecies *paratuberculosis* (Map) infection has been reported to be one of the useful diagnostic methods for bovine paratuberculosis. Several criteria for interpretation of IFN-gamma test using purified protein derivative (PPD) antigens and mitogens have been applied and discussed, however, it seems that
the generally accepted interpretation criteria for the IFN-gamma test are not established yet. In this study, the IFN-gamma test of whole-blood stimulation with mycobacterial PPDs and Concanavalin A (Con A) was evaluated with samples from experimentally infected calves and different age groups of cattle in Japan. Johnin and tuberculin PPDs were prepared from Map strain Kag-1, which had been isolated in Japan and adapted to the protein free medium, and M. bovis BCG strain Tokyo respectively. After 24 hours incubation of heparinized blood with PPDs or ConA, the culture supernatant samples were tested for IFN-gamma concentration using ELISA with monoclonal and polyclonal antibodies against bovine IFN-gamma. The concentrations of IFN-gamma were calculated according to the formula of the dose-response curve obtained from the same ELISA using a recombinant bovine IFN-gamma. After 1 to 2 months from inoculation of Map, IFN-gamma was detected by stimulation with johnin PPD (jPPD) in experimentally infected calves, thereafter those production of IFN-gamma have lasted for more than two years. The blood samples from healthy cattle showed higher concentration of IFN-gamma against the stimulation of ConA than those of PPDs. On the contrary, the stimulation with jPPD induced the highest IFN-gamma production in experimentally and spontaneously infected cattle compared to those of M. bovis PPD (bPPD) or ConA. On the basis of the results obtained from experimentally infected calves and more than 1,000 cattle from herds infected with or without paratuberculosis in Japan, the following criteria of the IFN-gamma test seems to be useful for the early diagnosis of bovine paratuberculosis. 1) IFN-gamma is detected by ConA stimulation (positive control), 2) concentration of IFN-gamma induced by jPPD is higher than that of ConA, 3) jPPD/bPPD ratio of IFN-gamma concentration is higher than 1.5, and 4) no-antigen control/jPPD ratio is less than 0.1. The samples that satisfied these all conditions are interpreted as IFN-gamma test positive.

Title Detection of Mycobacterium avium subspecies paratuberculosis by modified FASTplaqueTB bacteriophage assay

Author(s) De Buck J¹, Griffiths T², Barkema H³, Rioux K³.

Institution ¹ Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary; ² Department of Medicine, Faculty of Medicine, University of Calgary.

Presentation Poster

Abstract BACKGROUND: Diagnosis of infection with Mycobacterium avium subsp. paratuberculosis(Map), the causative agent of Johne's disease in ruminants, is currently performed by culture or serology. These tests are only reliable in advanced stages of the disease and, moreover, detection of Map by culture requires long incubation times. A commercial kit using mycobacteriophage D29 to detect M. tuberculosis in human sputum samples(FASTplaqueTBTM) has recently been adapted for rapid identification of viable Map in pure culture as well as in spiked milk samples. AIM: To evaluate the usefulness of this kit as a simple, rapid, and quantitative means to detect viable Map bacteria in fecal samples from veterinary sources. METHODS: (1) We first compared the susceptibility of different Map isolates to the bacteriophage provided in the kit, using only the FASTplaqueTBTM assay reagents. In total, 8 Map isolates were tested. Ten-fold dilutions were studied in triplicate with the phage lysis kit and in parallel by conventional culture on 7H11 agar plates allowing quantification of both plaque forming units (pfu) and colony forming units (cfu), respectively. (2) Using various processing and decontamination procedures, we then applied the mycobacteriophage method to detect Map in bovine fecal samples that were spiked with a phage-susceptible Map strain as well as in culture-positive (paraJEM Map culturing system and PCR confirmation) samples from bovine Johne's disease. RESULTS: While some of the isolates (5/8) gave almost equal numbers of cfu/ml and pfu/ml, other isolates (3/8) were poorly quantified with the bacteriophage assay as demonstrated by low pfu/cfu ratios. In fecal samples from cattle infected with Map, the modified FASTplaqueTBTM system was not successful.
in detecting viable Map bacteria, even in non-decontaminated specimens. This was also the case for Map-spiked fecal samples. CONCLUSIONS: Inhibitory components in bovine fecal matter and/or unsuitable decontamination procedures interfere with the detection of Map in feces by mycobacteriophage D29. Moreover, some isolates of Map have variable susceptibility to infection and lysis, and thus detection, by the D29 mycobacteriophage. Overall, these studies suggest that the routine use of this kit to detect viable Map bacteria might not be suitable for all types of samples without modification, especially where low bacterial counts are expected.

Title: Evaluation of fluorescence labelling of *Mycobacterium avium* subsp. *paratuberculosis* by carboxyfluorescein diacetate succinimidyl ester and carboxyfluorescein diacetate

Author(s): Aodongeril, Yoshihara K, Wang X, Wang H, Momotani Y, Mori Y, Momotani E.

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Presentation: Poster

Abstract: Background *Mycobacterium avium* subspecies *paratuberculosis* (Map) is an important pathogen causing ruminant paratuberculosis and human infection. Paratuberculosis is characterized by chronic granulomatous enteritis, persistent diarrhea, progressive wasting, and finally death, and has resulted in significant economic losses to the dairy and cattle industries worldwide. Furthermore, the bacteria are speculated to be the cause of human Crohn's disease. One of the difficult issues in diagnosis and research of the infection is their significant slow growth. Even by using specially enriched Herrold's egg yolk medium (HEYM) with Mycobactin J, it takes 3 to 4 months to see minimal colonies. However, in a susceptibility test for anti-bacterial substance, evaluation of disinfectant or bactericidal activity of macrophages, for example, we need to know their viability for efficacy as soon as possible. Carboxyfluorescein diacetate succinimidyl ester (CFDA) and carboxyfluorescein diacetate succinimidyl ester (CFDA/SE) labelling has been used previously to study the adhesion of labelled bacteria to host cells and the uptake of labelled substrates by various cells using flow cytometry analysis and viability test of several bacteria, however no study was reported on Map. Therefore, we investigated the application of a viability test of Map by CFDA or CFDA/SE. Results The results revealed that CFDA is a useful reagent as a fluorescent probe to determine the quantitative viability of Map and as a tracer. CFDA/SE labelling heat-killed Map as well as live bacilli. Incubation of Map with CFDA at 100 microM for 30 min was practically the optimal condition for the viability test. Conclusions CFDA staining with fluorescent measurement is a useful tool in various required tests of viability, such as the evaluation of antibiotics, disinfectant, other sterilization conditions, and the bactericidal effect of activated phagocytes for Map.

Title: Proteomic Analysis for Biomarkers for Johne's Disease in Sheep Serum by SELDI-TOP Mass Spectrometry

Author(s): Zhong L, Taylor DL, Whittington RJ.

Institution: Faculty of Veterinary Science, The University of Sydney, Camden, NSW 2570, Australia

Presentation: Oral

Abstract: Surface enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry has facilitated the discovery of disease specific protein profiles from different biological samples, such as serum and tissues, in a variety of diseases of man. These results have raised the possibility that protein profiles may become a powerful diagnostic tool and may be applicable to Ovine Johne's Disease (OJD).
the first phase of this study, SELDI-TOF MS experiments have been rigorously optimized by using sheep serum applied on four different ProteinChip® Array surfaces, in combination with nine different binding/washing buffers and five different sample dilutions. The reproducibility study showed the range for mean within-chip coefficient of variation for peak intensity determined from up to 18 peaks using sera from 8 sheep was 13% - 18% and for mass accuracy was 0.01% - 0.02%. Corresponding values between-chip were 13% - 23% and 0.02% - 0.03% respectively. Based on the results of the optimization experiment, a large scale biomarker discovery experiment has been conducted. We used SELDI-TOF MS to identify potential proteomic biomarkers from sheep serum that can differentiate between sheep infected with *Mycobacterium avium* subsp. *paratuberculosis*, uninfected sheep and those previously vaccinated with Gudair™. Univariate and two independent multivariate data analysis procedures: Linear Discriminant Analysis (LDA) and Classification and Regression Decision Tree (CART), have been used to develop classification models between contrasting populations. A panel of key polypeptides has been selected using both models for identification and further analysis. To identify the serum proteins found using SELDI-TOF MS, a number of chromatographic procedures, include gel filtration, affinity and ion exchange chromatography are being used in a protein purification scheme. A 13.6 kDa protein, transthyretin, which was down-regulated in both infected and vaccinated serum samples, has been identified by MS/MS.

**Title**
Pathological and bacteriological diagnosis of paratuberculosis in farmed red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) in Chile.

**Author(s)**
Paredes EA¹, Pradenas MV², Kruze JD², Jara MC², Collins MT⁴.

**Institution**
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**Presentation**
Poster

**Abstract**
Deer farming in Chile began in the 1960s. Currently there are about 3,000 fallow deer and about 7,000 red deer distributed mainly in southern regions of Chile. Most animals were introduced from Germany mainly for hunting purposes and meat production. The aim of this study is to report the first isolation of *Mycobacterium avium* subsp *paratuberculosis* (*Map*) in a Chilean deer farm established in 1996. Most animals were imported from a German deer herd with breeding stock but some came from Argentinean herds. The herd comprised 180 red deer (*Cervus elaphus*) and 200 fallow deer (*Dama dama*) which lived commingled but were separated by gender into adjacent but different pens. Diagnosis of paratuberculosis was based on clinical signs, gross pathology, histopathology, and isolation of *Map* from feces and tissues. An adult hind red deer died suddenly in poor body condition without any other clinical signs. At necropsy, samples taken for parasite examination were negative, however, thickening of the small intestine and hyperactive mesenteric lymph nodes resembling paratuberculosis were observed. Fecal and tissue samples were collected for bacteriology and histopathology. Two grams of feces and tissue were cultured for *Map* using HEY medium with and without mycobactin J, and incubated at 37 °C for up to 5 months. Two months later, four fallow deer of the same farm were euthanized because poor body condition, macroscopically examined, and fecal and tissue samples collected and processed as before for *Map* isolation. All Mycobactin-dependant colonies resembling *Map* were tested for *IS9900* using PCR technology. One out of 5 fecal samples and all tissue samples examined were positive for *Map*, and confirmed by PCR. Tissue samples of liver, ileum and lymph nodes were fixed in formalin 10% and stained with hematoxylin and eosin and Ziehl-Neelsen stains for histopathological examination. Gross lesions and histopathology were characteristic of paratuberculosis.
Thickening of the small intestine and calcification of the mesenteric lymphatic vessels were evident. Intense macrophage infiltration with many acid-fast bacteria in the lamina propria of the small intestine and mesenteric lymph nodes were observed. Necrotic focuses with acid fast bacilli were also present in the liver. These cases of paratuberculosis are the first reported in farmed deer in Chile.

**Title**
Pathology of ovine paratuberculosis in the North East of Portugal: A comparison of histopathology, faeces and tissue culture, polymerase chain reaction in blood, faeces and tissues, and serology

**Author(s)**
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**Presentation**
Poster

**Abstract**
Thirty adult sheep suspected of having clinical paratuberculosis were examined. Histopathological examination, bacterial culture of faeces and tissues, polymerase chain reaction (PCR) in blood, faeces and tissue samples, and antibody responses were used to diagnose infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Twenty-six of the 30 (86.7%) showed gross findings of intestinal mucosa thickening, and 20 out of 30 (66.7%) animals exhibited enlarged mesenteric lymph nodes. Twenty-one out of 30 (70.0%) animals showed lesions suggestive of paratuberculosis. Lesions associated with infection were classified as proposed by Pérez et al. (1996). Of the 21 sheep with histopathological lesions, 2 (9.5%) had focal lesions characterized by small granulomas. Multifocal lesions appeared in 3 (9.5%) animals with granulomas in the lymphoid tissue and in the intestinal lp. Sixteen (76.1%) sheep showed diffuse lesions. Animals were divided into different subtypes according to the main cell type present in the infiltrate and the amount of acid-fast bacilli: 9 sheep (52.9%) presented diffuse non-lymphocytic granulomatous enteritis and 8 (47.1%) showed diffuse lymphocytic granulomatous enteritis. MAP colonies were recovered from faeces and tissue samples from only that 2 and 6 of the 30 sheep respectively. MAP was detected in 4 sheep by faeces PCR (13.3%) and in 19 (63.3%) sheep by tissue PCR. PCR in blood revealed 7 (23.3%) infected animals. Three (10.0%) animals showed antibodies against MAP. Histopathological results were compared with other techniques such as culture, PCR and serology. A comparison between the histopathological results and the microbiological techniques was performed. Histopathological examination and PCR in tissues allowed a greater number of positive animals to be detected when compared with the results obtained by culture or serology. The results of this study suggested that histopathology and PCR in tissues were more sensitive than PCR in faeces, bacterial culture, or ELISA in the clinical detection of MAP.

**Title**
Association of disseminated *Mycobacterium avium* subspecies *paratuberculosis* infection with severity of granulomatous enteritis and mesenteric lymphadenitis in cattle

**Author(s)**
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In a considerable proportion of cattle infected with Mycobacterium avium subspecies paratuberculosis (MAP), the bacterium can be isolated from tissues other than intestine and associated lymph nodes, including certain tissues with potential for human consumption. Should evidence legitimize MAP as a food safety risk, methods will be needed to differentiate cattle with MAP infection confined to intestinal tissues from those with disseminated infection (DI). The objective of the present study was to determine if the severity of histologic lesions of small intestine and mesenteric lymph nodes could be used to classify DI status of cattle. Forty culled dairy cows from four MAP-infected herds were enrolled in the study. Conventional bacteriologic culture was performed on samples of feces collected immediately prior to euthanasia, and 15 tissue specimens collected during postmortem examination. Ileum, jejunum, mesenteric lymph node, and ileocolic lymph node were collected postmortem for histological evaluation. A grading system was developed to categorize the level of severity of granulomatous inflammation in these tissues; grades of 0 to 3 were assigned based on the quantity and micro-anatomical distribution of leukocytes. Cows were classified as "infected" if MAP was only isolated from feces, intestine, or gut-associated lymph nodes. Cows were classified as "DI" if MAP was isolated from other tissues. Twenty-eight (70%) of enrolled cows were infected. Twenty-one cows (75% of infected, 52.5% of all enrolled cows) had DI. The proportion of cows with DI increased with inflammation grade of each tissue. The sensitivity/specificity of granulomatous inflammation lesions that exceeded grade 1 for classifying DI status were 76/95%, 71/100%, 67/94%, and 62/82% for ileum, jejunum, mesenteric lymph node, and ileocolic lymph node respectively. The presence of DI in cows with low-grade or nonexistent intestinal lesions suggests that DI may occur in an early stage of disease. Since most cows with intestinal lesions of moderate or greater severity have DI, histologic evaluation of intestinal tissues may be a useful means to selectively identify and exclude a large proportion of cows with DI from the human food chain.
of the PCR amplicons of the isolates did not show much significant variation between the isolates. The allotted accession numbers for sheep 1, sheep 2, cattle and goat strains were DQ366928, DQ366929, DQ986325 and DQ986326 respectively. In conclusion, single strand conformational polymorphism can be a simple and effective method in differentiating isolates and could be further exploited by subjecting more isolates for characterization.

Title  Strain typing based on sequence polymorphisms in a surface exposed PPE protein of *Mycobacterium avium* subspecies *paratuberculosis*

Author(s)  Griffiths T², Rioux K², De Buck J¹.

Institution ¹ Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary; ² Department of Medicine, Faculty of Medicine, University of Calgary.

Presentation  Poster

Abstract  BACKGROUND: In the last two decades a variety of different molecular typing methods have been developed to differentiate strains of the genomically highly conserved *Mycobacterium avium* subsp. *paratuberculosis* (Map). The most successful techniques are based on insertion sequences, repetitive loci, comparative genomics or single nucleotide polymorphisms. In the latter class, AFLP, RAPD and PFGE have been applied with variable success. In this study a single Map gene was targeted that is coding for a member of the polymorphic PPE protein family for which roles in immune evasion, antigenic variation and virulence have been suggested. AIM: To examine whether polymorphic PPE proteins can serve as a means of differentiation of Map isolates. METHODS: We have identified at least four Map PPE proteins to be surface exposed on the cell wall of Map by enzymatic trimming of viable Map bacteria in specific in vitro conditions. One of the corresponding genes, Map1506 was sequenced from a collection of 60 isolates from different sources, hosts and typing profiles, including 7 isolates of bovine (C) and 6 ovine (S and I) variants representing most predominant IS900-RFLP profiles. IS1311 PCR-REA was performed on all isolates to determine their bovine (C) or ovine (S) subtype. Sequence data for the entire Map1506 gene was collected and compared. These sequencing results were used to select polymorphic regions in the gene to amplify for analysis by denaturing gradient gel electrophoresis (DGGE). RESULTS: Grouping based on the sequencing data corresponded in general to the C versus S subtyping by IS1311 PCR-REA. One of the Map1506 gene sequences differed substantially from the sequenced K10 strain with only 79% identity. Polymorphic regions of Map1506 were selected for analysis by denaturing gradient gel electrophoresis allowing visual discrimination of bovine and ovine *M. avium* subsp. *paratuberculosis* isolates as well as separation of ovine isolates into two subgroups (S and I). CONCLUSION: The Map1506 gene encodes a surface exposed polymorphic PPE protein with putative roles that are relevant to Map pathogenicity. Sequence polymorphisms in this gene are readily detectable by DGGE, and allow distinction of isolates correlating with the known C, S and I variants.
A molecular biological approach to producing a live vaccine for paratuberculosis

Vaccination has for many years been identified as a potentially cost-effective method for the control of paratuberculosis. However, current vaccines based on whole *Mycobacterium paratuberculosis* bacilli mixed with an oil adjuvant have major side effects, including large lesions at the injection sites and interference with diagnostic tests for bovine tuberculosis. In this presentation we outline the steps being undertaken using molecular biological methods, to produce a new, live vaccine based on the deletion of specific genes of the paratuberculosis organism. This strategy has been successfully followed for producing new tuberculosis vaccines. Two different approaches have been used to produce avirulent mutants. The first, was to make a library of mutants in virulent *M. paratuberculosis* through the use of the conditionally replicating shuttle phasmid phAE94 which contains the Tn5367 transposon. The mutant library was screened using *in vitro* culture systems including inability to multiply in minimal media, increased temperature sensitivity, carbon source preference and altered colonial morphology, all phenotypes that have been associated with loss of virulence in other pathogenic species. The library was also screened by looking for mutants with reduced ability to survive in cultures of bovine peripheral-blood macrophages. The second approach was to inactivate specific genes through homologous recombination. The genes selected for inactivation were those which have homologues in *Mycobacterium bovis* and whose inactivation in *M. bovis* produced mutants with good vaccine efficacy against bovine tuberculosis. A crucial step in developing live vaccines is to determine the virulence of potential vaccine strains in an animal model. The loss of virulence of selected mutants has been determined by the intravenous inoculation of 10⁸ bacilli into recently weaned goats. Initial studies showed that a range of different gut tissues were colonized with moderate numbers of *M. paratuberculosis* one year after being infected with virulent strains. In contrast, no or very low numbers of bacilli were isolated with mutant strains. Subsequent trials have shown that virulence can be determined by examining goats after a six month infection. Immune responses were monitored by measuring gamma interferon release to Johnin PPD and will be used for selecting strains for determining their vaccine efficacy. Vaccine efficacy will be determined in a goat vaccination / challenge model. Recently weaned BALB/c mice are being investigated as a possible alternative animal for the initial screening of mutants for loss of virulence. The reduction in the number of *M. paratuberculosis* bacilli in spleen and liver is associated with reduced virulence.

Techniques for allelic exchange in *Mycobacterium paratuberculosis*

The ability to inactivate specific genes in pathogenic organisms is an important requirement for determining the role of those genes in pathogenic processes and for producing attenuated strains as live vaccine candidates. We have investigated the usefulness of two homologous recombination techniques, for producing allelic exchange mutants of *Mycobacterium paratuberculosis*. In both techniques, a suicide plasmid construct is made containing a DNA fragment encoding a selected gene that is interrupted by insertion of a hygromycin resistance gene. In one technique, the suicide plasmid is delivered into a virulent strain of *M. paratuberculosis* by high-temperature electroporation, while in the other technique it is delivered in a specific strain.
temperature-sensitive phage construct. The transformed strains are then cultured on solid hygromycin-containing medium. Mutants that are antibiotic resistant are sub-cultured and characterized by PCR and Southern blotting to determine if allelic exchange of the active gene for the inactive gene has occurred. Two virulent \textit{M. paratuberculosis} strains were used for these studies; strain 989, a New Zealand strain isolated from cattle, and strain k10, the strain used for genome sequencing. When a direct comparison of the electroporation technique to the phage technique was made between these two strains for allelic exchange of two unrelated genes, the phage technique was found to be much more efficient. Efficiency, defined as the percentage of allelic exchange mutants to the number of antibiotic resistant colonies, ranged from 25\% to 91\% for the phage technique, and the efficiencies were moderately higher in strain 989 than in strain k10. In comparison, on the one occasion that electroporation was successful, the efficiency was only 10\%. Clearly, the phage technique is the preferably approach for allelic exchange in \textit{M. paratuberculosis}.

**Title**
Large Sequence Polymorphisms in \textit{Mycobacterium avium} subspecies \textit{paratuberculosis} (MAP) vaccine strains revealed by MAPAC microarray analysis

**Author(s)**

**Institution**
St George's University of London, United Kingdom.

**Presentation**
Oral

**Abstract**
As part of the European Commission 6th Framework Programme of research into paratuberculosis (ParaTBTTools) we designed and validated a combined 60mer oligonucleotide microarray for both the MAP and \textit{Mycobacterium avium} subspecies \textit{avium} strain 104 (MAA) genomes. Using the Inkjet in situ synthesised (IJISS) array system (Oxford Gene Technologies) and ortholog matching bioinformatics software we designed 15,000 candidate 60mer reporter probes and 15,000 SNP-containing mismatched pairs spanning both genomes. In cycles of optimisation against reference genomic DNA, we used these to prepare a finalised set of oligos having greatest specificity for their corresponding genes and minimising differences in signal strength between genes. The resulting array designated MAPAC comprised 4132 oligos reporting genes shared between MAP and MAA, 218 MAP specific genes, 952 MAA specific genes, 18 genes found only in MAP sheep strains, 19 MAP MIRU sequence regions and 58 MAP intergenic regions, together with 7 genes carried on the MAA plasmid pVT2. Initially we have used the array to explore genomic differences between MAP vaccine strains of differing origins and pedigrees. Weybridge vaccine strains including 316F obtained directly from the Veterinary Laboratories Agency (VLA), UK and indirectly from other European centres revealed differences between strains. Vaccine strain II from VLA was found to have a previously unreported large contiguous sequence deletion of 32,826bp encoding 33 ORFs. A strain of 316F which had originated from VLA in the 1970's and had subsequently been passed for use in vaccination between European centres was found to have a contiguous deletion of 26,830bp encoding 22 ORFs. By contrast, a strain designated 316F recently obtained from VLA and a human Crohn’s Disease isolate of MAP, lacked this deletion and appeared to be identical with MAP K10 in agreement with a previous report (Semret et al. 2006. J Clin Microbiol 44; 881-887). The deletions found in both MAP strain II and 316F affected regions of the genome predicted to be involved in pathogenicity. These findings may have implications for the safety of vaccine strains and emphasise the importance of comparative genomics in the definition and monitoring of MAP vaccines. Other MAP vaccine strains are currently being investigated.

**Title**
Genetic characterization novel Indian \textit{Mycobacterium avium} subspecies \textit{paratuberculosis} ‘Bison type’ isolate (S5) used to prepare vaccine and diagnostic kits.

**Author(s)**
Sohal JS\(^1\), Narayanasamy K\(^2\), Singh SV\(^1\).
Mycobacterium avium subspecies paratuberculosis (MAP) the cause of Johne's disease in animals is distributed world over and has also been associated with inflammatory bowel disease (IBD or Crohn's disease) of human beings. Despite low productivity (1/6 in Asian countries), huge animal (>450 millions) and human population (>1.3 billion), has not been extensively screened for the presence of MAP. Limited studies have shown that MAP was endemic in animal herds and flocks in the country. Preliminary characterization of MAP showed that native strain of MAP prevalent in domestic ruminants was 'Bison type' (Sevilla et al., 2005). Presently India lacks a referral laboratory to look into different aspects of research on Mycobacterium avium subspecies paratuberculosis in the huge population of domestic ruminants and human beings. Johnin has been used for last 40-50 years and was the only diagnostic reagent available to screen animals against Johne's disease. Johnin production was based on a strain of MAP imported from UK. Johnin production was discontinued due loss of strain in serial passages and has been restarted by importing a new strain of MAP, as there is no standard Indian MAP strain. Limited studies on diagnostics and vaccines developed utilizing antigens from strains prevalent outside the country were not as efficacious as to that indigenously developed utilizing antigens from native MAP 'Bison type' strain of goat origin. Therefore genetic contents including different markers (IS elements, short sequence repeats and large sequence polymorphisms) of native MAP strains were analyzed and compared with MAP K10 genome to have inference of differences in native MAP 'Bison type' isolate and to study the phylogeny of native isolates. Results of present study confirmed that MAP 'Bison type' (S5) genome has genetic differences in terms of SNPs, locus polymorphisms and genomic duplications and there is need to extensively analyze Indian MAP isolates to have insights in to their evolution and pathogenicity. In the present study different methods of the strain typing were also optimized for the first time in the country to guide geno-typing and futuristic molecular epidemiological studies.
sequencer ABI prisms 3700 DNA analyzer. Quality of sequencing data was analyzed using ABI sequence scanner v1.0 software and number of repeats at each locus was determined. All MAP isolates yielded detectable PCR product for G and GGT repeat loci. Sequence analysis of these 2 loci revealed that all isolates recovered from different host species were having same profile of SSR repeats, 7 G and 4 GGT (7g4ggt). No allelic variation among the isolates may be an indicative of interspecies transmission of Indian MAP 'Bison type' genotype among domestic and wild ruminant species (cattle, goat, sheep, buffaloes and blue bulls), located at CIRG campus and in the Mathura region of North India. Clinical data from our study indicate that Indian MAP 'Bison type' genotype is highly pathogenic for both domestic and wild ruminants (Hajra et al., 2005, Yadav et al., 2007, Kumar et al., 2007, Singh et al., 2005, Singh, 1998).

Title Amplified Fragment Length Polymorphism to investigate MAP in Italy

Author(s) Gorni C¹,², Taddei R³, Arrigoni N¹, Williams JL².

Institution ¹ CERSA-PTP, Livestock Genomics, Polo Universitario, via Einstein, Lodi, Italy; ² IDRA Laboratory ISILS-PTP, Polo Universitario, via Einstein, Lodi, Italy; ³ Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Centro di Referenza Nazionale per la Paratubercolosi - Piacenza, Italy.

Presentation Poster

Abstract To investigate the molecular variability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Italy, 8 different strains, collected in different regions and provinces, were analysed with Amplified Fragment Length Polymorphism (AFLP) technique. ATCC strain 19698, isolated in USA, was added to the analysis as a reference and to act as an "outgroup" in the analysis. The AFLP technique produces a large number of molecular markers through selective PCR amplification, with no need of prior sequence information or probe isolation. The information obtained from this analysis will improve the knowledge of the molecular variability of MAP, for which information on genomic sequence polymorphism is poor, and as a tool for epidemiological studies. Eight different AFLP primer combinations produced a total of 193 polymorphic fragments (88%) with an average of 24.12 fragments per primer pair and a range between 1 and 85. The PstG-MseTT primer combination produced 86 fragments, 85 of which were polymorphic (99%), and was able to differentiate all 8 strains in a single experiment. These preliminary data indicated that the AFLP approach is able to produce a sufficient number of polymorphic markers to distinguish between different MAP strains. A genetic distance matrix between MAP strains was generated using PHYLIP software package and UPGMA cluster analysis was performed on Nei-Li (1979) model. To obtain a robust tree, bootstrap was set at 1000 and the CONSENSE option selected. The UPGMA tree showed three different clusters of strains: the major one included five strains, the second grouped two strains and the third comprised an Italian strain, isolated in Cuneo, and ATCC 19698. There was no relationship between clusters and geographic locations where isolates were obtained. Additional epidemiology information identifying contacts between farms may explain the UPGMA data. Ongoing work is examining the variability of MAP strains within a restricted geographical region.

Title Genetic Association Study between Bovine NRAMP1 and CARD15 Genes & Infection by *M. a. paratuberculosis*

Author(s) Ruiz O¹, Manzano C¹, Iriondo M¹, Garrido J¹, Juste RA², Estonba A¹.


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Our research group aims to find DNA variations influencing resistance/susceptibility to paratuberculosis infection or Johne's disease in Friesian cattle. The focus is on genes related to innate immune system. Moreover, two years ago, we reported a genetic association between a microsatellite marker in 3'UTR region of bovine Nramp1 gene and the resistance to Johne's disease (8th International Colloquium on Paratuberculosis, Copenhagen, 2005). In the present study the analysis is extended to a second gene, Card15, previously associated with Crohn disease, and new polymorphisms in Nramp1 gene are studied. A total of 69 SNPs distributed in those genes (17 from Card15 and 52 from Nramp1) are analysed: (1) 28 out of those 69 SNPs were newly discovered by comparative sequencing in 14 bovine breeds; (2) the distribution of 18 out of those 69 SNPs that showed variation are described in our Friesian cattle population. The MAF (minor allele frequency) was higher than 0.01 in all cases and the whole panel of SNPs was in Hardy-Weinberg equilibrium. To evaluate the potential role of both candidate genes in determining the outcome of MAP infection, a case-control association study was performed in MAP naturally infected herds. Comparisons between cases (137 ELISA positive cattle) and controls (136 ELISA negative cattle) were performed for the 18 SNPs mentioned above. Three SNPs showed significant difference between the two groups: two ncSNP in Card15 gene (C12 p<0.01 and C25 p<0.002) and an intronic SNP in Nramp1 gene (N23 p<0.05). These results suggest that bovine Card15 and Nramp1 genes might be associated with natural resistance to MAP infection in cattle, or at least, the results might reflect linkage disequilibrium with another resistance conferring polymorphism nearby in the chromosome. Further studies are needed to clarify this point.

**Title**

Use of Single Nucleotide Polymorphisms in inh-A gene to classify *Mycobacterium avium* subspecies *paratuberculosis* into Types I, II and III.

**Author(s)**


**Institution**

1 Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain; 2 International Research Centre, Pentlands Science Park, Moredun Research Institute, Penicuik EH26 0PZ, Scotland, United Kingdom.

**Abstract**

*Mycobacterium avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*) is the causative agent of paratuberculosis (Johne's disease), a chronic inflammatory disease of the gastrointestinal tract that affects mainly livestock and wild ruminants. *M. a. paratuberculosis* isolates have been classified into three major groups by using large restriction fragment analysis (RFLP, PFGE) and rapid molecular techniques based on Single Nucleotide Polymorphisms analysis (SNPs) or). In this study the *inh-A* gene, an important gene in fatty acid biosynthesis, and previously classified as polymorphic, was analyzed for the detection of SNPs among the different *M. a. paratuberculosis* types. A panel of *M. a. paratuberculosis* strains from different types (I, II and III), different hosts (cattle, sheep and goat) and geographical origin (Spain, Scotland and Denmark) was selected. The complete *inh-A* gene from all strains was amplified by PCR, sequenced and then compared to detect the presence of SNPs. The sequencing analysis of the *inh-A* gene was able to classify all *M. a. paratuberculosis* strains into Types I, II and III. These results are in agreement with previous studies in which these strains were classified as well by *gyrA* and *gyrB* genes by using sequencing analysis. The SNPs were also used to design a restriction endonuclease analysis that allowed the classification of all *M. a. paratuberculosis* isolates into the three types with a simple PCR-REA analysis, this resulting in a fast and useful molecular technique. Therefore, the *inh-A* gene has been shown to be a valuable target to differentiate *M. a. paratuberculosis* isolates for epidemiological purposes.
Title Characterization of *Mycobacterium avium* subsp. *paratuberculosis* isolates by Large Sequence Polymorphisms (LSP) detection and hsp65 sequencing


Institution Departamento de Sanidad Animal, Laboratorio VISAVET, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid (Spain).

Presentation Poster

Abstract The complete genomes of two members of the *Mycobacterium avium* complex (MAC), *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and *Mycobacterium avium* subsp. *hominissuis*, have been recently published. Comparison between these two genomes of closely related bacteria has revealed a number of polymorphic genomic sequences between them, called Large Sequence Polymorphisms (LSP). Some of those LSPs are characteristic of bacterial subspecies, and can be used as diagnostic targets to correctly identify an isolate. Besides, some other LSPs can be used to distinguish between different types within one particular subspecies. In particular, three of those LSPs have been shown to distinguish *Map* isolates belonging to Types I and II. However, the LSP-profile of the Type III isolates of *Map* has not been determined yet. In the present study a selection of Spanish *Map* isolates recovered from goats and cattle from different geographic areas were selected. These strains were characterized as Type III or I/III by Pulsed-Field Gel Electrophoresis and PCR analysis aimed to the IS1311 element. Strains previously characterized as Type II recovered from cattle and goats were also included in the panel as controls. All these strains were analyzed using three-primer PCRs in order to demonstrate the presence/absence of LSPa4-II, LSPa20 and LSPa18. All isolates were further characterized by sequencing of the 65-kDa heat-shock protein gene (*hsp65*). All Type III and I/III isolates showed the same LSP-profile and *hsp65* sequevar than those described for Type I strains, thus confirming the relatedness between these two types. On the other hand, the analyzed Type II *Map* strains yielded the expected results in all PCRs, what corroborates the homogeneity within this cluster.

Title Draft genome sequence of an ovine *Mycobacterium avium* sub-species *paratuberculosis* isolate

Author(s) Paustian ML¹, Kapur V², Sreevatsan S³, Bannantine JP⁴.

Institution ¹National Animal Disease Center, USDA-ARS; ²Pennsylvania State University; ³University of Minnesota.

Presentation Oral

Abstract An isolate of *Mycobacterium avium* sub-species *paratuberculosis* (*M. paratuberculosis*) was cultured from the distal ileum of a sheep that had been diagnosed with Johne's Disease. Comparative genomic hybridization and short sequence repeat typing were used to characterize this isolate as typical of North American Johne's Disease sheep isolates. Genomic DNA was isolated and used as a template for sequencing on the Roche Genome Sequencer 20 System. Pyrosequencing resulted in nearly 18-fold coverage of the genome and the resulting sequence fragments assembled into 550 contigs. Directed PCR followed by Sanger sequencing was subsequently used to close gaps in the assembly, resulting in fewer than 100 contigs. Analysis of the sequence identified several regions encoding genes that lack homology to other sequenced mycobacteria as well as regions with no homology to all publicly available sequences. Several regions contained homologues to sequences from *Nocardia*, *Burkholderia*, and *Frankia* isolates and encoded a variety of metabolic enzymes and transport proteins. Previously reported large sequence polymorphisms were identified in the genome sequence as well as a
glycopeptidolipid biosynthesis gene cluster. The draft and finished sheep isolate sequences will be made available to the research community. It is expected that the completed genome sequence will impart novel insights into the unique biology of *M. paratuberculosis* sheep isolates as well as provide a basis for further comparative genomic studies.

**Title**
Molecular Characterisation of Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis*

**Author(s)**
Tripathi BN¹, Stevenson K².

**Institution**
¹Division of Pathology, Indian Veterinary Research Institute, Izatnagar-243 122 (UP) India; ²Division of Bacteriology, Moredun Research Institute, International Research Centre, Pentlands Science Park, Penicuik, Midlothian, Scotland, UK.

**Presentation**
Poster

**Abstract**
The objective of the present study was to characterise Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by pulsed-field gel electrophoresis (PFGE) and IS900 PCR. This technique has proved to be effective in detecting more polymorphisms than IS900-RFLP. In the present study, strains of MAP isolated from naturally occurring paratuberculosis infection in cattle (5) and goat (4) were characterised by PFGE. Isolates were successfully grown in Middlebrook 7H9 broth with ADC supplement and mycobactin J. All strains were found to have IS900 and F57 genes by PCR. PFGE analysis with *Sna* B1 revealed that the profiles of the cattle and goat isolates were identical and had a unique profile when compared with profiles currently in the PFGE database. PFGE analysis with *Spe* I demonstrated that all of the isolates were identical to *Spe* I profile 1 in the database. Studies are continuing to type the isolates by restriction fragment length polymorphism analysis. It was concluded that Indian isolates from cattle and goats were genetically similar but different from European strains. This study provides useful data that ultimately will facilitate control measures for the disease in India.

**Title**
New characteristic 'Molecular Signatures' to distinguish Indian and non-Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis*

**Author(s)**
Sohal JS¹, Subodh Swati², Singh SV¹, Narayanasamy K², Singh PK¹, Singh AV¹, Sheoran N², Sandhu KS².

**Institution**
¹Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - Farah, District - Mathura (UP); ²R & D Facility, Institute of Molecular Medicine, 254, Okhla Industrial Estate-III, New Delhi, 110020, India.

**Presentation**
Poster

**Abstract**
Paratuberculosis (Johne's disease) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is the most costly production disease of animals, has zoonotic concerns and invites trade restrictions. Johne's disease is endemic in Indian domestic livestock population (cattle, buffalo, goat and sheep) and wild animals. MAP has been recovered from cases of Crohn's disease, normal and in-contact animal attendance. India possesses huge population of animals however, per animal productivity is very low as compared to other countries. Despite test and cull policy, incidence of disease continues to increase, resulting in heavy economic losses that have never been estimated or realized. Lack of effective vaccine has increased the demand of devising preventive measures based on epidemiological information. In India work on the characterization of strain was started from 1999 with the help of Australian and Spanish laboratories (Sevilla et al., 2005, Whittington, 2001, personal communication) giving first indication that Indian MAP strains may be different from other known strains of MAP in the world. In the present study, based on genome
sequence of International Reference isolate (MAP K10), assays were designed to have insights of various molecular markers including IS elements, LSPs and SSRs of MAP 'Bison type' S5 of goat origin. In some of IS1311 loci a novel polymorphism was observed in MAP 'Bison type' S5, not seen in MAP K10 genome and MAP isolates from outside India. This is the first report of this polymorphism and so far these polymorphisms have not been observed in Indian and non-India strains. In the next stage presence of this polymorphism was tested in a panel of Indian MAP isolates (from different host species and diverse geographical regions) and non-Indian MAP isolates. Novel polymorphism was consistently present in all Indian MAP isolates tested despite differences in host species and diverse geographical regions and this polymorphism was not seen for non-Indian MAP isolates. Non-Indian MAP isolates were identical to International Reference isolate (MAP K10). Study provided unique characteristic molecular signature that can distinguish Indian MAP from other isolates. Study indicated the need for analyzing large number of Indian MAP isolates in order to confirm this polymorphism as a characteristic of Indian MAP isolates. This effort will provide new dimensions to paratuberculosis research in the country.

Title
Comparative analysis of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle, sheep and goats by short sequence repeat and pulsed-field gel electrophoresis typing

Author(s)
Sevilla I, Li L, Amonsin A, Garrido JM, Geijo M, Molina E, Kapur V, Juste RA.

Institution

Presentation
Poster

Abstract
*Mycobacterium avium* subsp. *paratuberculosis* (Map) causes paratuberculosis in animals and is suspected of causing Crohn's Disease in humans. Previous investigations have revealed a relative lack of genetic diversity amongst isolates. Combined with the slow growth of the organism in pure culture, strain differentiation among Map isolates has proved to be difficult and has limited the study of the molecular epidemiology of the disease. We here compare a set of 268 isolates from different hosts (cattle, sheep, goats, bison, deer and wild boar) that have been previously characterized for IS*1311* PCR-restriction endonuclease analysis and SnaBI-SpeI pulsed-field gel electrophoresis patterns with the more recently described short sequence repeat (SSR) analysis of locus 1 (G residue) and locus 8 (GGT residue). The results show that a total of nineteen different multi-locus SSR (SSR1_SSR8) types were identified amongst the 268 isolates. In terms of host species distribution, there were 13 SSR types identified from cattle, 6 from sheep and 3 from goat isolates. Cluster analysis with both PFGE and SSR based typing methods confirmed that Map isolates are genetically divided into two main groups, the cattle type and sheep type groups. Amongst isolates recovered from Spain, SSR type 7_4 accounted for the 54% of cattle isolates, while types 7_3 and 14_3 together accounted for the 29% of sheep isolates. Interestingly, amongst isolates recovered from goats, approximately the same proportion (43%) of isolates were typed as either cattle type (7_4) or sheep type (14_3). While the overall discriminatory power of both methods as calculated by Simpson's index of diversity (D) was almost the same (0.693 for PFGE and 0.691 for SSR), for both methods, comparative analysis revealed that the most abundant PFGE 1-1, 2-1 and 23-16 profiles were subdivided into 11, 7 and 4 different types, respectively. Similarly, isolates representing the most abundant SSR type (7_4) could be subdivided into 19 different PFGE profiles. Amongst isolates
recovered from sheep, there was a slightly higher discrimination with PFGE (D = 0.865) than with SSR (D = 0.775). Taken together, the results of our studies confirm the utility of the SSR approach as an easy and rapid method based on PCR and sequence analysis that requires only small amounts of sample to perform. The results also suggest that the addition of a third locus to SSR typing may help in increasing the discriminatory power of this method. Overall, the results of our comparative analyses suggest that, based on current methodologies available, a combined approach that includes IS1311 PCR-REA, SSR and PFGE provide the highest level of discrimination for Map strain typing. *Both authors contributed equally

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<th>Title</th>
<th>Survival, dormancy and the proteome of <em>Mycobacterium avium</em> subsp. <em>paratuberculosis</em> during the stress response to hypoxia and nutrient starvation</th>
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<tr>
<td>Author(s)</td>
<td>Gumber S¹, Taylor DL¹, Marsh IB¹², Whittington RJ¹⁺</td>
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<tr>
<td>Institution</td>
<td>¹ Faculty of Veterinary Science, The University of Sydney, Australia; ² Elizabeth Macarthur Agricultural Institute NSW Department of Primary Industries PMB 8 Camden, NSW 2570, Australia.</td>
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<tr>
<td>Presentation</td>
<td>Oral, winner of JDIP Award</td>
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<tr>
<td>Abstract</td>
<td>Few data exist on physiological adaptation of <em>M. paratuberculosis</em> (<em>Mptb</em>) in either the host or the environment. The responses of the two distinct strains of <em>Mptb</em> (C and S) to hypoxia and starvation were studied <em>in vitro</em> in this study. The growth pattern of <em>Mptb</em> during stress appeared similar to the dormancy response of other mycobacteria. The C strain was more resistant to starvation stress than the S strain. A total of 66 protein spots differentially expressed in response to starvation and/or hypoxic stress were selected and identified, providing the first functional assessment of the genomic differences known to exist between these strains. Differentially expressed proteins were classified based on biological function and 13 categories were identified including antioxidant enzymes, amino acid metabolism, fatty acid metabolism, ATP and purine biosynthesis, proteolysis, cell wall synthesis, oxidoreductase enzymes, protein synthesis, signal recognition, hypothetical proteins with putative function, hypothetical proteins with unknown function, cyanate hydrolysis, phosphate metabolism and cell division. These differentially expressed proteins are potential screening targets for future diagnosis, prevention and control of <em>M. paratuberculosis</em> infection and their identification will assist understanding the pathogenesis of the diseases caused by this organism.</td>
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<th>Title</th>
<th>Targeting differential expression to identify subspecies specific proteins for the diagnosis of <em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em> infections</th>
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<td>Author(s)</td>
<td>Hughes V¹, Smith S¹, Garcia-Sanchez A¹, Kerr K¹, Denham S¹, Sales J², Watkins C¹, Paustian ML³, Bannantine JP¹, Stevenson K¹</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>Global screening of genomes and proteomes provides a powerful tool for identifying differences between two or more closely related organisms. In this study we have used comparative proteomics to identify proteins responsible for the phenotypic variations in the two genetically similar subspecies IS901+ <em>Mycobacterium avium</em> and <em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em>. The advantage of this approach was that comparison of the proteomes of the two organisms would identify subspecies-specific proteins including the products of differential gene regulation that</td>
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would not be detected by a comparative genomics approach. Comparisons were made between the proteomes of the two organisms grown in vitro at both log and stationary phases of growth. Differentially expressed proteins were identified in both organisms and those found to be upregulated in *M. paratuberculosis* were further investigated by mass spectroscopy and Mascot analyses. The proteomes were compared from different strains of the organisms to ensure that the proteins identified were representative of the subspecies. Comparison with the in vivo proteome of *M. paratuberculosis* confirmed that the proteins were expressed during natural infection of the target species. The genes encoding the proteins of interest were cloned and expressed in *Escherichia coli* and the immunogenicity of the recombinant proteins determined to assess their potential as specific immunological reagents for diagnosis and epidemiological studies.

**Title** Analysis of Toll-like receptor gene expression in *Mycobacterium paratuberculosis* infected sheep

**Author(s)** Taylor DL, Zhong ZL, Di Fiore L, Begg DJ, De Silva H, Whittington RJ.

**Institution** Faculty of Veterinary Sciences, University of Sydney, Australia.

**Presentation** Poster

**Abstract** Recognition of microbial pathogens by the innate immune system is essential to development of an adaptive immune response and is dependent on signaling by Toll-like receptors (TLR). TLR are Pattern Recognition receptors (PRR) that recognize and respond rapidly to invading bacteria leading to the production of pro-inflammatory cytokines, radical oxygen species, nitric oxide and the induction of adaptive immunity. In parallel to this microbes have co-evolved mechanisms to avoid host responses, and may even exploit TLR induced innate responses to their own advantage. Elucidation of the mechanisms of pathogen recognition and host immune evasion by the TLR pathways is central to our understanding of how these factors contribute to pathogenesis in the susceptible host and for developing improved approaches to control, treatment and immunoprophylaxis of this important disease. In this study we have analysed the expression of eight ovine TLR genes in peripheral blood and several intestinal sites by comparative qPCR to determine if the levels of TLR expression is modulated in these tissues by disease. We have found significant changes in the expression of several TLRs at various sites. Additionally there appears to be a substantial difference in gene expression between lesion grades for some genes. The implications for diagnosis and pathogenesis of disease will be discussed.

**Title** Detection of *Mycobacterium avium* subsp. *paratuberculosis* from cattle feces using Loop-mediated isothermal amplification (LAMP).

**Author(s)** Enosawa M¹, Suzuki W², Kageyama S³, Minekawa H⁴, Notomi T², Onoe S¹, Mori Y⁴.

**Institution** ¹ Hokkaido Animal Research Center, Shintoku; ² Eiken Chemical Co., Ltd., Ohtawara; ³ Hokkaido Central Agricultural Experiment Station, Naganuma; ⁴ National Institute of Animal Health, Tsukuba; Japan.

**Presentation** Poster

**Abstract** We have reported the simple detection system of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) based on LAMP method, which was sensitive and specifically identify MAP DNA by means of white turbidity (J. Clin. Microbiol. 41:4359-65. 2003). Here we present the improved LAMP system with modified primer sets and additional denaturing step of DNA, which allows for a rapid detection of MAP in cattle feces. The new primer sets were designed from first one third region of IS900 sequence. As the additional step, the extracted DNA was denatured by heat-treatment and added to a reaction mixture. Using the new system, 10 copies of
cloned IS900 DNA or 0.005pg of genomic DNA from cultured MAP were detected within 30 minutes. It specifically distinguished MAP from *M. avium* or *M. scrofaeum*. Non-specific amplification was not observed within 120 minutes in the LAMP assay with 10 fecal samples which were obtained from Johne's disease free herd. Thus, the improved LAMP system showed high sensitivity and specificity for detecting MAP in fecal samples. In order to compare with the culture method using Herrold's egg yolk medium with mycobactin, LAMP system was evaluated using 40 cattle fecal samples obtained from MAP infected cattle herds. The samples were collected when the cattle were slaughtered, and were stored at 4°C for less than 3 days before conducting the culture test. Nineteen samples, including 9 culture-positive samples, were positive with LAMP system. On the other hand, twenty one LAMP negative samples were all culture-negative. The LAMP's higher sensitivity would allow for the detection of tiny amount of DNA of dead or dormant MAP cells potentially present in the culture-negative but LAMP positive samples. Additionally, real-time monitoring data of LAMP indicates that there is some correlation between a number of colonies on culture media and amplification time to reach the threshold turbidity. These results suggest that LAMP system is robust and useful method for the detection of MAP DNA, and will contribute to rapid and reliable diagnosis of MAP infected cattle.

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**Title**
Population analysis of fecal microbiota from cows infected with *Mycobacterium avium* subspecies *paratuberculosis*

**Author(s)**
Paustian ML¹, Fyock TL², Scupham AJ¹, Whitlock RH², Stabel JR¹.

**Institution**
¹ National Animal Disease Center, USDA-ARS; ² University of Pennsylvania.

**Presentation**
Poster

**Abstract**
*Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) is a gram-positive, acid-fast bacillus that is the causative agent of Johne's disease, a chronic infection of ruminant animals characterized by inflammation of the digestive tract leading to nutrient malabsorption and eventually death. Dramatic changes in the intestinal environment due to clinical disease are expected to result in alterations to the normal flora, which in turn will significantly impact the health and metabolic potential of the host animal. A molecular phylogenetic approach was used to identify changes in the microbiota of cows infected with *M. paratuberculosis*. DNA was isolated from archived fecal samples taken from cows before and after the observed onset of clinical disease, or from recent fecal samples taken as part of a dairy herd survey. The infection status of animals was determined by fecal culture and PCR. Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to characterize the organisms present in the fecal DNA preparations. Clustering of the T-RFLP results indicated that regardless of the amount of fecal shedding or the source animal, infected animals grouped together and separately from uninfected animals. The T-RFLP fragment profiles were then used to identify the bacterial genera present in each fecal sample. Actinobacteria (which includes *M. paratuberculosis*) increased from an average bacterial population component of 6.1% in uninfected animals to 13.5% in infected animals, while Firmicutes decreased from 33.5% to 27.1%. Overall Proteobacteria levels remained relatively stable between infected and uninfected animals at 34.6% and 36.9%, respectively; however, Gammaproteobacteria comprised 12.0% of this group in uninfected animals and 17.4% in infected animals. The implications of these observed changes in the microbiota will be further studied with a sequencing-based approach to establish the functional changes associated with this population shift. Elucidating the impact of host-pathogen interactions on commensal microorganisms has the potential to enhance our understanding of the disease process as well as provide novel approaches for diagnosis and treatment.
Title  Education for veterinarians and producers as part of the national control program.

Author(s)  McDonald J¹, Horn EA¹, Patton E², Collins MT¹.

Institution  ¹ Technology for Learning Center, University of Wisconsin-Madison; ² Wisconsin Dept. of Agriculture, Trade, and Consumer Protection; ³ Dept. of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin.

Presentation  Keynote

Abstract  Successful control programs depend on knowledgeable veterinarians and producers. In the United States we developed a multi-pronged approach to Johne's disease education, targeting regulatory veterinarians, as well as veterinarians in private practice, and producers of multiple species. For veterinarians, we developed the Online Johne's Disease Veterinary Certificate Program, consisting of seven modules covering the basics of Johne’s disease pathobiology and epidemiology, diagnostics and test interpretations, risk assessment, and management and control in dairy and beef operations. For practical application we created four virtual farm visits (dairy and beef) so that veterinarians can practice assessing the risk of Johne's disease occurrence and developing management plans for different types and sizes of operations with varying levels of disease prevalence. We also have modules that address Johne's disease in other species: goats, sheep, cervidae, camelids, and bison. For producers, we have revised the modules for the certificate program to specifically provide relevant information. These modules are organized by type of operation (dairy or beef) and species (goats, sheep, cervidae, camelids, and bison.) In addition, we are developing a series of four modules where producers talk to producers about the economic impact of Johne's disease and control efforts on their businesses. We will also report on the evaluation studies underway of both the certificate program and the Dairy Producer module. The purpose of the studies is to gain further insights into the impact of the respective education programs on veterinarians' and producers' knowledge and practice. In addition, we are assessing veterinarians' and producers' individual learning preferences, strategies, and activities, during and after their participation in the online education programs.

Title  Control of paratuberculosis in Sweden

Author(s)  Lewerin SS¹, Ågren E¹, Frössling J¹, Bölske G¹, Holmström A², Lindberg A¹, Szanto E⁴, Viske D¹.

Institution  ¹ National Veterinary Institute, SE-751 89 Uppsala; ² Swedish Animal Health Services, SE-121 86 Johanneshov; ³ Swedish Dairy Association, Box 210, SE-101 24 Stockholm; ⁴ Swedish Board of Agriculture, SE-551 82 Jönköping.

Presentation  Poster

Abstract  Legal framework: Paratuberculosis has been included in the Swedish Epizootic Act (SFS 1999:657) since 1952. According to this legislation, any suspicion that an animal (regardless of species) is infected by *M. paratuberculosis* is notifiable for animal owners, veterinarians or other professionals with animal contact. Moreover, the Swedish Board of Agriculture must investigate all suspect cases and take all necessary means to eradicate and prevent the spread of the infection, if confirmed. Investigations: Since the detection of paratuberculosis in an imported beef cow in 1993, a number of surveys have been conducted, and shown a very low prevalence of the infection in Sweden. In the 1990s, directed surveys by faecal culture and serology on all imported cattle and all herds with cattle of the limousin breed were carried out, as well as a post-mortem culture investigation of a random sample from abattoirs. Since 2000, three faecal culture surveys on a stratified random sample of the dairy cattle population have been conducted, and since 2004, culture samples are taken of all adult cattle submitted for necropsy. Another directed survey conducted in 2006-2007 included cattle herds that had imported since 1995. In sheep, annual
surveys have been conducted since 1993, until 2003 based on serology and since 2004 based on faecal culture. Moreover, culture samples are always taken if paratuberculosis is suspected in any of the numerous post-mortem investigations conducted in wildlife. Results: A total of 53 infected herds have been identified since 1993, the two most recent in 1998 and 2005. All cases have been linked to imported animals and none have been in dairy herds. An effort to estimate the probability of disease freedom based on the major surveillance components is underway. Voluntary measures: A voluntary control programme including the majority of all pedigree beef herds has been in place since 1998. This programme is based on regular faecal sampling and trade is restricted to herds with the same status in the programme. Moreover, additional samples for paratuberculosis on imported animals and in the exporting herd are requested by Swedish Farmers’ Disease Control Program, a voluntary import control organisation. Research: Studies on diagnostic methods, as well as epidemiological studies help form the basis of the Swedish control policy. In addition to international research, aspects relating to the Swedish situation must be included. The aspect of test specificity is especially important, as a positive test always leads to severe consequences for the herd. Other aspects of disease epidemiology and test sensitivity are, however, important for tracing of the infection from positive herds and optimising eradication measures. Import control: Although mandatory sampling of imported animals has not prevented the introduction of paratuberculosis to Sweden in the past, it has undoubtedly helped keep the prevalence at such a low level. However, trade aspects may lead to the cessation of this import control. It is of vital importance that some kind of import control remains in place unless exporting herds can be certified as having the same status as regards paratuberculosis as Swedish herds. Otherwise, the money and efforts spent on eradication measures so far will soon be wasted.

Title
A new approach in the compulsory fight against paratuberculosis - eradication of clinical cases

Author(s) Khol JL, Dünser M, Damoser J, Baumgartner W.

Institution 1 Clinic for Ruminants, Department for Farm Animals and Herd Management, University of Veterinary Medicine Vienna, Austria; 2 Austrian Agency for Health and Food Safety, Institute for Veterinary Disease Control Linz; 3 Austrian Federal Ministry of Health, Family and Youth.

Presentation Poster

Abstract Two statistical balanced studies concerning the seroprevalence of specific antibodies against MAP (Mycobacterium avium subsp. paratuberculosis) were performed in the years 1995 to 1997 and 2002/2003 in Austria. These two studies revealed a highly significant increase in Austrian cattle and cattle farms showing specific antibodies against MAP (Baumgartner et al., 2005). In April 2006 the Regulation of the Austrian Federal Ministry of Health and Women on monitoring and abatement of clinical paratuberculosis in ruminants (Paratuberculosis-Regulation) came in action. The regulation affects cattle, sheep, goats and farmed deer. Animals showing clinical signs of paratuberculosis have to be notified and separated. Blood and faeces of suspicious animals are taken by the district veterinarian and sent to the national reference laboratory for paratuberculosis of the Austrian Agency for Health and Food Safety. If severe emaciation of an individual is noticed during slaughtering organ samples including liver - and intestinal lymph nodes as well as parts of the small intestine have to be sent to the reference laboratory for laboratory examinations. The same actions have to be taken if signs of clinical paratuberculosis occur in culled or perished animals. Clinically ill animals which are MAP positive have to be culled. Furthermore hygienic precautions listed in the Paratuberculosis-Regulation have to be performed. Compensation for culled animals is paid by the government. Whenever an animal is showing clinical signs or emaciation is diagnosed as MAP positive the farm the animal is originating from has to be controlled for further clinical cases. Although only ruminants showing clinical paratuberculosis are affected by the regulation, it can

<table>
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<th>Title</th>
<th>Results from a Paratuberculosis surveillance by government regulation in Austria</th>
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<tr>
<td>Author(s)</td>
<td>Dünser M¹, Khol JL², Damoser J³, Baumgartner W².</td>
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<tr>
<td>Institution</td>
<td>¹ Austrian Agency for Health and Food Safety, Institute for Veterinary Disease Control Linz, National Reference Laboratory for Paratuberculosis; ² Clinic for Ruminants, Department for Farm Animals and Herd Management, University of Veterinary Medicine Vienna, Austria; ³ Austrian Federal Ministry of Health, Family and Youth.</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>Austria is one of those European countries where Mycobacterium avium subspecies paratuberculosis (Map) infection in cattle was given the status of a notifiable disease. In April 2006 control of paratuberculosis was based on a new ordinance, focused on the eradication of clinically affected cattle, sheep, goats and farmed deer. The costs for laboratory diagnosis and indemnity for the destruction of paratuberculosis diseased animals are provided by the Austrian government. Objective of this regulation is the detection and eradication of clinically diseased animals, which are the main source for further infections with MAP. Clinically suspicious animals are tested by ELISA for MAP specific antibodies in blood samples and by Realtime PCR for MAP specific DNA from feces or tissue samples in cases of culled or perished animals. Samples from 103 farms were sent to the National Reference Laboratory for paratuberculosis within the first year since the implementation of the paratuberculosis regulation. In 36 farms the clinically suspicious cases were confirmed by laboratory examinations. The significantly most affected breed was Limousin. Further examinations for molecular typing of isolated MAP strains will be carried out for epidemiological investigations.</td>
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<th>Title</th>
<th>Experimental control program for paratuberculosis in dairy cattle in the Veneto region</th>
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<tr>
<td>Author(s)</td>
<td>Pozzato N, Stefani E, Bottazzari M, Benini N, Cestaro F, Passarini G, Vicenzoni G.</td>
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<td>Institution</td>
<td>Istituto Zooprofilattico, Sperimentale delle Venezie IZSVe-Verona, Servizi Veterinari AZ.ULSS 20-Verona</td>
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<td>Presentation</td>
<td>Oral</td>
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| Abstract | In 2001, a survey to estimate the seroprevalence of paratuberculosis in dairy herds was carried out in the Veneto region (North Eastern Italy). This region has about 250,000 dairy cattle and is the 3rd milk producing area in Italy. The results of the survey showed 27% of the herds to be positive, and in those with 100 heads or more, the prevalence of infected animals reached 50%. For this reason, a three-year experimental control program for paratuberculosis focused on two issues was implemented: 1. application of biocontainment and biosecurity measures in order to prevent the introduction and spread of the disease; 2. in-farm management of the animals tested positive on the basis of a semiquantitative risk assessment taking farm productivity into account as well. Five infected herds from 70 to 200 cows were selected on the basis of the farmers' willingness to participate. The farmers enrolled
were asked to avoid introducing new animals during the study period, apply the management measures agreed, and provide program evaluation information when requested. The management measures applied at farm level were: · separate calving areas for positive and negative cows based on serological results at drying; · early separation of young calves from dams at birth; · use of colostrum from negative cows in calves born from positive dams; · use of different grazing areas for young calves and cows. Serological prevalence in each selected herd was evaluated by commercial ELISA at the beginning of the program. Some production phases (calving, pre-weaning calves, post-weaning calves, young heifers, pregnant heifers and lactating-dry cows) were defined and scored accordingly to the different risk of disease spreading. The management measures were applied after testing. All the >24 month old cows were tested serologically every six months and at drying, and whenever positive or doubtful results were observed, fecal culture and PCR were performed. Although culling of culture or PCR-positive animals was advised, the time-lag between test results and culling varied considerably both among herds and within the same herd depending on farmer attitudes and cow values. Although not all the farmers strictly applied all the measures specified in the program, after 3 years no more clinical cases were observed and average herd seroprevalence fell from 15.0% (6.6-25.7) to 4.6% (1.0-8.9).
and biocontainment measures tailored to individual farms was agreed between farmers and veterinarians.

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<th>Title</th>
<th>Cattle movements in to and out of Johne's infected beef suckler herds in Ireland.</th>
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<tr>
<td>Author(s)</td>
<td>Mullowney PC, Barrett D, Egan J, Fallon R, Blake M, Good M.</td>
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<td>Institution</td>
<td>Department of Agriculture, Food and Fisheries, 6E, Agriculture House, Kildare Street, Dublin 2, Ireland.</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>In the period 2002 to 2006 a total of 94 beef suckler herds submitted faecal samples to the Central Veterinary Research Laboratory, which yielded a culture positive result for Mycobacterium avium subspecies paratuberculosis (MAP). The composition of these herds and movement in to and out of them during this five-year period is analysed. These findings are compared with those from a control group of 243 suckler herds which gave ELISA negative results as part of a survey which was conducted to estimate the prevalence of paratuberculosis (Johne's disease) in the Irish cattle population. Analysis of the data showed that infected herds were significantly larger than non-infected herds but that there was no significant difference in movements in or out of the herds in the two groups. In the infected herds a large percentage were sold on to other herds rather than going directly to slaughter. In the control herds there were very few closed herds, those that did not buy in any animals. These two points would need to be addressed in herds establishing a control programme for the disease.</td>
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<th>Title</th>
<th>A Risk Assessment Approach to the Control of Johne's Disease</th>
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<tr>
<td>Author(s)</td>
<td>Duthie S, Orpin P, Snodgrass S</td>
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<td>Institution</td>
<td>¹ Biobest Laboratories Ltd, 6 Charles Darwin House, The Edinburgh Technopole, Milton Bridge, Penicuik, EH56 0PY, UK; ² Park Veterinary Group, 82-84 High Street, Whetstone, Leicester, LE8 6LQ, UK.</td>
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<td>Presentation</td>
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<tr>
<td>Abstract</td>
<td>In the UK, eradication and control of Johne's disease in cattle has been at the individual herd level, and a number of herds are accredited free of the disease as defined by Cattle Health Certification Standards (CHeCS). By necessity, the requirements to reach and maintain accreditation are onerous and involve the testing of all cattle in the herd over 2 years of age for antibodies to Map annually. Consequently, the majority of members of CHeCS programmes are pedigree herds selling breeding stock where a premium is commanded by high health status. We have developed a Johne's risk assessment (JRA) scheme, particularly directed to commercial herds where the burden of whole herd testing is not financially viable, as a rational approach towards investigating a herd's Johne's status. The JRA scheme is consistent with advice from the UK government and offers a logical classification of herd status according to the risk of presence of Map based on the purchase and clinical history over the preceding 10 years and the results of annual targeted sampling. Green, amber and red colour coding is used respectively to indicate herds with a low, medium or high risk of having cattle infected with Map and over time; herds have the opportunity to progress to a lower risk category if they purchase replacement stock solely from sources with a higher health status and have consistently negative targeted sampling results. The JRA scheme provides a framework for vets and farmers to investigate the Johne's status of a farm, and emphasises the impact of buying in replacement stock from herds with an unknown health status. If all laboratory results are clear, the farmer can work towards improving the herd's risk status in future years, however if targeted testing reveals evidence of Map infection, there is an impetus for vet and farmer to develop an</td>
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### Abstract

Faecal culture examination using the 0.75% HPC method was used for the control of paratuberculosis in the Czech Republic in cattle, sheep, goats, wild capricorn, antelope, moufflon, fallow and red deer. A total of 117,210 cultures from cattle and 12,968 cultures from wild ruminants were examined. The introduction of infected animals from other farms (in the main cases, the importation of highly pregnant heifers and the purchase of their progenies) was the main reason for the spread of paratuberculosis in the Czech Republic with 54 officially registered cattle outbreaks in 2006. Successful control was adopted in 19.3% of cattle herds, in one herd of Capricorn and one herd of moufflon. The main reason for the achievement of the successful outcome was the removal of all faecal culture positive animals from the herd, including their progenies, separate rearing of calves from old animals and stringent hygienic management. However, the control programme is not yet completed in 45.0% of herds. The cause of the prolonged and unsuccessful control programme was failure to remove all progenies of infected animals and feeding calves with mixed colostrum and/or unpasteurised milk. A radical control programme was applied in 20% of cattle herds and a few herds of wild ruminants (three farmed red deer and fallow deer herds, one antelope herd and three herds of moufflon). In the rest of the outbreaks the control programme was suspended for financial reasons and due to low motivation of farmers. Certification program based on milk examination by PCR is now under discussion and preparation. Supported by the grant No. MZE 0002716201 (MAg., Czech Republic).

### Abstract

Paratuberculosis is widespread in Denmark and a voluntary control programme was established in 2006, aiming at providing tools for farmers to control infections, and to ultimately reduce the prevalence in the country. Approximately 1150 (24%) of dairy farmers were enrolled in the programme by September 2007. Participating herds test all lactating cows four times/year by use of a milk antibody ELISA. The test results are recommended to be used for risk-based management of infectious animals. This risk-based approach is aimed to reduce the workload of herd managers, thereby making implementation of changes more feasible than if all cows had to be managed with increased awareness. The test results are also used for communication to farmers, which is a central part of the programme. Communication between farmers and advisors also takes place via risk assessments, which helps the farmers identify risk areas of transmission. The end result is that farmers are well informed and play a role in management of their herd health. Farmers are informed that the control programme in their herd is expected to last 6 to 8 years. Therefore, there is a continued need from farmers, their advisors and the central administration to identify tools and methods to
ensure ongoing enthusiasm. A surveillance component may be added to the programme at a later stage, but currently no officially recognised recommendations are available related to trade of live animals. The surveillance component may be a next step to maintain farmers in the programme and encourage more farmers to join.

**Title**
Results of the Dutch bulk milk quality assurance programme for paratuberculosis

**Author(s)**
Weber MF, van Schaik G, van Weering HJ.

**Institution**
GD Animal Health Service, PO Box 9, 7400 AA Deventer, The Netherlands.

**Presentation**
Oral

**Abstract**
In January 2006, a bulk milk quality assurance programme (BMQAP) for paratuberculosis in Dutch dairy herds was initiated. 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 was based on modelling studies presented at 8ICP and is run alongside the pre-existing 'Intensive Paratuberculosis Programme' (IPP), which aims at low-risk trade of cattle. The BMQAP starts with an initial assessment including a single herd examination. Test-negative herds enter a surveillance procedure consisting of biennial herd examinations ("green herds"). Test-positive herds enter a control procedure consisting of annual herd examinations and culling of test-positives ("red herds"). All herd examinations are done by ELISA. In the initial assessment and surveillance procedures, ELISA results may be confirmed by faecal culture (FC). The aim of this paper is to summarise progress within the BMQAP during its first 15 months. Initiation of the BMQAP increased the total number of participating herds from 1071 in December 2006 (IPP only), to 1711 herds in March 2007 (including 495 herds in the IPP and 1216 in the BMQAP). Results of the initial assessment of all 670 dairy herds that newly joined the BMQAP (i.e. did not shift from IPP to BMQAP) were analysed in detail. In 363 (54%) of these herds, >=1 cattle were ELISA-positive. In 202 of these 363 herds, confirmatory FC of ELISA-positives was performed. In 107 (53%) of these 202 herds, >=1 cattle were FC-positive. The observed proportion of test-positive herds was larger than estimated in our previous modelling studies, possibly related to a selection bias of participating herds. In conclusion, tailoring the Dutch paratuberculosis programmes towards the needs of various groups of farmers, by initiating the BMQAP alongside the IPP, largely increased the total number of participating herds.

**Title**
Johne's disease control program in Israeli dairy farms - prevalence in national level

**Author(s)**
Koren O.

**Institution**
Israel Dairy Board

**Presentation**
Poster

**Abstract**
The Israel Johne's disease control program (IJDCP) is operating since 2003. The program is voluntary and consist a herd management program and a whole herd testing by milk and/or serum Elisa and faecal culture of the seropositive or doubtful cows. In the last 4 years 224 dairy herds were tested (42000 cows) for at least once. The results of first testing indicate that half of the herds are lightly infected or not at all (0 to 2% seroprevalence). Some of these herds suffer sporadic clinical cases and had some positive cultures. 18% of the herds from the other half are considered heavily infected with 5% or higher seropositive cows, higher incidence of clinical cases and faecal culture positives. Of the above mentioned herds 121 were tested twice or more in one year intervals. Most Herds with low (0-2%) prevalence in the first test maintained their status on the second test. Herds with medium (2-5%) prevalence didn't show marked improvement in the 2nd test. A few High (5%<) prevalent Herds showed some improvement which thought to be technical due to
selection of positive cows, but the true picture was seen in the third (or higher) testing. Herds, which used a proper maternity pen, showed reduction from average of 4.6% in the first two tests to 3.4% positive seroprevalence in the 3rd test while herds, which didn’t do so increased their prevalence to 6.3%. On national level there is some improvement concerning herd seroprevalence. There are 13% heavily (5%<) infected herds in comparison to 19% at start point. These Heavily infected herds produced an average of 11423 L of milk in 2005 with 3.57% fat and 3.12% protein which is not different from the country average on the same year.

Title Prevalence of Mycobacterium avium subspecies paratuberculosis infection over the first three years of the U.S. National Johne's Disease Demonstration Herd Project
Author(s) Fossler CP, Lombard JE, Carter MA.
Institution United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services
Presentation Poster
Abstract The National Johne’s 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 67 dairy herds and 21 beef herds in 17 states. Adult populations in these herds range from 35 to more than 4,000 animals. All enrolled herds began with culture-confirmed Mycobacterium avium subspecies paratuberculosis (MAP) on the operation. Sufficient time since the start of the NJDDHP has now elapsed such that Johne's testing information on cattle born since the beginning of the project are becoming available. Using a generalized estimating equations approach to adjust for effects of herd and after adjustment for cow age, odds for fecal shedding at moderate to high levels were significantly less in the third year of the project compared to the first year (3rd Year OR=0.73 95% CI 0.56,0.96). Compared with the first year, significant differences were also noted for both dairy and beef herds by ELISA test results (Dairy: 2nd Year OR=0.80, 95% CI 0.65,0.97; 3rd Year OR=0.75 95% CI 0.62,0.91 Beef: 2nd Year OR=0.85, 95% CI 0.59,1.25; 3rd Year OR=0.36, 95% CI 0.24,0.55). These results to date suggest that herd prevalence has decreased since the beginning of the project.

Title Incidence of Mycobacterium avium subspecies paratuberculosis infection over four years of the U.S. National Johne's Disease Demonstration Herd Project
Author(s) Fossler CP, Lombard JE, Carter MA.
Institution United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services.
Presentation Poster
Abstract The National Johne’s Demonstration Herd Project (NJDDHP) in the United States was initiated to evaluate the long-term feasibility and effectiveness of management practices designed to control Johne's disease on dairy and beef cattle operations. The NJDDHP started in 2003 and includes approximately 90 beef and dairy operations in 17 states. All herds began with culture-confirmed Mycobacterium avium subspecies paratuberculosis (MAP) on the operation, and all herd owners agreed to make efforts to control exposure of young cattle to adult cow fecal contamination. A Cox Proportional Hazards model was used to evaluate incidence of fecal shedding while adjusting for effects of herd. Cows were divided into 3 cohorts: -2 = cows born 13-24 months prior to program participation, -1 = cows born 1-12 months prior to program participation, and 0 = cows born 0-11 months after beginning the program. Results to
Date indicate that after three years of follow-up, dairy cattle born since the beginning of the project had a significantly decreased risk of being fecal culture positive and of fecal shedding at moderate to high levels compared to cattle born 2 years prior to the start of the project (Fecal-culture positive: Cohort -1: HR 0.61, p=0.08; Cohort 0: HR 0.49, p<0.02; Fecal shedding at moderate-to-high levels: Cohort -1: HR 0.77, p=0.36; Cohort 0: HR 0.49, p=0.02). In beef herds, there was not a significant decrease in risk, but there are fewer enrolled beef herds. These results suggest that management efforts initiated since the beginning of the project were effective in reducing incidence MAP. However, further analysis is needed to identify those efforts that have the greatest effect on incidence.

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<td>Author(s)</td>
<td>Links IJ.</td>
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<tr>
<td>Institution</td>
<td>EH Graham Centre (NSW DPI &amp; Charles Sturt University), Wagga Wagga, NSW 2650 Australia.</td>
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<td>Presentation</td>
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<tr>
<td>Abstract</td>
<td>Ovine Johne's Disease (OJD) was first detected in sheep in Australia in 1980. Monitoring of sheep for OJD commenced in New South Wales (NSW) abattoirs in late 1999 as part of a national program, with approximately 70% of adult sheep slaughtered monitored annually. Since 1999, OJD has spread to infect more flocks in the High (48% of direct consignments positive in 2006) and Medium Prevalence Areas (30% positive) of NSW. However around half of the sheep flocks in NSW remain in the Very Low Prevalence area (0.5% positive in 2006). Inspection details, laboratory results and the origin of the sheep slaughtered were recorded in a Microsoft Access database. From 1999, OJD positive consignments from a single vendor (direct lines) were traced to their property of origin. In contrast, OJD negative consignments were initially only identifiable to locality and local government area. Tracing was streamlined by the introduction of the National Livestock Identification System (NLIS) in 2002 which required sheep consignments to be accompanied by a National Vendor Declaration (NVD). Recording of the Property Identification Code (PIC) on the NVD became compulsory from January 2006. This enabled direct lines to be precisely allocated to a property, locality, local government area or OJD prevalence area. Linking inspection details to the PIC database led to reporting of negative inspection results to producers from 2004. A list of GPS coordinates for all localities was added in 2006 which enabled accurate mapping for the first time. Mapping provides an excellent visual communication tool that has markedly improved the capacity to review progress with the national OJD program. This has been particularly important in consultation with sheep industry representatives regarding the location of prevalence area boundaries at the regional, state and national level. The results of monitoring from 1999 to 2006 will be presented including maps showing the changes in distribution over time.</td>
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<th>Title</th>
<th>A Comparison of Vaccination for Ovine Johne's Disease and Prevalence of Lesions Detected by Abattoir Monitoring in New South Wales</th>
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<td>Author(s)</td>
<td>Links IJ.</td>
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<tr>
<td>Institution</td>
<td>EH Graham Centre (NSW DPI &amp; Charles Sturt University), Wagga Wagga, NSW 2650, Australia.</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>Ovine Johne's Disease (OJD) was first detected in sheep in Australia in 1980. Monitoring of sheep for OJD commenced in New South Wales (NSW) abattoirs in late 1999 as part of a national program. Following the results of research trials in</td>
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NSW, Gudair® vaccine was approved by the National Registration Authority in April 2002 as an aid to the control of OJD. Previously the vaccine had been limited to use under a special permit restricted to heavily infected flocks (>5% deaths annually). The wider availability of OJD vaccine changed the emphasis of Property Disease Management Programs from reliance solely on management strategies. Research demonstrating no untoward outcomes following vaccination of adult sheep in a heavily infected flock was pivotal in encouraging many producers to undertake whole flock vaccination. The major emphasis was, however, on vaccination of lambs at less than 16 weeks of age in infected and at-risk flocks. From January 2001 to December 2006 6.86 million doses of vaccine were sold in NSW. It is estimated around 70% of replacement sheep were being vaccinated annually in the High Prevalence area and 30% in the Medium Prevalence area. Field officers report that clinical disease is now rare in vaccinated flocks in the High Prevalence Area previously suffering high death rates. While this is generally attributed to vaccination, the role of better management strategies, including strategic culling of heavily infected mobs or age groups, and environmental factors (including drought) remains unclear. Abattoir monitoring provides an independent measure of the level of OJD infection present in the sheep population of NSW. This presentation will compare the uptake of vaccine with the prevalence of lesions detected in monitoring of adult sheep for OJD from November 1999 to December 2006, based on the OJD prevalence area and local government area from which the sheep were derived.
eliminate Johne's disease from their herds, are the first point of contact for producers who wish to access the benefits of the Package. Since its inception in July 2004, 105 producers have accessed this service and in excess of A$2 million has been allocated to eligible producers. Government veterinarians work in partnership with the counsellors by providing sound technical advice upon which to base the disease eradication program in each herd. Outcomes The Beef Only status has improved risk awareness of Johne's disease resulting in increasing numbers of beef producers sourcing replacement breeding cattle from these herds. A recent external review of the Package has confirmed the program is addressing most of the earlier negative impacts on owners of infected and suspect herds, by reducing the social impact of BJD on herd owners, the economic impact, and following eradication reduces the trade impacts associated with regulation. Participation in the Package is also improving producer understanding about animal health risks.

Title Paratuberculosis diagnosis and control in Thailand


Presentation Poster

Abstract Paratuberculosis in Thailand was first observed in an imported bull in 1981 and the criteria of diagnosis were based on chronic diarrhea and pathological examination. Since 1987, the serological testing has been carried out using complement fixation test (CFT) for detecting an antibody against Mycobacterium avium subsp. paratuberculosis (MAP). From 1987 to 1996, the testing was focused on dairy infected herds at which the percentage of sero-positive results was ranged from 0.58% - 13.17%. During 1997-2006, serum samples from both of dairy and beef cattle throughout the country were conducted for serological surveys. The result of sero-positive animals was 0.37%-1.53%. Thirty heads of sero-suspected cattle tested during 1991-2005 were examined. Fourteen of 30 cattle were diagnosed as paratuberculosis based on serological tests, bacterial culture and/or pathological examination. Among those confirmed infected animals, 6 out of 14 (42.86%) cattle were imported from foreign countries. In addition to the serological routine diagnosis, the fecal samples were also collected for bacterial detection by acid-fast staining. The result showed 24 positive samples out of 3,970 fecal samples, including 12 fecal samples from the infected cattle. For a successful control of paratuberculosis, the field veterinary official were trained, and they were expected to play an important role in giving advices to farmers who will eventually succeed the program. At present, control of paratuberculosis has been accomplished by performing bovine serological survey throughout the country in order to evaluate the disease status. For those sero-positive cattle, fecal samples have to be collected for acid-fast staining and/or cultivation. Therefore, paratuberculosis is included, together with brucellosis and tuberculosis, to the National Control Program.

Title Present approach to Johne's disease control in Japan and the feature of its incidence

Author(s) Kobayashi S, Tsutsui T, Yamamoto T, Nishiguchi A.

Institution Epidemiological Research Team, National Institute of Animal Health, Japan

Presentation Oral

Abstract Johne's disease has been one of the notifiable diseases since 1971 in Japan and all the detected animals are culled compulsorily with compensation by the government. The annual reported cases started to increase in 1980s and reached more than 200 by 1996.
Therefore, in addition to this passive surveillance, active surveillance has been in action following the amendment of the Law in 1997. In the active surveillance, all the targeted cattle had to be tested at least once in every five years. Infected cattle are practically detected the ELISA of two sequential positive results (56%), the agent isolation by fecal culture (41%) or the microscopic confirmation of the agent in fecal samples from animals with clinical signs (3%). Affected farms are subject to the monitoring for a certain period to assure the free status of the disease by periodic testings. Since the active surveillance started, the number of annual cases reached more than 1,000 in 2004, and then it shifted to about 800 and 1,200 in 2005 and 2006, respectively. However, this trend was influenced by the difference in intensiveness of the surveillance each year in each prefecture and nationwide annual detection rate (number of detected cattle / number of tested cattle) has been stable as 0.1-0.2%. The government has organized the Johne's Disease Advisory Committee since 2005 for evaluating epidemiological situation of the disease and advising on its control measures.
<table>
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<th>Title</th>
<th>Review of prevalences of paratuberculosis in farmed animals in Europe</th>
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<tr>
<td>Author(s)</td>
<td>Nielsen SS¹, Toft N².</td>
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<tr>
<td>Institution</td>
<td>¹ Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark; ² Danish Meat Association, Kjellerup, Denmark.</td>
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<tr>
<td>Presentation</td>
<td>Oral</td>
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<tr>
<td>Abstract</td>
<td>Multiple studies have been carried out to assess the prevalence of infection with <em>Mycobacterium avium</em> subsp. <em>paratuberculosis</em> (MAP) in farmed animals. However, most studies are not directly comparable because different diagnostic tests were used. True prevalences can be calculated from apparent prevalences if test-accuracy estimates for the diagnostic test used are available. The objective of the present study was to conduct a review of MAP prevalences among farmed animals in Europe. Data about prevalence of MAP in all farmed animal species were included from a variety of literature databases. Information on target population and study design, tests used and apparent prevalences was recorded, and subsequently true prevalences were calculated when possible. A full critical review of the included studies indicated that although a wide range of studies have been conducted, credible true prevalences could often not be calculated. Based on a few studies in which the prevalences appeared plausible, it was concluded that prevalences of MAP would have to be guesstimates based on available data. Among cattle, approximately 20%, or a minimum of 3 to 7% were infected in several countries. Between-herd prevalence estimates appeared to be &gt;50%. No countries appear to have published sufficient information to state that they have a low or a zero prevalence of MAP infections. In goats and sheep the only within-herd prevalence guesstimates were 14% and 2%, respectively, but these figures were based on Norwegian populations only. The between-herd prevalence guesses were &gt;23%, based only on figures from Switzerland.</td>
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<th>Title</th>
<th>A Serological Survey of Paratuberculosis in Finnish Suckler Herds</th>
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<tr>
<td>Author(s)</td>
<td>Seppänen J, Seuna E, Pelkonen S.</td>
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<tr>
<td>Institution</td>
<td>Microbiology Unit and Kuopio Research Unit, Department of Animal Disease and Food Safety Research, Evira, Finnish Food Safety Authority, Mustialankatu 3, 00790 Helsinki, Finland</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>In Finland, symptomatic paratuberculosis was recorded for the first time in 1992 since the early 1900s. During the years 1992-2000 paratuberculosis was detected in five beef herds. After that paratuberculosis was not found in beef cattle, and it has never been detected in dairy herds. This serological survey will give information on the spread of paratuberculosis among beef cattle in Finland. The results will be used in risk analysis of paratuberculosis: to evaluate the risk of beef cattle for spreading paratuberculosis to dairy herds, to plan control measures to combat paratuberculosis, and to assess the need of a specific control program. About 14,500 blood samples from about 1450 suckler herds were collected at slaughter in 2003 - 2005 to survey bovine viral diarrhoea virus. From these samples, those from over three-year old animals are selected to determine antibodies to paratuberculosis. With the help of the identification number, we can find the age, breed, sex and potential foreign origin of the animal in the national data system Elite. About one third of the samples are from cattle over three years old. The serum samples are examined with ELISA test (Parachek) and the results will be presented in the poster. This study is supported by the Walter Ehrström Foundation.</td>
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| Title | Investigation of paratuberculosis status based on comparative analysis of serology and |

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faecal culture in a dairy herd in Thuringia (Germany)

Author(s) Ebert MN¹, Munjal SK², Siebert W³, Schau U³, Donat K¹.

Institution ¹ fzmb GmbH, Research Centre for Medical Technology and Biotechnology, Department of Veterinary Diagnostic, Erfurt; ² Dentognostics GmbH, Jena; ³ Animal Health Service of Thuringia, Weimar, Germany.

Presentation Poster

Abstract
In Thuringia, a federal state of Germany, paratuberculosis is being monitored by a voluntary control programme. Within the framework of this programme, a dairy herd of about 400 cattle with clinical problems of untreatable chronic diarrhea was tested for the first time to detect paratuberculosis. The objective of the present investigation was to determine the status of paratuberculosis in this herd, to analyse the diagnostic significance of an absorbed ELISA and the faecal culture, and compare it with age and lactation. Cattle older than 24 months (n=279) were included in the study. The ELISA included a preabsorption step with Mycobacteria phlei to detect specific antibodies to Mycobacterium avium ssp.paratuberculosis in serum. Faecal culture was carried out on HEYM supplemented with mycobactin J. In faecal culture (gold standard), 93 cows (33%) were detected as shedders and in ELISA, 39 cows (14%) were positive or doubtful. Of the 93 shedders, only 31 cows (33%) were classified as positive or doubtful by the ELISA. Almost half of the shedders were in the first or second lactation period. No obvious differences in age of cows between serological negative and positive shedders were observed. The average milk production of shedders was reduced by approximately 1000 kg per lactation period, which corresponds to 10% of the milk production. There was a high prevalence of paratuberculosis in the herd. Faecal culture is still the most suitable method to determine the individual status of paratuberculosis in a herd as most of the shedders were not detected by ELISA. In view of the economic losses through reduced milk production, a herd management based on testing of the individual animals by faecal culture was found necessary to institute an effective control programme for paratuberculosis in infected herds.

Title Survey on paratuberculosis prevalence in dairy herds of the Lombardia Region (Italy)

Author(s) Arrigoni N¹, Cammi G¹, Losini I¹, Taddei R¹, Tamba M², Belletti GL¹.

Institution ¹ Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Centro di Referenza Nazionale per la Paratubercolosi - Piacenza, Italy; ² Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Centro Emiliano-Romagnolo Epidemiologia Veterinaria- Bologna, Italy.

Presentation Poster

Abstract
391 dairy herds, stratified for size and province, were selected with the purpose of estimating paratuberculosis herd prevalence in the Lombardia region, a Northern Italian Region where 15.000 dairy herds and 45% of the national milk production are concentrated. The prevalence of infected herds was estimated by a commercial ELISA test (Institut Pourquier) on individual blood samples of 38,487 dairy cattle over 12 months. The percentage of herds showing at least one seropositive animal was 43.7%, while the percentage of seropositive cows was 2.6%. On the basis of the performances of ELISA test on blood (sensititity 45%, specificity 99%), the herds that didn't reach a herd specificity of 95% were tested for confirmation by fecal culture on the seropositive cows. The resulting estimated herd prevalence was 19.2%.

Title Paratuberculosis in Sheep from Serra da Estrela Region, Portugal

Author(s) Vala H¹, Santos C¹, Esteves F¹, Albuquerque T², Afonso A², Botelho A², Seixas C³, Amado A².
This work is part of a Project, started in September 2004 and with the duration of three years, aiming the study of the incidence of Paratuberculosis in sheep herds in one of the best Portuguese cheese production region, Serra da Estrela. Thirteen sheep herds were selected and submitted to an epidemiological inquiry focused on the herd size, animal movement, clinical history and sanitation measures, type of bedding and manure management and human involvement. Several diagnostic methods were used and correlated with each other: clinical signs, serology, pathological lesions at necropsies, histopathological and immunohistochemical tests, Ziehl-Neelsen staining, direct PCR and bacteriological culture. The epidemiological inquiry revealed that factors such as: the great size of the herds, poor herd management and sanitary conditions, type of bedding, the absence of suspected animals sequester, as well as common transhumance routes, favoured the spreading of the disease. A serological survey by ELISA and AGID tests performed in 2 562 animals, revealed that 234 samples (9.1%) were positive in ELISA and 30 (1.2%) in AGID. Twenty seven from 34 animals submitted to necropsies presented clinical signs compatible with the disease. Histopathological lesions were present in 21 animals, and in 18 of them acid-alcohol resistant bacilli were observed. Only two animals were tested by immunohistochemical and showed positive results. From 28 PCR positive samples collected at necropsies, *Mycobacterium avium* subsp. *paratuberculosis* was isolated in 20 of them. The correlation between all the diagnostic tests used was performed. *Mycobacterium avium* subsp. *paratuberculosis* isolates are being molecular typed by single nucleotide polymorphisms of *gyrA* and *gyrB* genes. Paratuberculosis is endemic in sheep herds in Serra da Estrela region and special measures are being taken in order to control further spreading of the disease between herds.

**Title**
Factors associated with *Mycobacterium avium* subsp. *paratuberculosis* seroprevalence in sheep in Trás-os-Montes e Alto Douro, Portugal

**Author(s)**
Coelho AC1*, Pinto ML1, Silva S1, Coelho AM4, Aires A4, Garrido JM3, Rodrigues J1,2, Juste RA5.

**Institution**
1 Departamento de Ciências Veterinárias, Universidade de Trás-os-Montes e Alto Douro, Apartado 202, 5001-911 Vila Real Codex, Portugal; 2 CECAV Portugal; 3 Direcção de Serviços Veterinários da Região Norte, Divisão de Intervenção Veterinária de Vila Real - Núcleo do Corgo, Lugar de Codeçais, 5000-421 Vila Real, Portugal; 4 Departamento de Fitotecnia e Engenharia Rural, Universidade de Trás-os-Montes e Alto Douro, Apartado 202, 5001-911 Vila Real Codex, Portugal; 5 NEIKER (Instituto Vasco de Investigación y Desarrollo Agrario), Departamento de Sanidad Animal, Berreaga, 1, 48160 Derio, Bizcaya, España.

**Presentation**
Poster

**Abstract**
The aim of this study was to investigate the risk factors for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) seroprevalence in sheep in the Trás-os-Montes e Alto Douro region, Portugal. A structured questionnaire was used to collect information. The effects on seroprevalence of several variables such as: individual characteristics; farm management practices; farm characteristics; animal health; and available veterinary services were evaluated. This information was used in a multivariable logistic regression model to identify risk factors for MAP seropositivity. Univariable analysis was used to screen the variables used in the logistic regression model. Variables that showed *p*<0.15 were retained for the multivariable analysis. Fifteen variables were associated with seropositivity in univariable analysis. Multivariable logistic regression model identified a number of variables as risk factors: when the sheep were a pure local breed and/or a cross of a local breed (OR=2.02; 95% CI: 1.28, 3.18); a herd size of between 31-60 head (OR=2.14; 95% CI: 1.34, 3.41); culling
during the Spring-Summer season (OR=1.69; 95% CI: 0.93, 3.08); the use of an anti-parasitic treatment such as Ivermectin as the only anti-parasitic medication (OR=5.60; 95% CI: 1.85, 16.99); use of Albendazole with other anti-parasitic treatment (OR=3.89; 95% CI: 1.71, 8.89); use of associations of Closantel and Mebendazol (OR=2.83; 95% CI: 1.13, 7.09). Contrastingly, a number of factors which appeared to offer protection against paratuberculosis came to light. These were: ensuring the winter housing period lasts for less than six months; not spreading manure in fields to be used for grazing; and preventing access to areas where manure is stored. Considering the paucity of epidemiological reports in the region and the absence of any data concerning factors related to either the prevention or the spread of the disease, our results could make a useful contribution towards the prevention of ovine paratuberculosis in the area.

Title
Seroprevalence of bovine paratuberculosis in dairy cattle herds in the Mexico-U.S. border area in Baja California, Mexico.

Author(s)

Institution
Instituto de Investigaciones en Ciencias Veterinarias, Universidad Autónoma de Baja California, Mexicali, BC 21000, México.

Presentation
Oral

Abstract
Several clinical cases of paratuberculosis in dairy cattle and sheep were recently confirmed in our laboratory, nevertheless the accurate prevalence data for northern Mexico is lacking. A total of 303 individual blood samples from 37 dairy cattle herds in Tijuana, Baja California, Mexico were analyzed to estimate the seroprevalence of bovine paratuberculosis. These samples are representatives of approximately 20,000 cows belonging to the local Dairy Producers Association in Tijuana, Baja California (AGLPLT). They were taken as a part of a seroprevalence survey of major infectious abortive diseases during the period from December 2006 to March 2007. Herd size ranged approximately from 100 to 1000. Collected sera were subjected to in-house ELISA test by using commercial PPA antigen and control reagents (Allied Monitor Inc., USA). Ninety-one samples were also tested by a commercial kit (Institut Pourquier, France). For the in-house ELISA, 28 sera gave positive results, 27 were suspicious, and 248 were negative. Apparent prevalence of infection at the animal level was 9.2% (95% I.C. 6.5-13.0%). Using a sensitivity value of 58.8% and a specificity of 95.4% (as claimed by the manufacturer) the overall true prevalence in dairy cattle in Tijuana was estimated as 8.6% (95% I.C. 3.2-15.9%). Apparent herd-level prevalence was 43.2% (16/37, 95% I.C. 28.7-59.1%) when the herds with at least 1 positive animal were considered as positive. When the herds with only 2 or more seropositive animals were considered positive, the prevalence was shifted to 21.6% (8/37, 95% I.C. 11.4-37.2%). These results are consistent with the precise information of the disease infection in California (Adaska and Anderson, 2003). Since the importation of the cattle from US to Mexico is currently limited and aggressive culling is not employed in the local herds, the immediate implementation of disease control program is necessary to prevent the economic loss due to the disease propagation in this region.

Title
Epidemiology of Johne's Disease in organised dairy farms of Punjab (India) and analysis of associated risk factors

Author(s)

Institution
Department of Epidemiology and Preventive Veterinary Medicine, College of Veterinary Sciences, Guru Angad Dev Veterinary Animal Sciences Univeristy, Punjab, India.
The control and eradication of Johne's disease is hindered by the prolonged incubation period, presence of undetected subclinical cases, absence of specific diagnostic tools and lack of knowledge of strain diversity. The present study was conducted to understand the epidemiological distribution of the disease and to analyze associated risk factors in the state of Punjab (India). The organized dairy farms (n=30) distributed under different ecological zones were selected randomly and minimum of ten animals from each farm were sampled. Milk and serum samples were subjected to ELISA for detection of Mycobacterium avium subsp paratuberculosis specific antibodies whereas the milk samples were also subjected to IS 900 based PCR. The apparent prevalence based on serum ELISA (sELISA), milk ELISA (mELISA) and milk PCR was found to be 15.6, 16.3 and 14.0% respectively. The degree of association between different tests revealed low degree of agreement between sEISA and mELISA (kappa value 0.326) however, high degree of agreement was noticed between mELISA and PCR (kappa value 0.682). The present study employing ELISA and PCR revealed high prevalence of the disease in the region in contrast to low prevalence recorded earlier based on histopathology and intradermal johnin testing. The results of the study suggested the usefulness of antibody detection test (ELISA) for screening of affected farms followed confirmation using PCR for identification of animals shedding the organism. The risk factors associated with the disease were identified using binary logistic regression analysis by SPSS (Statistical Package for Social Sciences) program.

Title
Real time estimates of prevalence of Bovine Johne's Disease in dairy cattle herds in Mathura region of North India using fecal culture and indigenous ELISA kit and characterization of Mycobacterium avium subspecies paratuberculosis by IS 900 PCR

Author(s)
Mishra P, Singh SV, Bhatiya AK, Singh PK, Singh AV, Sohal JS.

Institution
Dept. of Microbiology, Veterinary University, Mathura, UP, INDIA; Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - FARAH, District - MATHURA (UP), INDIA.

Presentation
Poster

Abstract
Prevalence of Bovine Johne's Disease (BJD) was estimated in dairy cattle herds (Hariana breed) exhibiting weakness and emaciation, using 2 sensitive tests. Cattle belonged to government (DDD Farm) and private dairy farms (Krishna Balram Gosha, Malwiya Gosha, Panchayati Gosha and cows reported at Kothari Veterinary Hospital) in Mathura district (UP) of North India. Serum and fecal samples from 120 cattle were screened by indigenous ELISA kit and fecal culture. ELISA kit, (using protoplasmic antigen from native Mycobacterium avium subsp. paratuberculosis 'Bison type' strain of goat origin), originally developed for goats and standardized in bovines was evaluated for field use in bovine herds (Hariana breed of cattle), exhibiting clinical disease. JD is endemic in animals in this region; therefore, cows in strong positive category were considered sero-positive. IS900 PCR was used to characterize colonies of Mycobacterium avium subsp. paratuberculosis (MAP). Prevalence of MAP was 20.8 and 28.3% by ELISA kit and fecal culture, respectively. There was agreement in 79.1% cases in 2 tests and 14.1 and 6.6% positives were detected exclusively in fecal culture and ELISA, respectively. Sensitive and specificity of ELISA kit was 50.0% and 90.6% respectively and were comparable to commercial kits available for surveys of BJD. Screening of on 224 dairy cattle using this kit as 'herd screening test', sero-prevalence of MAP in dairy herds was 23.6%. Individually, 36.3, 35.0, 16.0, 29.0 and 33.3% cattle were positive from Krishna Balram Gosha, Malwiya Gosha, DDD Farm, Panchayati Gosha and Kothari Veterinary Hospital, respectively. The 229 bp band obtained from amplification of DNA was characteristic and similar to control DNA from MAP 'Bison type' strain of goat origin (characterized previously on the basis of IS 1311 PCR-REA by Sevilla et al., 2005). Of the 34 DNA isolated from colonies in fecal culture, 85.2% were
amplified by IS900 PCR. Prevalence of MAP was high in clinically suspected dairy cattle in North India. Indigenous ELISA kit was validated with respect to fecal culture and utility as 'Field Herd-screening test'.

Title: Evaluation of indigenous ELISA kit with tissues culture and PCR for the estimation of Ovine Johne's disease (OJD) in farmer's flocks in India

Author(s): Singh PK, Singh SV, Singh AV, Sohal JS.

Institution: Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - FARAH, District - MATHURA (UP), INDIA.

Presentation: Poster

Abstract: Information on Ovine Johne's Disease (OJD) is limited in the farmer's flocks in India. Target tissues of 39 sheep belonging to farmer's flocks were screened by tissue culture, direct tissue PCR and ELISA kit to estimate the presence of Mycobacterium avium subspecies paratuberculosis (MAP). Indigenous ELISA kit, originally developed for the screening of goats, was adapted for screening of sheep and was evaluated with respect to tissue culture and PCR. Soluble protoplasmic (PPA) antigen from MAP 'Bison type' strain cultured from a terminal case of JD in goat was used in ELISA. Decontaminated tissues pellets from intestines and mesenteric lymph nodes (MLN) were processed for DNA isolation and IS900 PCR. Positive DNA samples on amplification yielded specific 229 bp band. Of the 78 target tissues from farmer's flocks, live cultivable MAP was recovered from 64.1% sheep (41.0 in intestine and 38.9% MLN), respectively. Screening of tissues by specific IS900 PCR, 48.7% sheep were positive (33.3% each in intestine and MLN). Culture and PCR together detected 69.2% sheep positive for MAP (64.1%. in culture and 48.7% in PCR). Screening by ELISA kit, 46.1% were positive and together with culture kit detected 69.2% sheep positive (46.1% in ELISA and 64.1% in culture). On applying kappa statistics to direct tissues PCR and indigenous ELISA kit with 'Gold standard' tissue culture method, showed 'substantial agreement' both for direct PCR and ELISA kit. ELISA kit also showed 'substantial agreement' with tissues culture. Using, 3 tests (culture, PCR and ELISA), 74.3% sheep were detected positive. In combinations of 2 tests, 69.2, 69.2 and 66.6% sheep were positive in culture and ELISA, culture and PCR and ELISA and PCR, respectively. Independently, 64.1, 48.7 and 46.1 sheep were positive in culture, PCR and ELISA, respectively. Screening of 39 serum samples by indigenous ELISA kit, 46.1, 30.7, 12.8, 7.6 and 2.5% sheep were in strong positive, positive, low positive, suspected and negative categories with respect to Johne's disease status (S/P ratio). The 46.1% sheep in strong positive category were considered positive for JD. Of these 18 (46.1%) positive sheep, 88.8 and 61.1% were positive in culture and PCR, respectively. The sensitivity and specificity of ELISA kit (goat based) in sheep was 66.6% and 83.3%, respectively.. Unlike 'sheep' genotype the sheep population in India were infected with MAP 'Bison type' genotype, which could be easily recovered from sheep tissues.

Title: Status of Mycobacterium avium subsp. paratuberculosis infection in farm and farmer's goats in India by screening of target tissues using culture, PCR and evaluation of indigenous ELISA kit

Author(s): Singh PK, Singh SV, Singh AV, Sohal JS.

Institution: Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - FARAH, District - MATHURA (UP), INDIA.

Presentation: Poster

Abstract: Study aimed to estimate presence of Mycobacterium avium subspecies paratuberculosis (MAP) in farm and farmer's goatherds (sacrificed for meat), in
Mathura region of North India and to evaluate indigenous ELISA kit, with respect to tissues culture and PCR. Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* was 51.7, 37.9, and 46.5% in the goat population in North India, using tissues culture, tissues PCR and ELISA kit, respectively. Prevalence was 65.2, 39.1 and 65.2% in farmers and 42.8, 37.1 and 34.2% in farm herds using culture, PCR and ELISA kit, respectively. In culture MAP was recovered from 39.6 and 32.7% of intestine and MLN tissues, respectively. In PCR 25.8% intestine and 24.1% MLN were positives. Tissues culture and PCR together detected 62.0% infected goats (51.7% in culture and 37.9% in PCR). In ELISA kit 46.5% goats were positive. Using 3 tests combination (tissues culture and PCR and ELISA kit), 68.9% goats were positive for MAP infection. Whereas, using 2 tests combinations, 62.0, 63.7 and 58.6% goats were positive in Culture and PCR, Culture and ELISA and PCR and ELISA, respectively. In S/P ratio, 46.5% goats were positive in ELISA kit, of which 74.0 and 55.5% were detected in culture and PCR, respectively. Collectively in low positives, positives and strong positives categories, 81.0% goats were detected by kit, of which 53.1 and 44.6% were detected positives in culture and PCR tests, respectively. In farm goats, true prevalence of MAP (culture and PCR) was 54.2% (51.4% goats each were detected in Culture and ELISA and PCR and ELISA combinations). However, in farmer's herds 73.9% goats were positive for MAP infection (true positives). In culture and ELISA and PCR, 82.6 and 69.5%, goats were positive, respectively. On applying kappa to direct tissues PCR and ELISA kit with 'tissue culture method, showed 'substantial agreement' both for direct PCR and ELISA kit. ELISA test also showed 'substantial agreement' with direct PCR. Sensitivity and specificity of ELISA with respect to culture and PCR, was 66.6 and 75.0% and 68.1 and 66.6%, respectively. When, PCR was compared with culture, the sensitivity and specificity was 57.1 and 73.3%, respectively. The 39 sheep sacrificed for mutton production were also sampled from slaughterhouse, Mathura. Evaluation of combined data on 97 animals (58 goats and 39 sheep); ELISA kit had 65.4 and 78.5% and 63.4 and 66.0%, sensitivity and specificity with respect to culture and PCR. PCR with culture had 60.0 and 80.9% of sensitivity and specificity, respectively

**Title**
Seroprevalence of paratuberculosis in selected population of ruminants in India

**Author(s)**
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**Institution**
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**Presentation**
Poster

**Abstract**
The occurrence of paratuberculosis in the Indian ruminant population has been reported since early 1930s, which has been mainly based on the skin johnin test, faecal smear examination and at times pathology and serology. The objective of this study was to know the seroprevalence of paratuberculosis in selected population of ruminants from different part of India using a highly specific commercial ELISA kit (Institut Pourquier, France). The sensitivity of assay was tested on sera from naturally infected and serial sera from experimentally infected goats and sheep. All these animals were confirmed to be positive on the basis of bacterial culture and/ or histopathology. The sensitivity on experimental sera was 56% (28/50) whereas on natural sera, it was 73.3% (22/30). The naturally infected animals were mostly in the clinical stages, whereas experimentally infected animals had moderate and severe infection of paratuberculosis. The overall sensitivity was found to be 62.5%. The specificity of the assay was about 98% based on 98 sheep and goat sera from slaughterhouse, whose MLN tissue samples were negative on bacterial culture. A total of 1822 sera from large and small ruminant population were screened by the ELISA and found to be positive in 22.45%, 20%, 11.6% and 4% of 414 indigenous cattle, 264 cross-bred cattle, 465 sheep and 359 goats, respectively. Cattle sera originated from central-west, northern and southern part of India, and sheep and goat sera were from...
organised farm of Rajasthan and Uttar Pradesh states of India, respectively. A total of 320 buffalo sera from central west (n= 80) and northern (n= 240) part of India did not show antibody prevalence. Though incidence of paratuberculosis has been reported in buffaloes, this ELISA kit has not been evaluated on buffalo sera in India. It was concluded that paratuberculosis was prevalent in many parts of India.

Title Prevalence estimates of *Mycobacterium avium* subspecies *paratuberculosis* in animals, milk, milk-products and human beings in India

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Institution Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - FARAH, District - MATHURA (UP), INDIA.

Presentation Poster

Abstract Poor reporting of Johne's disease (JD) in India is mainly due to lack of diagnostic kits. Information on prevalence of JD in domestic ruminants was extracted from literature, compiled and presented here. Part A reports prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in domestic livestock in North India, using sensitive culture, ELISA kit and PCR tests and includes the work on Johne's disease by us (1985 - 2007), at Central Institute for Research on Goats (CIRG). Part B includes works of other Indian workers from 1940. Retrospective information (Part A and B), was collected from published works and less accessible records like thesis/dissertation, project reports and annual reports of institutes. Published data includes serum samples submitted to Microbiology lab (CIRG), for screening of suspected cattle (26) and buffaloes (15), from Indian Veterinary Research Institute, Bareilly, UP and on 69 samples from prospective cattle bulls (24, Sahiwal and Hariana breed) and Buffalo bulls (45 Murrah breed), earmarked for purchase from Rohtak district of Hariana from farmer's herds. These animals were screened using indigenous serum ELISA kit. Using Milk culture the presence of MAP in milk of lactating goats and cows (including commercial milk and milk products) in South UP and Punjab was 5.0-91.6%. Using milk ELISA kit, prevalence was 37.3-54.7% and 18.1-88.4%, in goats and dairy cattle, respectively from South UP and Punjab. Sero-prevalence of MAP on the basis of serum ELISA kit was 8.5-65.2%, 11.1-61.9%, 17.3-42.6% and 8.6-46.7% in goats, sheep, cattle and buffaloes, respectively in South UP, West UP, Punjab, Rajasthan and Tamil Nadu states of country. Prevalence on the basis of fecal culture, was 52.1-80.0%, 13.3-37.5%, 28.3-96.1%, 23.8% and a case report in goats, sheep, cattle, blue bulls and Hog deer, respectively in South UP, Punjab and North India. On the basis of tissues culture in South UP, recorded presence of MAP was 22.2-65.2%, 42.3-58.9% and 48.0% in goats, sheep and buffaloes, respectively. Using IS900 PCR, the presence of MAP in different samples and animal species was recorded. By using colony PCR, the DNA from different sources was characterized as MAP. Vohra (2005), reported 37.9% prevalence of MAP in vaginal secretions by culture from post parturient farm goats in South UP. Singh (1998, reported 11.3% prevalence of MAP, by fecal culture, in 12 farms of goats in North India in 1998. The prevalence was highest in Jamunapari farm of Etawah (13.4%). Using microscopic examination of fecal samples of farm goats, variable prevalence has been reported by authors (10.6-82.6%) in South UP and North India. Microscopic examination of target tissues of farm young goats in South UP by Hajra (2003), recorded 31.0% (74) samples positive. In fecal samples of farm sheep, 10.4% animals were reported positive. Reported prevalence was 17.3-96.1% and 8.6-50.0% in cattle and buffaloes, respectively in North India. Part B: prevalence in cattle and buffaloes reported by other workers in India was 1.4-66.6% and 4.9-8.5%, respectively. Similarly in goats and sheep, reported prevalence of Johne's disease by other workers in India and Nepal was 2.0-14.1% and 9.7-24.0%, respectively.

Title Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* by PCR in blood sheep in the North East of Portugal

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**Presentation** Poster

**Abstract** Very little was known on the epidemiology of ovine paratuberculosis in the North-East of Portugal until a recent serological study in the Trás-os-Montes e Alto Douro region showed a high flock and moderate sheep prevalence. In order to improve and confirm the seroprevalence data and to explore other diagnostic alternatives, a study based in detection in blood of MAP DNA was carried out. A total of 150 flocks were included in this survey. In order to limit the costs for each flock a set of five clinically healthy sheep and another one of five clinically suspect paratuberculosis sheep were sampled. In healthy sheep only blood were taken to pooled PCR and individual ELISA. From clinically suspected sheep both faeces and blood were taken. Faeces were submitted to pooled PCR and isolation on LJ and Middlebrook 7H11 and blood to absorbed individual ELISA and pooled PCR. One thousand and five hundred sheep were examined in total. Results with each method were compared to each other in order to estimate complementary sensitivity, agreement and cost efficiency. PCR detected MAP DNA in 56 (18.7%) pool samples. The overall individual prevalence of MAP was calculated to range from 6.4 to 15.4%. MAP was found in 20.7% pooled samples from apparently healthy animals and in 16.7% pooled samples from suspect animals. Fecal isolation and PCR showed an overall prevalence of 0.9% and 1.9% respectively. Agreement between pooled methods was generally low, but for healthy versus suspect blood PCR which was moderate. Healthy and suspect pooled ELISA showed a similar average estimate for flock and individual prevalence but costs were much lower. The results of this study also suggest that the health status is not closely related to the true level of infection with MAP.
previously fecal culture positive with only one cfu of MAP on one of four tubes of HEYM. These three positive fecal samples may have represented transient "pass-through" MAP from other cattle in the herd. Of the 20 fecal culture positive cattle at the time of slaughter, 9 were characterized as heavy shedders in the fecal samples with over 100 cfu MAP/tube and the same 9 cows massively infected in each of the four tissues examined with more than 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. The other 3 cows had modest levels of MAP in several tissues. Of the 156 cattle with all negative fecal cultures prior to culling, 58/156 (37%) had at least one positive sample at slaughter. Of these 58 cattle, 25 were culture positive only on one sample, 11 on two samples, 12 on three samples, 6 and 4 samples and 4 cows positive on all five samples. An intestinal Inn 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. While 18/58 (31%) cattle had the next higher tissue level of MAP ranged between 10 and 100 total cfu MAP for the 20 tubes, suggesting a heavier and or repeated doses over time as adults. Conclusions. Cattle shedding more than a few colonies of MAP or had multiple positive fecal cultures had multiple tissues positive at high MAP concentrations. An estimated 35% of always culture negative cattle will have positive tissues at slaughter with a wide spectrum of MAP concentrations suggesting a moderate level of adult infections. Acknowledgement: Financial support for this work was provided by a cooperative USDA Agricultural Research Service (58-1265-3-115) grant.

Title | Time from shedding of Mycobacterium avium subsp. paratuberculosis to occurrence of ELISA-positive cows
---|---
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Institution | Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark.
Presentation | Poster
Abstract | Infections with Mycobacterium avium subsp. paratuberculosis(MAP) are characterised by long incubation periods. Diagnosis using repeated ELISA testing is preferred in the Danish control programme to detect infectious animals. Therefore, the time from testing positive by ELISA to MAP shedding was studied. Repeated ELISA and FC results were available from 1892 dairy cows. The cows were divided into five shedding groups based on the FC results: Non-shedders (NS, nNS=1507); potential transient shedders (TS, nTS=40); intermittent shedders (IS, nIS=116); low shedders (LS, nLS=142); and high shedders" (HS, nHS=87). Cows were defined as TS if a positive FC was followed by three negative tests; IS if had more than one positive FC, but no successive positive samples and the last sample was negative; LS and HS if had a series of positive samples, and FS if had a minimum of one sample with > 50 CFU / g faeces. The time between entering the shedding group to testing positive by ELISA was assessed by a generalised additive model. The results showed that 15 to 20% of cows in the three shedding groups IS, LS and HS were positive by ELISA one year prior to entering the shedding group. Among IS, 50% were ELISA positive when shedding was detected, whereas among low and high shedders 60% were ELISA positive when entering the shedding group. One year after detection of MAP shedding, 80 to 90% in the three groups had positive ELISA results. Only 9 (10%) of the HS cows were shedding > 50 CFU prior to being ELISA-positive. Among the TS-group, 40% had positive ELISA-reactions, of which most occurred 0 to 3 years after entering the TS-group. The latter result indicates that many cows that were classified as transient shedders were probably infected and should have been classified as intermittent shedders. In conclusion, although many shedding cows are detected by ELISA prior to shedding, a large proportion may only become
ELISA-positive after shedding has started, but the amount of bacterial shedding is at low levels until the animal become ELISA-positive.

Title Epidemiological survey of *Mycobacterium avium* subspecies *paratuberculosis* isolates in Europe

Author(s) Karen Stevenson¹, Linda May¹, Susan Denham¹, Ian Heron¹, Lucia de Juan², Julio Alvarez², Gerald Friedrich Gerlach³, Karen Dohmann¹, Ivo Pavlík⁴, Marketa Kopečná⁴, Peter Willemsen⁵, Douwe Bakker⁵, Virginie Thibault⁶, Franck Biet⁶, Alastair Greig⁷.

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Presentation Oral

Abstract A panel was assembled of 168 field isolates of *Mycobacterium avium* subspecies *paratuberculosis* isolated from 19 different host species from the Czech Republic, Finland, Germany, Greece, The Netherlands, Norway, Spain and the United Kingdom. The panel was typed by pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism and hybridisation to IS900 (RFLP-IS900), mycobacterial interspersed repetitive repeats and variable number tandem repeat (MIRU-VNTR) and amplified fragment length polymorphism. A total of 17 BstEII profiles were detected by RFLP-IS900 analysis and the C1 profile was found to be the most predominant in Europe. Thirty one different multiplex PFGE profiles were detected using SnaBl and SpeI and the most widely distributed profile was [2-1]. Twenty five different MIRU-VNTR types were detected with INMV1 and 2 being the most widely disseminated. A few strains were found to be restricted to specific geographic locations although larger numbers are required to determine if this is significant. No evidence was found for species-specific strains and where details were available, wildlife isolates on a single farm were found to be identical to those of cattle on the same farm suggesting interspecies transmission. A comparison of the discriminatory power of the various techniques indicated that PFGE was the most discriminatory followed by MIRU-VNTR and if both of these techniques were combined the discriminatory power was sufficient for epidemiological surveys.

Title Molecular typing of *Mycobacterium avium* subsp *paratuberculosis* strains from different Chilean domestic and wildlife animal hosts.

Author(s) Salgado MÁ¹,², Herthnek D³, Kruze JD¹, Pradenas MV¹,², Böliske G³.

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Presentation Poster

Abstract Paratuberculosis (Johne's disease) is a chronic granulomatous enteropathy of ruminants. It affects primarily cattle, sheep, and goats and is caused by
Mycobacterium avium subsp. paratuberculosis (Map). Paratuberculosis is a common disease in many countries worldwide, and its effects on production can be economically significant. In Chile paratuberculosis has been reported in cattle, sheep and more recently it has been described in goats. Preliminary data suggests that the infection is also present in Chilean wild animals such as guanacos, as well as in some other introduced wild animal species. Nevertheless, there is a lack of molecular epidemiological data on the type of strains existing in Chile. A deep knowledge of paratuberculosis infection transmission between and within both domestic and wildlife host species should be the basis to set up a national control programme. The classification of Map isolates using genomic typing methods provides some understanding of the infection. A method that detects a stable variation at base pair 223 in the IS1311 using polymerase chain reaction with restriction endonuclease analysis (PCR-REA) provides a fast and easy way to differentiate between cattle and sheep Map strains. This IS1311 PCR-REA analysis was used to detect genetic differences among 28 Map isolates from cattle (19), goats (9), guanacos (3) and deer (1) from different regions of Chile. All isolates were C-type and probably of bovine origin. These results showed no genetic differences between Chilean Map isolates from different geographic and host sources. Tracing with regard to these factors are epidemiologically important. The study described isolates of Map from different Chilean animal host species using IS1311. The typing result for the guanaco isolates indicates that these animals have not been infected from the sheep, more likely from cattle or goats. PCR-REA as a simple and rapid test that can be used on a range of diagnostic samples for the confirmation of paratuberculosis and will be of benefit in control and eradication programmes for this disease.
### Abstract

*Mycobacterium avium* subspecies *paratuberculosis* (Map) is the known cause of Johne's disease of ruminants and has been implicated as a cause of Crohn's disease in humans. Previous work has shown that Map is present in untreated water entering water treatment works (WTWs) in Northern Ireland. The work reported here was directed at extending this study by developing both molecular and culture methods for detecting Map and using them to conduct a limited survey of two local WTWs. These have the same source water, Lough Neagh, but have different water treatment systems viz. WTW1 is based primarily on slow sand filtration (SSF) while WTW2 is based on dissolved air floatation (DAF). The SSF process incorporates a schmutzdecke which is a biologically active 'dirty layer' responsible for most of the bactericidal effects while DAF causes particulate matter, including microorganisms, to flocculate and rise to the surface where they are physically removed. This work not only allowed the efficiency of the water treatment processes to kill or remove Map to be determined but also compare the two respective water treatment systems. The survey was carried out over a 9-month period to take account of seasonal effects and husbandry practices. The molecular method used was based on centrifugation, filtration and in-house immunomagnetic separation (IMS) followed by conventional and real-time PCR, the latter based principally on the IS900 insertion element. The method was calculated to have a sensitivity of 10 Map cells ml-1. Map was found throughout both WTW processes from source water to final treated water. No definite concentration of the organism was found at any particular stage. It is recognized that the PCR method employed does not distinguish between viable and non-viable cells. It is hoped that the culture methods that have been performed in parallel with these PCR assays will shed light on this question. It is also hoped that laboratory biofilm studies will provide more fundamental information on the behaviour of Map during both water treatment processes. Since water is one of the possible routes of transmission, the outcome of this work should contribute to a more meaningful risk assessment of the public's exposure to Map and hence inform on possible intervention strategies.

### Title

**Distribution of Mycobacterium avium subspecies paratuberculosis in the Lower Florida Keys**

### Author(s)

Manning EJB¹, Pedersen K², Corn J³.

### Institution

¹ University of Wisconsin-Madison, USA; ² University of Georgia, USA.

### Presentation

Poster

### Abstract

Johne's disease was first diagnosed in an endangered Florida Key deer (*Odocoileus virginianus clavium*) in 1996 and six additional Key deer deaths were documented from 1998 to 2004. We investigated the geographic distribution of *Mycobacterium avium* subspp. *paratuberculosis* in the Lower Florida Keys from February 2005 through May 2006 via collection of blood and fecal pellets from 51 live-captured deer, collection of 550 fecal samples from the ground, and by necropsies of 90 carcasses. Tissue and fecal samples also were submitted from 30 raccoons (*Procyon lotor*), 3 feral cats (*Felis catus*), an opossum (*Didelphis virginiana*), and a Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*). *Mycobacterium avium* subspp. *paratuberculosis* was identified in 23 Key deer fecal samples collected from the ground, tissue samples from two clinically ill Key deer, and from the mesenteric lymph node of a raccoon. Recovery of Map from multiple samples confirms the presence and persistence of the micro-organism on Big Pine Key, Munson and Little Palm Islands. Supplemental feeding of the key deer occurs on these islands; all previous cases of Johne's disease reported since 1996 have occurred in these locations. The organism appears to be limited to this relatively small geographic area within the range of Key deer and evidence of the infection in non-ruminant animals is scant. **Key words**: Florida Keys, Johne's disease, Key deer, *Mycobacterium avium* subspp. *paratuberculosis*, *Odocoileus virginianus clavium*, paratuberculosis, raccoon
Surveillance for *Mycobacterium avium* subspecies *paratuberculosis*

**Author(s)** Manning EJB¹, Cushing H¹, Sleeman J¹, Rohm JH², Sims JP², Sanchez SS², Gerhold R³, Keel MK³.

**Institution** ¹ University of Wisconsin-Madison, USA; ² Virginia Department of Game and Inland Fisheries, USA; ³ University of Georgia, USA.

**Presentation** Poster

**Abstract** Following numerous reports of emaciated and scouring adult free-ranging deer in Virginia, Johne's disease was diagnosed in a 2 year old free-ranging white-tailed deer (*Odocoileus virginianus*) based on histopathology and culture of *Mycobacterium avium* subspecies *paratuberculosis* from frozen hepatic tissue. Clinical and pathologic findings were consistent with advanced Johne's disease: emaciation; diarrhea; severe, chronic, diffuse granulomatous colitis with acid-fast bacilli within macrophages. These findings are consistent with previous reports of Johne's disease in cervids. Subsequent targeted surveillance of nine emaciated adult deer with diarrhea as well as active surveillance of 65 asymptomatic deer for *Mycobacterium avium* ss. *paratuberculosis* using culture for multiple tissue types plus serology did not confirm any additional cases of infection. This appears to be an isolated case of Johne's disease in a free-ranging white-tailed deer, and deer from this region do not appear to represent a reservoir for the organism. The origin of infection was most likely domestic animals. Stressors such as high deer population density and low nutritional quality of the habitat may have contributed to the development of clinical disease in this case. Clinical symptoms identical to what is seen with Johne's disease in numerous animals are insufficient evidence to establish a diagnosis of widespread *M. paratuberculosis* infection in a free-ranging deer population. Key words: Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis*, *Odocoileus virginianus*, White-tailed deer.

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**Title** *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in semen and organs of a breeding bull

**Author(s)** Khol JL¹, Beran V², Kralik P², Tesinska I¹, Aurich A¹, Pavlik I¹, Baumgartner W¹.

**Institution** ¹ Clinic for Ruminants, Department for Farm Animals and Herd Management, University of Veterinary Medicine Vienna, Austria; ² OIE Reference Laboratory for Paratuberculosis, Veterinary Research Institute Brno, Czech Republic; ³ Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Medicine Vienna, Austria.

**Presentation** Poster

**Abstract** Consecutive semen samples of a breeding bull suffering from paratuberculosis were collected over a period of one year and tested for the presence of *MAP*. Furthermore blood, faecal and after culling organ samples were collected. Semen was tested for *MAP* by IS900 and *F57* Real-time PCR, ELISA was used for the detection of specific antibodies in blood, faecal and organ samples were tested by culture on Herrold's Egg Yolk Medium and Ziehl-Neelsen staining. All faecal samples taken during the trial were positive for *MAP* by culture, blood ELISA also gave a positive result at all times. At necropsy the bull showed typical signs of advanced paratuberculosis. *MAP* was detectable in multiple organs including the intestine, intestinal lymph nodes and reproductive organs. *MAP* was detected by IS900 Real-Time PCR in all 9 (100%) semen samples and in 6 of 9 (66.6%) samples using the *F57* Real-Time PCR. semen quality, especially viability of the sperm, was poor in collected samples. The finding that all semen samples were positive for the period of one year in the described case, underlines the potential risk for the spread of *MAP* by natural or artificial insemination. The financial support was provided by grants No. MZE0002716201 of the Ministry of Agriculture of the Czech Republic and
**Title**  
In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*  

**Author(s)**  
Whittington RJ, Windsor PA.  

**Institution**  
Faculty of Veterinary Science, University of Sydney, Australia.  

**Presentation**  
Oral  

**Abstract**  
*Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) causes Johne's disease in ruminants. Disease control programs aim to break the faecal-oral cow-calf transmission cycle through hygienic calf rearing and removal of cows shedding *Mptb* from the herd, but these programs do not take account of congenital infection. The aims of this study were to determine the prevalence of foetal infection in cattle and to estimate the incidence of calves infected via the in utero route following meta-analysis. 9% (95% c.l. 6-14%) of foetuses from subclinically infected cows and 39% (20-60%) from clinically affected cows were infected with *Mptb*. The true rates of infection would be higher than these figures suggest due to incomplete sensitivity of culture methods. The incidence of calf infection derived via the in utero route was estimated to be in the range 0.44 to 1.2 infected calves per 100 cows p.a. in herds with within-herd prevalence of 5% and 3.5-9.3 in herds with 40% prevalence. In utero transmission of *Mptb* could retard the success of disease control programs if the opportunities for post natal transmission via colostrum/milk and environmental contamination were able to be controlled. The immunological consequences of foetal infection will be discussed in the context of diagnosis and vaccination.

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**Title**  
Acidification of raw cow milk and effects on the culturability of *Mycobacterium avium* subsp. *paratuberculosis*  

**Author(s)**  
Mutharia LM, Raymond M.  

**Institution**  
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**Presentation**  
Poster  

**Abstract**  
A source of *Mycobacterium avium* subs. *paratuberculosis* (MAP)-free milk for calf feeding is needed to control MAP transmission to calves, and is a key component in the success of the national Johne's disease (JD) control programs. JD affects up to 30% of Ontario Dairy herds and similar rates are reported for dairy and beef herds across Canada. Calves are most susceptible to infection when they ingest the bacterium in colostrums and milk contaminated either in the mammary glands or post harvest with feces of cows with JD. At the present, installing pasteurization systems, or purchasing pasteurized and commercial milk replacers to feed calves are the only options available to producers. For producers, these options present added economic costs, since they buy commercial products while discarding readily available colostrums or waste (non-saleable) milk. In this study, raw milk cow milk and colostrum seeded with cultured clinical MAP strains was employed to investigate the following, (i) the pH necessary and the minimum contact time required to achieve log-reduction in MAP viability, (ii) whether MAP bacteria subjected to acidic treatments were actually 'killed' or could resuscitate once milk is stored, neutralized or diluted, (iii) whether acidification destroyed milk immunoglobulins. Here we discuss our results on the effect of acidification on MAP culturability. Our results show that acidification affected the recovery of MAP from milk and decreased by up to 70% the culturability of the bacterium. HPC decontamination and antibiotic treatment further decreased the culturability of MAP bacteria. In our hands, acidification significantly reduced the cfu of MAP in milk. Neutralization of the milk prior to recovery of the bacterium did not counteract the effect of acidification. It remains to be determined
whether acidification affects the nutritional quality of the milk and the functions of the colostrum immunoglobulins.

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<td>Author(s)</td>
<td>Saito F, Nakaoka Y, Kogishi K, Kato K, Wakamatsu T, Mikami Y</td>
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<td>Institution</td>
<td>1 Kyoritsu Seiyaku Corporation; 2 Hokkaido Tokachi Livestock Hygiene Service Center; 3 Hokkaido Rumoi Livestock Hygiene Service Center; 4 Hokkaido Hidaka Livestock Hygiene Service Center</td>
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**Abstract**

Paratuberculosis, caused by Mycobacterium paratuberculosis, is an important chronic enteric disease of domestic cattle. The major routes of infection are the oral ingestion of contaminated manure, milk, and colostrum. Infection by contaminated colostrum is the most important route. This route has three times higher rate of infection than from ordinary milk (Robet et al, 1995). Prevention of infection via contaminated colostrum will play an important role towards controlling Paratuberculosis. Development of colostrum pasteurization protocols which inactivates pathogens without affecting important beneficial fractions of colostrum is the objective of the studies.

1. Establishment pasteurization of colostrum

Pasteurization of milk is commonly done at 62-65°C for 30 minutes, however similarly treating colostrum results in coagulation. The investigative objective is to develop practical methodology and method(s) capable of deactivating pathogens such as *M. paratuberculosis*, *Salmonella* spp., leukaemia virus etc., without inactivating the beneficial colostrum fractions such as immunoglobulin. In our studies, results indicated that pasteurizing colostrum at 60°C for 30 minutes meets our objective and also avoids coagulation.

1. Development of "Colostrum pasteurizer" devise

A prototype "Colostrum Pasteurizer" which can be easily and routinely utilized by farmers was developed and tested. The prototype had a maximum capacity of treating ten liters of colostrum per cycle. Ten liters of colostrum to be pasteurized is poured into a vesicle and placed into the water tank. The vesicle contents are stirred continuously during pasteurization process. The temperature, duration, and stirring are automated to achieve the optimal 60°C for 30 minutes and shutdown.

1. Using "Colostrum Pasteurizer" at dairy farms

Modifications to prototype were made and a commercial model has been marketed since November, 2005. At present, there has been no report of transmission of Paratuberculosis from colostrum treated by "Colostrum Pasteuriser". Field reports indicate the decrease of the frequency of diarrhea in calves fed pasteurized colostrum. These reports suggest the use of "Colostrum Pasteurizer" reduces incidences of diarrhea by deactivating diarrhea causing pathogens. Prevention of infections through contaminated colostrum will contribute to Paratuberculosis eradication program in Japan. Additionally, pasteurized colostrum will prevent other infectious diseases caused by contaminated colostrum such as leukaemia and decrease calf diarrhea cases by decreasing total bacterial count in colostrum.

<table>
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<th>Title</th>
<th>Bayesian methods for assessing genetic similarity and familial aggregation of paratuberculosis in beef cattle of unknown pedigree</th>
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<tbody>
<tr>
<td>Author(s)</td>
<td>Osterstock JB, Fosgate GT, Cohen ND, Derr JN, Roussel AJ</td>
</tr>
<tr>
<td>Institution</td>
<td>College of Veterinary Medicine and Biomedical Sciences; Texas A&amp;M University; College Station, TX, USA</td>
</tr>
</tbody>
</table>
Abstract

Describing familial aggregation of infectious disease is an important first step in identifying genetic components of disease resistance. Traditional measures of familial aggregation utilize subjects of known pedigree in family-based designs to compare disease frequency between family members of cases and family members of controls. Restricting the study-base to populations with known pedigree structures in paratuberculosis research may introduce selection bias if study populations are not representative in regards to other risk factors for disease. We developed an alternative approach for identifying groups of genetically similar individuals to compare disease frequency. Texas beef cattle herds were selected for sampling based on breeder surveys, veterinarian referral, and clinical cases admitted to the Texas Veterinary Medical Center. Diagnostic samples were collected from animals (n = 2,622) >2 years of age and evaluated for paratuberculosis using 2 ELISAs (Herdcheck®, IDEXX Laboratories; Parachek®, Prionics) and fecal culture. Additionally, whole blood was preserved for genotyping. All animals positive on at least 1 test and 3 herd-matched controls were selected for genotyping. A separate group of animals of known pedigree relationships were sampled for genotyping to assess validity of clustering methods. Cases, controls, and pedigreed animals were genotyped using a panel of 12 microsatellites previously described for parentage testing in cattle. Bayesian methods (Structure v2.2) were employed to identify the optimal number of clusters of genetically similar individuals in the sample population and to probabilistically assign individuals to clusters. The proportion of parent-offspring pairs assigned to the same cluster among the pedigreed animals was evaluated to validate clustering methods. Conditional logistic regression was used to compare proportion of test positive animals among clusters controlling for herd of residence. Analysis of cluster results indicated that 9 clusters was optimal for this population. Using the cluster with the lowest proportion of test-positive animals as the referent group, the odds of having a positive test were significantly greater for 3 clusters (Cluster 2 OR 36.4, 95% CI 3.1 to 430.4; Cluster 7 OR 7.4, 95% CI 1.0 to 12.0; Cluster 9 OR 5.9, 95% CI 1.8 to 19.4) compared to the referent cluster. Of the 9 animals positive for Mycobacterium avium subsp. paratuberculosis on fecal culture, 5 were assigned to cluster 7. These results support the hypothesis that there are differences in genetic susceptibility to paratuberculosis test-positivity that can be quantified for beef cattle of unknown pedigree using genetic markers to assemble groups of genetically similar individuals. Employed methods demonstrate a unique approach to describing familial aggregation of disease in cattle. Clusters with the disparate odds can be targeted for further study of genetic susceptibility and may contribute to the understanding of the pathogenesis and genetic resistance to paratuberculosis in cattle.

Title

Effects of infection by Mycobacterium avium paratuberculosis on fertility of dairy cows.

Author(s)

Marcé C, Beaudeau F, Bareille N, Seegers H, Fourichon C.

Institution

Unit of Animal Health Management, Veterinary School of Nantes, INRA, BP 40706, 44307 NANTES Cedex 3, France.

Presentation

Oral

Abstract

The effects of infection by Mycobacterium avium paratuberculosis (MAP) on performance have to be estimated in order to assess its economic impact. This study aimed at quantifying the variation in fertility of dairy cows according to their MAP-infection status. The hypothesis of an indirect effect was set. Fertility was measured by the non-return rate at first and second services. A non-return was defined as the absence of another artificial insemination (AI) after the first one while the cow was still present. Three different statuses were defined based on both individual and herd results: positive cow, negative cow in a negative herd and negative cow in a positive herd. 27 612 AI from 72 135 cows in 1 470 herds were studied by logistic regression after adjusting on known factors influencing reproduction. Non-return rate
was higher for infected cows compared to negative cows from negative herds (OR of 1.14, or +3.2 point of % of non-return rate). This increase was higher for parity 1 cows (OR of 1.20, or +4.4 point of % of non-return rate) compared to older cows. The effects were lower when comparing positive cows to negative cows in the same herds. Looking at these observations, the hypothesis of MAP-effect based on the relation between MAP-infection, production and reproduction is formulated. Due to the lack of protein absorption in the intestine, the milk production is reduced. In the subclinical stage of the disease, this could lead to a lower negative energy balance that could be associated with improved fertility.

Title  Association between Johne's Disease Milk ELISA Test Result and Milk Production and Breed in Canadian Dairy Cows
Author(s)  Sorge US, Kelton DF, Sears W.
Institution  Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.
Presentation  Oral
Abstract  Various studies have shown that cows with clinical and subclinical Johne's disease (JD) are likely to produce between 4 and 24% less milk than test negative cows. The objective of this study was to quantify the difference between the milk production of JD milk ELISA positive cows and test negative cows, as well as to evaluate variations in the test results due to breed. The data included the test day information from 47,418 cows from 817 CanWest DHI herds in Ontario, Manitoba, Saskatchewan, Alberta and British Columbia. The cows were tested between March 2005 and April 2007 with the AntelBio Johne's Milk ELISA. The optical density (OD) cut points for a suspect or positive test result were set at > 0.065 and > 0.1, respectively. A mixed model was fitted to investigate the association of a positive milk ELISA test with the estimated cumulative 305 day milk production. Only factors with a p-value < 0.01 were retained in the model. A second model was fitted to examine the relationship between breed and observed test result. The SAS Glimmix procedure was used, including only herds with at least one positive cow. Herd was included in both models as a random effect. The number of tested cows per herd varied between 1 and 342 (Interquartile range: 35 -71). The breeds were Holstein (n= 44,420), Jersey (n= 2,078), Brown Swiss (n= 361), Ayreshire (n= 308), Guernsey (n=141), and Milking Shorthorn (n= 110). The majority of the cows (n= 46,515; 98.1%) tested negative, while 753 tested positive (1.59%) and only 150 cows (0.32%) had suspect test results. Therefore, positive and suspect cows were combined into one JD positive group. The percent of positive cows by province varied from 0.74% to 2.27% (p < 0.0001). The average 305 day milk production was 9,696 kg/ cow. After adjusting for breed, lactation number, season of calving, somatic cell count, days in milk on test day as well as several interactions among these factors, cows that tested positive for JD produced 748 kg or 7.7% less milk over the estimated 305 day lactation compared to milk ELISA test negative cows (p < 0.001). The between breed comparison was adjusted for the lactation number, province, days in milk on test day and somatic cell count. Guernsey and Jersey cows were 3.9 and 2.3 times as likely to test positive for JD as Holstein cows (p <= 0.0001). These findings indicate differences in JD prevalence across the 5 western most provinces in Canada. Furthermore, cows testing suspect or positive with the milk ELISA test produced significantly less milk than their negative herd mates and cows of the Channel breeds were more likely to test positive than Holsteins. Further research is needed to examine the differences in dairy herd management practices among the provinces that might explain the variations in the JD prevalence, and the cause for the variation in the test results among the different dairy breeds.

Title  Bulk milk contamination by Mycobacterium avium subsp. paratuberculosis and related risk factors
52 dairy herds, infected with *Mycobacterium avium* subsp. *paratuberculosis* (Map), were submitted to repeated bulk milk sampling (an average of 3.5 samples/herd). On 183 samples, tested by culture and nested-PCR, 20 (11%) resulted positive for Map. 11 herds on 52 controlled (21.2%) registered at least one positive sample. The positivity appears strongly correlated to the herd prevalence. In the risk factor analysis for milk contamination, both the paratuberculosis herd prevalence and the hygienic measures to control the fecal contamination were taken into consideration; the risk of milk contamination appears directly related both to the infection prevalence in the herd and to udder hygiene. In contrast, neither the ideal hygienic measures in routine milking, nor proper milk filtration were effective in preventing the presence of Map in milk.
### Title
Reduced incidence of Johne's disease in dairy cattle herds on a long-term herd management program

### Author(s)
Ferrouillet C¹, Wells SJ¹, Hartmann W², Godden S¹.

### Institution
¹ Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN, USA; ² Minnesota Board of Animal Health, St. Paul, MN, USA.

### Presentation
Poster

### Abstract
The objective of this prospective longitudinal field study was to describe changes in the incidence of seroconversion, fecal shedding, and culling of dairy cows with clinical signs of Johne's disease in 6 Minnesota (USA) dairy herds participating in the Johne's Disease Demonstration Herd Project from 2000 through 2005. Implementation of this program was evaluated using an annual herd risk assessment and adult cattle were tested annually using serum ELISA and bacterial culture of feces to evaluate progress made using the control program through time. After 6 years of follow-up, there was a significant reduction in the incidence of seroconversion, fecal shedding and cows with clinical signs of Johne's disease. For 3 herds achieving recommended management changes with a risk assessment score under 30 in the last year evaluated, test results from the control program were consistent with a reduced risk of infection in calves. Further investigations including controlled trials are underway to evaluate if specific management interventions recommended within the Johne's disease control program are effective in preventing new MAP infections.

### Title
The economical value of Mycobacterium avium subsp paratuberculosis fecal shedding and culling due to clinical Johne's disease on Minnesota dairy farms

### Author(s)
Raizman EA¹, Fetrow J², Wells SJ².

### Institution
¹ School of Veterinary Medicine, Purdue University West Lafayette IN; ² College of Veterinary Medicine, University of Minnesota, Sat Paul MN.

### Presentation
Poster

### Abstract
Introduction: The National Animal Health Monitoring System (NAHMS, 1996) has estimated that the annual cost to infected U.S. dairy operations is over $100 per cow in inventory, with higher costs of more than $200 per cow in inventory per year in herds with high infection levels. These estimations however were performed more than 10 years ago and were based on serum ELISA test results, which is known to have less sensitivity than bacterial fecal culture, and therefore can bias any prevalence estimation. The scientific literature provides limited information about the economical impact of Mycobacterium paratuberculosis (Map) fecal shedding in dairy cattle on lactation performance. Quantification of the monetary impact of Map fecal shedding or clinical JD on lactation performance is critical to participation by dairy cattle producers in JD control programs, because it enhances the relationship between stage of disease and economic loss. This information will allow dairy producers to make appropriate management decisions within their operations regarding implementation of control measures to decrease herd JD prevalence. The objective of this study was to evaluate the economical cost of Mycobacterium paratuberculosis (Map) fecal shedding prior to calving and of cows that were culled due to clinical Johne's disease (CCJD) during the subsequent lactation. Material and Methods: 1,050 cows from 2 Minnesota dairies were enrolled where fecal samples were obtained during the close-up period. Milk production, clinical diseases (other than CCJD), and reproductive performance data were recorded for each cow. The model was built in an Excel ® spreadsheet where we took into consideration the following parameters: Loss of value across lifetime of a cow: average loss in value per lactation (US$105), average slaughter value ($450), etc; Income over feed cost during the lactation (IOFC): milk price per pound ($0.14 milk price/lb), dry matter intake feed cost feed ($0.08/lb), cost to support maintenance in a milking cow/d ($1.76) and cost of feed/lb
of milk/d above maintenance ($0.03); Reproduction managements costs: cost of an extra day open above a baseline of 85 days ($2.5), and cost of an insemination ($12).

For diseases costs we considered only treatment, labor, and milk discard costs. Results: Among culled cows, mean culling loss of fecal negative and positive cows was US$779 and $727 (p>0.05). The cost per cow in the herd, however, was $265 and $543 for fecal negative and positive cows, respectively. Among culled cows mean IOFC for negative and positive cows was $1200 and $840 (p<0.01) and among non-culled cow IOFC for fecal negative and positive cows was $1960 and $1680, respectively (p<0.05). Mean IOFC for CCJD was $1075 and for light, moderate and heavy fecal shedding cows $1460, $960, and $370, respectively. Cost of disease for culture negative and positive cows was not significantly different. Mean reproduction cost for fecal negative cows was significantly higher than for fecal positive cows ($145 vs. $43; p<0.001), probably because of early culling of culture positive cows.

Conclusions: The losses due to lower lactation performance and early culling from the herd should alarm dairy producers and motivate them to implement the appropriate control measures for the disease. Results of this study should be incorporated into educational programs that emphasize the importance of JD control and prevention.

Title Optimizing management of infectious cows only - simulations indicate an effective and more feasible control strategy

Author(s) Kudahl AB1, Nielsen SS2.

Institution 1Faculty of Agricultural Sciences, Dep. Animal Health, Welfare and Nutrition, University of Aarhus, Blichers Allé 20, P.O. Box 50, DK-8830 Tjele; 2Faculty of Life Sciences, Dep. Large Animal Sciences, University of Copenhagen, Grønnegårdsvej 8, DK-1870 Frederiksberg

Presentation Poster

Abstract In February 2006 the Danish Cattle Federation initiated a national voluntary control programme "Operation Paratuberculosis" ("Operation PTB"). The programme focuses on closing transmission routes, because it has been demonstrated to be essential for the control of paratuberculosis in cattle herds. However, closing transmission routes is also difficult to practice, because persistence and much extra labour is needed. This amount of labour is reduced in "Operation PTB" which implies diagnostics of all cows 3-4 times per year by milk-ELISA, and - in cooperation with the herd health advisor - a contingency plan for changes in management and housing systems is made in order to reduce transmission of PTB. Transmission routes between calves and all cows being diagnosed as infectious should be broken effectively, and the most infectious cows culled before the next calving. The improvement of management is thus focused only on cows having been diagnosed as infectious instead of on the whole herd. Thereby the labour and time needed for improving management are reduced considerably. The expected long-term effects of this strategy compared to alternative strategies were evaluated by simulation studies with the herd-simulation model PTB-Simherd. Scenarios were simulated in a herd with 200 cows (500 replications), an initial herd prevalence of 25% and an otherwise typical Danish herd management. The simulated results of following "Operation PTB" were a reduction of prevalence from 25% to 5% after 5 years and less than 1% after 8 years, which makes this strategy just as effective in reducing prevalence as if management had been optimized for all cows in the herd. If no action was taken to control PTB, the prevalence would increase to 75% after 10 years. If transmission routes were not broken by improving management, but infectious cows were still culled within the next calving, the prevalence continued to increase to 26-39% depending on how quickly the infectious cows were culled after having the diagnosis. The economy of "operation PTB" compared to the economy of optimizing the management of all cows (which implies more labour hours, but no costs for tests) depends directly on the hourly rate and the time spent on optimizing management. If the extra workload per calving is assumed to be 1 hour, "Operation PTB" is the economically most attractive option in Denmark whenever the hourly rate exceeds 7 euro (9 $).
<table>
<thead>
<tr>
<th>Title</th>
<th>A multi-species approach to control Johne's disease in New Zealand</th>
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<tr>
<td>Author(s)</td>
<td>Heuer C, Glossop JC, Jackson R, West DM, Wilson PR.</td>
</tr>
<tr>
<td>Institution</td>
<td>EpiCentre, Massey University, Priv Bag 11222, Palmerston North 4442, New Zealand.</td>
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<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>Johne's disease (JD) has significant economic impact on deer and to some extent on dairy cattle production while little is known about its effect on beef, sheep and dairy goat enterprises in New Zealand. Since <em>Mycobacterium avium</em> subtype <em>paratuberculosis</em> (MAP) causes considerable economic loss and is a hypothesised human health hazard, considerable development efforts focus on the control of Johne's disease in New Zealand. This presentation summarises current research findings and evidence of inter-species transmission including domestic livestock and wildlife species. Options for control involving multi-species farming practices are evaluated leading to major new research objectives for the next five years. Highlights of current evidence include a quantification of production loss and risk factors for clinical JD in dairy cattle, the impact of co-grazing with sheep and cattle and other risk factors on clinical JD in deer, evidence for a virulence difference between sheep and cattle strains, and performances of current and new diagnostic tests to detect sub-clinical infection with MAP.</td>
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<th>Title</th>
<th>Association of farm management and soil risk factors with ovine Johne's disease in Australia</th>
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<tr>
<td>Author(s)</td>
<td>Dhand NK, Eppleston J, Whittington RJ, Toribio JLML.</td>
</tr>
<tr>
<td>Institution</td>
<td>Faculty of Veterinary Science, The University of Sydney, Australia.</td>
</tr>
<tr>
<td>Presentation</td>
<td>Poster</td>
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<tr>
<td>Abstract</td>
<td>Farm management and soil risk factors are known to effect ovine Johne's disease (OJD) prevalence, but little is known about their impact after adjusting for each other. This study aimed to evaluate both sets of factors simultaneously in 92 sheep flocks in Australia in 2004-05. Pooled faecal samples were collected from an identified cohort of sheep in every flock to estimate OJD infection status and soil samples from the paddocks grazed by this cohort of sheep to measure soil characteristics. A questionnaire was administered to farmers by face-to-face interview to obtain information about husbandry and management factors. Multivariable ordinal logistic regression, generalised- and general-linear mixed model analyses were conducted to test the simultaneous association of management and soil factors with OJD. Both farm management and soil risk factors were significant in the final models. OJD prevalence was higher in sheep whose dams were maintained at a higher stocking rate and had lower condition scores during lambing. Prevalence was also higher in flocks that grazed sheep along the roads shared by neighbours and that had adopted some OJD control practices. Soil organic carbon%, an indicator of soil organic matter content, had a positive linear association with OJD prevalence. Our results suggest that both farm management and environmental factors are important in the epidemiology of OJD. Implications of the study results for OJD control programmes will be discussed.</td>
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<th>Title</th>
<th>Safety and Efficacy of Silirum® Bovine Johne's Disease Vaccine in an Experimental Bovine Challenge Model</th>
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<tr>
<td>Author(s)</td>
<td>Sweeney RW(^1), Whitlock RH(^1), Bowersock TL(^2), Pruitt GW(^2).</td>
</tr>
<tr>
<td>Institution</td>
<td>(^1) University of Pennsylvania School of Veterinary Medicine, Kennett Square PA USA; (^2) Pfizer Animal Health, Kalamazoo MI USA.</td>
</tr>
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</table>
The objective of this study was to evaluate the safety and efficacy of a killed *Mycobacterium paratuberculosis* vaccine (Silirum®, Pfizer Animal Health) using a bovine experimental infection model. Twelve newborn Holstein male calves were randomly assigned to one of two groups. The vaccinated calves (*n*=6) received a single dose of vaccine, administered subcutaneously in the side of the neck, at 14 days of age. Control calves were sham vaccinated with placebo. All calves were given an oral challenge of 109 CFU live field strain *Mycobacterium paratuberculosis* (MAP), administered on days 35 and 36 of age. Body temperature and injection site diameter were measured periodically following vaccination. Blood samples were collected at various time points for measurement of antigen specific release of Interferon-gamma by peripheral blood cells (Bovigam®, Prionics) and for ELISA testing for detection of serum antibodies against MAP (Paracheck®, Biocor). Calves were euthanized at 98 days of age and 32 tissues collected for culture of MAP, using both solid media (Herrold’s Egg Yolk Media) and liquid media (MGIT®, Becton Dickinson). Total number of tissues that were culture positive, as well as the total number of CFU/calf on HEYM were compared for the two groups. For liquid culture, time to signal positive (TTP) was compared for the two groups, with a more rapid TTP indicating a higher concentration of MAP in the original sample. MAP-induced IFN-gamma release by prescapular lymph node cells cultured in-vitro was measured. Following vaccination, there was a transient rise in body temperature (approximately 1 degree F), significantly different from control, on Days 1 and 2 following vaccination, with a return to baseline on Day 3. Vaccinated calves had visible swelling at the injection site that persisted throughout the study, but swellings were not painful and did not develop drainage. Vaccination sites were culture-negative for MAP at the conclusion of the study. ELISA testing for serum antibodies to MAP gave negative results for all calves in both groups. Vaccinated calves had significantly higher IFN-gamma release by peripheral blood cells, and by prescapular lymph node cells, compared with controls. Vaccinated calves had significantly reduced colonization of tissues by MAP, compared with control calves, whether measured by CFU/calf in the HEYM system or TTP in the liquid media system. There were on average, 22 tissues positive per calf in vaccinated calves compared with control, and TTP in liquid culture was 10 days longer on average for vaccinated calves. All of the above differences were significant at *P*<0.05. We conclude that Silirum® vaccine was associated with reduced tissue colonization by MAP when administered to calves.
relevant aspects regarding farmer acceptance, TB diagnosis interference and other adverse effect, milk production, and culling/replacement rate effects. Vaccination was applied to all animals present in the farm at the moment of joining the trial, and then to all heifer calves intended for replacement during their first month of life. A clinical follow-up was performed at one month post-vaccination at the beginning, and then blood and faecal samples were taken on a yearly schedule. All cullings were reported, and sample from the ones slaughtered within the Basque Country were taken for pathology and bacteriology. Records from the breeders association regarding milking monthly controls, total lactation and days of lactation were obtained and analyzed comparing pre-and post-vaccination data. No relevant clinical effects were observed apart from the formation of a small nodule at the point of inoculation that tended to disappear or at least decrease after a few months. Comparative tuberculin testing one year and more after vaccination did not yield any positive results for bovine TB. Up to now, clinical cases have been reduced from all farms, 152 animals older than 24 months have been culled or fallen stock since the vaccination, we sampled 22.37% (34/152) of them and clinical paratuberculosis was diagnosed only in a Holstein cow and in 4 Jersey cows, that is 14.7% (5/34). We have to take into account that the Jersey breed is more susceptible and the hygiene practices of this farm were deficient. The most clear effect has been on culling rate reduction of first calving cows which was 45.82% according to milking records. Regarding the rate of excretion a reduction of 10.87% of positives by PCR was observed 12 months post-vaccination. Nevertheless, no general reduction was observed by faecal culture, but the 18.75% of the whole positive animals detected in the first sampling were heavy shedders, which disappeared 12 months after vaccination. Milk production was increased by an average 709 kg per cow after vaccination, but varied greatly according to number of lactation and farm. Given the trial design it cannot solely attributed to paratuberculosis control achieved with the vaccine. In summary, vaccination in commercial farms has brought back to normal figures the replacement rate and has probably increased the overall milk production per cow without relevant adverse effects both in clinical and TB-diagnostic terms.
precise the frequency of meat Map contamination and the long term effects of vaccination on it. The relationship between paratuberculosis and Crohn's disease has been discussed along the time and there are several studies which link both diseases. Although the main way of introduction of the mycobacteria in the human food chain seems to be the milk and dairy products we can’t reject the meet as a possible source of infection. Even though in Crohn's disease the bacteraemia has been described, this fact has not received enough attention in the study of the paratuberculosis. In this way, we will present our results proving the bacteraemia in Johne's disease and the presence of viable Map in muscle. At the moment 31 dairy cattle from vaccinated and 17 from not vaccinated herds have been analyzed. All these animals were slaughtered or fallen stock and were sampled collecting faeces, different sections of gut tissues, mesenteric lymph nodes, muscle and blood. The diagnosis was based on histopathological and microbiological studies. On the one hand the lesions were classified following a previous described classification and on the other hand microbiological studies were based on isolation on Löwenstein Jensen and Herrold Egg Yolk media added with Mycobactine J. Regarding the histopathological classification no difference has been detected between vaccinated and unvaccinated animals probably due to the short time from the vaccination. In the studied group we have observed that the presence of Map in faeces and tissues was 50% lower in vaccinated animals. Moreover we detected that the proportion of heavy faecal shedders was reduced 60% comparing the two groups. Two positive blood cultures and one positive muscle culture were obtained from three animals with clinical symptoms of paratuberculosis, which confirms the role of the bacteraemia in the spread of Map in infected animals, so this aspect of the pathogenesis should be considered in future studies.

Title
Johne's disease vaccination: a valuable tool in managing Johne's disease

Author(s)
Patton E1, Knust B2, Konkle D1, Bohn J3, Wells SJ2.

Institution
1 Wisconsin Department of Agriculture, Trade, and Consumer Protection, Madison, WI, USA; 2 Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN, USA; 3 Northwest Wisconsin Veterinary Service, Amery, WI, USA.

Presentation
Oral

Abstract
In this clinical trial, 3 commercial dairy herds vaccinated every other heifer calf against Johne's disease using a conditionally licensed commercial vaccine until two cohorts with the greater of the following were obtained: 10% of the adult herd or 50 head per cohort. Each herd participated in an annual Johne's disease risk assessment and herd management plan and had made efforts to reduce the risk of Johne's disease transmission prior to initiating this project. Baseline prevalence estimates indicated that the three herds were moderately to heavily infected with Johne's disease. Fecal samples from heifers from the cohort groups were collected at first calving and at the 90 day pregnancy check at each subsequent lactation and tested using bacterial culture with liquid media. After at least one test per cohort cattle, heifers from the vaccinated cohort had significantly fewer positive fecal cultures than the non-vaccinated cohort (relative risk 0.32; p value < 0.01). The concentration of fecal shedding and clinical disease, although not statistically significant at this point of the study, both showed a trend toward lower levels of fecal shedding in vaccinated cohorts as compared to the non-vaccinated cohorts. These preliminary data suggest a protective role for Johne's disease vaccine in combination with management changes in moderate to heavily infected herds.

Title
Changes in the prevalence of Map shedding following the commencement of paratuberculosis control programs using Gudair TM vaccine in Australian merino flocks

Author(s)
Eppleston J1, Windsor P2, Sergeant E3, Whittington R2.
Institution 1 Central Tablelands Rural Lands Protection Board, Bathurst, Australia; 2 The University of Sydney, Camden, Australia; 3 AusVet Animal Health Services, Orange, Australia.

Presentation Poster

Abstract We previously reported the results of Australian research demonstrating the efficacy of vaccination with GudairTM for controlling the impact of paratuberculosis in local Merino sheep flocks. Vaccination of lambs between 1 and 4 months of age in 3 high prevalence flocks reduced the prevalence of clinical disease by 90%, delayed the onset of faecal shedding by 12 months and thereafter reduced both the prevalence of shedders and the number of bacteria shed by 90 %, compared to unvaccinated control lambs. However, this trial involved only one generation of lambs that were part of flocks with a high prevalence of infection. In this paper we report on a follow up trial designed to monitor changes in shedding across 4 generations of vaccinated lambs in 12 flocks that varied in their initial disease prevalence. In the first two generations of vaccinates, 8 of the 12 flocks have experienced a substantial reduction in disease prevalence as measured by faecal culture 3 and 4 years post vaccination, compared to unvaccinated sheep of the same age measured 2 years previously. However, in the remaining four flocks this disease reduction has not been evident. The evidence suggests that management factors in addition to vaccination are required in some flocks to reduce the prevalence of infection.

Title An outbreak of Johne's disease in a newly established commercial goat farm leading to heavy losses and closure

Author(s) Singh SV, Singh AV, Singh PK, Sohal JS.

Institution Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO - Farah, District - Mathura (UP), India.

Presentation Poster

Abstract Johne's Disease has been frequently reported from organized herds of government, semi-government and state government (Mathur et al., 1981, Kumar et al., 1988, Koul et al., 1989, Sharma et al., 1987, Srivastava and More, 1987, Singh et al., 1998, Ram Kumar et al., 1998, Tripathi and Parihar, 1999, Goswami et al., 2000) and in farmer's herd from slaughtered goats (Kumar, 2004) in India. JD is endemic in goats, sheep, cattle and buffaloes population of the country (Singh et al., 1996, Kumar, 2002, Singh et al., 2007 a, b, c, d, Kumar et al., 2007 a and b, Yadav et al., 2007). However, Information on outbreaks of JD in goats is not available in India. Study aimed to diagnose a rare outbreak of severe weakness and diarrhea in a newly established commercial goat farm using indigenous ELISA kit and fecal culture and suggest possible control measures. Fecal and serum samples were collected randomly from kids (15) and adult goats (20) belonging to a herd of 100 goats suffering with weakness and diarrhoea. Fecal samples were processed for isolation of MAP using HEYM with mycobactin J. Serum samples were screened by indigenous ELISA kit. Of the 35 fecal samples, 77.1% were positive in culture in less than 2 months of incubation. Individually, 80.0% young kids (3-6 months of age) and 75.0% adult goats were positive. None of the samples were contaminated during incubation and no fast growing mycobacterial colonies appeared. Majority of the cultures were pauci-bacillary (65.7%) and only 11.4% were multibacillary or in super shedder category. Of the 35 goats, 40.0% were sero-positive in plate ELISA kit. Comparative evaluation of fecal culture with ELISA kit showed that fecal culture detected, 27 (77.1%), goats as compared to 14 (40.0%) sero-possitive in ELISA kit. Independently, 48.5% and 11.4% cases were detected by fecal culture and ELISA kit, respectively. The sensitivity and specificity of plate ELISA kit was 37.0% and 50.0%, respectively. Diminished humoral response due to anergy in advanced clinical stage may be responsible for lower sensitivity of ELISA. Outbreak was confirmed as that of Johne's disease caused by MAP. For control, farmer was advised vaccination-using
indigenous MAP strain, which was highly fruitful and improved the condition of animals, when again visited the herd after 2 months.

Title
Recovery in advance clinical cases of Johne's disease in naturally infected goats using a highly efficacious indigenous vaccine made from novel Indian strain of *Mycobacterium avium* subsp. *paratuberculosis* 'Bison type'

Author(s)
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Presentation
Keynote

Abstract
Few countries using vaccines have effectively decreased incidence of clinical JD. In this study efficacy of indigenous inactivated vaccine containing highly virulent field strain of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) 'Bison type' was compared with 'Commercial Inactivated Vaccine' for the recovery from clinical JD in naturally infected goats. A total of 71 adult, weak, infected (culture and sero-positive), ready to cull goats belonging to farm units of Central Institute for Research on Goats (CIRG), Makhdoom ( endemic for JD), were randomly divided into 3 groups. 'Group-A' of Indigenous vaccine, 'Group- B' of Commercial vaccine and 'Group- C' of Sham Immunized' controls. After vaccination, physical condition, body weight gained, fecal shedding of MAP, humoral and cell-mediated immune responses, mortality and morbidity etc., were monitored for 7 months. At the end of trial there was marked overall improvement in body condition of the vaccinated animals (Group A and B) as compared to 'Group-C'. Body coat showed marked improvement in shining, pliability, smoothness, regained luster and re-generation of hairs. Many of the goats kidded, delivering healthy kids and marked improvement in milk production. Average body weights gained per animal were significantly high (p<0.05) in the goats of Group-A as compared to Group B and C. Mortality and morbidity rates (due to diarrhea, weakness and Johne's disease) were lower in 'Group-A' goats as compared to 'Group-B' and 'Group- C' goats. Gross lesions in goats died and necropsied from vaccinated groups (Group A and B) during the study showed regression of lesions of JD and regeneration of fat, while 'Sham immunized' goats had frank lesions of JD. Lymphocyte transformation test (LTT) (indicator of cellular response) showed higher stimulation of PBMCs in 'Group-A' animals with MAP antigen as compared to PBMCs in 'Group-B' and 'Group-C' goats. Concentration of nitric oxide, (cellular response), was also higher in 'Group-A' goats followed by 'Group-B' and 'Group-C'. Goats of both vaccinated groups also had higher protective antibody titer in comparison to 'Sham-immunized' goats. Both the vaccines reduced fecal shedding remarkably but indigenous 'Bison type' vaccine was more effective. However, few of the very advance cases of Johne's disease did not recover and died eventually during the study and at necropsy had frank lesions of JD. Overall both the vaccines were effective in restricting MAP infection and JD in clinically infected goats but indigenous vaccine using native MAP 'Bison type' strain of goat origin was superior.

Title
Comparative efficacy of an indigenous 'Inactivated vaccine' using field strain of *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' with a commercial vaccine for the control of Caprine Paratuberculosis in India: a challenge trial

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Presentation
Poster
Abstract

Johne's disease (JD) is endemic in farm and farmer's goatherds in India. Despite using test and cull method for the control of JD in last 25 years, prevalence of JD was not reduced instead it increased in a farm goatherd. Study compared the efficacy of 'indigenous vaccine' using native 'Bison type' strain of Mycobacterium avium subspecies paratuberculosis (MAP) of goat origin with a 'Commercial Vaccine' against JD by involving challenge trials. It is the first indigenously developed vaccine against caprine JD in India. A total of 85 kids, 4-6 months in age were randomly divided in to 3 groups. Vaccinated groups ('Group-A' and 'Group- B' received 1 ml of vaccine subcutaneously) and Sham-Immunized ('Group-C' received 1 ml of sterile PBS). After 75 days post vaccination, goats in the 3 groups were challenged, (except 5 in each group for monitoring of CMI response), with live MAP 'Bison type' culture. Four goats each, males and females of Barbari and non-Barbari breeds, from 3 groups were sacrificed at 200 days post challenged to evaluate the carcass characteristics with respect to vaccine and challenge response on goats of different groups. Samples (Blood, serum and fecal for Lymphocyte transformation test, ELISA and MAP shedding, respectively) and data (live animal trait, mortality, experimental sacrifice for carcass evaluation and microscopic and macroscopic lesion) were collected and analyzed. Results of body weights gained during study periods (14 months) were analyzed breedwise and sexwise using ANOVA. Significant improvements were seen in the performance of vaccinated groups over 'Sham-Immunized' group. Goats of 'Group-A' gained higher (not significantly) body weights over both 'Commercial' and 'Sham-Immunized' groups. Mortality was observed only in 'Sham-Immunized' group (12%). Goats of both vaccinated groups had greater cell mediated immune response than 'Sham-Immunized' group throughout the period of vaccination trial. Humoral immune response also was higher in both vaccinated groups with significantly high rate of sero-conversion in vaccinated goats as compared to 'Sham-Immunized'. Results of post challenged fecal culture showed significant reduction (P<0.005) in excretion of MAP in both vaccinated groups than in 'Sham-Immunized'. A number of external (body confirmation) and internal traits of sacrificed goats for carcass characteristics, (fat deposition, gross lesion etc.) were analyzed. There was significant improvement in both the traits in case of vaccinated groups than 'Sham-Immunized' group. However, 'indigenous vaccine was superior in some aspects over commercial vaccine.

Title
Quick and early response to vaccination and improvements in production traits in a naturally infected breeding farm of Jamunapari goats in India using indigenous vaccine against Johne's disease

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Presentation
Poster

Abstract
Paratuberculosis or Johne's disease caused by Mycobacterium avium subspecies paratuberculosis is an infectious disease of ruminants, responsible for huge economic losses in animal production systems and has potential to affect international trade (OIE, 2004). In terms of various production losses (livestock traits) affected by the Johne's disease (JD) include; milk production, body condition, body weights, reproductive efficiency and culling rate of goats. Disease is endemic in goats in the northern region of India. At present country lacks control measures (vaccine) against JD. Endemicity of the MAP infection in Jamunapari farm has resulted in heavy losses due to morbidity, mortality and untimely culling of animals on the basis of Johne's disease and other health problems. So far the efforts to control disease in this farm of high milk yielding goats of Jamunapari breed, on the basis of screening of animals (general herds or suspected goats) by fecal examination / fecal culture and culling has not been successful, instead prevalence of disease continued to increase. Goats were under same system of management since last 5-8 years. Indigenous inactivated vaccine developed using native strain of Mycobacterium avium subspecies
paratuberculosis (MAP) 'Bison type' has been used to control disease in the breeding farm of Jamunapari goats (endemic for Johne's disease). Herd-level assessment was performed to check the efficacy of indigenous vaccine containing highly pathogenic field strain of MAP 'Bison type'. In Jamunapari herd 526 goats with high prevalence of JD (significantly high annual morbidity, mortality and culling) were vaccinated with 1 ml of indigenous vaccine subcutaneously, in the 3rd week of September, 2006 and were monitored for different physical traits (morbidity, mortality, body weight, milk yield, kidding rate, birth weight of born kids) and humoral response. PER and post-vaccination data for same period and season was compared. First visible and significant response was in coat colour, texture, skin luster and regeneration of hair, within one month of vaccination. There was significant improvement in body weights gained in feedlot experiments and general herds, age at first kidding, milk yield at 90 days interval, ready to culled and stunted kids, morbidity, mortality and humoral immune response. There was improvement in birth weights, body weights gained at 3 months, kidding percent and litter size. Indigenous vaccine showed overall improvement in all the production scores and traits and immune response in the goatherd of Jamunapari breed, after vaccination.
**Title**  
*Mycobacterium Paratuberculosis* and Crohn's Disease: That's What Epidemiology is All about

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**Presentation**  
Oral

**Abstract**  
*Mycobacterium avium* subsp *paratuberculosis* (Map) is the causative agent of Johne's disease (JD), a chronic and progressive intestinal disease in ruminants, which imposes large direct and indirect productivity losses on affected farms. Crohn's disease (CD) in humans has been characterized as a chronic, relapsing, and remitting inflammatory process of the digestive tract with protein losing enteropathy, general malabsorption and steatorrhea. Numerous studies have shown that genetic and environmental factors are the basis for the pathogenesis of CD. Twin studies and familial aggregation have provided compelling evidence for the heritable nature of CD, and strong familial pattern has been observed in CD patients. It has been reported that the lifetime risk for developing CD in the sibling of an affected person is approximately 30-40 times greater than that in the general population. To date, several human genes and loci have been identified that may contribute to CD such as the IBD1. There are several histopathological and clinical similarities between JD and CD. Because of these similarities, a mycobacterial cause of CD has been sought for more than 90 years! Although early studies did not detect Map in tissues from patients with CD by conventional staining and culturing techniques, in the late 80's researchers could isolate Map from tissue samples from patients with CD after many months of incubation, leading to renewed interest in a mycobacterial origin of CD. Recent serologic studies have demonstrated that up to 83% of CD patients showed evidence of serum antibodies to Map. However, molecular mimicry that can serve as target for cross-reactive immunity in CD has been recently described. To date, several antibodies have been identified in the serum from CD patients such as for *Pseudomonas fluorescens*, and *E. coli*. These antibodies are regarded as signals of abnormal responses to innate or foreign proteins, because they do not usually exist in the healthy population. The frequent use of the DNA insertion element IS900 as a tool for accurate identification, in addition to bacterial culture, has yield provocative but inconsistent results, probably because PCR cannot differentiate between viable Map and Map DNA. In these studies, 13%-100% of CD patients tested positive to Map. Other studies, however, have been unable to demonstrate Map DNA in CD tissue. One potential problem with these results is that some primers designed from IS900 can cross-react with closely related IS901 and IS902. Nevertheless, in addition to CD patients, studies have detected Map also in control subjects. It is possible that low incidence rates in CD patients' tissues may be more a reflection of the widespread and ubiquitous occurrence of mycobacterial organisms, than an indication that Map is a causative agent in CD. It has been also demonstrated that lactating mothers with CD shed Map in their breast milk. Unlike in JD, where Map can be detected in almost any clinical case, reports are mixed as to the presence or absence of Map at the site of the intestinal lesions. There is no sound epidemiological evidence that links exposure to Map to an increased incidence of CD disease, even though cows with clinical paratuberculosis do shed viable organisms in their milk at low levels (50 CFU/50ml milk), and the consumption of inadequately pasteurized dairy products has been a major source of concern for the potential spread of Map to humans. Standard temperatures and times for heat pasteurization of milk, especially either via standard holder methods (63.5°C for 30 minutes) or high-temperature, short-time methods (71.7°C for 15 seconds), have been shown to be insufficient to kill all Map in milk. In Great Britain it has been demonstrated that Map DNA is present in milk samples obtained from retail markets. On the other hand, some studies indicate that through adequately pasteurized dairy products (72°C for 15 seconds), the transmission of viable Map from animals to humans via can be made even less likely, thus minimizing any remaining potential concern that it may act as a zoonotic agent in CD. The culture, however, of viable Map from pasteurized retail milk samples, raised the concern that milk may become contaminated post pasteurization. Nevertheless, the detection of
Map DNA in retailed milk raised the obvious question whether people with lifetime or childhood intense exposure to dairy cows are more susceptible or more resistant to CD? In cattle, Map infection occurs in a very early age but clinical signs do not develop until at least 2 years of age. If Map were to behave in a similar manner in humans, it would be extremely difficult to associate exposure events occurring during childhood with the subsequent development of CD, perhaps many years later. No clinical evidence has shown an increased incidence of CD in farmers or agricultural workers associated with dairy herds with a high incidence of JD. Nor has eating organs or tissues from infected animals been documented to cause infection of humans with Map. Two studies one from England and another from Israel find no association between exposure to dairy or even to JD cows and CD. Another case-control study from the UK, found that the consumption of pasteurized milk was associated with reduced risk for CD (OR=0.82). Finally, a recent study in Ontario, Canada performed a systematic review of the literature on the subject, weighing in on the current evidence for or against a causal relationship. So far, despite the volume of research conducted to address this issue, the evidence for an association between JD and CD has not been strong. Because CD is a rare disease the appropriate epidemiological approach would be a large-population case-control study, looking for potential risk factors with strong association. These and other study results, even when supporting the presence of Map in CD patients, require us to reflect on two basic concepts in epidemiology: association and causality. An apparent association between Map and Chron's does not imply casual relationship. The issue of causality can only be addressed by examining disease onset in relation to exposure to the putative pathogen. Temporal association, one of the important criteria for causality, makes us wonder what came first? The egg or the hen i.e. Map or CD? Is it possible that Map is only an opportunistic bacteria? In the absence of suitable animal models, epidemiological studies are the most effective means of determining whether Map plays a causative role in the etiology of CD. As a conclusion it is suggested that although solid evidence insinuate an association between CD and Map, the proclamation of Map as the causal agent of CD and therefore JD as a zoonotic disease requires not only time, but large population epidemiological studies. Reference available from the author upon request.

Title
High prevalence of Mycobacterium avium subspecies paratuberculosis (MAP) DNA in the blood of healthy human blood donors and the effect of treatment with chronic anti-MAP antibiotic therapy in patients with Inflammatory Bowel Disease (IBD).

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Presentation
Oral

Abstract
Background: Although controversial, there is evidence that IBD may be caused by infection with MAP. Recently, three agents (5-ASA, methotrexate and 6-MP) used to treat IBD because of clinical efficacy, but without an accepted mechanism of action, have been shown to inhibit MAP growth in culture. The purpose of this study was to determine the prevalence of MAP DNA in the blood of healthy controls and patients with IBD, to determine the influence of chronic treatment with these anti-MAP antibiotics and to evaluate the associations between MAP DNA prevalence, disease activity and patterns of treatment by geographical location. Methods: The blood of
100 healthy individuals and 246 patients with IBD was evaluated for MAP DNA using nested PCR. Statistical analysis was by the Fischer Exact Test or Pearson Correlation as necessary. Results: MAP DNA was detected in 47% (47/100) of the healthy controls and in 16.3% (40/246) of all subjects with IBD (p<0.0001). MAP DNA was found in 15% (37/246) IBD patients, who were receiving any anti-MAP antibiotic therapy. The lowest MAP DNA frequency was observed with combined methotrexate, sulfasalazine, 6-Mercaptopurine or Ciprofloxacin therapy 3.1% (1/32) (p<0.02). The group receiving azathioprine (a precursor of 6-MP) combined with prednisolone was 42% (5/12) MAP DNA+, compared to the group with azathioprine without prednisolone that were 10.5% (4/38) MAP DNA+ (p<0.03). MAP DNA prevalence varied by geographical location and showed a correlation with disease activity and pattern of treatment (p<0.001). Conclusions: Analogous to leprosy, our data show an unsuspectedly high incidence of MAP DNA in healthy human blood donors. Chronic use of anti-MAP antibiotics is associated with a significantly lower incidence of MAP DNA, possibly an indication of therapeutic efficacy. The use of prednisolone is associated with an increased prevalence of MAP DNA. This may indicate prednisolone immunosuppression, and/or reflect IBD disease activity. The geographical variation may reflect different IBD therapy by local physicians and may become a useful indicator of therapeutic efficacy. These data are compatible with an etiological role of MAP in IBD that could be comparable with leprosy where for every case of clinical leprosy, >100 asymptomatic individuals shed *M. leprae* DNA.
Title Isolation, identification and characterization of *Mycobacterium avium* subspecies *paratuberculosis* from multiple clinical samples of Crohn's disease patients in India using microscopic examination, culture, ELISA and PCR tests

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Presentation Poster

Abstract In recent years, Crohn's disease (CD, chronic, relapsing inflammatory condition of bowel) has been reported with increasing frequency in India. However, etiology of CD still remains obscure. Present study investigated association of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in cases of CD using microscopic examination, culture, ELISA and PCR tests. A total of 18 samples (5 stool, 5 biopsies, 5 serum and 3 blood clot) were collected from 5 patients, diagnosed suffering with CD in the Gastroenterology department of All India Institute for Medical Sciences (AIIMS), New Delhi. Samples were screened for the presence of MAP and its antibodies. Isolation of MAP was done as per method of Whipple et al., (1991) with some modifications (Singh et al., 1998), on HEY medium with Mycobactin J. ELISA was performed as per method described by Singh et al., (2007) with some modification. Antigens derived from MAP 'Bison type' (S 5) of goat origin and MAP 'Bovine' strain were used at 0.1 microg / well and 2 microg / well concentration in ELISA, respectively. None of the stool, biopsies and blood clot sample were positive for acid-fast bacilli. Of the 5 CD patients, 4 (80.0%) were positive for MAP in culture of both biopsies and stool samples. MAP was isolated from 2 (66.6%) of 3 blood clots left after harvesting serum from CD patients. Same individual were also positive for MAP in stool and biopsies cultures. Majority of MAP colonies grew around 120 days of incubation on Herrold's egg yolk medium (HEYM) with mycobactin J (Allied Monitor, Inc, USA). Primary colonies were identified on the basis of cultural characteristics, bacterial morphology, slow growth, mycobactin J dependency, acid fastness and finally IS900 PCR. All CD patients were positive for MAP antibodies using antigen from MAP 'Bison type' strain. Using MAP 'Bovine' antigen detected only 2 (40%) of 5 CD patients positive for MAP. 'Bison type' antigen had better correlation with culture (stool and biopsies) from CD patients. Present study indicated that MAP may play an important role in the pathogenesis of CD, ELISA developed using MAP 'Bison type' S 5 antigen can be used as mass screening test for MAP infection in human. The study is the first report of association of patients MAP with CD in human beings, in India.

Title Association of *Mycobacterium avium* subspp. *paratuberculosis* with Type-I diabetes, a possible trigger

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Presentation Oral

Abstract *Mycobacterium avium* subspecies *paratuberculosis* (Map) is a zoonotic pathogen whose association with Crohn's disease in humans is under scrutiny. To investigate its association with other chronic diseases where the involvement of a persistent pathogen as Map could be the trigger Forty-six diabetic patients were recruited along with 50 healthy people as control. Map was searched in the PMBC by a specific PCR targeting IS900. Sequence product confirmed identity by sequencing. Also, all the diabetic patients revealed significant humoral immune responses to two recombinant Map antigens and the whole cell lysate of the Map bacilli. A total of 29 blood samples
out of 46 were found to be positive for Map specific PCR (63%) whereas only 8 out of the 50 healthy control samples (16%) generated a positive signal. Extremely significant humoral responses to recombinant HbHA and GSD proteins and the whole cell lysates of the Map bacilli were recorded in T1DM patients as compared to healthy controls. We report presence of Map DNA and Map specific antibodies in the blood of Type1 Diabetes Mellitus (T1DM) patients in an endemic setting like Sardinia. Finding evidence of Map involvement in T1DM is perhaps a novel finding that might serve as a foundation stone in establishing an infectious aetiology for T1DM.

Title	Thiopurine drugs (azathioprine and 6-mercaptopurine) inhibit Mycobacterium paratuberculosis growth in vitro

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Presentation
Oral

Abstract
The in vitro susceptibility of human and bovine-origin Mycobacterium paratuberculosis to the thiouprine drugs 6-mercaptopurine (6-MP) and azathioprine (AZA) were established using conventional plate counting methods and the MGIT 960 ParaTB culture system. Both 6-MP and AZA had antibacterial activity against M. paratuberculosis; isolates from Crohn’s disease patients tended to be more susceptible than were bovine-origin isolates. Isolates of Mycobacterium avium, used as controls, were generally resistant to both AZA and 6-MP even at high concentrations (≥ 64.0 µg/mL). Among rapidly growing mycobacteria, M. phlei was susceptible to 6-MP and AZA whereas M. smegmatis strains were not. AZA and 6-MP limited the growth of, but did not kill, M. paratuberculosis in a dose-dependent manner. Anti-inflammatory drugs in the sulfonamide family (sulfapyridine, sulfasalazine, and 5-amino-salycilic acid (mesalamine)) had little or no antibacterial activity against M. paratuberculosis. The conventional antibiotics azithromycin and ciprofloxacin (CPX) used as control drugs were bactericidal for M. paratuberculosis, exerting their killing effects on the organism relatively quickly. Simultaneous exposure of M. paratuberculosis to 6-MP and CPX resulted in significantly higher CFUs as compared to use of CPX alone. These data may partially explain the paradoxical response of Crohn's disease patients infected with M. paratuberculosis to treatment with immunosuppressive thiopurine drugs i.e., they do not worsen with anti-inflammatory treatment as would be expected with a microbial etiologic pathogen. These findings also should influence the design of therapeutic trials to evaluate antibiotic treatments of Crohn's disease: azathioprine drugs may confound interpretation of data on therapeutic responses both antibiotic-treated and control groups.

Title	The effect of chemical and physical stress factors on Mycobacterium avium subsp. paratuberculosis

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Presentation
Oral

Abstract
Mycobacterium avium subsp. paratuberculosis(MAP) is considered as a highly resistant species among mycobacteria towards various stress factors. The purpose of this study is to assess a resistance profile for MAP towards inactivation factors relevant in food processing, such as an increased temperature, UV-light pulses, chlorine dioxide and lactic acid. Two strains of MAP, a reference strain and a bovine isolate from faeces, have been subjected to the heat treatment (60-90°C at 10s, 20s
and 30s intervals), the chlorine dioxide treatment (6-12 ppm, 2 min. interval), and the lactic acid treatment (25, 50, 75 and 100 mg/ml, 2 min. interval). Two repeated cycles of inactivation by UV-light pulses with the same strains have been performed in order to induce resistant variants (number of pulses: 10-120). Controls are included in all experiments. The results of inactivation have been measured by the time to detection in liquid MGIT media and by the log mean CFU/ml on solid HEYM media. A not so clear effect of an increased temperature on MAP has been observed within the time intervals, even some kind of activation has been shown from the curves at 60-80°C. With UV-light pulses, only mild inactivation has been observed for both the first and the second repeated experiments, depending on the strain. On the other hand, when using chlorine dioxide, a strong effect of inactivation for the chosen range of concentrations has been demonstrated. Similarly, a strong effect of lactic acid on MAP has been documented. The reference strain, probably more sensitive than the bovine isolate, does not grow after some treatments on solid HEYM, but it grows in liquid MGIT. Thus, the use of the MGIT culture system may be promising for such studies or perhaps for primary isolation of the etiological agent of paratuberculosis. This work was supported by the "PathogenCombat" grant No. FOOD-CT-2005-007081 (Brussels, EC).

Title
Sero-prevalence of Mycobacterium avium subsp. paratuberculosis in spontaneous cases of abortions in human beings in North India using native and commercial protoplasmic antigens in indigenous ELISA kit

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Presentation
Poster

Abstract
Mycobacterium avium subsp paratuberculosis (MAP) is an important veterinary pathogen, implicated in Sarcodosis and Crohn's disease. MAP infection in the uterus and placenta may leads to congenital infection and abortion in animals. Present study aimed to estimate association of MAP with spontaneous cases of human abortions in North India. Indigenous ELISA kit (using native protoplasmic antigen (NPA) from MAP 'Bison type' genotype of goat origin), originally developed for screening of goats was adapted for screening of MAP infection in human beings (Singh et al., 2005). The purified protoplasmic antigen (PPA) of MAP 'Bovine' origin was procured from Allied Monitor, Inc., (USA) and used in place of NPA in this ELISA kit for screening of serum samples. Kit 1 and kit 2 were developed using NPA and PPA, respectively. Fifty serum samples (20 from Bareilly region of Uttar Pradesh and 30 from Punjab) from spontaneous cases of human abortions in North India were screened and 34.0 and 30.0% patients were positive by Kit 1 and Kit 2, respectively. Of the 20 samples (7 from District Female Hospital, 8 from Nildev Hospital, and 5 from Suvidha Hospital) from Bareilly region, 30.0% were positive by each Kit (Kit 1 and it 2). Individually 14.2, 50.0, 20.0% and 14.2, 37.5, 40.0% samples were positive in Distnct Female Hospital, Nildev Hospital and Suvidha Hospital by kit 1 and kit 2, respectively. While of the 30 sample from Punjab (Gurpreet Nursing Home), 36.6 and 30.0% samples were positive in kit 1 and 2, respectively. On comparative evaluation, 2 kits together detected total 42.0% patients positive for MAP antibodies. The 22.0% samples were detected as positive by both the kits. The agreement (positive and negative) between two kits was 80.0%. The 12.0% and 8.0% patients were detected independently by kit 1 and 2, respectively. In another study kit 1 and kit 2, detected, 100.0 and 40.0% serum samples positive from CD patients. The 80.0% patients were positive in stool culture and MAP colonies were characterized using IS900 PCR (personal communication). High sero-response to both MAP antigens (34.0% for NPA and 30.0% for PPA) indicated the contamination of patients with MAP of possible animal origin. Further isolation and genotyping is needed to confirm these findings.
| Title | Is *M. avium* subspecies *paratuberculosis* (MAP) the cause of multiple "autoimmune" and "inflammatory" diseases in man? Inferences from the anti-MAP activity of methotrexate, 6-MP, 5-ASA and thalidomide, on MAP in culture. |
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| Institution | VAMC Bronx USA |
| Presentation | Oral |
| Abstract | BACKGROUND: We have shown that the "immuno-modulators" methotrexate and 6-MP and the "anti-inflammatory" 5-ASA inhibit MAP growth (www.PLoSONE.org) and concluded that their most plausible mechanism of action in several idiopathic diseases is as antiMAP antibiotics. Thalidomide is an "immunomodulator" used in multiple "auto-immune" and "inflammatory" diseases and the mycobacterial diseases leprosy and tuberculosis. We now test the hypothesis that thalidomide inhibits MAP growth. METHODS Thalidomide (±) and (+) and (-) and its two components, phthalimide and 1-hydroxy 2,6 piperidine dione (HPD) were evaluated in culture of two strains each of MAP (ATCC 19698 [bovine] & Dominic [human]) and M. avium subspecies avium (ATCC 25291 & 101.) We used a radiometric (14CO2 Bactec®) detection system. Inhibition is indicated by "percent decrease in cumulative Growth Index" (%-DcGI) from control. RESULTS: Phthalimide has no dose dependent inhibition on any strain. There was no dose dependent inhibition on either M. avium strain with thalidomide or its components. With the two MAP strains, there is dose dependent inhibition with thalidomide (±); Dominic (31%-DcGI) and ATCC 19698 (26%-DcGI) at 64µg/ml. Thalidomide (+) is more inhibitory than (-). HPD is, on a weight for weight basis, the most inhibitory agent evaluated; Dominic (46%-DcGI) and ATCC 19698 (44%-DcGI at 64µg/ml) CONCLUSIONS: We show in vitro heretofore-undescribed inhibition of MAP growth by racaemic thalidomide. Thalidomide (+) is more potent than (-). Of thalidomide's two moieties, phthalimide has no antiMAP activity and HPD is the active component in inhibiting MAP growth. We suggest that since 1942, initially with 5-ASA, the medical profession has unknowingly been treating MAP infections. These data are compatible with our concern that MAP is zoonotic. We conclude that all idiopathic "autoimmune" and "inflammatory" diseases, empirically treated with medications that we show are active against MAP, should now be evaluated for MAP as the etiological agent. |

| Title | The coupling of culture enrichment with real time quantitative PCR (qPCR) to detect viable *Mycobacterium avium* subsp. *paratuberculosis* |
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| Presentation | Poster |
| Abstract | Considerable attention has been given to milk and dairy products as potential vehicles of *Mycobacterium avium* subsp. *paratuberculosis*(Map) transmission to the food chain. Surveillance of such products for the presence of Map has been hindered by difficulties in culture methodology. Molecular detection methods for Map, including endpoint PCR (duplex, multiplex and nested) and real time quantitative PCR (qPCR) have been widely reported. However, these methods cannot differentiate between DNA recovered from dead and viable cells. The method assessed in this study involved addition of test sample to enrichment broth, removal of initial sub-sample for Map-DNA extraction/qPCR followed by enrichment and a subsequent DNA extraction/qPCR assay. A significant reduction in the qPCR Ct value following enrichment is indicative of Map growth/recovery. Samples containing < 2 cfu/ml Map were enriched into Middlebrook 7H9 broth, Dubos broth and a customised enrichment buffer. |
broth. Following Map-DNA extraction using the Adiapure® kit and real time PCR (IS900 and F57), Map was undetected at time zero but was detected in 7H9 enrichment medium after 7 d (Ct: 36); 14 d (Ct: 25); 21 d (Ct: 20). Enrichment in Dubos medium and the customised broth resulted in slower rates of growth and qPCR detection. Raw milk cheeses (n = 14) were subject to PANTA-supplemented 7H9 enrichment/qPCR. No viable Map were detected (enrichment (>100 d)/qPCR) despite a number of the samples revealing Map presence prior to enrichment. All samples were negative by culture (7H10 and HEYM medium) after 20 wk incubation suggesting no viable Map present in cheese samples. Map- spiked pasteurised and raw milk samples were heat treated and subsequently enriched. Decreases in Ct values (qPCR) in the enriched pasteurised samples after 5 wk was indicative of recovery and growth of heat-treated Map cells whereas enriched raw milk samples presented problems for the PCR assay. This enrichment-qPCR strategy may represent a methodological option to screen samples for the presence of viable Map rather than liquid culture which can rely on continual monitoring, subculture and confirmation.

Title  Lethality of the milk spray-drying process for *Mycobacterium avium* subsp. *paratuberculosis* using a model system
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Presentation  Poster
Abstract  This work was undertaken to determine the efficacy of the milk spray-drying process for killing *Mycobacterium avium* subsp. *paratuberculosis* (Map). In the absence of pilot plant equipment with the necessary safety containment facilities a tripartite approach was adopted. Firstly, using *M. smegmatis* as a surrogate for Map and pilot plant equipment the organism was subjected to evaporation heat treatments which are a precursor to the spray-drying stage of milk powder production. *Mycobacterium smegmatis* did not survive evaporation treatments ranging from 75ºC for 15s to 85ºC for 4 min. Secondly, *M. smegmatis* was found to have a significantly higher (P<0.05) D value (D638.71 min) than Map (D636.78 min) at 63ºC using milk concentrate as the heating menstruum. Thirdly, milk powder from 3 plants within Northern Ireland was surveyed over a year period for Map using PCR assays based on the IS900 insertion element and culture. Although 9.5% (18/190) samples were positive by PCR no culture positives were obtained. This work provides credence for considering spray-drying as one strategy for dealing with milk suspected of containing viable Map and destined for human consumption.

Title  Evaluation of Real Time and End point PCR for the Detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in artificially-contaminated Raw and Pasteurised Dairy Products.
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Presentation  Poster
Abstract  Dairy products, such as cheese and yoghurt, are potential vehicles of transmission of *Mycobacterium avium* subsp. *paratuberculosis* (Map) to the food chain. In this study a method has been developed for the extraction and detection of Map DNA from raw and pasteurised dairy products; Gouda (semi-hard), Danish Blue (semi-soft) and
Munster (soft) cheese, along with plain full fat yoghurt, artificially-contaminated with three Map strains:- NCTC 8578, Niebuell and NIZO B2962. Cheese and yogurt samples were spiked with a range of Map inocula. Map-DNA was recovered from homogenised (sodium citrate-based) cheese and yogurt samples using the Adiapure® kit (Adiagene, France). Template DNA was assayed by real time PCR, targeting both IS900 and F57 genetic elements (ABI 7500) in addition to end point PCR using P90/P91 primers and the Adiavet® kit (Adiagene, France). Initial inoculum levels were determined by plate culturing on Herrold's Egg Yolk and Middlebrook 7H10 media. In real time PCR assays, higher sensitivities were achieved by probes targeting IS900 than the f57 target. Using the former, Map was detectable at < 20 cfu g⁻¹ in the cheeses Danish Blue and Munster and yogurt whereas Map extraction and subsequent real time detection was less sensitive for the harder Gouda cheese. This may imply a more efficient Map-DNA extraction from the matrices having a higher water content. For all matrices and Map strains assessed the endpoint Adiavet® kit provided a higher rate of detection/ improved sensitivity than endpoint derived P90/P91 (both based on IS900) PCR. However, the real time PCR assay represented a more sensitive molecular detection method than either end point PCR assay. The real time PCR method coupled with the Adiapure Map-DNA extraction kit represents a reproducible, sensitive and convenient assay for the detection of Map-DNA from a range of raw and pasteurised dairy products.

Title: Estimation of presence of Mycobacterium avium subspecies paratuberculosis in un-pasteurized (individual and pooled milk) and commercial pasteurized milk and milk products in India and its characterization using culture, ÉLISA and PCR

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Presentation: Poster

Abstract: Mycobacterium avium subspecies paratuberculosis (MAP) the cause of Johne's disease in ruminants and Crohn's disease in human beings escapes pasteurization temperature and liquid milk has been subject of intense research. India though is the highest milk producer also has largest population (403.8 million) of dairy animals in the world, however, status of MAP in un-pasteurized and pasteurized milk and milk products was not known. This pilot study was the first attempt to know presence of live cultivable MAP in branded pasteurized milk and milk products and un-pasteurized milk marketed in 3 major cities of North India for human consumption, using 3 sensitive diagnostic assays (culture, ELISA and PCR). Specific IS900 PCR was used to characterize MAP from positive cultures. Of the 43 samples screened by 3 tests, culture was most sensitive (58.1%) followed by PCR (23.2%) and ELISA (4.6%). In culture, 43.7, 72.2 and 55.5% un-pasteurized milk, pasteurized milk and milk products were positive. The 44.1, 34.8 and 20.9% were positive in culture of fat, sediment and both together, respectively and 12.1 and 87.8% cultures were multi (>10 colonies) and pauci-bacillary, respectively. Colonies first appeared on 45 DPI and continued to appear up to 120 DPI. PCR was used for screening of decontaminated pallets (Fat and sediment) of un-pasteurized and pasteurized milk and milk products, 6.2, 38.8 and 22.2% samples were detected positive, respectively. Specific IS900 PCR confirmed all the positive cultures as that of MAP. ELISA detected 12.5% lacto-antibodies in un-pasteurized raw milk samples only. Pasteurization improved the recovery of MAP in culture and PCR. Presence of MAP in the un-pasteurized milk indicated that livestock population was infected and may be cause of low productivity of the Indian livestock. In view of the increasing human population the dairy products are in high demand. High presence of MAP in the pasteurized milk and milk products are potential threat to human contamination with MAP in India.
Detection of *Mycobacterium avium* subsp. *paratuberculosis* DNA in commercially pasteurized cow's milk in Italy.

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**Poster**

**Abstract**

To confirm the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in commercially pasteurized cow's milk, several studies were carried out in European and American countries. In our research we examined 22 samples of retail HTST milk, collected from supermarkets in 3 Italian regions (Lombardia, Emilia-Romagna and Lazio) to verify the presence and the vitality of MAP. The cultures from the pasteurised milk were monitored for growth in Herrold's medium with Mycobactin J and PANTA antibiotics, with previous chemical decontamination on HPC 0,75%. The comparative evaluation of method DNA extraction between Adiapure-Adiagene and Qiagen kit was carried out. The first is based on the capture bacteria by means of magnetic uncoated beads and lysis by chemical-mechanical destruction, whereas with the second method bacteria were destroyed only by chemical lysis. The Quiagen kit results were less sensitive than the Adiapure-Adiagene. The extracted DNA from milk samples was assayed by magnetic uncoated beads (Adiagene-Adiapure) and after the lysis of bacteria obtained by chemical and grinding destruction with glass beads (Q-Biogen, FAST-PREP). To detect the presence of MAP genetic material, we developed IS 900 PCR multiplex (Adiavet-Adiagene) and IS 900 PCR nested home-made using the following primers: P90/P91 and P24/P25. The PCR products were analysed by agarose gel electrophoresis (1.5% agarose). The integrated procedure combining optimal DNA isolation and IS 900 PCR methods detection had a reproducible detection limit of about 10 MAP cells per ml when a starting sample volume of 10 ml of MAP-spiked milk was analyzed. A sample was considered positive when amplified product was noted at 229 bp for the IS 900 primers. All 22 cartons samples of pasteurized cow's milk, produced in Italy and purchased in different supermarkets, have been found culture negative. Only one milk sample resulted positive to commercial PCR kit and PCR home-made too. This milk sample from raw milk was collected from small dairy farms in the province of Rieti, which has the highest herd prevalence of paratuberculosis in the Latium region (52%, ±5%-CI 95%). This is the first report of MAP detection in retail milk in Italy, but now it is necessary to verify the vitality in dairy food (milk and cheese). For this reason further investigations are in progress relating to recovery of MAP from milk using more sensitive cultural method as non-radiometric MIGIT system without chemical decontamination of milk sample and successive typing by molecular techniques.
Animal challenge models are critical to evaluate potential vaccine candidates and to study host immune responses to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. Virtually all researchers have developed their MAP challenge model independently of others, resulting in a high degree of variability. The need to standardize challenge models for vaccine efficacy studies was a conclusion reached in August 2005 at the International Colloquium for Paratuberculosis "Role of Vaccination" workshop, held in Copenhagen, Denmark. An international expert committee of Johne’s Disease (JD) researchers was convened to review and develop guidelines for JD challenge studies in multiple animal species. Members of the committee included Murray E. Hines II, Judith R. Stabel, Raymond W. Sweeney, Frank Griffin, Adel M. Talaat, Douwe Bakker, Geart Benedictus, William C. Davis, Geoffrey W. de Lisle, Ian A. Gardner, Ramon A. Juste, Vivek Kapur, Ad Koets, Jim McNair, Greg Pruitt, Robert H. Whitlock. Parameters essential for the development of long term and acute infection models were outlined and harmonized to provide a template JD challenge model for cattle, goats, sheep, cervids, and mice. The intent was to develop and propose international standard guidelines for models that would gain acceptance worldwide. The consensus guidelines for models developed by this committee included recommendations for experimental challenge studies listed by animal species for strains of *Mycobacterium avium* subsp. *paratuberculosis* used, challenge dose, dose frequency, age of challenge, route of challenge, preparation of inoculum, method of quantifying MAP in the inoculum, experimental animal selection, quality control and minimal experimental endpoints. These models will be useful to study host-pathogen interactions, host immunity at the local and systemic level, and for evaluating vaccine candidates and therapeutics.