Abstracts from Oral and Poster presentations at the

7th International Colloquium on Paratuberculosis

Bilbao, Spain

June 11-14, 2002

Table Of Contents

Section 1 : Etiology

Section 1 : Etiology

Section 2 : Pathogenesis

Section 2 : Pathogenesis

Section 2 : Pathogenesis

Section 3 : Immunology

Section 3 : Immunology

Section 4 : MAP Culture

Section 4 : MAP Culture

Section 5 : Molecular Biology

Section 5 : Molecular Biology

Section 5 : Molecular Biology

Section 6 : Public Health

Section 6 : Public Health

Section 7 : Epidemiology and Control

Section 7 : Epidemiology and Control

Section 7 : Epidemiology and Control

Section 7 : Epidemiology and Control

Section 7 : Epidemiology and Control

Title Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. avium reveals potential diagnostic sequences.

Author(s) Bannantine JP[1*] , Zhang Q[2] , Li L[2] , Kapur V[2] .

Institution [1] National Animal Disease Center, USDA-ARS, Ames, IA, USA. [2] University of Minnesota, St. Paul, MN.

Abstract The genetic similarity between *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) and other mycobacterial species has confounded the development of *M. paratuberculosis*-specific diagnostic reagents. Random shotgun sequencing of the *M. paratuberculosis* genome in our laboratories has shown greater than 98% sequence identity with *M. avium* subsp. *avium* (*M. avium*) in some regions. However, an in silico comparison of the largest annotated *M. paratuberculosis* contigs, totaling 2,658,271 bp with the unfinished *M. avium* genome has revealed 27 predicted *M. paratuberculosis* coding sequences that do not align with *M. avium* sequences. BLASTP analysis of the 27 predicted coding sequences (genes) shows 24 do not match sequences in public sequence databases such as Genbank. These novel sequences were examined by PCR amplification with genomic DNA from eight mycobacterial species and eight independent isolates of *M. paratuberculosis*. From these analyses, 21 genes were found to be present in all *M. paratuberculosis* isolates and absent from all other mycobacterial species tested. One region of the *M. paratuberculosis* genome contains a cluster of eight genes, arranged in tandem, that is absent in other mycobacterial species. This region spans 4.4 kb and is separated from other predicted coding regions by 1,408 bp upstream and 1,092 bp downstream. The gene upstream of this eight gene cluster has strong similarity to mycobacteriophage integrase sequences. The GC content of this 4.4 kb region is 66%, which is similar to the rest of the genome, indicating this region was not horizontally acquired recently. Southern hybridization analysis confirms that this gene cluster is present only in *M. paratuberculosis*. Collectively, these studies suggest that a genomics approach will help in identifying novel *M. paratuberculosis* genes as candidate diagnostic sequences.

Title Characterisation of IS901 integration sites in the *Mycobacterium avium* genome.

Author(s) Inglis NF[*] , Stevenson K, Heaslip DG, Sharp JM.

Institution Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Scotland, UK.

Abstract Data is presented on the identification and characterisation of 14 chromosomal integration loci of the insertion element IS901 on the *Mycobacterium avium* (cervine strain JD88/118) genome. Thirteen of these integration loci have been mapped to their corresponding positions on individual contigs of the *Mycobacterium avium* (strain 104) genome (TIGR unfinished genome-sequencing project). One further integration locus, which could not be correlated with strain 104 (an IS901- strain), was shown to consist of at least 2 copies of IS901 lying in tandem. Nucleotide sequence analysis of 5(and 3(flanking sequences revealed putative ORFs on both plus- and minus-strands that are interrupted as a direct consequence of IS901 integration. One of the plus-strand ORFs was positively identified as the catalase HPII (katE) gene although no recognisable -10 or -35 promoter sequences could be detected upstream of the initiation codon. Database searches using an additional 65 plus-strand ORFs and a further 74 minus-strand ORFs revealed no significant homology with any other known genes. A consensus IS901 insertion target sequence compiled from 11 integration sites was in broad agreement with earlier reports that were based on only 2 such loci.

Title A PCR test based on a novel DNA sequence distinguishes between sheep and cattle strains of *Mycobacterium paratuberculosis*.

Author(s) Collins DM[*] , De Zoete M, Cavaignac SM.

Institution AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand.

Abstract In New Zealand, goats and deer can be infected with isolates of *Mycobacterium paratuberculosis* of either the cattle or the sheep type. For epidemiological purposes, it can be desirable to know which type is involved. While these two types can be distinguished by RFLP analysis using IS900 or by PCR amplification of IS1311 followed by restriction endonuclease analysis, a simple PCR test would be advantageous. We have identified a 0.3 kb DNA fragment from an isolate of the sheep type that contains a tandem copy of a 12 bp sequence. The same DNA fragment but without the tandem copy of 12 bp was found to be present in an isolate of the cattle type. One flanking sequence of the 0.3 kb fragment differs between the two types. A PCR test utilising these differences was constructed and has been applied to sheep (4 isolates) and cattle (3 isolates) types of *M. paratuberculosis* and also to *M. avium* (5 isolates). Primers specific for the cattle type gave a PCR product only for isolates of the cattle type and primers specific for the sheep type gave a PCR product only for isolates of the sheep type. Neither primer set gave a product for isolates of *M. avium*. Because of the nature of the DNA sequence differences between the sheep and cattle isolates it should be possible to construct a multiplex PCR that distinguishes clearly between cattle and sheep isolates in a single reaction. This test should be useful in any situation where it is important to distinguish quickly and cheaply between isolates of the cattle and sheep types.

Title Characterization of chemically generated *M. avium* subsp. *paratuberculosis* cell wall deficient forms (spheroplasts).

Author(s) Hines II ME* , Styer LE.

Institution University of Georgia, College of Veterinary Medicine, Tifton Veterinary Diagnostic and Investigational Laboratory.

Abstract Cell wall deficient forms (CWD, spheroplasts) genetically indistinguishable from *M. avium* subsp. *paratuberculosis* have been isolated from patients with Crohn's Disease and Sarcoidosis. These CWD organisms may be important in the pathogenesis of these diseases, as well as Johne's disease in other animal species. However, CWD forms are extremely difficult to isolate and cultivate. When cultured in vitro, they generally revert to cell wall competent forms. Since sufficient quantities of CWD could not be obtained for experimentation, Naser and co-workers recently developed a method to chemically generate large numbers of *M. avium* subsp. *paratuberculosis* CWD. In this study, chemically generated *M. avium* subsp. *paratuberculosis* 19698 CWD were compared to cell wall competent *M. avium* subsp. *paratuberculosis* 19698 organisms. Organisms were evaluated by electron microscopy, chemotype profile (using matrix solid phase dispersion and thin-layer chromatography), silver stained SDS-PAGE gels, and Western blots. Marked differences in organism morphology, chemotype profile, presence of proteins and glycosylated compounds, and recognition of antigens by Johne's Disease positive and negative bovine control serum were detected between CWD and cell wall competent forms of *M. avium* subsp. *paratuberculosis* 19698.

Title Factors affecting the survival of *Mycobacterium paratuberculosis* in soil.

Author(s) Schroen CJ, Kluver PF, Butler K, McDonald WL, Hope AF, Condron RJ* .

Institution Victorian Institute of Animal Science. 475 Mickleham Road Attwood 3049, Australia.

Abstract To eliminate Johne's disease from an infected farm or to prevent transmission, it is essential that susceptible animals are not exposed to an environment contaminated with *Mycobacterium paratuberculosis*. *M. paratuberculosis* is capable of persisting in the environment for long periods due the high lipid content in the cell wall and the metabolic inactivity of the organism. Physical factors that could influence the survival of *M. paratuberculosis* in soil including temperature, pH, exposure to ultra-violet light and moisture content were investigated under controlled conditions. The experiment involved trays of contaminated soil randomised 2 x 2 x 2 x 2 x 3 factorial (48 unique treatments) featuring implied replication. Four naturally occurring soils with each combination of high or low pH and organic matter were mixed with faeces from a cow with clinical Johne's disease. The experimental temperature, moisture and ultra-violet conditions were applied to soil in individual trays and survival of *M. paratuberculosis* was

measured by proportional recovery using double incubation and Bactec culture methods. Dry soil and high soil temperature (30ºC) were the most significant factors in reducing the recovery of *M. paratuberculosis* from soil. Wet and dry cyclic conditions resulted in intermediate recovery of *M. paratuberculosis* compared to soil exposed to either wet or dry conditions. The effect of soil pH was minor but results were confounded with soil organic matter and soil type. Ultra-violet exposure appeared to have no direct effect on survival of *M. paratuberculosis* in soil however summer periods of high sun exposure with consequent elevated soil temperatures and dry conditions will be most effective in reducing the persistence of *M. paratuberculosis* in the environment. In strategies for on-farm control of Johne's disease consideration needs to be given to farm environments where poor drainage or permanent water and shading would enhance the survival of *M. paratuberculosis*.

Title Effects of ammonia treatment on viability of *Mycobacterium avium* subsp. *paratuberculosis* inoculated in low moisture roughage.

Author(s) Katayama N* , Kamata GS, Yokomizo GY.

Institution Shizuoka Toubu Livestock Hygiene Service Center, Nippon Veterinary and Animal Science University, National Institute of Animal Health.

Abstract Self-supplying forage might be a possible source of the infection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in a farm where paratuberculosis has been diagnosed. We investigated the effects of ammonia treatment under various conditions on the viability of MAP inoculated in low moisture roughage. One hundred gm of ground alfalfa hay was put into a resin bag(180x240mm), and was used as a container for ammonia treatment. Ammonia treatment on a laboratory scale was performed by adding NH4OH and a filter paper containing inoculum into it before sealing. The viability of MAP was decreased in conditions more advantageous to chemical reactions that promote high concentration, high temperature, and a prolonged high moisture treatment period. However, by the addition of 1 and 2% concentrations of ammonia treatment, MAP was observed to survive under low temperature and low moisture conditions, with its number decreasing under conditions in which material moisture or temperature was kept higher than it. At a 3% concentration, except for the 1st day of 10 and 20% moisture conditions, since any survivor of it was not observed. Since weak correlation coefficients between viability and material moisture, processing temperature, ammonia concentration, the number of treatment dates were determined as -0.39, -0.38, -0.34, and -0.26, respectively, the following methods are for reducing the viability rate are presented in the descending order of their effectiveness: adding water to material > increasing of processing temperature > increasing of ammonia concentration > extension of processing period.

Title Protein expression by nonreplicating persistent *Mycobacterium paratuberculosis*.

Author(s) Sung N* , Collins MT.

Institution University of Wisconsin-Madison, School of Veterinary Medicine, Department of Pathobiological Sciences, 2015 Linden Dr. Madison, WI 53706, USA.

Abstract

Background.

Mycobacterium paratuberculosis (Mptb) persists in host macrophages and in the environment. Protein expression patterns may vary depending upon its growth phase.

Purpose.

To contrast protein expression of Mptb ATCC 19698 in persistent, stationary and reactivation growth phases.

Methods and materials.

Mptb was cultured in Middlebrook 7H9 broth with OADC, Tween 80 and mycobactin J at 37°C. After 5 years in undisturbed upright tissue culture flasks with tightly closed caps the cells were viable, nonreplicating and thus considered persistent. Cells were harvested from the

sistent phase cultures, from reactivated cultures (cells from the persistent phase cultures put to new media) and from stationary growth cultures (i.e., 4 week old cultures from frozen seed lot). Proteins from the three growth phases were evaluated by SDS-PAGE.

Results.

Persistent Mptb cells were reactivated when inoculated into 7H9 medium with OADC, but not in modified Watson-Reid or 7H9 medium enriched with glycerol and dextrose. Soluble proteins extracted from the persistent Mptb yielded 8 protein bands on SDS-PAGE gels. However, about 25 protein bands were seen from the stationary growth phase cells and the reactivated Mptb cells. Expression of a 40 kDa protein was higher and expression of two proteins (27 and 23 kDa) was lower in reactivated Mptb than in cells from the stationary culture.

Conclusions.

Mptb persists in a viable state for 5 years or longer. Fewer soluble proteins were found in persistent phase cells. Persistent Mptb resumes growth in 7H9 medium enriched with OADC, but not in 7H9 with glycerol and dextrose or in modified Watson-Reid medium. This finding implies that fatty acids may be needed to support reactivation of persistent Mptb.

Title Effect of Lacticin on the growth of *Mycobacterium avium* paratuberculosis (MAP).

Author(s) Murphy P[1*] , Hill C[2] , Auty M[1] , Ross P[1] .

Institution [1] Teagasc Moorepark Fermoy Co.Cork. [2] University College Cork Ireland.

Abstract

Introduction.

The bacteriocin, Lacticin 3147 has been shown to be highly effective in controlling the growth of Gram positive pathogens such as Listeria monocytogenes and Staphylococcus aureus. Lacticin is a dipeptide produced by Lactococcus lactis which attaches to microbial cell walls creating pores in the cytoplasmic membrane leading to cell death. The purpose of this study was to determine if MAP, with its resistant cell wall was susceptible to inactivation by lacticin.

Materials and methods.

MAP (ATCC 19698) was grown in static and stirred culture at 37ºC in 7 ml bijou containers. 3 ml volumes (in triplicate) of Middlebrook medium were inoculated with MAP (10^5-10^6 cfu/ml) in the presence and absence of lacticin (1280 AU/ml). Negative controls containing lacticin but no inoculum were also included. At selected time intervals 0.1ml was pipetted to the wells of a microtitre plate and the absorbance at 620nm measured.

Results.

In stirred culture and in the absence of lacticin there was no evidence of growth of MAP up to day 11. Growth was detected when measured on day 21 and increased steadily thereafter resulting in a fine suspension with a minimum of deposit. Comparable data generated in the presence of lacticin indicated absence of growth under these conditions. The growth curve for the organism was less uniform in static culture a reflection of the pellicle nature of growth under these conditions and in this instance lacticin appears to retard rather than prevent growth.

Discussion.

Mycobacteria have a complex exterior making them resistant to many antimicrobial agents including antibiotics whose action is usually species specific. Some mycobacteria have a tendency to form clumps and in this form they may have increased resistance to inhibitors. It is possible that reduced clumping of cells in stirred culture makes MAP more susceptible to inhibition by the bacteriocin.

Title Molecular characterisation of *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from goats.

Author(s) de Juan L[1*] , Mateos A[1] , Domínguez L[1] , Sharp JM[2] , Stevenson K[2] .

Institution [1] Dpto. Patología Animal I. Fac. Veterinaria. U.C.M., Madrid, Spain. [2] Moredun Research Institute, Edinburgh, UK.

Abstract Molecular characterization of *Mycobacterium avium* subspecies *paratuberculosis* strains is a helpful tool for a better understanding of the epidemiology. Therefore, several molecular techniques have been tested for their suitability to differentiate M.a.p strains isolated from different animal species in the last decade. The objective of this study was to characterise caprine *M. a. paratuberculosis* strains from different geographical locations by Pulsed Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism and hybridisation to IS900 (RFLP-IS900) and IS1311 Polymerase Chain Reaction-Restriction Enzyme Analysis (PCR-REA) to determine the genetic variation amongst the caprine isolates and the relationship with strains isolated from other animal species. The study included 38 caprine isolates of M.a.p from Spain (n=25), Scotland UK (n=11) and Norway (n=3). All the isolates were subjected to PCR analysis for IS1311 combined with restriction analysis (Hinf I and Mse I) according to Marsh et al. 1999. All the samples studied were found to be of the cattle (C) type with the exception of two Spanish strains that were of the sheep (S) type. DNA for PFGE and RFLP analysis was prepared from stirred broth cultures of M.a.p isolates and restricted with SnaB I and Spe I (for PFGE) and BstE II and Pvu II (for RFLP-IS900). Combination of PFGE and RFLP results obtained with both restriction enzymes gave a total of 12 and 6 'multiplex' profiles, respectively. Novel profiles were identified with both techniques. The data were subjected to phylogenetic analysis, which revealed that the caprine isolates have an interesting genetic relationship with respect to strains isolated from other animal species. Attendance to this Congress was sponsored by the EU-funded project FAIR6-CT98-4373.

Title Molecular characterisation of pigmented and non-pigmented isolates of *Mycobacterium avium* subspecies *paratuberculosis*.

Author(s) Stevenson K[1] , Hughes VM[1*] , De Juan L[2] , Inglis NF[1] , Wright F[3] , Sharp JM[1] .

Institution [1] Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ Scotland, UK. [2] Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense de Madrid, Av. Puerta de Hierro s/n, 28040 Madrid. [3] Biomathematics and Statistics Scotland, Scottish Crop Research Institute, Dundee DD2 5DA, Scotland, UK.

Abstract Five pigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis* were examined by pulsed-field gel electrophoresis (PFGE), IS900 restriction fragment length polymorphism (IS900-RFLP) and by IS1311 polymorphism analysis using PCR. All of the pigmented isolates exhibited one of three distinct PFGE profiles with SnaB I designated 9, 10 and 11 and with Spe I designated 7, 8 and 9, which generated three multiplex profiles designated [9-7], [10-8] and [11-9]. All of the pigmented isolates had the same IS900-RFLP BstE II and Pvu II profiles. The IS900-RFLP BstE II profile was new but the IS900-RFLP Pvu II profile corresponded to Pvu II type 6 of a sheep strain described by Cousins et al. (Aust. Vet. J. 2000 78:184-190). IS1311-PCR analysis typed all of the pigmented isolates as sheep (S) strains. The genetic relationship between pigmented and non-pigmented isolates was investigated using multiplex PFGE data from the analysis of both the five pigmented isolates and 88 non-pigmented isolates of *M. avium* subsp. *paratuberculosis* from a variety of host species and geographic locations. It was possible to classify the isolates into two distinct types designated Type I, comprising the pigmented isolates, and Type II comprising the non-pigmented isolates that exhibit a very broad host range.

Title Strains characterization by PCR and RFLP from *Mycobacterium avium* subsp. *paratuberculosis* isolates of red deer with paratuberculosis.

Author(s) Verna A[1*] , Morsella C[1] , Zumárraga M[3] , Gioffre A[3] , Romano M[3] , Cataldi A[3] , Paolicchi F[1,2] .

Institution 1 Laboratorio de Bacteriología, Departamento de Produccion Animal, EEA INTA. 2 Facultad de Ciencias Agrarias, Universidad Nacional Mar del Plata, CC 276, Balcarce (7620), Argentina. 3 Instituto de Biotecnologia, CNIA-INTA Castelar.

Abstract It is important to develop the polymerase chain reaction (P.C.R.) and the restriction fragments of longitude polymorphism (R.F.L.P.) technologies to identify genome segments since they constitute quick, sensitive, and specific methodologies for the detection of *Mycobacterium avium* subsp. *paratuberculosis*, a microorganism that produces animal Paratuberculosis and that is related to Crohn's disease in humans. Objective: isolate *M. paratuberculosis* strains and to apply a P.C.R. protocol to confirm the IS900 insertion sequence, by R.F.L.P the genetic pattern of the strains isolated and to analyze cellular and extracellular proteins expressed in *M. paratuberculosis*. Twelve *M. paratuberculosis* strains, the strains isolated developed only in mycobactin Herrold medium, showing their characteristic dependence. The analysis by P.C.R. was positive starting from strains developed in the culture medium. When faeces were inoculated with a *M. paratuberculosis* strain, the detection rate was of 100%, demonstrating the highest specificity for IS900 in a 1:1000 dilution of the problem sample, but it was negative starting from the raw sample (faeces). All the isolates revealed an identical R.F.L.P. pattern, with a 217-bp probe located to the right of the cut of the BstE11 endonuclease. All of the isolates analyzed from this deer farm possessed R.F.L.P.-BstE11 "A" pattern. Inmmunoblotting detected 65 kDa (thermal shock), 42 kDa, 35 kDa, and 28 kDa protein antigens, from the bacterial extracts as well as directly from the tissues corresponding to such an isolate from an animal with clinical symptoms and characteristic lesions of Paratuberculosis in the organs. The negative results of the immunoblotting of most of the strains cultured could be because the mycobacterial growth was not enough to detect the proteins. However, these tests could be the basis of a program to control Paratuberculosis in deer, especially if multiple tests are used to ensure that as many as possible of the infected animals are detected.

Title Isolation and analysis of nine novel *M. avium* subsp. *paratuberculosis* specific single-copy genetic elements.

Author(s) Klitgaard K.

Institution Danish Veterinary Institute.

Abstract *Mycobacterium avium* supsp. *avium* (*M. avium*) and *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) share a genetic identity of more than 95% and presently, only a few *M. paratuberculosis* specific fragments have been described. However, the fundamental differences in habitat and virulence between *M. avium* and *M. paratuberculosis* seem to indicate, that factors exist, that condition these differences. It is possible, that identification of such genetic factors will offer an opportunity to improve existing diagnostic tests and perhaps allow new insight to the mechanisms of the disease Paratuberculosis. In this experiment the method of subtractive hybridisation of *M. paratuberculosis* against *M. avium* was used to isolate nine novel *M. paratuberculosis* specific sequence fragments of between 318 and 596 bp. Database search revealed little or no similarity with other mycobacteria, including *M. avium*. For each of the nine fragments specific primers were designed and assessment by PCR demonstrated, that the fragments, isolated by subtractive hybridisation, were present in the type strain (ATCC 19698) and a number of field strains of *M. paratuberculosis* but absent from *M. avium* and a number of mycobacterial species found in the environment. In Southern blots, all nine fragments appeared to represent probes capable of distinguishing between *M. avium* and nine other mycobacteria. RT-PCR with fragment specific primers resulted in amplified product of the expected size for all except one of the subtracted elements. This result seems to indicate, that eight of the nine fragments origin from areas of the genome that are expressed in vitro. The presence of open reading frames in these putative in vitro expressed *M. paratuberculosis* specific genetic elements implies, that some of these fragments origin from genes that encode *M. paratuberculosis* specific proteins and consequently will be of interest to isolate and characterise further - both with respect to possible antigens or virulence genes.

Title Integrated proteomics approach to identification of novel antigens in *M. avium* subspecies *paratuberculosis*.

Author(s) Shiell BJ, Beddome G, Vaughan JA, Stiles PL, Stewart DJ, Michalski WP* .

Institution CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Abstract With genome sequencing now almost routine in application, interest has expanded to the information embedded in the DNA sequence. Recent advancements in protein sciences and the technological development in proteomics allow exploration of gene products and functions on a larger scale. The availability of the almost complete *M. avium* subspecies *paratuberculosis* genome information and improved proteomics platforms provides an opportunity for the accelerated identification of antigens suitable for improved diagnosis and possibly prevention of Johne's disease. Currently, in our laboratory, an integrated proteomics approach consisting of liquid-phase (preparative isoelectric focusing and gel elution) and solid-phase (1- and 2-dimensional electrophoresis) separation platforms are employed in the study of secreted antigens of *M. avium* and *M. avium* subspecies *paratuberculosis* suitable for improved diagnosis. The liquid phase separation approach allows continuous monitoring of IFNg release assay activity (BOVIGAM™, CSL Animal Health Ltd) of the fractionated protein samples whereas 2-dimensional electrophoresis provides high-power resolution of individual components. Amino acid sequencing and mass spectroscopy are employed in the identification of potentially active proteins. Secreted proteins were derived from "time-course" grown cultures of *M. avium* (2 to 10 weeks) and *M. avium* subspecies *paratuberculosis* (7 to 34 weeks) and are being analysed using the integrated approach. A number of antigens have been already identified and tested in the IFNg release assay in conjunction with recombinant antigen screening.

Title Characterisation of MIRUs loci in Map and other *M. avium* spp.: application to PCR based genotyping.

Author(s) Sidi-Boumedine K* , Bull TJ, McMinn EJ, Skull A, Hermon-Taylor J.

Institution Department of Surgery, St George's Hospital Medical School, London UK.

Abstract Mycobacterial interspersed repetitive units (MIRUs) are short repetitive DNA elements found as tandem repeats in intergenic regions of mycobacterial genomes. MIRUs display variations in tandem repeat copy numbers and exhibit minor sequence variations between repeat units. Although MIRUs may be useful for fingerprinting, their function remains largely unknown, however it has been proposed that they may play a role in genomic structure and rearrangements, differential translation of genes, mRNA stability and transcriptional termination. Using a 53bp MIRU consensus sequence from Mtb BLAST searched onto *Mycobacterium avium* subspecies *avium* (Maa) 104 genome we have identified 25 MIRU loci each containing 1-5 tandem MIRU copies. Using this data we BLAST searched a Maa MIRU consensus onto the *Mycobacterium avium* subspecies *paratuberculosis* (Map) genome and identified a total of 16 MIRU loci, 9 of which contained single MIRU with 100% homology to Maa. Seven MIRU were found to display variations in arrangement and copy number compared to the corresponding loci in Maa. Based on these polymorphic loci, we have developed a PCR-based typing method for Maa, *Mycobacterium avium* subspecies *silvaticum* (Mas) and Map strains. When MIRU PCR was applied to a panel of Maa, Mas and Map this showed 100% differentiation of species and 4 different profiles amongst Map strains. Results of MIRU PCR on Map vaccine strains also demonstrate a unique MIRU profile. This work demonstrates a new heterogeneity of Map strains and provides a new method for the molecular identification of Maa, Mas and Map.

Title Standardization of amplified fragment length polymorphism for *Mycobacterium avium* subspecies *paratuberculosis*.

Author(s) Kiehnbaum L* , Amonsin A, Wells SJ, Kapur V.

Institution University of Minnesota, USA.

Abstract The molecular ecology of *Mycobacterium avium* subspecies *paratuberculosis* in the U.S. is poorly characterized. A discriminatory, reproducible method of DNA fingerprinting is needed to

elucidate the clonal diversity of *M. paratuberculosis*. However, current methods of DNA fingerprinting *M. paratuberculosis* are generally labor-intensive, relatively slow, and do not yield high discriminatory power. The development of amplified fragment length polymorphism (AFLP) in a commercially available kit has introduced a fast, reproducible, highly discriminatory method for DNA fingerprinting. AFLP uses restriction digestion of the whole genome with two enzymes, ligation of adaptors to create unique ends, a non-selective PCR amplification, and a selective PCR amplification using fluorescent dye-labeled primers resulting in a completely computerized output. Objectives of this study were to standardize AFLP for use on *M. paratuberculosis* and compare it to a currently available method, multiplex PCR using IS900 (MPIL). Twenty-seven bovine isolates of *M. paratuberculosis* on agar slants from a small, Johne's-infected herd in Pennsylvania, U.S.A, were received and grown again in liquid culture. DNA was extracted using enzymatic lysis and standard phenol-chloroform extraction methods. MPIL was modified from the previously published protocol. Three selective primer combinations were ascertained to be optimal for AFLP, they are named A, B, and C here. Using MPIL, all 27 isolates belonged to the M1 subtype. For AFLP, dendrograms were created using the Dice coefficient, UPGMA, and the similarity cut-off set at 95%. A, B, and C individually yielded six, five, and eight different profiles, respectively, for the 27 isolates. The concordance values are good for comparisons between each primer combination. In conclusion, AFLP was faster, easier to use, and more discriminatory for DNA fingerprinting of *M. paratuberculosis* compared to MPIL for the isolates in this study.

Title Molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* based on variable numbers of tandem DNA repeats.

Author(s) Nishimori K[1*] , Uchida I[1] , Tanaka K[1] , Nishimori T[1] , Tachibana S[2] , Nakaoka Y[2] , Uemura Nobuko [2] , Tamada Y[2] , Inahara K[2] , Jinma K[2] .

Institution [1] National Institute of Animal Health, Japan. [2] Livestock Hygiene Service Centers of Hokkaido, Japan.

Abstract Fingerprinting method based on variable numbers of tandem DNA repeats (VNTR) in 17 loci of *Mycobacterium avium* subsp. *paratuberculosis* genome was developed using BLAST search and PCR. 7 allele profiles were detected in 184 isolates. They show relatively homologous profiles compared with those of reference strains of *M. avium*. It was suggested that this method opened the rapid and easy way to the construction of digital databases for molecular epidemiology studies of not only *M. avium* subsp. *paratuberculosis* but also *M. avium*.

Title RFLP analysis of *Mycobacterium avium* subsp. *paratuberculosis* for the study of molecular epidemiology.

Author(s) Bartos M[*] , Svastova P, Yayo Ayele W, Machackova M, Pavlík I.

Institution Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic.

Abstract A total of 1 750 strains of *Mycobacterium avium* subsp. *paratuberculosis* isolated from cattle, sheep, goats, wild ruminants (moufflon, deer etc.), non-vertebrates, small vertebrates (cat, mouse, rabbit etc.), external environment, milk from cattle, moufflon, deer, fallow deer and other animals and Crohn's patients were examined by the use of standardised RFLP method with two restriction endonucleases (RE) PstI and BstEII, and standard IS900 probe prepared by PCR a non-radioactive labelled. Strains were obtained from Europe (15 countries), USA (13 states), Canada, Africa (2 countries), Australia, and New Zealand. After digestion by RE PstI, 15 RFLP types (A-O) were detected; whereas digestion by RE BstEII resulted in 34 RFLP types (C1-C29, I1-I2 and S1-S3). A combination of both BstEII and PstI RFLP results revealed o total of 40 RFLP types. We have analysed the similarity of certain BstEII RFLP types and noticed that some RFLP profiles might have been a mixture of several different strains. Such isolates were subcultivated and progeny from single bacterial colony was analysed. By this way one "new" BstEII RFLP type was identified as mixture of two "known" RFLP types. For more detailed differentiation of PstI RFLP types three different hybridisation probes were developed. As RE PstI digests inside of the element IS900. Due to this fact one probe was designed so that it

covered both part of the digested fragments (total probe) and two half-probe hybridised to different halves of IS900. A combination of hybridisation by these three probes enabled to identify new RFLP subtypes inside original types. RFLP typing was used for the epidemilogical studies in animal and human populations, in the environment and for the study of localisation of the causal agent in the host organism's tissue. Supported by the grants FAIR6-CT98-4373 and QLK2-CT-2000-00928 (Brussels, EC).

Title Bovine macrophage gene expression during the phagocytosis of *M. paratuberculosis*.

Author(s) Tooker BC* , Coussens MJ, Coussens PM.

Institution Department of Animal Science and Center for Animal Functional Genomics, Michigan State University, East Lansing, MI, USA.

Abstract *Mycobacterium paratuberculosis* (*M. paratuberculosis*) is a facultative intracellular bacteria and the causal agent of Johne's disease in cattle. *M. paratuberculosis* and other Mycobacteria (in general) have the ability to survive and proliferate within the phagosomes of host macrophage cells by arresting vesicle maturation. How Mycobacteria are able to accomplish this unique survival tactic is not well understood. Recently, our group has taken a functional genomics approach to determine if phagocytosis of Mycobacteria affects macrophage gene expression and how these interactions may lead to reduced phagosome maturation. DDRT-PCR was used, as an initial unbiased high throughput-screening tool that allows the macrophage to "tell" us which genes may be of interest during the general process of phagocytosis and during the phagocytosis of *M. paratuberculosis* specifically. We have compared the RNA expression profiles of macrophage cells at a 60-minute time point during a negative control of no phagocytosis with the expression profiles of RNA from macrophages during phagocytosis of *E. coli*, *M. paratuberculosis* and latex beads (a positive phagocytosis control). To date we have identified over 380 separate amplicons representing genes whose expression appears to change during the general process of phagocytosis. A subset of amplicons has been cloned and subjected to dot-blot and Northern blot hybridizations to confirm differential expression observed by DDRT-PCR. Amplicons exhibiting differential expression by Northern hybridization were then subjected to direct DNA sequencing and BLAST analysis for probable identification. Our initial results indicate that macrophage gene expression profiles change dramatically during the general process of phagocytosis and that gene expression profiles during the phagocytosis of *M. paratuberculosis* are distinctly different from gene expression profiles during phagocytosis of a readily degradable bacteria such as *E. coli*. Genes discovered thus far appear involved in a variety of functions such as energy metabolism, calcium binding, cell signaling and macrophage migration.

Title Gene Expression Profiling of Cattle Infected with *M. paratuberculosis*.

Author(s) Coussens PM* , Sipkovsky S, Abouzied A, Wiersma K, Suchyta S.

Institution Michigan State University Department of Animal Science and Center for Animal Functional Genomics.

Abstract A bovine-specific cDNA microarray system was used to compare gene expression profiles of peripheral blood mononucler cells (PBMCs) from control uninfected, clinical Johne's disease positive, and subclinical Johne's positive Holstein cows. PBMCs from control, cows and an early clinical Johne's disease positive cow responded similarly to the general mitogen ConA, with activation of 119 genes, of these, 104 were identical between the two animals. Stimulation of PBMCs from the control uninfected animal with *M. paratuberculosis* had little effect on immune cell gene expression (activation of 6 genes involved in phagocytosis). Stimulation of PBMCs from the clinical Johne's disease positive animal with *M. paratuberculosis*, however caused significant (fold change > 1.5, $P < 0.05$) down-regulation in expression of over 30 immune cell genes and activation of many others (>100). Analysis of a second, more severely affected animal validated these initial results. Exposure of PBMCs from the subclinical Johne's disease positive cows to *M. paratuberculosis* resulted in significant (fold change >1.5, $P < 0.05$) activation of numerous genes. In sharp contrast to the clinical infected animal PBMCs, there were no genes whose expression was repressed following exposure of PBMCs from the subclinical animals to *M. paratuberculosis*. In fact, the pattern of immune cell gene activation observed in PBMCs from the subclinical Johne's positive cows exposed to *M. paratuberculosis* was extremely similar to that of PBMCs exposed to ConA, a general T cell mitogen. Thus, within the confines of a small sample set, the overall response of PBMCs from cows with clinical Johne's disease to *M. paratuberculosis* are dramatically different than the response of PBMCs from cows with subclinical Johne's disease. In addition, our results demonstrate that the response of PBMCs from subclinical Johne's disease positive cows to *M.*

paratuberculosis is extremely similar to PBMCs stimulated with ConA.

Title Phenotypic characterisation of macrophages in paratuberculosis lesions.

Author(s) Valheim M* , Storset AK, Aune L, Press C McL.

Institution Norwegian School of Veterinary Science, Oslo, Norway.

Abstract Mycobacteria ingested by macrophages are able to inhibit phagosome maturation and phagosome lysosome fusion and the subsequent degradation of internalised material. The activation of macrophages and presentation of mycobacterial antigens to T-lymphocytes is important for the elimination of mycobacteria and is mainly mediated through the secretion of IFN-gamma. Mycobacteria can interfere with this process by affecting macrophage MHC expression. The aim of this study was to examine the histological and immune- and enzyme histochemical phenotypes of macrophages in the paratuberculosis lesions of goats experimentally infected with *M. a. paratuberculosis*. The granulomatous lesions in the small intestine of goats consisted of large macrophages with round to oval nuclei and abundant pale stained cytoplasm. These lesions were predominantly located in the lamina propria and submucosa of the intestinal wall. Few to many acid-fast bacilli were observed in the macrophages. The macrophages in these granulomatous lesions showed a low level of expression of MHC compared with macrophages in areas of the lamina propria without lesions. A further comparison of these two macrophage populations revealed low levels of expression of CD68, a marker for lysosomal membranes, and strong reactivity for acid phosphatase in macrophages of granulomatous lesions. The present study demonstrates clear phenotypic differences between macrophages in granulomatous lesions and in adjacent non-affected areas. These differences suggest that the functional status of macrophages is altered in mycobacterial lesions and may reflect inhibition of antigen presentation and changes in phagosome and lysosome function.

Title Comparative expression profiling in the three defined forms of ovine paratuberculosis.

Author(s) Watkins CA[2*] , Gossner A[2] , Jones DG[1] , Sharp JM[1] , Hopkins J[2] .

Institution [1] Moredun Research Institute. Pentland Science Park, Bush Loan, Midlothian EH26 0PZ. [2] Department of Veterinary Pathology R(D)SVS, Summerhall, University of Edinburgh, Edinburgh, Scotland EH9 1QH.

Abstract Paratuberculosis (Johne's disease) is a chronic intestinal condition of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map). Map gives rise to three different forms of intestinal pathology:

1. Infected but asymptomatic - showing no sign of clinical disease.
2. Paucibacillary or tuberculoid form, affecting about 30% of clinical cases - is a granulomatous disease with a marked lymphocyte infiltrate, few mycobacteria and high T cells numbers
3. Multibacillary or lepromatous form, affecting 70% of clinical cases - is characterised by presence of a macrophage infiltrate containing many mycobacteria and few T cells.

Several studies have described some of the immunological features that are unique to each pathological form. In this study we hope to build on these findings. In paratuberculosis, as in tuberculosis and leprosy, the response of the macrophage is thought to be pivotal to the outcome of the bacterial infection. Using "functional genomic" (microarray) we aim to characterise the transcriptome signature of alveolar macrophages, derived from sheep showing the three forms of Map. This technology will test two hypotheses:

1. There are intrinsic differences in the way that macrophages respond to Map infection in the three forms of the disease (asymptomatic, paucibacillary and multibacillary).
2. Differences in the immuno-inflammatory gene expression in gut-associated

lesions are directly related to the different pathological forms of the disease.

To address these two hypotheses, we have designed an immuno-inflammatory oligonucleotide microarray, which will measure the expression of more than 450 different ruminant immuno-inflammatory genes in the three forms of the disease. In developing this microarray we have produced a first generation chip; the Ruminant Immuno-inflammatory Gene Reference Array (RIGRA). This is being used for initial validation and to examine intrinsic variations in gene expression.

Title Differential expression analysis by microarray of MAP resident within protozoa.

Author(s) Bull TJ[1*] , Hinds J[2] , Butcher P[2] , Sidi-Boumedine K[1] , McMinn EJ[1] , Skull A[1] , Hermon-Taylor J[1] .

Institution [1] Department of Surgery, St Georges Hospital Medical School, London, UK. [2] Wellcome Trust/MRC Bacterial Microarray Facility, St Georges Hospital Medical School, London, UK.

Abstract *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an intracellular pathogen. The differential expression of MAP genes resident in cells that are chronically infected with MAP could highlight the molecular mechanisms behind the pathogenesis of MAP disease. We constructed a microarray of specific PCR products from a bank of MAP specific genes, mycobacterial housekeeping genes and genes previously associated with mycobacterial pathogenesis. MAP strains were introduced into 75cm^3 flask cultures of Acanthamoebae polyphaga, allowed to ingest over 3- 4 days, then treated once with 100mg/ml Amikacin for 2 hours and incubated at RT. Control MAP cultures were grown for the same period at RT in amoebic culture medium only. After 4-8 weeks amoebic cultures were differentially lysed and mRNA extracted from the MAP. Equal quantities of mRNA from extracellular and intracellular MAP cultures were mixed. cDNA was generated using a random primer set, flourescently labelled with either Cy3 or Cy5 and hybridised to microarrays at 65ºC overnight. These were then given a high stringency wash and read using a dual laser 428 scanner. Signals were normalised to 16SrRNA dilution series and ratios of intracellular/extracellular signals read and calculated using the Genespring software. Results show a significant increase in expression of genes immediately downstream of IS900 elements confirming the presence of an active mycobacterial promoter associated with intracellular induced expression of p43 in IS900 and a highly significant increase in expression of genes associated with the GS cassette, the desA1 gene and MAP specific genes associated with IS900 Locus 6. The significance of these genes in MAP pathogenesis will be discussed.

Title Pathogenesis of Johne's disease; a possible role of cell-wall deficient forms of *M. avium* subspecies *paratuberculosis*.

Author(s) El-Zaatari FAK[*] , Hulten K, El-Zimaity HMT, Collins M, Graham DY.

Institution Baylor College of Medicine and VA Medical Center, Houston, TX, University of Wisconsin, Madison, WI, USA.

Abstract

Background.

M. avium subspecies *paratuberculosis* (MAP) is the cause of Johne's disease and has been implicated in Crohn's disease. Cell wall deficient (CWD) forms have been suggested as the pathogenic form of the organism in humans. It is uncertain if CWD forms are present in tissue of animals with Johne's disease. We used a previously developed an in situ hybridization assay for CWD MAP to study animals.

Purpose.

To determine whether CWD MAP were present in diseased animals and to validate the in situ hybridization assay in animal tissue.

Methods.

Paraffin-embedded tissues (intestines, liver and lymph nodes) from 36 coded archival specimens (representing 14 cows and one sheep) were tested by in situ hybridization assay using a digoxigenin-labeled IS900 probe. Positive and negative controls were beef tissues injected with MAP CWD forms and acid fast bacilli, respectively.

Results.

Six of the 10 JD infected animals were positive for MAP (60% sensitivity); 4 intestinal and 2 intestinal/lymph nodes. The single positive liver specimen originated from a cow with JD whose intestinal specimen was also positive. None of the 5 control cows was positive (100% specificity).

Conclusion.

The presence of CWD Map in animals with JD may explain why MAP isn't readily cultured from some animals and/or why pathology is seen in tissues like the liver when no acid fast bacilli are detected. CWD forms may represent a highly evolved survival strategy as the lack of the bacterium's outermost structural layer could assist in evading or delaying the host's defense system, thereby allowing it to quietly establish itself within the host.

Title The *Mycobacterium avium* subspecies *paratuberculosis* 35 kDa protein plays a role in invasion of bovine epithelial cells.

Author(s) Bannantine JP[1*] , Huntley JFJ[1] , Stabel JR[1] , Bermudez LE[2] .

Institution [1] National Animal Disease Center, USDA-ARS, Ames, IA, USA. [2] Kuzell Institute, CPMC, San Francisco, CA.

Abstract *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) enters intestinal epithelial cells of cattle and other ruminants via a mechanism that remains to be fully elucidated. In this study, we observed that the *M. paratuberculosis* 35-kDa protein, also termed major membrane protein (MMP), plays a role in invasion of bovine epithelial cells. The gene encoding the major membrane protein (MMP) was cloned and expressed as a fusion protein with the maltose binding protein (MBP/MMP) in *E. coli*. Rabbit antisera were raised against a *M. paratuberculosis* whole cell sonicate and MMP-specific antibodies were purified from rabbit sera by affinity chromatography. Immunoelectron microscopy of *M. paratuberculosis* bacilli labeled with MMP-specific antibodies shows that the protein is localized to the surface of these bacteria. Cattle with Johne's disease produced antibodies against MMP but did not produce IFN-g, suggesting the protein elicits a humoral but not cell-mediated immune response. Both anti-MMP antibodies and MBP/MMP protein inhibited *M. paratuberculosis* invasion of cultured MDBK cells by 30%. Similar invasion experiments with *M. paratuberculosis* incubated in low oxygen tension, a condition simulating in the intestine, decreased invasion by 60%. Collectively, these data show that the 35-kDa protein is a surface exposed protein that plays a role in invasion of epithelial cells. From these studies, we suggest that the major membrane protein is a virulence factor of *M. paratuberculosis* that may be important in the initiation of infection in vivo.

Title Effects of supplemental energy on periparturient immunosuppression in dairy cows with Johne's disease.

Author(s) Stabel JR* , Goff JP, Kimura K, Harp J, Whitlock RH.

Institution USDA-ARS-National Animal Disease Center, Ames, IA 50010. New Bolton Center, University of Pennsylvannia, Kennett Square, PA.

Abstract Paratuberculosis is associated with a long latent period of infection before clinical signs of disease develop. Stressors may influence the progression to more clinical disease in infected animals. Stress such as parturition and lactation may result in significant suppression of host immunity. This may contribute to the progression of Johne's disease from a subclinical to more clinical state in cows following the periparturient period. The present study was designed to evaluate if feeding supplemental energy to dairy cows during the periparturient period would

alleviate some of the immunosuppression typically noted and ultimately allay presentation of clinical signs of paratuberculosis. Twelve dairy cows in late gestation were assigned to a treatment group receiving silage (n = 6) or silage plus additional feed (n = 6). Diets were fed for 3 weeks pre- and post-calving and blood and fecal samples were obtained 2 days per week during the 6-week period. Milk samples were obtained each day during the 3 weeks post-calving. Lymphocyte blastogenesis, in vitro Ig production, neutrophil iodination and IFN-g were performed on blood samples. Flow cytometric analysis for homing receptors on immune cells was performed on blood and milk samples. Fecal and milk samples were cultured for viable organisms using HEYM and BACTEC. Parturition reduced immune function with notable reductions in all immune function tests including neutrophil assays, ELISA, IFN-g, lymphocyte blastogenesis, and in vitro Ig production. Expression of LPAM-1, a mucosal homing receptor, was reduced on blood T cell subsets after calving but increased on milk T cell subsets. Energy supplementation moderated cell-mediated immunity by reducing lymphocyte blastogenesis and IFN-g production but enhanced neutrophil iodination during the periparturient period. These data suggest that parturition has a significant effect on immune function parameters including diagnostic tests for paratuberculosis and energy supplementation did not preclude the immunosuppression during the peripaturient period.tion did not preclude the immunosuppression during the peripaturient period.

Title Experimental infection of *Mycobacterium avium* subsp. *paratuberculosis* in IL-18 deficient Mice.

Author(s) Momotani E[*1] , Bari ASM [1] , Aodongeril[1] , Buza JJ [1] , Hikon H [1] , Tsuji N [2] , Takeda K[3] .

Institution [1] National Institute of Animal Health, 3-1-5, Kan-nondai, Tsukuba 305-0856 Japan. [2] National Institute of Agrobiological Sciences, Tsukuba, Japan. [3] Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan.

Abstract To understand the role of IL-18 in the pathogenesis of paratuberculosis, we studied on experimental infected IL-18ko mice. Histo- and immunohisto-pathological changes, clinical findings, expression of inflammatory cytokines and chemokines were studied. 10^7 CFU of *M. avium* subspecies *paratuberculosis* (ATCC 10698) (*M. paratuberculosis*) were injected intraperitoneally, and examined in 1, 2, 3, 5 weeks after inoculation. Clinically, IL-18ko mice showed progressive emaciation after the infection. Hepatic epithelioid cell granulomas were formed in both groups. The number of granuloma in Ko mice was statistically higher than wild type mice. In IL-18ko mice number of the granuloma increased until 3 weeks. And then the granulomas showed maturation with large epithelioid cells. Epithelioid cell granuloma in spleen of ko mice was much severe than those in wild type mice. Granulomatous enteritis was not observed in both group in 5 weeks study, however mesenteric lymph nodes showed severe granulomatous change. Pattern of inflammatory cytokines and ckemokines were measured by RT-PCR. Role of the IL-18 in the granuloma formation and host defense will be discussed.

Title Cellular and humoral immune responses against AhpC and AhpD from *M. avium* subsp. *paratuberculosis*.

Author(s) Olsen I[1*] , Storset AK[2] , Wiker HG[3] , Reitan LJ[1] .

Institution 1 National Veterinary Institute. 2 Norwegian School of Veterinary Science. 3 National Institute of Public Health, Oslo, Norway.

Abstract AhpC and AhpD are detoxifying enzymes involved in the protection against oxidative stress. The enzymes are constitutively expressed in *M. avium* subsp. *paratuberculosis* as opposed to other mycobacteria. The enzymes are immunogenic and can differentiate between animals infected with bovine tuberculosis and paratuberculosis in ELISA. The aim of the current study was to further characterise the immune response against these proteins including epitope mapping of AhpC using overlapping synthetic peptides. Experimentally infected calves with subclinical disease were used for measuring IFN-g production, lymphocyte proliferation and T-cell epitope mapping. Naturally, infected cattle and goats in addition to immunised rabbits where used for B-cell epitope mapping. Several of the infected calves had IFN-g production and

lymphocyte proliferation responses against AhpC and AhpD at various time points after infection. The calves with the strongest responses were used to identify T-cell eptiopes. An epitope causing lymphocyte proliferation was located near the C terminal end of the protein while several peptides induced low levels of IFN-g production. Two B-cell eptiopes were localised and these were recognised both by infected cattle, infected goats and immunised rabbits showing consistency between the species in recognition of B-cell epitopes. One of the B-cell epitopes was in the same area as the epitope that induced lymphocyte proliferation. These results show that AhpC and also AhpD are immunogenic proteins that can identify cattle with subclinical paratuberculosis and may thus be useful in diagnostic testing.

Title Assessment of diagnostic tools in young calves experimentally infected with *M. avium* subspecies *paratuberculosis* (ATCC 19698)

Author(s) Marché S[1*] , Walravens K[1] , Perier AF[1] , Rosseels V[2] , Huygen K[2] , Godfroid J[1] .

Institution [1] Veterinary and Agrochemical Research Centre, Brussels. [2] Mycobacterial Immunology, Pasteur Institute of Brussels, Belgium.

Abstract In order to assess the performances of paratuberculosis diagnostic tests, 5 young calves (1-2 weeks old) were experimentally infected by oral route. The animals were infected with 10 mg (10^8 CFU) of *M. avium* subspecies *paratuberculosis* (ATCC 19698) per day during 10 days. Samples were taken periodically. The Cell Mediated Immunity (CMI) was assessed by the Interferon gamma assay and the lymphoproliferative responses after in vitro stimulation of blood samples with avian and bovine PPD's. Humoral responses were detected by an absorbed ELISA. Detection of *M. avium* subspecies *paratuberculosis* in faeces was done by classical mycobacteriology (HEYM + Mycobactin). Our results can be summarized as follows:

1. the animals showed fluctuating CMI responses in both Interferon gamma and lymphoproliferation assays. It took more than 12 weeks post-infection before some animals were consistently classified M. avium subspecies paratuberculosis positive by the Interferon gamma;
2. no detectable humoral responses could be evidenced, so far;
3. we were unable to culture M. avium subspecies paratuberculosis from faecal samples of these animals, although all tests and reagents passed the quality control and
4. no paratuberculosis clinical signs could be seen, until now.

All together these results emphasises the difficulties of an early and specific detection of *M. avium* subspecies *paratuberculosis* infected animals although a combination of available tests was used. More, our results suggest that the absence of positive serology as well as the absence of fecal excretion of *M. avium* subspecies *paratuberculosis* at the group level, could jeopardise "paratuberculosis-free" certification programs based on those techniques.

Title A comparison of the virulence of strains of *Mycobacterium avium* subsp. *paratuberculosis* isolated from different host species.

Author(s) Stevenson K[1*] , Schock A[1] , Sales J[2] , Sharp JM[1] .

Institution [1] Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Scotland, UK. [2] Biomathematics and Statistics Scotland, King's Buildings, Edinburgh, Scotland, UK.

Abstract A mouse model for paratuberculosis has been optimised and used to compare the virulence of strains of *M. a. paratuberculosis* isolated from different host species. C57/BL6 mice were inoculated with different strains of *M. a. paratuberculosis* previously characterised by pulsed-field gel electrophoresis and restriction fragment length polymorphism analysis (cervine, bovine, pigmented ovine, non-pigmented ovine and human). Control mice were inoculated with heat-killed cervine strain, PBS or IS901+ M.avium. A necropsy was performed after 8 weeks. Body, spleen and liver weights were recorded and the livers were examined histopathologically for the presence of granulomas and cultured to determine the presence of viable organisms. Data

will be presented to show the different virulence characteristics observed between the various strains.

Title Longitudinal follow up of calves experimentally infected with *M. paratuberculosis*.

Author(s) Koets AP[1*] , Langelaar MFM[1] , Hoek A[1] , Bakker D[3] , Müller K[2] , van Zijderveld F[3] , van Eden W[1] , Rutten VPMG[1] .

Institution [1] Immunology Division, Institute of Infectious Diseases and Immunology. [2] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. [3] Central Institute Animal Disease Control (CIDC), Lelystad, The Netherlands.

Abstract

Introduction.

Bovine paratuberculosis is caused by infection of young calves with *Mycobacterium paratuberculosis*, and results in chronic granulomatous infection of the ileum. The aim of the present study was to perform a longitudinal follow up of immunological and microbiological parameters of calves experimentally infected in the first month of life.

Materials and methods.

White blood cell counts and differentiation were done on whole blood samples. PBMC were isolated from blood samples of 20 experimentally infected calves and 10 uninfected control animals. The PBMC were phenotyped by flow cytometry using a panel of 8 monoclonal antibodies. Lymphoproliferation assays, with a panel of 6 mycobacterial antigens were used for evaluating T cell function. Fecal culture was performed to aqcuire data on the mycobacterial load.

Results and conclusion.

The results obtained during the first 3 years of infection indicated that minor responses in the PBMC population of infected calves can be detected within 3 months after infection, followed by an exponentially rise 1 year post infection to reach a plateau. By the end of the third year, the indications of a decrease in cell mediated immune responses became evident. The immunophenotyping of the PBMC showed differences in the gamma-delta T cell and B-cell frequencies between the infected and the control group. Comparisons of lymphocyte stimulation and bacterial load in the infected group indicated a dose-response relationship. Definite conclusions on whether the observed immunological responses are the correlates of protective immunity awaits completion of this ongoing study.

Title Effect of IL-12 on immune response to paratuberculosis vaccination in calves.

Author(s) Uzonna J, Chilton P, Whitlock R, Scott P, Sweeney R[*] .

Institution U. of Pennsylvania School of Veterinary Medicine.

Abstract Although currently available paratuberculosis vaccines may reduce the incidence of clinical disease, they may not prevent infection with *M. paratuberculosis*. The purpose of these experiments was to evaluate the immune response to commercially available vaccine (Strain 18) compared with vaccine prepared from a field isolate. The adjuvant effect of IL-12 administration was also evaluated, as was the timing of vaccination in relation to challenge exposure to *M. paratuberculosis* organisms. Calves were vaccinated with Strain 18 vaccine +/- hrIL-12 at 2-7 days of age and given an oral challenge with field strain *M. paratuberculosis* 3 weeks later. Other calves were given the oral challenge at 2-3 days of age, then vaccinated (Strain 18+/- hrIL-12) one week later. Finally, some calves were vaccinated with heat-killed field strain *M. paratuberculosis* +/- hrIL-12 at 2-7 days of age, then given an oral challenge 3 weeks later. Calves were euthanized at 49 days, and 40 tissues collected for mycobacterium culture on HEYM. Lymph node cells from ileocecal lymph node and prescapular lymph node (draining vaccination site) were cultured in-vitro, stimulated with *M. paratuberculosis* antigen, and IFN-g concentration in supernatant determined by ELISA.

Vaccination in all 3 experiments resulted in high concentrations of IFN-g from prescapular and ileocecal lymph node cultures. Inclusion of hrIL-12 with the vaccine seemed to have no significant effect on IFN-g production by lymphocytes, although it did result in reduced IL-4 gene expression in draining lymph node. Severity of infection (n of colonies *M. paratuberculosis* recovered by culture) was reduced when vaccination was given before challenge exposure (both commercial and field-strain vaccines) compared with vaccination after challenge. Also, field strain vaccine showed better protection than commercial vaccine. Finally, some calves vaccinated with IL-12 before challenge were culture-negative in all tissues, while none of the calves vaccinated after challenge were completely protected.

Title Paratuberculosis vaccination response in cattle, sheep and goats

Author(s) Geijo MV* , Garrido JM, Sevilla I, Aduriz G, Juste RA.

Institution NEIKER (Instituto Vasco de Investigación y Desarrollo Agrario), Dpto. de Sanidad Animal. Berreaga 1, 48160 Derio. Bizkaia. Spain.

Abstract Diagnostic and control criteria for paratuberculosis are based on the assumption that all ruminant species have a similar response to the infection. However, there is some evidence that infections in each species have quite different features, like the different strains involved in cattle and sheep or variations in the pathology in each species. In order to compare the immune response in natural conditions, we designed an study involving cattle, sheep and goats comparing the effects of vaccination according to age of vaccination and paratuberculosis status of the herd. Three age cohorts (15 days, 6 months, and 1.5 to 2 years) in one farm with recorded cases of paratuberculosis and another without were tested by PPA3-ELISA, g-IFN ELISA, and blood PCR each 6 months for 1.5 years. For each age, 5-9 animals were vaccinated and 5-9 were not. Individual antibody and g-IFN ELISA results at the first control were substracted from results at subsequent controls in order to have comparable data for all species. Overall, goats had significantly ($p<0.01$) smaller changes in the antibody response during the period of study, while bovine had larger changes in the g-IFN response. By PCR Map was, at least, as frequently detected in PTB-free than in PTB-affected herds, both before and after vaccination. These results indicate that Map distribution might not be related to a previous clinical history of paratuberculosis in the herd, and that there are different patterns of paratuberculosis immune responses according to species, age, and clinical history.

Title MERKAL AWARD LECTURE: Expression library immunization of mice identifies five clone pools that offer protection against challenge from *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Huntley JFJ[1*] , Stabel JR[2] , Bannantine JP[2] .

Institution [1] Department of Veternary Pathology, Iowa State University, Ames, IA USA. [2] National Animal Disease Center, ARS-USDA, Ames, IA USA

Abstract Johne's disease is a chronic granulomatous infection of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*). Clinical disease is characterized by weight loss, diarrhea, decreased milk production, and ultimately death. To date, there is no effective treatment for paratuberculosis and current vaccines do not prevent infection, but rather delay the onset of clinical disease. Furthermore, currently available vaccines interfere with bovine tuberculosis skin testing and paratuberculosis diagnostic tests. In an effort to identify protective *M. paratuberculosis* genomic sequences, an expression library of *M. paratuberculosis* was generated and subdivided into 78 pools of clones. Each clone pool, which contained an average of 1500 clones, was evaluated by PCR with a set of seventeen known *M. paratuberculosis* sequences. C57BL/6J mice (6 week old, male, 20 grams) were immunized with 2 ìg of DNA in the abdominal area with each clone pool via gene gun delivery. The mice were boosted three weeks after initial immunization and inoculated in the tail vein with live, virulent *M. paratuberculosis* (strain 19698, 10^8 bacteria per mouse) two weeks following the boost. Protective effects of each clone pool were evaluated based on culture of viable bacteria from liver, spleen, mesenteric lymph node, and ileum obtained at necropsy three

months post-challenge. Five of twenty-six clone pools examined thus far have demonstrated at least a hundred-fold reduction in *M. paratuberculosis* colonization in mice when compared to other clone pools and nonvaccinated, infected control mice.

Title Characterisation of a putative virulence factor of *Mycobacterium avium* subspecies *paratuberculosis*.

Author(s) Heaslip D, Stevenson K[*] , Sharp JM.

Institution Moredun Research Institute, Pentlands Science Park, Penicuik, Scotland, UK.

Abstract A 34kDa protein expressed in vivo by *M. a. paratuberculosis* exhibits an overall similarity to HtrA, a trypsin like serine protease, of several gram positive and gram negative bacteria. HtrA mutants of several pathogenic bacterial strains have demonstrated attenuation in both in vivo and in vitro models of infection. To investigate the potential role that the 34kDa protein plays in the virulence of *M. a. paratuberculosis*, the entire open reading frame was cloned into a mycobacterial shuttle vector under the transcriptional control of the *M. tuberculosis* hsp60 promoter. The construct was introduced into *M. smegmatis* mc2155, and protein expression detected by western blot using a monoclonal antibody raised against the 34kDa putative serine protease. *M. smegmatis* mc2155 expressing the recombinant protein exhibited increased survival within ovine ex-vivo alveolar macrophages at 24 hours post infection. Further work has centred on the identification and characterisation of the transcriptional signals responsible for the expression of the 34kDa putative serine protease. This work was funded by grants from the European Commission and the Scottish Executive Environment and Rural Affairs Department.

Title Interactions between antigen presenting cells and *Mycobacterium paratuberculosis* 70 KD heat shock protein.

Author(s) Langelaar M[1,2*] , Koets A[1] , Muller KV[2] , v.Eden W[1] , Noordhuizen J[2] , Howard C[3] , Hope J[3] , Rutten V[1] .

Institution [1] Div. of Immunology, [2] Dep. of Farm Animal Health, Fac. of Vet. Medicine, Utrecht, The Netherlands. [3] Institute for Animal Health, Compton, UK.

Abstract

Introduction.

Cytotoxic T lymphocyte (CTL) responses have been shown to contribute to immunity against intracellular pathogens. However, no information is available on the role of CTL in immunity to bovine paratuberculosis, caused by the intracellular pathogen *Mycobacterium paratuberculosis* (Mp.). To study paratuberculosis specific CTL responses we aim to use the cross-priming abilities of heatshock protein (Hsp70) to transport antigen into MHC class I pathway of antigen presenting cells (APC). As a first step, the specific interaction of Mp. Hsp70 with APC is illustrated in the present study.

Material and methods.

Purified recombinant Mp. Hsp70 protein was conjugated to FITC to study binding and uptake of the Hsp70. FITC-conjugated BSA or OVA were used as a negative control. Unlabeled Hsp70, OVA and alpha2-macroglobulin were used in competition studies. Bovine monocytes were isolated by magnetic separation using CD14 labeled magnetic particles. The CD14 positive cells were cultured in culture medium, to obtain macrophages, or culture medium supplemented with bovine rIL-4 and rGM-CSF, to obtain dendritic cells. The murine RAW264.7 macrophage cell line was used for comparison. Cells were analysed using confocal microscopy, and flow cytometry.

Results.

When APC are incubated with Hsp70-FITC, a clear interaction with the protein can be visualized which can be specifically inhibited using alpha2-macroglobulin.

Conclusion.

Bovine APC clearly show interaction with Mp. rHsp70 molecules similar to that observed in murine model systems, indicating uptake also occurs via the alpha2-macroglobulin receptor. For the use of Hsp70 as a tool to induce and study bovine CTL responses, proof of receptor mediated uptake of rHsp70 by APC is a first important step.

Title Expression of inflammatory cytokine mRNA in lymphoid tissue from swine experimentally infected with *Mycobacterium avium* serovar 2.

Author(s) Hines II ME* , Frazier KS.

Institution University of Georgia, College of Veterinary Medicine, Tifton Veterinary Diagnostic and Investigational Laboratory.

Abstract Swine mycobacteriosis (tuberculosis) is a common cause of carcass condemnation in major swine production areas. Once the infection is established in a swine herd it is difficult to effectively prevent or eliminate the disease. Evaluation of in situ mRNA cytokine profiles of lymphoid tissue in swine mycobacteriosis has not previously been performed. The inflammatory cytokine mRNA expression of inflammatory cytokines (TNF, IL-1, IL-6 and IL-8) in lymphoid tissues of swine experimentally infected with *M. avium* serovar 2 was compared to non-infected swine lymphoid tissue and evaluated by morphological localization of cytokine mRNA using in situ hybridization at 160 days post-infection A marked increase in TNF mRNA expression, mild increase in IL-8 mRNA expression and mild increase in IL-1 mRNA expression was detected in mandibular lymph nodes from infected swine compared to non-infected swine. A mild increase in IL-6 mRNA expression was also observed in tonsils from infected swine. Cytokine mRNA was detected in macrophages and lymphocytes primarily within cortical follicles and adjacent mantle zones. The inflammatory cytokine mRNA expression profile appears altered in infected swine lymphoid tissue possibly by local factors present on, or secreted by, *M. avium*. Altered cytokine expression could delay or prevent an appropriate immune response capable of effective clearance and prevention of disease. These results suggest that alterations in cytokine mRNA expression are important in the pathogenesis and clinical course of swine mycobacteriosis. (Am J Vet Res 2000; 61:1487-1491)

Title Flow cytometric evaluation of cytosolic calcium changes in J774 cells infected with *Mycobacterium avium* subspecies *paratuberculosis*.

Author(s) Hostetter J* , Steadham E.

Institution Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University.

Abstract *Mycobacterium avium* subspecies *paratuberculosis* (Map) is an intracellular pathogen of monocytes and macrophages. Increased cytosolic calcium is a early event following phagocytosis which is involved in multiple signaling cascades including activation of kinases, cytoskeletal alterations, and phagolysosome development. Our laboratory is developing an assay for detecting changes in cytosolic calcium concentrations in macrophages using flow cytometric techniques. The hypothesis of this study is that cytosolic calcium levels do not significantly increase following uptake of Map into J774 cells. We prepared suspension cultures of J774 macrophages and incubated them with the fluorescent calcium indicator Fluo-4 (Molecular Probes, Eugene, OR) for 20 minutes at 37ºC. Fluo-4 can cross cell membranes and after binding to cytosolic calcium is strongly fluorescent. By flow cytometry we determined the baseline level of Fluo-4 fluorescence in noninfected cells. Next, we incubated cells with either the ionophore ionomycin, Map, or zymosan A, and monitored Fluo-4 fluorescence for 6-10 minutes. As expected, following the addition of ionomycin there was a consistent increase in Fluo-4 fluorescence. We did not demonstrate a significant increase in Fluo-4 fluorescence after addition of Map to the J774 cell suspensions; however, we did detect increases in Fluo-4 signal following addition of zymosan A. These results of this study suggest that following uptake of Map into J774 cells there is no significant change in the cytosolic calcium.

Title Cytokine gene expression in different types of granulomatous lesions in ileal tissues of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Tanaka S[1*] , Sato M[1] , Yokomizo Y[2] .

Institution 1 Kyushu Research Station, National Institute of Animal Health. 2 National Institute of Animal Health.

Abstract The granulomatous lesions in bovine paratuberculosis have been classified into 2 types, i.e. lepromatous type and tuberculoid type. Lepromatous type lesions are categorized as diffuse granulomas composed of many macrophages and epithelioid cells bearing large numbers of mycobacteria. Tuberculoid type lesions are categorized as focal granulomas consist of small numbers of macrophages and many lymphocytes with no or scant numbers of bicilli. In this study, we compared the cytokine gene expression between the two types of granulomatous lesions in ileal tissues. Ileal samples were obtained from noninfected control cows (n = 5: noninfected group) and infected cows (n = 6) that were diagnosed by ELISA or fecal culture test, and were processed for RT-PCR and in situ hybridization to detect cytokine mRNAs. All of infected cows were in the early stages of disease without clinical signs, and the tuberculoid lesions were observed in 4 cows (tuberculoid group) and the lepromatous lesions were in 2 cows (lepromatous group). Among cytokines examined, interleukin-2 (IL-2) and Th2 type cytokines, IL-4 and IL-10, were expressed more in lepromatous group than that in tuberculoid group. The expression of IL-12, however, was not different among the two groups and noninfected group, yet IL-18 was expressed lower in the lepromatous group than that in the tuberculoid group and noninfected group. In addition, IL-1 beta and tumor necrosis factor-alpha (TNF-alpha) were expressed more in the lepromatous group than that in the tuberculoid group. On the contrary, expression of Th1 type cytokine, interferon-gamma (IFN-gamma), was more in the tuberculoid group than that in the lepromatous group. These results indicate that the formation of lepromatous lesions or tuberculoid lesions may be influenced by alterations in Th1/Th2 type cytokine production, and IL-18 may play an important role in a Th1 to Th2 switch.

Title Interferon-gamma and antibody responses in cattle prior to shedding of *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Huda A[1,2*] , Jungersen G[2] .

Institution 1 Danish Dairy Board, Brørup, Denmark. 2 Danish Veterinary Institute, Copenhagen, Denmark.

Abstract

Introduction.

Bacteriological culture of faeces is the most commonly used diagnostic test for paratuberculosis in Denmark. With the hypothesis of a shift in immune response from Th1 to Th2 preceding progression of paratuberculosis into the shedding stages, measures of cellular immunity is expected to decrease parallel to an increase in serological response prior to diagnosis by culture. The purpose of the present study was to compare cellular immune response with antibody response in the subclinical stages in cattle naturally infected with paratuberculosis before their first culture-positive faeces sample.

Materials and methods.

Cattle were tested simultaneously and repeatedly for cellular immune response, antibody response and faecal excretion. Twenty-nine cattle of 1 month to 49 months of age, selected from 9 dairy herds were sampled 3 to 8 times during a 2-year period. Whole blood samples were stimulated with johnin PPD (PPDj), and secreted interferon-gamma was assayed by the BOVIGAM ELISA. Serum samples were tested by an in-house absorbed indirect IgG ELISA using a commercially available antigen (Allied Monitor, USA), and decontaminated faeces samples were inoculated on modified Löwenstein-Jensen medium.

Results and discussion.

The measures of cellular immunity were decreasing in 19 (66%) of 29 cattle prior to diagnosis by faeces culture; of these, 14 (74%; 48% of total) cattle had simultaneously increasing serological response. Thus, the hypothesis of a shift from Th1 to Th2 response was in the

present study only confirmed by approximately half of the culture-positive cattle. Circumstances not taken into account in this study could influence the results. Culture-positive results could occur because of passive excretion (false-positive), faeces samples contaminated with moulds inhibit in-vitro growth of the pathogen (false-negative results prior to a positive sample), or peripheral measures of cellular immune response may not accurately reflect local immune responses in the intestine more closely related to excretion of the pathogen.

Title Gamma-Interferon responses to avian and Johnin purified protein derivatives in blood and biopsies of the prescapular lymph node from sheep infected experimentally with *Mycobacterium paratuberculosis*.

Author(s) Gwozdz JM.

Institution Victorian Institute of Animal Science, Attwood, Australia.

Abstract The objective of this study was to compare the production of gamma-interferon (IFN-g) in response to Avian and Johnin Purified Protein Derivatives (PPD) in blood and biopsies of prescapular lymph nodes (PLN) from sheep infected experimentally with *Mycobacterium paratuberculosis*. Six of 12 lambs, aged from 1.5 to 2 months, were inoculated orally with *M. paratuberculosis*. The other six non-inoculated lambs constituted negative controls. Twelve months after inoculation blood for an agar gel immunodiffusion test (AGID), enzyme-linked immunosorbent assay (ELISA) and for the IFN-g assay were collected, and sheep were humanely killed. After euthanasia, punch biopsies of the PLN for the IFN-g assay and intestinal and mesenteric lymph node samples for histopathological examination and culture were obtained from each sheep. Samples of blood and PLN biopsies were stimulated with two antigens; Avian and Johnin PPD. Among the six sheep dosed with *M. paratuberculosis*, four had bacteriological evidence of infection, of which two had unequivocal paratuberculosis lesions. The PLN and blood IFN-g assays gave positive reactions in six inoculated animals, regardless of the type of antigen used as a stimulus. In comparison, the AGID and ELISA detected three and four inoculated sheep, respectively. None of the six non-inoculated sheep had histological evidence of paratuberculosis or tested positive by culture, AGID, ELISA or the IFN-g assay on samples of blood stimulated with Johnin PPD. One non-inoculated animal tested positive in the Johnin PPD PLN IFN-g assay. The blood IFN-g assay and PLN IFN-g assay in which Avian PPD was used as an antigen gave positive reactions in two and three non-inoculated sheep, respectively. Differences between IFN-g responses in blood and PLN biopsies to Avian PPD and Johnin PPD are discussed.

Title *M. paratuberculosis* specific production of IL-10 after whole blood stimulation is correlated with specific IFN-gamma production and non-stimulated IL-10 levels.

Author(s) Jungersen G[1*] , Huda A[1] , Grell SN[1] , Howard CJ[2] .

Institution 1 Danish Veterinary Institute, Copenhagen, Denmark. 2 Institute of Animal Health, Compton, UK.

Abstract IL-10 is one of the major immune regulatory cytokines, inhibiting synthesis of a number of cytokines including IFN-gamma. IL-10 producing T cells have been reported to be specific immune suppressors in progression of anergy in tuberculosis (Boussiotis et al., 2000). The shifting immune response from an early cellular mediated type to a humoral response in development of paratuberculosis warrants comparison between specific IFN-gamma and IL-10 production over time in this disease.

Materials and Methods.

Twenty-five cattle, aged 3 to 54 months, were selected from 2 herds infected with *M. avium* subsp. *paratuberculosis* and 3-10 blood samples from each were collected over a 2-year period. On the day of collection, heparinized whole blood was cultured overnight with johnin PPD (PPDj) and PBS (Jungersen et al., 2002). Culture supernatants were analysed for IFN-gamma by the BOVIGAM(r) ELISA and for IL-10 by a chemiluminescent monoclonal sandwich ELISA (Kwong et al., 2002). IFN-gamma contents were calibrated against the kit

positive and negative controls, while IL-10 levels were calculated from a recombinant bovine IL-10 standard curve. PPDj specific IFN-gamma and IL-10 responses were calculated by subtracting values in nil (PBS) stimulated wells from PPDj stimulated wells.

Results and discussion.

PPDj specific IL-10 levels were correlated ($P<0.0001$, $r = 0.33$) with specific IFN-gamma production. However, in contrast to IFN-gamma, a strong ($P<0.0001$, $r = 0.75$) correlation was also observed between IL-10 levels in nil and PPDj stimulated samples, indicating some IL-10 production in cultures ""ex vivo"", presumably cells having been stimulated in vivo and synthesis continuing in culture wells. IL-10 levels in the nil cultures were, however, not correlated with specific IFN-gamma production. Although paratuberculosis specific IFN-gamma and IL-10 thus was produced in PPDj stimulated cultures, no clear role in the relation between individual animals' IFN-gamma and IL-10 responses over time could be deduced.

Title Identification of genes involved in macrophage response to *M. paratuberculosis*.

Author(s) Coussens PM* , Tooker BC, Wertz J, Abouzied A.

Institution Michigan State University Department of Animal Science and Center for Animal Functional Genomics.

Abstract Gene expression profiling using a bovine-specific cDNA microarray system has revealed a subset of genes (6) activated by exposure of bovine peripheral blood mononuclear cells to live *M. paratuberculosis*. Subsequent analysis gene expression by real-time PCR and Northern blot hybridization has suggested that several of these genes are strongly activated in macrophages by uptake of E.coli within 60 minutes of phagocytosis. The kinetics of gene expression when *M. paratuberculosis* is phagocytosed appear to be severely delayed relative to E.coli. Our results suggest that *M. paratuberculosis* is capable of entering macrophages in a manner that prevents the immediate activation of these cells and may have implications for how *M. paratuberculosis* and other mycobacteria survive in host macrophages.

Title A longitudinal evaluation of the interferon-g test in penned cattle, sheep and goats following infection with either bovine or ovine strains of *Mycobacterium paratuberculosis*.

Author(s) Stewart DJ[1*] , Vaughan JA[1] , Stiles PL[1] , Tizard MLV[1] , Prowse SJ[1] , Michalski WP[1] , Jones SL[2] .

Institution [1] CSIRO Livestock Industries. Australian Animal Health Laboratory, Private Bag 24, Geelong, 3220, Victoria, Australia. [2] CSL Limited, 45 Poplar Road, Parkville, 3052, Victoria, Australia

Abstract Two longitudinal experiments involving Friesian cattle, Merino sheep and Angora goats (5 animals per group) challenged with either bovine or ovine strains of *Mycobacterium avium* subspecies *paratuberculosis* have been conducted over a period of 48 and 31 months, respectively. The experiments are still in progress. The age of the cattle, sheep and goats challenged with the bovine Johne's disease (BJD) strain was 6 weeks, 6 months and 5 months, respectively whereas those challenged with the OJD strain were 14 weeks, 10 months and 10 months. Four doses of BJD or OJD cultured bacteria ($1x10^{10}$ - $2x10^{10}$ per ml) and either BJD or OJD mucosal tissue (15 - 20 g) were given orally at weekly intervals for 4 weeks. Control animals for each of the species were dosed with broth diluent. Blood samples for the bovine gamma interferon test (BOVIGAM™, CSL Limited) and the Johne's absorbed EIA (PARACHECK™, CSL Limited) and faecal samples for conventional culture and radiometric culture (BACTEC™, Becton Dickinson) were taken pre-challenge and monthly post-challenge. There were differences between the species in their bacteriological and immunological responses to infection. Goats appear to be the most susceptible to BJD infection than cattle or sheep in terms of clinical disease, seroconversion and persistent bacterial shedding. Goats also appear to be more susceptible to BJD than OJD. The BJD affected goats developed persistently

elevated interferon-g and antibody responses around the time that shedding commenced with interferon-g declining to baseline levels in the terminal stages of the disease. The presence of high background interferon-g levels in the control cattle, obtained from different farms, in both the BJD and OJD experiments made it difficult to interpret the results of the test in the challenged cattle groups. However, the results do indicate that for sheep the interferon-g test may be potentially useful for determining if a flock has been exposed to OJD.

Title Time course of interferon-g response to E.coli expressed recombinant antigens by *Mycobacterium avium* subspecies *paratuberculosis* infected animals.

Author(s) Tizard MLV* , Bruce K, Stiles PA, Vaughan J, Beddome G, Michalski WP, White J, Duch C, Davis A, Stewart DJ.

Institution CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Abstract Many antigens within the protein/peptide complement of Johnin purified protein derivative (PPD) and cell free culture supernatants of *Mycobacterium avium* subspecies *paratuberculosis* are capable of eliciting a strong interferon-g release response by lymphocytes from ruminants early in the course of Johne's disease. Since these preparations are complex, ill defined and hard to reproduce it is of value to identify individual gene products that may contribute to the overall response. An *E. coli* based expression system has been used to express and purify a range of protein antigens using poly-histidine tags. The purified recombinant proteins have been used to stimulate whole blood preparations and using the BOVIGAM™ assay (CSL Animal Health Ltd) to determine interferon-g release in a range of animal species, of differing infection states and at different time points post-infection. This has been performed within the reference frame of a time course of bacteriological, immuno-assay and clinical observations. Interferon-g release has been observed in response to four of fourteen antigens assessed. Two antigens, AhpC and BrfA, elicited strong responses in a small number of animals across a number of time points. A number of these antigens, including AhpC, have been assessed by expression in a baculovirus/insect cell culture system. These materials currently show no benefit over the *E. coli* derived materials. The purification/assay system proved robust with no animals showing responses to either control recombinant proteins or potential contaminants from the *E. coli* system. Qualitative comparison of native purified antigens and antigens the expressed and purified from an *E. coli* or *M. smegmatis* background will be performed. This approach is being utilized in conjunction with proteomic analysis and expression library screening.

Title Upregulation of TGF-beta and IL-10 in the clinical stage of Johne's disease in cattle.

Author(s) Stabel JR* , Khalifeh MS.

Institution USDA-ARS-National Animal Disease Center, Ames, IA 50010.

Abstract Johne's disease is an important disease that results in great economic losses for both dairy and beef production in the US and worldwide. The disease progresses through distinct stages, a subclinical stage where there are no clinical signs and a clinical stage that is characterized by progressive symptoms associated with chronic shedding of high levels of bacteria in the feces along with severe diarrhea and concomitant weight loss. The host immune response to *M. paratuberculosis* is paradoxical with strong cell-mediated immune responses during subclinical stages and strong humoral responses during clinical stages of the disease. It is possible that immune modulation of an effective cell-mediated immune response in paratuberculosis may play an important role in disease progression. We hypothesized that the clinical stage of Johne's disease is mediated by production of cytokines such as TGF-beta and IL-10 that downregulate IFN-gamma production and interfere with an effective cell-mediated immune response. Therefore, ileum, ilealcecal junction, ilealcecal lymph node and mesenteric lymph node tissues from healthy, subclinical or clinical animals were collected and analyzed for the presence of TGF-beta, IL-10 and IFN-gamma mRNA by quantitative RT-cPCR. The results show that TGF-beta and IL-10 mRNA levels in animals that have progressed to the clinical

stage of disease is higher than that found in subclinical or healthy animals, whereas, IFN-gamma is higher in subclinical animals. A change in the balance of cytokines at the site of infection may have an important effect on the microbicidal activity of macrophages.

Title Immune responses after oral inoculation of weanling bison or beef calves with a bison or cattle strain of *Mycobacterium paratuberculosis*.

Author(s) Stabel JR* , Palmer MV, Whitlock RH.

Institution USDA-ARS-National Animal Disease Center, Ames, IA. New Bolton Center, Kennett Square, Pennsylvannia.

Abstract Paratuberculosis is endemic in domestic and wild ruminants worldwide. Little is known about the potential for one ruminant species to act as a vector of transmission of infection to another species. Recently, paratuberculosis has been diagnosed in a population of captive bison in the western United States. Although bison develop signs of unthriftiness and suffer from severe weight loss, other clinical signs are lacking. In addition, it is difficult to culture *M. paratuberculosis* from feces of infected bison for definitive diagnosis. We designed the following study to compare host immune responses and pathologic changes in beef calves and bison calves after challenge with either a cattle or bison strain of *M. paratuberculosis*. In the first study, 6 bison and 6 beef calves were orally inoculated over a 2-week period with a cattle isolate of *M. paratuberculosis*. In a second study, 6 bison and 6 beef calves were similarly inoculated with a bison strain of *M. paratuberculosis*. Throughout each of the studies, blood and fecal samples were taken monthly for a 6-month infection period. Tissue samples were obtained at necropsy for culture and histopathologic analyses. Results from this study demonstrated that bison calves were more susceptible to tissue colonization than beef calves, regardless of bacterial strain. Although lesions were minimal they were most apparent in the jejunum and distal ileum. Interferon-gamma responses were noted in some calves by one month post-inoculation and were sustained longer in beef calves after challenge with the bison isolate. Antibody was not detected in either beef or bison calves during the 6-month infection period. These results indicate that the host response to strains of *M. paratuberculosis* may differ between ruminant species.

Title Relationship between the microelements Copper, Zinc, Iron, Selenium, and Molybdenum and Paratuberculosis in meat cattle.

Author(s) Perea J[2] , Cseh S[1,2] , Verna A[2] , Morsella C[2] , Paolicchi F[1,2*] .

Institution [1] Departamento de Producción Animal, EEA INTA Balcarce. [2] Facultad de Ciencias Agrarias, Universidad Nacional Mar del Plata, CC 276, Balcarce (7620), Argentina.

Abstract The deficiency of some minerals in blood could predispose to the onset of Paratuberculosis in cattle. With the objective of studying such relationship, samples of blood, serum, and feces were obtained from 90 bovine adults without clinical symptom of two meat herds of the Province of Buenos Aires, Argentina. Drinking water and pasture samples were taken. Serum was processed by the absorbed ELISA to identify seroreactors, while feces were individually cultured in tubes containing Herrold medium with and without mycobactin, plus pyruvate and antibiotic, and were observed during four months to development of *Mycobacterium avium* subsp. *paratuberculosis* (Map). In serum, copper, zinc, and iron concentration was quantified by spectrophotometry of atomic absorption and selenium concentration, by activity of peroxidase glutathione. In pasture, copper, zinc, iron, and molybdenum concentration was measured. The pH of water and the concentration of sulfates were determined. Of the 90 animals, 16 (17.7%) were positive to absorbed ELISA, of these, 6 (37.5%) were positive to Map, of these, 4 (66.6%) were deficient in copper (x:0.4 ug/ml) and selenium (x:20.8 UGPx/gHb), while their values of iron (x:1.4 ug/ml) and zinc (x:1.3 ug/ml) were normal. Another 6 (6.66%) animals suspicious through absorbed ELISA and positive to Map culture, showed low values of copper (x:0.6 ug/ml). Iron (1391.4 ppm) and molybdenum (2.4 ppm) values in pasture were high, what would affect copper absorption at ruminal level. Zinc (34.8 ppm) and copper (7 ppm) values, as well as sulfates (213 mg/L) were normal in water, with an alkaline pH. Selenium deficiency and

primary or secondary deficiency copper due to the presence of antagonists, as iron, molybdenum, or sulfates, could indicate predisposition to the development of Paratuberculosis in meat cattle.

Title Serologic and pathologic characterization of infection by *Mycobacterium avium* subsp. *paratuberculosis* in red deer from Argentina.

Author(s) Verna A [1*] , Morsella C [1] , Casar A [1] , Paolicchi F [1,2] .

Institution [1] Laboratorio de Bacteriologia, Departamento de Produccion Animal, EEA INTA. [2] Facultad de Ciencias Agrarias, Universidad Nacional Mar del Plata, CC 276, Balcarce (7620), Argentina.

Abstract The farming of deer is now widespread throughout Argentina and Paratuberculosis has been identified in red deer (Cervus elaphus) in a herd in the Province of Buenos Aires. In this study we examined three hundred and thirty two animals of different ages (8 to 84 months), selected at random and divided into seven groups, 50 % females and 50 % males each. Another eight seropositive animals were selected because they had clinical signs of Paratuberculosis. Blood for indirect absorbed ELISAs (ELISA 1: bovine anti-IgG, and ELISA 2: deer anti-IgG), Immunodiffusion test (AID) were collected. Feces and tissues for isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map) were cultured on Herrold's with and without mycobactin. Results showed that the ELISA 2 test detected a significantly higher percentage of reactor animals than AID. There were no differences in the percentage when the ELISA 1 test was compared with AID and the ELISA 1 with ELISA 2. In turn, ELISA 1 detected a higher percentage in the stratum of 12 and 84 months of age. Analyzing the three tests, it was proved that the highest incidence of seropositive animals occurred in the group of 84 month old males. The absorbency values of both ELISAs were highly correlated (r=0.865). The ELISA 1 test had a sensitivity of 83.3% and the AID test turned out to have a sensitivity of 33%. Twelve strains of Map were isolated. In the mesenteric lymph nodes of the animals with Paratuberculosis there was moderate to severe infiltration with macrophages (MO) and Langhans' giant cells (LC). The kind of lesion observed was similar to the IIIb and IIIc types established for small ruminants. All ZN smears were positive for MO and LC lymph nodes and intestine sections were positive to immunohistochemistry, showing the higthlest specificity to Map inside MO and LC.

Title Anatomopathological early findings in kids experimentally inoculated by oral route with caprine and ovine *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Chávez-Gris G[*] , Torres-Ramos E, Alarcón A.

Institution Departamento de Patologia. FMVZ. UNAM.

Abstract Anatomopathological lesions and the immune cellular and humoral response were evaluated in experimentally infected kids with Map obtained from goat and sheep. Twenty nine kids from a flock considered paratuberculosis free were used, divided in three groups: group A with 9 kids infected with Map from goat, group B with 9 kids infected with Map from sheep and group C with 11 kids as a control group. At 13 days post infection (dpi) 5 animals were immunizated respectively and 6 animals from the group C. The humoral and cellular response were evaluated employing Agar Gel Immuno Diffusion (AGID) and intra-dermal test (IDT) using in this last one PPDa and PPDb at different periods, anatomopathological studies were realized too. Serological results (AGID) in all groups showed similar humoral immune responses, at 29 dpi were positive diminishing at 182 dpi; positive response was stronger in the immunizated animals in comparison with non-immunizated. IDT showed similar response to both antigens (PPDa and PPDb) in immunizated animals, being stronger in comparison to infected animals in the group A and B although in the last group only was observed response to PPDb. At the anatomopathological studies focal granulomatous lesions were observed in the ileocecal valve and ileum in the animals slaughtered from the groups A and B including immunizated and no-immunizated animals being similar in severity and distribution at 120 dpi, mean while at 188 dpi lesions were more severe and extensive in jejunum. In this study the possibility of map infection in the same species and among species is evident. Also, immunization effect on development of lesions at least in early phases no showed differences in comparison with infected animals, to the contrary humoral and cellular responses were stronger in immunizated kids.

Title Pathological forms and peripheral immune responses in natural bovine paratuberculosis.

Author(s) Pérez V[1*] , González J[1] , Geijo MV[2] , Reyes LE[1] , Garrido JM[2] , Corpa JM[3] , García-Pariente C[1] , García-Marín JF[1] .

Institution [1] Dpt. Patología Animal: Medicina Animal (Anatomía Patológica). Facultad de Veterinaria. Universidad de León. 24071 León. Spain. [2] NEIKER, Instituto Vasco de Investigación y Desarrollo Agrario. Berreaga, 1. 48160 Derio. Bizkaia. Spain. [3] Dpt. Sanidad Humana y Animal. Universidad Cardenal Herrera-CEU. Moncada, Valencia. Spain.

Abstract In small ruminants, paratuberculosis has been demonstrated to be an spectral disease in which infected animals can show a variety of lesions, from focal, located exclusively in the intestinal lymphoid tissue to diffuse forms, either multibacillar or lymphocytic. This pathological forms have been shown as closely related to the immune responses. In cattle, diffuse lesions have been commonly described but not the rest of the forms. A total of 90 adult cattle, with or without clinical signs, from farms having clinical cases of paratuberculosis have been studied by histopathological methods. Samples were taken from the different parts of the gut and jejunal, ileal and ileo-caecal lymph nodes. In 84 of them, serological tests were performed, and in 36 cattle g-IFN was determined. Microbiological cultures were performed in 18 of the animals. Lesions were classified as follows: focal (small groups of macrophages mainly in the ileal and ileocaecal lymph nodes, n=35), multifocal (small granulomata in the intestinal mucosa, without modifying remarkably the histological architecture; n=7) and diffuse forms (extensive granulomatous infiltrates classified, according to the cellular types, in multibacillar, n=8; lymphocytic, n=2; and intermediate -sharing characteristics of both forms; n=8-). Mycobacteria were isolated in animals showing all the types of lesion. Positive serological responses were observed in all the cattle with multibacilar forms, and in high percentages of animals with intermediate or multifocal lesions; positive g-IFN responses were noted mainly in cattle with focal lesions, but also in animals with no lesions. In conclusion, a pathological spectrum was found in bovine natural paratuberculosis, but, in contrast to small ruminants, it is poorly defined, with large number of cattle with diffuse lesions not clearly identified between the classical two types. It is remarkable the importance of ileal lymph nodes in the location of focal lesions, instead of the intestinal lymphoid tissue, as in sheep. Correlation between pathological forms and immune responses has been also demonstrated in this work. Supported by grants 1FD1997-224 and AGL2001-0309 (GAN) from MCYT.

Title Developing an experimental infection model for Johnes disease in sheep to test vaccine efficacy and immune reactivity.

Author(s) Begg DJ[1*] , Frank J[1] , Griffin T[1] , Mackintosh CG[2] .

Institution [1] Disease Research Laboratory, Microbiology Department, University Of Otago, Dundin, New Zealand. [2] Agresearch, Invermay Research Centre, Mosgiel, New Zealand.

Abstract During the development of a sheep infection model for Johnes disease (Jd) the following parameters were studied: The age of lambs at challenge: animals challenged between 4 and 14 weeks appeared equally susceptible to infection. Dose of bacteria required to establish an infection: doses ranging from 10^6 to 10^{12} cfu were tested. The optimal range involved challenge doses between 10^8 and 10^{10} cfu. Infection could be established with as few as two challenge doses. The strain of microorganisms: different laboratory isolates produced variable results in establishing infection. Microorganisms directly isolated from tissue were superior to laboratory passaged cultures. Outgrowth by fast growing avirulent bacteria may explain the reduced efficacy of laboratory cultured microorganisms. Route of infection: oral inoculation or direct instillation into the tonsilar crypt, was suitable to infect animals. The development of a robust standard infection model is essential both for vaccine efficacy studies and a better understanding of the critical pathways of immune reactivity in animals.

Title Oral vaccination for paratuberculosis in calves.

Author(s) Uzonna J[*] , Whitlock R, Scott P, Sweeney R.

Institution University of Pennsylvania School of Veterinary Medicine.

Abstract The present commercial vaccines against *Mycobacterium paratuberculosis* do not prevent infection. One of the reasons for this may be the route of administration-parenteral route may favor strong systemic immunity but may not induce mucosal (enteric) immunity. We carried out studies aimed at targeting/enhancing the enteric immune response by oral vaccination. Two groups of calves were vaccinated on their first day of birth orally with heat-killed field strain *M. paratuberculosis* containing 8×10^8 and 8×10^7 CFU (high and low dose respectively). The calves were challenged with two dose of live organisms (8×10^8 CFU) on days 21 and 22 post immunization. Calves were euthanized at day 42 and 40 tissues were collected for mycobacterium culture. Spleen, prescapular and ileocecal lymph node cells were isolated, cultured in vitro in the presence of heat-killed *M. paratuberculosis* antigen or concanavalin A (Con A) and the concentration of IFN-g in supernatant was measured by commercial ELISA kit. Although immunization enhanced the systemic IFN-g response (PBMCs and prescapular lymph node), it did not enhance enteric IFN-g response beyond that induced by infection alone. Neither vaccination nor infection caused any significant change in Con-A-induced production of IFN-g by cells from spleen, prescapular and ileocecal lymph nodes. Paradoxically, immunization with low, but not high dose, caused a significant reduction in both the number of bacterial colonies and total number of colony positive tissues. The data suggest that oral vaccination may be a means of targeting and enhancing local enteric immunity against *M. paratuberculosis*, and that protection may be vaccine dose-dependent.

Title Development of a plasmid DNA based *M. paratuberculosis* sub-unit vaccine, coding for immunodominant T cell antigens identified in mycobacterial culture filtrate.

Author(s) Rosseels V[1*] , Scalan V[1] , Walravens K[2] , Marché S[2] , Godfroid J[2] , Huygen K[1] .

Institution [1] Mycobacterial Immunology, Pasteur Institute of Brussels. [2] Veterinary and Agrochemical Research Centre, Brussels, Belgium.

Abstract Paratuberculosis is responsible for major economic losses -particularly in the dairy sector- because of reduced milk production in affected animals. It is estimated that in Belgium, 10 % of the dairy farms are infected with M. ptb. The main mode of transmission within herds is through

ingestion of faecal contaminated milk. As the existing vaccine, composed of whole, killed mycobacteria, interferes with the PPD skin test used for the diagnosis of bovine tuberculosis, its use is limited and submitted to the approval of the veterinary services. A sub-unit vaccine, not interfering with bovine PPD skin test, could offer a solution to this problem. Mycobacterial culture filtrates (CF) are a rich source of secreted and surface exposed protein anti-gens and they have been reported to contain major protective antigens for tuberculosis (1). Using this information, we have analyzed CF from M. ptb ATCC 19698, grown as a surface pellicle on synthetic Sauton medium, for the presence of immunodominant T cell antigens. CF proteins were separated according to their molecular weight in 30 fractions using the "Whole Gel Eluter" (Biorad) and these fractions were tested for their capacity to elicit positive lymphoproliferative and IFN-gamma responses in PBMC cultures from naturally and experimentally infected cattle and in spleen cell cultures from experimentally infected mice. Several fractions in the 25-40 kD m.w. induced strong T cell responses. Highest IFN-gamma responses were found to fractions containing the M. ptb. homologues of the Ag85 complex, which ranks among the most promising TB vaccine candidates (2, 3). We therefore cloned the genes encoding the Ag85A and Ag85B proteins from M. ptb in eucaryotic expression vectors and tested the plasmids for immunogenicity in C57BL/6 and BALB/c mice. Strong Ag85 specific IFN-gamma and IL-2 responses could be induced by plasmid immunization. Protection studies in these and in mutant beige mice are in progress.

Title Immune response, pathological features and bacteriological findings after vaccinating a goat flock.

Author(s) Domínguez M C, Trigo F, Cervantes R, Suárez F, Del Río J, Chávez-Gris G.

Institution Departamento de Patologia. FMVZ. UNAM.

Abstract A study to compare the humoral and cellular immune responses, bacteriological and anatomopathological findings after vaccination a goat flock infected with paratuberculosis was performed. The humoral immune response in vaccinated (Group V) and non-vaccinated animals (Group NV) was temporally measured seven times by Agar Gel Immunodifussion (AGID), Enzyme-Linked Immunosorbent Assay (ELISA) and Counter Immuno-Electrophoresis (CIE). The cellular immune response was evaluated three times by delayed-type hipersensitivity skin reactions using PPD-A and PPD-B. Postmortem and bacteriologic studies were carried out in all animals that died or were culled during the study. With the 3 serological tests, Group V showed an increse in the humoral immune response between 15 and 60 days after vaccination. Group V also developed a strong a constant cellular immune response after vaccination, when compared with Group NV. However, paratuberculosis lesions in both groups were similar in severity and amount of acid-fast bacilli. Bacterial isolation was obtained was obtained from feces and tissue samples from both groups. The humoral immune response seemed to interfere with the serodiagnosis just for a brief period, while the cellular immune response was stronger and possibly would modify the course of the lesions and diminish the mycobacterial load in infected goats in a larger period of time.

Title In utero transmission of OJD.

Author(s) Lambeth C[1*] , Reddacliff L[1] , Windsor P[1] , Abbott K[2] , McGreggor H[2] , Whittington R[1] .

Institution [1] NSW Agriculture, Elizabeth Macarthur Agricultural Institute, PMB 8, Camden NSW 2570. [2] University of Sydney, Department of Clinical Sciences, Private Bag 3, Camden, NSW 2570.

Abstract Studies in cattle have demonstrated that Johne's disease can be transmitted from cow to calf in utero. Similar studies have not been carried out in sheep. Many of the current control practices in use assume that lambs are born uninfected, making it an important question to be answered. Post mortems were carried out on an infected, pregnant mob of 145 sheep on a property near Golbourn, NSW. The mob was previously screened by gel test, DTH skin test, gamma-interferon test and faecal culture. The following samples were collected from each sheep: ileocaecal valve, terminal ileum, ileocaecal lymph node, mesenteric lymph node, cotyledon, supramammary lymph node, milk/colostrum. And from the foetuses: terminal ileum (including ileocaecal valve), ileocaecal lymph node, mesenteric lymph node, blood. Tissues were collected for both histopathological examination and culture. A total of 46/145 (31.7%) ewes were culture positive, with 40/145 (27.6%) showing histopathological lesions. The foetal tissues from all infected ewes were cultured as well as controls to give a total of 80 foetuses. In addition tissues were collected from 4 pregnant, clinical cases in post mortems carried out at University of Sydney. These were added as there were few clinical cases in the mob from Golbourn. Cultures and histopathological examination of the foetal tissues is currently underway, and complete results will be discussed.

Title T-cell responses of calves infected with *M. a. paratuberculosis*.

Author(s) Storset AK[1*] , Hasvold H[1] , Olsen I[2] , Djønne B[2] , Larsen HJS[1] .

Institution [1] Department of Pharmacology, Microbiology and Food Hygiene, The Norwegian School of Veterinary Science, P.O.Box 8146, N-0033 Oslo, Norway. [2] National Veterinary Institute, Oslo, Norway.

Abstract The T-cell subsets taking part in the responses of eight calves infected with a caprine isolate of *M. a. paratuberculosis* were studied. Blood and faeces were sampled at regular intervals to monitor the cells responding to PPDp in vitro and to detect faecal excretion of *M. a. paratuberculosis*. The IFN-gamma response of whole blood to in vitro PPDp stimulation was higher in all the infected animals than in a group of age matched control animals from three months post infection and onwards. Six of the eight infected calves excreted bacteria to faeces during the third and fourth month post infection. The CD4+ cells were the major IFN-gamma producers as shown by intracellular staining. However, in addition to CD4+ cells, both CD8+ and gamma delta TcR+ cells from the infected animals up regulated IL-2 receptor (IL-2R) in response to in vitro PPDp stimulation. Of the gamma delta TcR+ subpopulations, mainly the WC1+ cells have been studied in mycobacterial infections of ruminants. However, as the WC1-CD2+ cells are the most abundant gamma delta TcR+ subpopulation in the intestine, the functions of these cells were studied further. While the WC1+ gamma delta TcR+ cells had a high level of IL-2R in peripheral blood, which was sustained by PPDp stimulation in vitro, the CD2+ gamma delta TcR+ cells had lower levels of IL-2R in peripheral blood, but up-regulated the IL-2R expression through in vitro PPDp stimulation. These results indicate that the CD2+ and the WC1+ gamma delta T-cells have different kinetics of activation and may play different roles in the immune response against mycobacteria.

Title Diagnosis of Paratuberculosis (PTB) in young cattle: Evaluation of humoral and cellular immunity in a heifer cohort during the first 8 months of age.

Author(s) Antognoli MC[1] , Salman MD[1*] , Goodell G[1] , Hirst H[1] , Hyatt D[1] , Martin BM[2] .

Institution [1] Animal Health Population Institute, College of Vet Med And Biomedical Sciences, Colorado State University, Fort Collins Co, 80523-1676. [2] USDA, APHIS:VS, NVSL.

Abstract A combination of tests based on detection of cellular and humoral responses were evaluated in a naturally exposed calf cohort over 8 months and compared to both fecal culture and PCR. The objectives of this study were:

1. To evaluate the validity of intradermal tuberculin tests, gamma interferon and ELISA in a cohort of calves with reference to fecal culture and PCR results.
2. To compare the findings from CMI or antibody-based tests in calves born from ELISA positive, suspect, and negative dams.
3. To explore the association between the PTB status of the dams (determined by serology, fecal culture, PCR in milk and feces) and the status of their respective heifers (determined by humoral and CMI-based tests, and fecal culture/PCR).

Two dairy herds with 11% and 2% seroprevalence of PTB infection were the source of study heifers. A cohort of heifer calves born from sero-positive, suspect and negative dams was followed up until 30 months of age with the purpose of characterizing the cellular and humoral immunity of naturally exposed individuals. Enrollment of heifers started in November 2000 and is currently on going. Preliminary descriptive results on 308 dams and heifers indicate the following:

1. Skin tests reactions in calves are more numerous when testing is performed between 4 and 6 months of age.
2. The majority of the skin test reactions correspond to the cervical injection site rather that the caudal fold site.
3. The ELISA in calves remains negative despite the repetitive intradermal injection of M. avium subsp. paratuberculosis PPD.
4. There is a significant loss of heifers born from ELISA positive dams due to

perinatal death or premature culling because of repetitive pneumonia or delayed growth.
5. Skin test reactions in calves seem not to be associated with the dam's ELISA status.

Title Development of a p35-based ELISA assay for the specific detection of *M. avium* subsp. *paratuberculosis* (Map) infection in cattle with Johne's disease.

Author(s) El-Zaatari FAK[1*] , Collins MT[2] , Huchzermeier R, Graham DY.

Institution [1] Baylor College of Medicine and VA Medical Center, Houston, TX. [2] University of Wisconsin, Madison, WI. 3 Syracuse Bioanalytical, Inc., Ithaca, NY.

Abstract

Background.

Early diagnosis of paratuberculosis in cattle is essential for controlling the spread of Johne's disease. Available tests lack sensitivity. We have shown that a recombinant p35K antigen was Map specific by immunoblotting.

Purpose.

To develop a purified p35-based ELISA and p35-immunoblots and to evaluate their efficacy compared to commercial ELISAs.

Methods.

Map-specific p35 protein was purified as a histidine-tagged fusion protein. A purified p35-based ELISA was developed and optimized and its efficacy evaluated on 44 reference sera. The results were compared to that of commercial ELISA assays and immunoblotting. The efficacy of the ELISAs was evaluated on sera from 100 cattle in a suspected infected herd.

Results.

Sera from 12 cattle with clinically advanced JD were positive by all assays. Sera from 10 Map-free cattle and 3 cows artificially inoculated with multiple doses of viable Map were negative by all assays. For sera from cows with subclinical infection, p35-ELISA was positive in 13/19 (68%) as compared to 42%, 47% and 53% for the most commonly used commercial ELISAs, respectively. One additional sample (14/19; 74%) was positive only by p35 immunoblotting. If the p35 assay was used in parallel with one commercial assay, the sensitivity increased to 79% (15/19). The specificity and the positive predictive values of the tests were 100% and the negative predictive values (NPV) of p35-ELISA and the better commercial-ELISA assays were 62.5% and 52%, respectively. When testing the 100 serum samples from cattle in the infected herd, the p35-ELISA was comparable but not significantly different from commercial ELISAs.

Conclusion.

Based on this limited initial evaluation, the p35-ELISA shows promise as a potentially more sensitive and specific test for paratuberculosis. The addition of p35-based immunoblot test further improved detection of subclinical cases. Evaluation of p35-ELISA on a larger set of well-characterized sera is warranted.

Title Improving detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle by anamnestic ELISA.

Author(s) Taddei S[*] , Cavirani S.

Institution Dipartimento di Salute Animale, Sezione di Malattie Infettive degli Animali, Università di Parma, Italy.

Abstract A practical approach to the indirect diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle is performed by ELISA test. Nevertheless, ELISA has been criticized as not being sufficiently sensitive because gave positive results after bacterial

excretion in feces has began. A commercially available indirect ELISA test (IDEXX) allows to discriminate serum samples on the basis of three possible results: negative (net sample OD (15% of net positive control OD), positive (net sample OD (30% of net positive control OD) and doubtful (net sample OD values ranging from 15 to 30% of net positive control OD). To improve test sensitivity and to differentiate doubtful animals as positive or negative, an anamnestic ELISA was applied. At the aim, 300 animals aged 2 - 4 years, of which 100 medium-strong-negative (net sample OD (5% of net positive control OD), 100 weak-negative (net sample OD values ranging from 5 to 15% of net positive control OD) and 100 doubtful, as detected by ELISA, were intradermally inoculated with johnin. In addition, 300 animals aged 2 - 4 years, of which 100 medium-strong-negative, 100 weak-negative and 100 doubtful were kept as non-inoculated controls. After 20 days post-inoculation, sera were collected and subjected to ELISA (anamnestic ELISA). 54 doubtful and 20 weak-negative animals became positive after johnin, otherwise no medium-strong-negative animals seroconverted. Only 22 doubtful and 4 weak-negative controls became spontaneously positive at second sampling. Statistical analysis (chi-square) pointed out a significant difference of seroconversion rate between johnin-treated and controls for either doubtful or weak-negative animals. Therefore, anamnestic ELISA may be considered a promising tool to improve serologic detection of paratuberculosis infection in cattle.

Title Validation of *Mycobacterium paratuberculosis* antibody detecting ELISAs.

Author(s) Van Maanen C* , Koster C, van Veen B, Kalis CHJ, Collins MT.

Institution Animal Health Service. The Netherlands.

Abstract

Background.

Several serological tests (ELISA, CFT, AGIDT) are available for the detection of antibodies against *M. paratuberculosis*. For the detection of antibodies in cattle ELISAs are most widely used. The reproducibility and sensitivity/specificity of ELISAs are considered better than those of alternative tests. Additionally, ELISAs are less laborious and therefore allow high throughput testing. Herd sensitivity and herd specificity are important criteria for certification programs. To calculate these parameters, it is of utmost importance to determine test characteristics like sensitivity, specificity and reproducibility accurately. Therefore, we performed an extensive comparative validation study of five commercially available ELISAs.

Methods.

Sensitivity, specificity, diagnostic performance, and reproducibility of commercially available ELISAs for detection of antibodies against *M. paratuberculosis* were evaluated using different serum panels from Dutch, American and French cattle populations. Relative sensitivity was evaluated taking faecal shedding as a gold standard (288 sera from faecal shedders from different origin in total). Specificity was evaluated using in total 2135 sera originating from repetitively faecal culture-negative herds from different origin. Diagnostic performance of tests was evaluated by ROC analysis, and effects on sensitivity and specificity of parallel testing schemes were evaluated. Additionally, detection limit, reproducibility (within and between plate variance) and other technical criteria were evaluated.

Results.

Results are too extensive to present in this abstract. However, clear differences in overall sensitivity, specificity and diagnostic performance were found between ELISAs. Also, for subpanels of different origin differences in specificity were found, possibly reflecting regionally relevant cross-reactions. Results of four out of five ELISAs were strongly correlated, whereas results of one ELISA showed low correlation coefficients with all other tests. Parallel testing using the latter ELISA with one of the other ELISAs would theoretically yield a higher combined sensitivity but a lower combined specificity. The feasibility of such a testing scheme, based on the underlying data, will be discussed besides the other parameters investigated.

Conclusions.

Most of the commercial ELISAs evaluated had similar overall accuracy but important differences were observed that must be considered when selecting the best ELISA kit for a

laboratory.

Title Likelihood ratios provide a rational and practical method for quantitative use of ELISAs for paratuberculosis.

Author(s) Collins MT.

Institution Dept of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison.

Abstract

Background.

ELISA optical density (OD) values are a measure of antibody concentration. Transforming OD values to positive or negative interpretations based on a single cut-off value ignores the clinical value of the magnitude of the ELISA result. Likelihood ratios (LHRs = [sensitivity/(1-specificity)]) calculated on sera from well characterized cases and controls allows the magnitude of ELISA results to be used to estimate the probability the tested animal has or does not have paratuberculosis.

Purpose.

Calculate LHRs for a wide range of ELISA S/P values.

Methods.

LHRs were determined using the a serum antibody ELISA for Johne's disease (IDEXX) on 143 bovine sera from culture- or histopathology-confirmed cases of paratuberculosis and 2,973 bovine sera from animals in herds proven free of *M. paratuberculosis* infection (herds negative on whole-herd fecal culture a minimum of eight times at intervals of 6 months or more). ELISA ODs were transformed to S/P values as per the kit manufacturer's directions. Sensitivity and specificity were calculated for S/P values 0.0 to 2.0 in intervals of 0.05 S/P units.

Results.

LHRs ranged from 1 to 583. LHR (y-axis) was plotted against ELISA S/P value cut-off (x-axis). These data pairs fitted a line defined by a power function with an r2 value = 0.94.

Conclusions.

The LHR algorithm can be used to estimate the probability of *M. paratuberculosis* infection.

Example

A cow with an ELISA S/P of 0.40 has an LHR of 41:1 (odds the cow is infected); converted to probability this is 97.6%. Infection probability estimates are more accurate if the pre-test probability of infection is included in the calculation. Comparison of LHR values on the 143 cases of paratuberculosis to other test results catalogued in a repository database showed that LHR values are directly related to the percentage of cattle positive on other tests for paratuberculosis.

Title Monitoring Johne's ELISA test results across laboratories.

Author(s) Dargatz D, Byrum B, Collins M, Goyal S, Hietala S, Jacobson R, Kopral C, Martin B, McCluskey B, Tewari D.

Institution USDA:APHIS Centers for Epidemiology and Animal Health.

Abstract Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), has been estimated to cost the U.S. dairy industry more than $200 million annually. Serological tests for antibodies play a significant role in control programs for MAP. To document the expected variability and identify sources of variability that could be minimized by adoption of standardized techniques a study was designed. Five laboratories using the IDEXX ELISA for MAP were recruited to participate in the pilot. Three serum samples, one negative (NC), one positive (P), and one high positive (HP), were aliquoted and lyophilized. Each laboratory received the three samples. After reconstitution the 3 serum samples were included on all plates

when client samples were being tested. Each plate or each run was treated as a separate observation. Overall 868 plates were run representing 5 kit lots. The data were analyzed graphically to observe variation over time and among kit lots. In addition, a random effects model was used to estimate the contribution of each of the factors (lab, kit lot, well, day-to-day) to the overall variability in S/P ratios. Modeling the S/P ratio for the P sample showed that the largest amount of variation (37.5%) was attributed to kit lot followed by random error (27.0%) and inter-laboratory variation (18.3%). Variation among the S/P values for the HP sample showed a different pattern with random variation accounting for 55.0% followed by test date (21.4%) and laboratory (17.1%). These data should be compared to other ELISA based tests to determine comparability in terms of overall amount of variation in the outcome and the proportional distribution of variation attributed to specific factors. Routine monitoring of variation in ELISA results using a standard panel of sera on a national or international basis would help discover sources of error and improve the overall quality control for paratuberculosis serology.

Title Validation of the interferon-g test for diagnosis of ovine Johne's disease: sensitivity and specificity field trials.

Author(s) Stewart DJ[1*] , Stiles PA[1] , Whittington RJ[2,8] , Lambeth C[2] , Windsor PA[2,8] , Reddacliff LA[2] , McGregor H[3] , Dhungyel OP[3] , Cousins DV[4] , Francis BR[4] , Morcombe PW[4] , Butler R[5] , Salmon DD[6] , Jones SL[7] .

Institution [1] CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, 3220. [2] NSW Agriculture, Elizabeth Macarthur Agricultural Institute, Camden NSW 2570. [3] University of Sydney, Department of Veterinary Clinical Sciences, Private Bag 3, Camden, NSW 2570. [4] Animal Health Laboratories, Department of Agriculture, South Perth, Western Australia 6151. [5] Department of Agriculture, Merredin, Western Australia 6415. [6] Riverina Rural Lands Protection Board, Deniliquin, NSW 2710. [7] CSL Limited, 45 Poplar Road, Parkville, 3052 Australia. [8] Current address: University of Sydney, Department of Veterinary Clinical Sciences, Private Bag 3, Camden, NSW 2570.

Abstract Field trials are underway to validate the interferon-g test for diagnosis of ovine Johne's disease prior to it being considered for endorsement and adoption as an official diagnostic test. The test kits (BOVIGAM™, PARACHECK™) and Johnin PPD for these trials are being provided by CSL Limited. The interferon-g test specificity trials will involve 6 flocks of Merino sheep on uninfected properties in New South Wales, Victoria, Western Australia and South Australia. Specificity trials on 2 flocks with 120 sheep and 3 age classes (adult ewes, yearlings and weaned lambs) in each flock have been completed. An interferon-g test sensitivity trial has been conducted in collaboration with NSW Agriculture on an infected commercial Merino flock, containing 146 ewes, with follow-up diagnostic bacterial culture and histopathology at slaughter. The interferon-g test is also being evaluated, in collaboration with the University of Sydney, in a 3 - year longitudinal study on a naturally infected experimental Merino flock of sheep. This trial will provide information on the interferon-g responses of 420 sheep at 6-monthly intervals during the trial period and their final infection status following autopsy. The results of the specificity trials on 2 flocks so far sampled and the sensitivity trial will be discussed. The trials for the validation of the interferon-g test in sheep are being funded by Meat and Livestock Australia.

Title Diagnostic tests for Johne's disease in red deer in Victoria.

Author(s) Schroen CJ[1] , Roche MJ[2] , Gwozdz J[1] , Bradley TL[1] , Jones S[3] , Condron RJ[1*] .

Institution [1] Victorian Institute of Animal Science 475 Mickleham Road Attwood 3049 Australia. [2] Department of Natural Resources and Environment Warrnambool 3280 Australia. [3] CSL Ltd Poplar Road Parkville 3052 Australia.

Abstract Johne's disease is recognised as an emerging problem in red and fallow deer in many countries. Prior to 1999 Johne's disease had not been identified in deer in Australia. Recently, however,

five red deer herds in Victoria have been confirmed to be infected with the bovine strain of *Mycobacterium paratuberculosis*. These included two herds with high prevalence of infection and significant clinical disease in yearling animals. In particular mobs, more than 60% of animals were infected with *M. paratuberculosis* when tested by faecal culture and/or culture of tissues at slaughter. To minimise the risk of spread of Johne's disease between farms several Australian livestock industries have implemented Market Assurance Programs (MAP) which involve testing statistically significant samples of animals and adopting appropriate management practices to avoid introduction of infected animals into the herd. For the purpose of implementing a MAP for the deer industry, an ELISA and faecal culture have been evaluated. Three different conjugates were optimised for use in absorbed ELISA using plates and diluents from a commercial Johne's disease kit (Parachek). Pooling of faeces has also been examined to determine the sensitivity and feasibility of detecting infected animals from pools of various sizes. The receiver operating curves and performance of anti-deer, protein G and anti-bovine conjugates were similar although the protein G conjugate appeared to have a marginally higher relative sensitivity. Pooling of faeces for culture provides an opportunity to reduce the cost of herd testing.

Title Targetting of in vivo expressed antigens of *Mycobacterium avium* subsp. *paratuberculosis* for the production of monoclonal antibodies.

Author(s) Hitchings E[1*] , Rowe MT[1,2] , Grant IR[1] , Ball HJ[2] .

Institution [1] Department of Food Microbiology, Queen's University of Belfast. [2] Veterinary Sciences Division, Department of Agriculture and Rural. Development for N. Ireland, Belfast, N. Ireland, UK.

Abstract The aim of this study was to produce monoclonal antibodies (MAbs) to in vivo expressed antigens of *Mycobacterium avium* subsp. *paratuberculosis* (Map) that would be useful for the development of assays for the detection of the organism in both disease and food specimens. Eight BALB/C mice were injected with live Map to cause infection. The intention was to stimulate an immune response that would include novel antibodies to in vivo expressed antigens that would be absent from mice immunised with culture-grown dead cells. Hybridoma fusions produced from the spleen cells of these Map-infected mice were screened with antigen derived from the intestines of animals naturally infected with Map. Several hybridomas were selected on the basis that they reacted by ELISA to the Map-infected bovine tissue antigen but not to that from normal tissue. Subsequent testing of the cloned lines failed to differentiate between additional antigens derived from healthy and Map-infected bovine small intestine. In addition, examination of the livers from the infected mice showed little or no sign of granulomatous lesions that would have been indicative of Map infection. Therefore no Map-specific MAbs were obtained. Since there was little evidence to demonstrate that Map mouse infection had occurred, this failure cannot necessarily be attributed to the scientific approach. Mouse infection, demonstrated by granulomatous liver lesions, had been observed in previous experiments, and the reason for failure in this experiment is unclear. The approach towards obtaining MAbs to Map-specific in vivo expressed antigens will continue by attempting to achieve such expression in Map cells cultured in macrophage.

Title Evaluation of a (lipo)polysaccharides absorbed ELISA for the serological diagnostic of *M. paratuberculosis*.

Author(s) Walravens K[1*] , Marché S[1] , Malbrecq S[1] , Rosseels V[2] , Huygen K[2] , Godfroid J[1] .

Institution [1] Veterinary and Agrochemical Research Centre, Brussels. [2] Mycobacterial Immunology, Pasteur Institute of Brussels, BELGIUM.

Abstract It has been demonstrated that lipopolysaccharides and polysaccharides of *Mycobacterium avium* subsp. *paratuberculosis* (Mptb) are major B-cell antigens in ruminants and therefore are components of interest for the development of serological diagnostic tests. We have extracted (lipo)polysaccharides antigens from *Mycobacterium bovis* BCG, *Mycobacterium phlei* and Mptb. Electrophoresis analysis showed that the major component of this antigen

preparation had an apparent MW corresponding to that described for the lipoarrabinomannan (LAM). Analysis of the reactivity of serum from animals experimentally infected with Mptb or *M. bovis* or sensitized to *M. phlei* showed strong cross-reactive responses against the LAM antigens tested even if intensity of the responses observed were greater against autologous LAM antigen. Cross-absorption studies showed that at least some epitopes seemed to be recognized by antibodies specific for the different Mycobacteria. Development of an ELISA test for the diagnostic of Mptb was performed using Mptb LAM for the plate sensitization and *M. phlei* LAM for the absorption step. This ELISA was evaluated on serum samples from animals originating from paratuberculosis, tuberculosis or control herds. Our results suggest: i) that the absorption step was essential for the specificity of the test; ii) and comparison with conventional commercial diagnostic tests showed at least similar performances. It was also observed that with the tests used, it was not possible to distinguish between *M. bovis* and Mptb infected animals.This work was partially funded by grants from the Belgian Ministery of Agriculture (DG6).

Title Reactivity of recombinant SOD from *Mycobacterium avium* subspecies *paratuberculosis* in the IFN-g test.

Author(s) Beddome G, Bruce K, Shiell BJ, Stiles PL, Michalski WP*.

Institution CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Abstract The mechanisms of immunity, pathogenesis and the molecular genetics of *M. avium* subspecies *paratuberculosis* are little known and only a few immunogenic proteins have been described. The identification and characterisation of novel antigens is of paramount importance for improved early diagnosis. Superoxide dismutases catalyse the dismutation of superoxide radical-ions and have been postulated to protect mycobacteria against oxidative stress. Secreted SODs have been identified in *M. tuberculosis* and *M. avium* subspecies *paratuberculosis* "in vitro" cultures. The objective of this study was to assess reactivity of Mn-containing SOD in a cell mediated immunity assay (IFNg release assay, BOVIGAM™, CSL Animal Health Ltd). Mn-containing SOD was identified in cell free supernatants of early cultures of *M. avium* subspecies *paratuberculosis*. Purified native SOD and its recombinant version expressed in *E. coli* were tested in the BOVIGAM™ assay. Fractions containing both native and recombinant SOD showed weak but significant activity in a small number of animals. To determine the true reactivity of *M. avium* subspecies *paratuberculosis* SOD, recombinant protein was purified to a homogeneity level sufficient to remove residual contaminants of *E. coli* origin. The purified SOD showed no activity when re-tested in the BOVIGAM test.

Title Sera from *M. avium* (Ma) infected guinea-pig cross-reacts with *M. paratuberculosis* (Mptb) 34 kDa antigen.

Author(s) Willemsen PTJ*, Riepema KH, Ruuls R, Bakker D.

Institution Central Institute Animal Disease Control (CIDC), Lelystad, The Netherlands.

Abstract Diagnosis of paratuberculosis is severely hampered by the lack of specific and sensitive diagnostic methods. In particular, serological tests are still of limited value partly because of cross-reactivity with related bacteria causing false positive reactions. The report on the immunodominance of a 34-kDa protein in sera of animals with Johne's disease promised the development of a new serological diagnostic tool. The more this new antigen contained species specific B-cell epitopes, facilitating the discrimination between infections with Mptb and related mycobacteria from the avium complex. In this study we showed that sera from quinee-pig infected with Ma recognized purified Mptb 34 kDa-antigen as well as a fusion-protein containing a C-terminal fragment of the 34 kDa-antigen containing "supposed Mptb specific" B-cell epitopes. We therefore conclude that claims on species-specific serological assay's based on the the 34 kDa antigen should be reconsidered.

Title Correlation between seropositive reactors to paratuberculosis and to pseudotuberculosis in sheep.

Author(s) Krt B[1] , Ocepek M[1*] , Pislak M[1] , Juntes P[1] , Pogacnik M[1] , Cvetnic #[2] .

Institution [1] Veterinary faculty, Gerbiceva 60, 1115 Ljubljana, Slovenia. [2] Croatian veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia.

Abstract

Introduction.

It is known that bacteria from Corynebacterium and Mycobacterium genus have some antigenic determinants in common and therefore serological cross-reactions may occur. In this study serological reactions in paratuberculosis and pseudotuberculosis infected flock were compared.

Material and methods.

In the flock where paratuberculosis and pseudotuberculosis were confirmed bacteriologically, sera of 519 animals were tested. For the detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* commercial ELISA kit (IDEXX, Sweden) was used whereas for pseudotuberculosis serology ELISA was developed in our laboratory.

Results and discussion.

In paratuberculosis test 38 samples were positive and 17 were doubtful. 42 samples were positive in pseudotuberculosis test (7 of them highly positive) whereas 60 samples were doubtful. Among 42 pseudotuberculosis positive sera 43% were positive and 14% were doubtful in paratuberculosis test. The percent of paratuberculosis positive animals was increased in cases of pseudotuberculosis highly positive sera to 71%. In pseudotuberculosis doubtful sera 10% were paratuberculosis positive whereas in pseudotuberculosis negative sera only 3% were paratuberculosis positive. Our results suggest that special care should be taken by interpretation of paratuberculosis positive serology in the flocks infected with *Corynebacterium pseudotuberculosis*. The work was supported by Ministry of Agriculture, Forestry and Food of the Republic of Slovenia.

Title Reliability of serological methods for paratuberculosis diagnosis in cattle.

Author(s) Couquet C.

Institution Laboratoire Vétérinaire Départemental de la Haute-Vienne. Av. Pr. J. Leobardy. 87000 Limoges. France.

Abstract The control of paratuberculosis is becoming a major issue for French breeders. The Laboratoire Vétérinaire Départemental of Haute-Vienne has been involved for years in routine controls of the status of high genetic value herds by serological testing and coproculture. The aim of this study is to compare the performances of three commercially available ELISA tests that may be used in large scale control programs. The specificity was evaluated on sera (n=250) coming from closed herds declared as negative following controls by coproculture every six months that did not gave any positive result for at least 6 years. Tests A, B and C gave specificities of 100%, 97.7% and 67% respectively (doubtful considered as negative). As there is no perfect method allowing paratuberculosis diagnosis on living animals, the sensitivity was evaluated on sera coming from all the animals (n=90) of a herd considered as infected on the basis of frequent coproculture positive results within the herd. Test A detected 23 positive sera, test B detected 32, test C detected 56 (doubtful considered as negative). Test A and B did not detect always the same sera in the infected herd suggesting some differences on antigens used for the preparation of the plates. Distribution of optical densities suggested that a better sensitivity (36 sera detected instead of 23) could be obtained for test A without altering specificity just by lowering the cut-off. The apparently good sensitivity of test C is difficult to interpret because of its very poor specificity: a lot of the extra positive sera may be false positive. The detectability was evaluated by serial dilutions of a pool of sera coming from coproculture positive animals. Test A detected of a two fold higher dilution of the pool of positive sera than test B and C which were equivalent. This work suggests that strong efforts must be done to standardise serological reagents for paratuberculosis and create panel of reference sera for the evaluation of performances of the different techniques.

Title A rapid serologic test for the detection of antibodies to *Mycobacterium paratuberculosis* with applications for bovine practitioners

Author(s) Jackson TA, Schroeder C* , Cecchetti P, McCrann M.

Institution Production Animal Services Research & Development, IDEXX Laboratories, Westbrook, Maine, USA.

Abstract

Introduction.

An ELISA test has been developed on the IDEXX SNAP™ device to detect antibodies to *Mycobacterium avium* subsp. *paratuberculosis* (M.pt.), the causative organism of Johne's disease in ruminants. Initial validation studies have been completed utilizing bovine serum as the specimen type. This SNAP test format has potential applications as in-clinic assay for large animal veterinarians who require a rapid test result (22') for symptomatic or suspicious animals. The purpose of the study in this report was to evaluate the performance of this new M. pt. ELISA by testing populations of dairy cattle (n = 1276).

Materials and Methods.

Sera were tested with the prototype test system and with a USDA-licensed microtiter plate antibody test kit. Quantitative data were recorded for the SNAP test platform by taking densitometric readings of the diagnostic spot at the completion of the test protocol. These values were compared to the S/P ratios yielded by the microtiter-plate technique by regression analysis.

Results.

The percent agreement in test results between the two assay methods utilized for herd groups defined by test histories were as follows: known infected herds - 94.4%; presumed negative herds - 99.1%; herds of unknown status - 96.8%. Regression analysis of quantitative data shows a significant correlation between the two techniques utilized, ($R2 = 0.73$; $p < 0.0001$; 95% CI). Ninety-six point six percent (96.6%) of the sera tested (1232 of 1276) yielded an agreement in serologic status as determined by the two test methods evaluated.

Conclusions.

The relative sensitivity and specificity of a new ELISA test was measured against an established technique. These data provide evidence of a significant correlation in performance between the new SNAP test and the microtiter-plate format. Further, the prototype SNAP test yields specimen dispositions which are consistent with the known source-herd histories for the bovine populations studied.

Title Comparison of different indirect ELISA methods on reference cattle

Author(s) Garrido JM* , Aduriz G, Geijo MV, Sevilla I, Juste RA.

Institution NEIKER (Instituto Vasco de Investigación y Desarrollo Agrario), Dpto. de Sanidad Animal. Berreaga 1, 48160 Derio. Bizkaia. Spain.

Abstract The ELISA test, although lacking sensitivity for subclinical infections, is a very convenient diagnositic method because it is quick and inexpensive. In order to determine the relative performance of each of four commercially available ELISA protocols, we carried out an assay where four of these tests (A, B, C, D) plus a locally modified PPA3 based ELISA (NEIKER) were compared. We used two sets of sera. One consisted of 20 suspect fecal culture confirmed cases, and 174 animals from a herd with no history of clinical paratuberculosis, nor serological reactors in the last 5 years. The other set was composed of 201 adult cattle killed in a slaughterhouse on which tissue culture and PCR and histopathology of the ileocecal valve and lymph node were used for classification into infected or non-infected. All the 395 sera were submitted to the NEIKER test, but only the first set was used for the five tests comparison. Overall, the NEIKER ELISA showed a sensitivity of 54.4% and an specificity of 93.2%, with an 87.6% agreement (kappa: 0.49, $p<0.05$) with the reference scoring. On the first set, our ELISA showed 100% sensitivity and specificity, but only ELISA A was close to the performance of NEIKER test showing a sensitivity of 95.0% and an specificity of 100% with a 95.5% (kappa: 0.97, $p<0.05$) agreement. The other tests showed a sensitivity ranging from

32.3% to 88.9% and an specificity ranging from 30.0% to 98.9%, with an agreement ranging from 47.5% to 96.9%. These results indicate that there might be great variability in ELISA performance according to the antigen and procedure used.

Title Agar gel immunodiffusion test (AGID) evaluation for detection of bovine paratuberculosis in Rio de Janeiro, Brazil.

Author(s) Ferreira R[1] , Fonseca LS[1] , Lilenbaum W[2*] .

Institution [1] Department of Medical Microbiology, Universidade Federal do Rio de Janeiro, Brazil. [2] Department of Microbiology, Universidade Federal Fluminense.

Abstract The aim of this study is to evaluate the AGID serological test for detection of antibodies anti-*M. paratuberculosis* and its possible adoption as diagnostic method in our field conditions. Bovine serum samples from dairy farms situated in Rio de Janeiro State, Brazil, were screened for the presence of antibodies against *M. paratuberculosis* using three different ELISA tests (PARACHECK, HERDCHECK, ELISA-PPA). Forty-eight randomly selected sera were evaluated by an agarose gel immunodiffusion (AGID) test using Protoplasmatic Paratuberculosis Antigen (PPA). AGID results were compared to the standards - the result of the three ELISA tests, and the specificity and sensitivity were calculated. From 48 sera tested for AGID, 14 (29.17%) were positive and 34 (70.83%) were negative. AGID sensitivity was 57% with two false-positive reactions, and specificity was 92.5% with nine false-negative results. The positive predictive value was calculated in 85.7% for a confidence interval of 95%. Thus, due to its low sensitivity and acceptable specificity rates, we do not agree with the recommendation of using AGID tests as a screening but only as a confirmatory diagnostic method for clinical suspects animals.

Title An ELISA for detection of bovine paratuberculosis.

Author(s) Ferreira R[1] , Fonseca LS[1] , Oelemann WMR[2] , Lilenbaum W[3*] .

Institution [1] Department of Medical Microbiology, Universidade Federal do Rio de Janeiro, Brazil. [2] Department of Immunology, Universidade Federal do Rio de Janeiro, Brazil. [3] Department of Microbiology, Universidade Federal Fluminense.

Abstract Paratuberculosis (Johne's disease) is a chronic enteritis that affects ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis*. The disease is spread worldwide and causes important economic losses. In Brazil, *M. paratuberculosis* has been isolated, but there are no statistic studies about its prevalence. In this study, 200 sera selected from animals held at dairy farms in Rio de Janeiro State, Brazil, were tested for the presence of antibodies specific for *M. paratuberculosis* using an in-house ELISA test. This test employs a protoplasmic paratuberculosis antigen (PPA - Allied Monitor) as capture antigen and a monoclonal anti-bovine IgG conjugated to alkaline phosphatase (Sigma). Eighty-seven (43.5%) samples were reactive in the PPA-ELISA and 113 (56.5%) were negative. The results were compared to those obtained with the commercial ELISA test PARACHEK (CSL) and showed a sensitivity of 61.6% and a specificity of 70.2%, with 38.4% (33/86) of false negatives and 29.8% (34/114) of false positives. The use of the PPA-ELISA showed to be feasible and can be helpful for herd diagnosis to identify foci of Johne's disease.

Title Relation between the ELISA optical densities and the histopathological findings in an ovine flock suspicious to paratuberculosis

Author(s) Martínez RG*, Chávez-Gris G.

Institution Departamento de Patología. Facultad de Medicina Veterinaria y Zootecnia. UNAM.

Abstract A total of 549 female, adult sheep, between 1 and 4 years old were tested for serum antibodies evidence of *Mycobacterium avium* subspecies *paratuberculosis*, using the absorbed

linked inmunosorbent assay (ELISA). Fifty one animal gave positive results to ELISA. Seventeen positive animals and three negative animals were necropsied. Gross lesions were recorded and samples for histopathological examination were collected fron ileocecal valve, ileum, jejunum, and mesenteric and ileocecal lymph nodes. Tissues were fixed un buffered 10 % formalin. Sections were stained with haematoxylin and eosin, and by the Ziehl-Neelsen method to detect acid-fast bacteria. Paratuberculosis lesions in ileocecal valve, ileum and jejunum were categorised according to a description of lesions associated with natural paratuberculosis in sheep. From an examination of 200 macrophages in eight fields (40x) of the mucosa with lesions of each sample tissue, were used to determinate the percentage of infected macrophages with mycobacteria. Eight fields of the same cells (100x) were observed to quantificate the mean number of mycobacteria per macrophage, grades as 0 (none), 1 (1-10), 2 (10-60) or 3 (>60). The relations between the serum ELISA optical densities values, the number of mycobacteria per macrophage, the lesion category and the body condition were analysed. The ELISA optical density range values 0.700 to 1.145, was divided into five equal intervals 1(0.700-0.789), 2(0.789-0.878), 3(0.878-0.967), 4(0.967-1.056), 5(1.056-1.145). The classifaction types of lesion of each tissue sample, the mean number of mycobacterias per macrophage and the body condition were asigned to their respective interval and the results are discussed.

Title Comparison of milk- and serum ELISA for detection of paratuberculosis in dairy cattle.

Author(s) Klausen J, Huda A* , Ekeroth L, Ahrens P.

Institution Danish Veterinary Institute, Copenhagen and Danish Dairy Board, Brörup, Denmark.

Abstract

Introduction.

Available tests for diagnosing paratuberculosis are based on detection of either the pathogen or of the host's immune response to this. Detection by bacteriological culture of faeces samples requires minimum 8 weeks of incubation; thus, detection of antibody response by ELISA would be advantageous for diagnosis of paratuberculosis. Here a milk ELISA and a serum ELISA for detection of antibodies against *Mycobacterium avium* ssp. *paratuberculosis* were evaluated.

Materials and methods.

Milk and serum were obtained concurrently from 6 dairy herds infected with *Mycobacterium avium* ssp. paratuberculosis and from 2 non-infected dairy herds. The two ELISAs were compared with CF test and culture.

Results and discussion.

At a cut-off value of 7 OD%, all 6 culture positive herds were found positive in the serum ELISA, whereas one of the 6 herds was found negative in the milk ELISA. All 6 culture positive herds were found positive in the CFT. In the 2 culture negative herds, the serum and the milk ELISA deemed all serum samples negative at this cut-off value, whereas 4 serum samples from one of these herds were found positive in the CFT. The highest cut-off value enabling the milk ELISA to record all 6 culture positive herds as positive was 4 OD%. The highest cut-off value enabling the serum ELISA to record all 6 culture positive herds as positive was 17 OD%. Individual sample sensitivity and specificity of the ELISAs at the cut-off values of 4, 7 and 17 OD%, respectively, were estimated. At 17 OD%, both ELISAs were almost equally effective in detecting infected herds. In a control programme, an ELISA would be a good first step for identifying the affected herds. The use of milk samples instead of the serum samples would be more convenient, as milk samples are easier to collect.

Title Binding of protein G to non-domestic hoofstock immunoglobulin and application in serodiagnosis of Johne's disease.

Author(s) Kramsky JA* , Manning EJB, Collins MT.

Institution Dept of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI USA.

Abstract Lack of serologic assays validated for non-domestic hoofstock species (Order Artiodactyla) makes diagnosis and control of infectious diseases challenging. As one example, diagnosis of Johne's disease in captive and free-ranging artiodactylids is limited to culturing the organism from tissue or feces. This procedure is slow and costly. However, the need for a labeled, species-specific secondary antibody (conjugate)precludes use of an ELISA assay to detect antibody in animals as diverse as artiodactylids. Protein G reportedly binds IgG in most mammals to varying degrees and thus may potentially serve as a universal conjugate. A Johne's disease ELISA with a conjugate capable of recognizing antibody from a multitude of species was investigated for use in artiodactyl. Twelve species representing three families within Order Artiodactyla were included (addax, antelope, bison, bontebok, kudu/nyala, llama, oryx, sheep, white-tail deer, elk, muntjac, impala), plus bovine (positive control) and chicken (negative control). A protocol for immunoglobulin (Ig) enrichment from serum was optimized in bovids and utilized for all other species. Binding assays were performed to assess the ability of protein G to bind Ig from artiodactyl species. A prototype ELISA assay for the detection of antibodies to *Mycobacterium paratuberculosis* in artiodactyl species, using the protein G conjugate, was developed. Sera from animals with documented paratuberculosis infections and from animals considered free of paratuberculosis infection were obtained. The prototype artiodactyl protein G ELISA was able to distinguish among samples containing *M. paratuberculosis* antibodies and those that did not. With further assay optimization and use of appropriate species' controls, ELISA OD cutoffs to classify negative and positive serum samples may be determined. Characterization of the binding ability of protein G to Ig of these species indicates its potential use as a conjugate in ELISAs for any disease, regardless of the causative organism (solid phase antigen).

Title Infection of ruminants by uncultivable strains of *Mycobacterium avium* subsp. *paratuberculosis* in the Czech Republic.

Author(s) Machackova M[1*] , Lamka J[1,2] , Yayo Ayele W[1] , Parmova I[3] , Svastova P[1] , Amemori T[1] , Pavlík I[1] .

Institution [1] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic. [2] Charles University, Faculty of Pharmacy, Hradec Kralove, Czech Republic, [3] State Veterinary Administration, Prague, Czech Republic.

Abstract During the survey of paratuberculosis in cattle, sheep, goats and wild ruminants (red deer, roe deer, fallow deer, moufflon, wild goat), tissue samples of the small intestine and corresponding lymph nodes of 307 animals with Ziehl-Neelsen microscopy positive results were examined. In 251 heads of cattle *M. paratuberculosis* was isolated from 250 (99.6%) animals within 1.5 to 3 months of incubation ("relatively fast-growing" strains). On the contrary, from the rest of 56 other ruminant species 34 (60.7%) were found positive for *M. paratuberculosis* within 2 and 3 months of incubation and within 4 and 9 months of incubation ("relatively slow-growing" strains). More than 50% of these isolated strains has grown between the upper limit (4 - 9 months). Tissue culture was negative in 9 (100%) moufflons, 2 (50.0%) fallow deer, 1 (25.0%) red deer, 8 (25.8%) sheep, in non of 5 goats and in 2 (66.6%) wild goat. Any relationship between occurrence of uncultivable strains of *M. paratuberculosis* strains and age or sex of ruminants was not revealed. A total of 12 "relatively slow-growing" strains isolated from sheep, domestic and wild goats were examined by standardised RFLP analysis. Four RFLP types were identified of cattle strains (n=11): A-C10, B-C1, B-C2, and E-C1 and one RFLP type was found in sheep strain (n=1): C-S1. No relationship was observed among RFLP types of above mentioned 12 "relatively slow-growing" strains and 1 500 "relatively fast-growing" strains in our database. Supported by the grants No. QLRT - 2000 - 00879 Brussels, EC, QD1191 Min. of Agriculture, Czech Republic and No. J13/98-1160002 MSMT, Czech Republic.

Title Bacteriology of Johne's Disease in Cattle, Sheep, Goats and Rabbits.

Author(s) Vaughan JA[*] , Stewart DJ, Stiles PL, Lenghaus C, Tizard M, Michalski WP, Prowse SJ.

Institution CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Abstract Bacteriologic detection of *Mycobacterium avium* subspecies *paratuberculosis* (Mptb) was used to confirm the shedding patterns, infection status and final disease profile of cattle, sheep, goats and rabbits in 3 infection experiments. Cattle, sheep and goats were orally dosed (4 doses at weekly intervals) with up to $2x10^{10}$ cfu of a wild-type bovine Mptb isolate or with 15-20g of bovine intestinal mucosal tissue from a naturally Johne's Disease (JD) infected cow. Faecal culture was performed pre-challenge and for 4years post-challenge. Similarly a further group of cattle, sheep and goats were orally dosed with up to $2x10^{10}$ cfu of a wild-type ovine Mptb isolate or with 15-20g of ovine intestinal mucosal tissue from a naturally JD infected sheep. Faecal culture was carried out pre-challenge and for 31 months post-challenge. Finally adult and juvenile rabbits were given oral infective doses of 10^8 cfu weekly on 3 occasions with faecal and autopsy tissue culture being used to monitor disease progression for up to 2 years. The BACTEC system of radiometric culture was used together with conventional culture for the recovery of Mptb from faecal specimens and post mortem tissues. The Growth Index was determined weekly using the BACTEC 460 and samples taken from vials initially with a GI>100 and transferred to 4 varieties of Herrolds egg yolk agar along with modified Middlebrooks 7H10 slopes. Ziehl-Neelson stained smears, Mycobactin J dependency and PCR were used as confirmatory tests. Constant, intermittent and non-shedders were detected and both high and low Mptb colony numbers were observed. Mptb was cultured and confirmed from a variety of post mortem tissues from animals manifesting different stages of JD infection. Radiometric bacteriologic culture increased the recovery of bacterial colonies for both the bovine and ovine strains of Mptb.

Title An International Proficiency study for culture or PCR detection of *Mycobacterium*

avium sp. *paratuberculosis* in bovine fecal samples.

Author(s) Whitlock RH* , McAdams SC, Fyock TL, Sweeney RW.

Institution University of Pennsylvania, School of Veterinary Medicine, New Bolton Center, 382 West Street Road, Kennett Square, PA 19348 USA.

Abstract This study was designed to evaluate the performance of culture methods and media types used for the isolation of *Mycobacterium avium* sp. *paratuberculosis* (MAP) in laboratories around the world. Recently, there have been advances for the detection of MAP including PCR, as well as enhancement of the standard culture method, liquid culture and the use of various types of solid media. This study compares the performance of these techniques in an effort to determine the most sensitive culture method and media currently available. Each participant received 30 coded fecal samples and 60 tubes of Becton Dickinson Herrold's Egg Yolk Media (HEYM). The fecal set consisted of 4 negative and 26 culture positive animals: 7 high, 8 moderate, 5 low and 6 intermittent shedders. Each laboratory was instructed to culture these samples according to their current protocol and inoculate two tubes of the BD HEYM and two tubes of their standard media. Each lab was required to submit 25 tubes of their media for a comparison using the standard three-day culture technique. Of the 30 laboratories results have been received from 16. In 60% of the samples the in-house media detected more positives. In 10% the BD HEYM detected more positives. In 30% the two medias were equal. The highest number of positives detected via standard fecal culture was 23/30 with an average of 16. Of the 16 labs from which results have been received five labs performed the liquid culture. The highest number of positives detected via liquid culture was 19/30 samples with an average of 13. Two labs performed diagnostic PCR on the fecal sample directly. One lab detected 53% and the other detected 43%. The results from the second part of this study to evaluate the media from each lab will not be available until May 2002.

Title A model to estimate optimal pool-sizes of fecal samples to detect *M. paratuberculosis* in dairy herds.

Author(s) van Schaik G* , Stehman SM, Schukken YH, Rossiter CR, Shin SJ.

Institution Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853, USA.

Abstract Pooled fecal samples can be a cost-effective and specific way to test a herd for *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The Animal Health Diagnostic Laboratory at Cornell University currently uses a liquid-culture method for rapid detection of MAP in bovine fecal samples. The objective of the study was to obtain the pool-size with the lowest cost given the herd size, expected prevalence in the herd, and the goal of the farmer. A stochastic spreadsheet model was developed to obtain estimates for the costs of pooled fecal sampling for pool-sizes between 1 and 30 cows. It was assumed that the whole herd is tested and that all animals in the herd are divided into equal sized pools. Moreover, the sensitivity (Se) of the test for pooled samples was supposed identical for heavy shedders when pool-size increased but the pool Se decreased for low and moderate shedders with a Log-formula per extra animal in a pool. The specificity (Sp) is set at 0.999. The number of positive pools in a herd was randomly determined based on the expected MAP prevalence. A pool was considered "high positive" when it contained at least one heavy shedder, "moderate positive" when it contains at least one moderate shedder but no high shedders and "low positive" when it only contains one or more low shedders. The individual samples of a positive pool were retested to identify the shedder in the pool and the total number of tests determined the total costs. The model showed that the optimal pool-size differed depending on the objective of the farm, the herd size, and the prevalence. An increased pool-size decreased the costs, increased the variation around the costs, and decreased the probability to detect the shedders. Pooling is not useful in smaller (<250 cows), low-prevalence (<5%) herds. In larger herds and herds with a higher prevalence, pooling is more cost-efficient than individual samples.

Title Diagnostic sensitivity of pooled fecal culture for *Mycobacterium paratuberculosis* in

dairy herds.

Author(s) Gardner IA[1*] , Anderson RJ[2] , Shin S[3] , Whitlock RH[4] .

Institution [1] Department of Medicine and Epidemiology, School of Veterinary Medicine. University of California, Davis CA, 95616, U.S.A. [2] California Department of Food and Agriculture, Animal Health Branch, Modesto, CA, 95351, CA, U.S.A. [3] Diagnostic Laboratory, Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca NY, 14853, U.S.A. [4] Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, PA 19348, U.S.A.

Abstract Pooled fecal culture is being considered as an alternative to ELISA testing for determination of herd *M. paratuberculosis* status and identification of groups of animals warranting individual fecal culture. In the USA, fecal culture is the only officially-recognized test yet it is cost-prohibitive to do individual fecal cultures for all cows in large dairy herds. In an experimental study, the sensitivity of pooled fecal culture (pools of size 5 and 10) ranged from about 30% to 100% and was strongly dependent on pool size and whether cows were shedding many (heavy shedders) or few (light shedders) *M. paratuberculosis*. The present study was done to evaluate the accuracy of pooled fecal culture (pools of size 10) compared with ELISA testing and individual fecal culture under field conditions. We studied 29 dairy herds that were clients of a single dairy practice in the Central Valley of California. Herds ranged in size from 300 to 1500 cows and all herds except one had not been previously tested for *M. paratuberculosis*. In each herd, sixty lactation-2 or older cows were randomly selected and blood and feces were collected for ELISA testing and culture, respectively. Feces was frozen at -70 until pooled for fecal culture. Pooled fecal cultures were done by 2 methods (TREK and traditional culture methods). ELISA data indicated that at least 27 of 29 herds were likely infected. Culture results are pending.

Title Comparative evaluation of the MGIT and BACTEC systems for the culture of *Mycobacterium avium* subsp. *paratuberculosis* from milk.

Author(s) Grant IR[1*] , Kirk RB[2] , Hitchings EIJ[1] , Rowe MT[1,2] .

Institution [1] Department of Food Microbiology, Queen's University of Belfast. [2] Department of Agriculture and Rural Development for N. Ireland, Belfast, N. Ireland, UK.

Abstract The non-radiometric Mycobacterium Growth Indicator Tube (MGIT) culture system has not previously been evaluated for the culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from milk. A study was undertaken to compare the detection capabilities and detection times of the MGIT system with those of the radiometric BACTEC system currently used in our laboratory. UHT milk samples were spiked with a range of MAP concentrations (10-10^7 cells/ml). MGIT tubes supplemented with MGIT OADC, MGIT PANTA and mycobactin J, and BACTEC vials supplemented with PANTA and mycobactin J, were inoculated with 500 microlitres of triplicate milk samples at each MAP concentration. MGIT tubes were read manually using a UV transilluminator and BACTEC vials on the BACTEC 450TB machine. Time to detection of growth in days was recorded for each system. A corresponding BACTEC count was determined from the BACTEC growth index readings using the published formula of Lambrecht et al. (1988). A further experiment assessed the recovery of MAP from milk by both culture systems following decontamination with 0.75% (w/v) cetylpyridinium chloride for 5 h. In the absence of any decontamination step both the MGIT and BACTEC culture systems were capable of detecting growth from milk samples containing 10-100 MAP cells within 30-40 d. The correlation coefficient between MGIT and BACTEC detection times was 0.826. MGIT detection times tended to be shorter than BACTEC detection times when low numbers of MAP were present in a milk sample and slightly longer when high numbers of MAP were present. After decontamination only milk samples originally spiked with > 10^2-10^3 MAP exhibited growth in both culture systems. Decontamination caused a significant reduction (mean 1.44 log) in numbers of viable MAP in all spiked milk samples. Overall, the MGIT system was shown to have similar detection capabilities to the BACTEC system for recovering MAP from milk.

Title Rapid enumeration of viable *Mycobacterium paratuberculosis* in milk.

Author(s) D'Haese E[1*] , Dumon I[2] , Werbrouck H[2] , Wiszniewska A[2] , Herman L[2] , Nelis HJ[1] .

Institution [1] Laboratory for Pharmaceutical Microbiology,Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium. [2] DVK-CLO Ghent, Brusselsesteenweg 370, B-9090, Melle, Belgium. 3 Department of Food Hygiene, Warmia and Masuria University of Olsztyn, Poland.

Abstract *Mycobacterium paratuberculosis* (MAP) is suspected of playing a role in human Crohn's disease and of surviving industrial pasteurization of milk. Although PCR and culture methods are able to detect MAP in milk, no method for the rapid detection of viable whole cells in milk has been established thus far. We have developed a method based on solid phase cytometry (SPC) to enumerate MAP in 50 ml of spiked pasteurized milk within a single working day. SPC is an innovative technique which allows the enumeration of fluorescently labelled bacteria as single cells on a membrane filter. The SPC procedure consists of a membrane filtration, the labelling of bacteria on the membrane filter with an Ar laser excitable fluorescent dye, the automated counting in 3 min by the ChemScan apparatus (Chemunex, Ivry-sur-Seine, France) and a visual confirmation by means of an epifluorescence microscope. An extensive sample clean-up was developed in order to render the milk filterable and to eliminate the predominant background flora. This pretreatment consisted of a chemical milk destruction, several centrifugation steps, decontamination with 0.75% cetyl pyridinium chloride, an enzymatic treatment, a prefiltration step and a purification by means of hydrophobic C8 polymeric beads. In the actual SPC procedure, the MAP retained on the membrane filter were fluorescently labelled using the non-specific viability substrate ChemChrome V6 which stains all living microorganisms. Therefore, the selectivity of the method depends on sample pretreatment only, i.e. milk destruction, decontamination and isolation on C8 beads. For spiked pasteurized milk inoculated with 10^3-10^5 CFU of MAP recoveries between 10-50% were obtained. On-going research focuses on a selective labelling procedure and the detection of MAP in naturally contaminated milk.

Title Effect of media supplements on growth of *Mycobacterium paratuberculosis*.

Author(s) Raymond M, Mutharia L[*].

Institution Department of Microbiology, University of Guelph. Guelph. ON N1G 2W1. Canada.

Abstract A survey of the literature reveals that several broth and agar /solid media containing a variety of supplements are used in isolation and culture of *Mycobacterium paratuberculosis*. Commonly used solid media include Herrold's egg yolk media (HEYM), Lowenstein-Jensen (LJ), Middlebrook 7H10. Broth media include, Dubos broth (DB) and Middlebrook 7H9. All media are supplemented with mycobactin J and variably contain glycerol, bovine serum and detergents such as Tween-80. In this study we attempted to identify the combination of media and supplements supporting the fastest and/or most abundant growth of recent fecal isolated *M. paratuberculosis* strains within a 6-week period. The test supplements included, egg yolk, lecithin, pyruvate, vitamins, casamino-acids, glycerol, Tween-80, Tyloxapol, bovine serum, mycobactin-J or bovine lactoferrin, hemoglobin, transferin, and hemin. The results suggest that the type and concentration of detergent, egg yolk and glycerol had most significant effect on growth of the bacterium, and that Dubos media does not support growth of *M. paratuberculosis*.

Title Growth and Detection of *Mycobacterium paratuberculosis* with BACTEC MGIT 960 system.

Author(s) Beaty PS.

Institution Becton Dickinson.

Abstract The BACTEC MGIT 960 system was evaluated for the growth and detection of

Mycobacterium avium subspecies *paratuberculosis*. The isolation and relative enumeration of *M. paratuberculosis* shed in the manure of ruminants is indicative of the presence and onset of active Johnes disease. Currently, the culture of *M. paratuberculosis* from manure is performed using a variety of solid media including LJ slants and Herrold's Egg Yolk agar. This evaluation is concerned with optimizing a Middlebrook based formulation for the growth and detection of *M. paratuberculosis* using the BACTEC 960 instrument. The basal Middlebrook medium was modified to evaluate the addition of various peptones, bovine serum albumen, mineral salts and osmolarity modifications. The tests included at least three strains of *M. paratuberculosis*, as determined by strict mycobactin-dependent growth, as well as other strains of *M. avium*. *Mycobacterium tuberculosis* was also included in media evaluations. The results show that the basic Middlebrook formulation typically used to grow and detect *M. tuberculosis* is not optimized for the growth and recovery of *M. paratuberculosis*. The data indicates that careful selection of BSA and the addition of certain peptones stimulates the growth and metabolism of *M. paratuberculosis*. This will greatly enhance the potential for using the BACTEC MGIT 960 system as a fully automated diagnostic tool for Johnes disease in ruminants.

Title Evaluation of culture media for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from Cheddar cheese.

Author(s) Donaghy JA[1*] , Totton NL[1] , Rowe MT[1,2] .

Institution [1] Food Science Division (Food Microbiology), Department of Agriculture and Rural Development for N. Ireland. [2] Department of Food Science, Queens University Belfast, Belfast, N. Ireland, UK.

Abstract There is a paucity of information on the incidence and survival of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in cheese. The presence of a starter and non-starter microflora may present difficulties for the detection of Map during cheese ripening. Several culture media were evaluated for the detection of Map in Cheddar cheese manufactured with different starter cultures. Serial dilutions of four commercially used Cheddar starter cultures - R603, R604, YY80 and EZAL MM101 were either subjected to a decontamination step (0.75% w/v hexadecylpyridinium chloride (HPC), 5 h at 18ºC) before inoculation onto test media or inoculated directly onto test media. The test media used were: Herrold's Egg Yolk Medium (HEYM) with and without antibiotics (vancomycin (V), amphotericin B (A) and nalidixic acid (N)); Middlebrook 7H10-PANTA agar and BACTEC 12B radiometric medium. In addition, triplicate samples of 10-wk old commercially prepared cheddar cheese made with each starter and laboratory-prepared Cheddar, manufactured with starter YY80 (10-wk) and EZAL MM101 (10-wk and 14-mth), were inoculated (without decontamination) onto the same test media. Without a decontamination step, growth of each starter, except EZAL MM101, occurred on HEYM without antibiotics but not on any of the other media, so inclusion of antibiotics (VAN or PANTA) in media may suppress Cheddar cheese starter cultures sufficiently so as to eliminate a decontamination step. In commercially and laboratory prepared cheeses growth was observed on HEYM-VAN medium for cheese prepared with all starters except EZAL MM101. Therefore HEYM-VAN may not be a suitable medium for the isolation/detection of Map from Cheddar cheese prepared under uncontrolled microbiological conditions. The medium 7H10-PANTA did not support the growth of Cheddar cheese microflora when cheese was prepared with any of the starter cultures. Therefore, in order to monitor the persistence of Map during Cheddar manufacture and ripening the use of 7H10-PANTA medium is recommended.

Title Investigation of two Swedish farms with mould overgrowth in faecal cultures.

Author(s) Holmström A[*], Bölske G, Bergström K, Mattsson R, Sternberg S.

Institution Swedish Animal Health Service.

Abstract

Introduction.

The Swedish control programme for paratuberculosis is based on faecal culture. With this

method, mould overgrowth is sometimes a problem, especially if affecting a large number of samples from a herd. This was the case with two beef herds in the programme. The mould identified in most of the samples was *Pseudallescheria boydii* (anamorph state, *Scedosporium apiospermum*).

Methods and materials.

When the problem with mould overgrowth remained after repeated sampling in the two herds, further investigations were undertaken. Samples from feed and straw were examined for the presence of mould. The farmers were interviewed about feed quality, animal health etc. An intensive programme for faecal sampling was suggested. This was not deemed practical and, instead, faecal samples were taken on 3 occasions in each herd.

Results.

Samples taken from feed in herd A showed low numbers of mould in both silage and straw while herd B had moderate numbers of mould in the straw and high numbers in the silage. None of the feed samples yielded growth of P. boydii. In herd A 53% of the faecal samples taken at the same time as the feed samples were overgrown by mould and another 20% yielded some mould growth. In most cases the mould was P. boydii. From herd B, 43% of the faecal samples were overgrown by mould, while another 18% yielded some mould growth. In this herd most cases of overgrowth was due to *P. boydii*, but a number of samples also yielded growth of other mould species.

Discussion.

For practical reasons, most large beef farms are sampled during the cold season, when the animals are indoors and easier to catch and handle. However, the risk of mould overgrowth may be higher during this period. Faecal samples from animals on pasture are thought less likely to be overgrown by mould. This study could, however, not substantiate that theory.

Title Reduction of the contamination rate of fecal cultures of *Mycobacterium avium* subspecies *paratuberculosis*.

Author(s) van Weering HJ* , Koene MGJ, Hesselink JW, Weber MF.

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

Abstract Strategies to eradicate paratuberculosis from cattle herds are based on the effective detection and removal of infected animals and improvement of animal husbandry to prevent new infections. Bacteriologic culture of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from fecal samples is the most definitive method to detect infectious animals. Also in the Dutch program for Map unsuspected herds, culture of fecal samples is a key element. A disadvantage of fecal culturing is that the method is time consuming, requiring up to 16 weeks of incubation, labour intensive and thus expensive. Besides contamination by other bacteria and fungi is another problem with fecal cultures of Map. Contamination rates may be as high as 30%. As a result of these contaminated cultures, sampling and culturing have to be repeated, which also means loss of time and more costs. This high contamination rate undermined the motivation of farmers to participate in the Dutch paratuberculosis program. Therefore a project was started with the aim to reduce the contamination rate to below 10 %.

Title Re-testing cattle following contamination at different incubation stages of fecal culture for *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Weber MF* , Oosterhuis V, Koene MGJ, Kalis CHJ.

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

Abstract Culture of *Mycobacterium avium* subspecies *paratuberculosis* (Map) from fecal samples is a key element in many paratuberculosis control programs. Contamination of fecal cultures by other bacteria and fungi at different stages of incubation is a major problem and results in the need to collect and culture new samples from the animals concerned. However, re-testing

animals following contamination of fecal samples at a late stage of incubation may be rather inefficient. The likelihood that these animals are fecal shedders is reduced, because Map colonies were not detected at an earlier stage. Therefore, the results of re-sampling and culturing of feces of 2532 cattle after contamination of a previous individual culture were analyzed in a retrospective study over the period April 1997 - June 1999. Re-sampling was done within 12 months after the initial sampling. The employed fecal culture technique has been described previously. Of the 2532 contaminated initial samples, 16% were found contaminated at four weeks of incubation, 53% at 8 weeks, 14% at 12 weeks and 17% at 16 weeks. Re-testing of animals following contamination at 4, 8, 12 and 16 weeks of incubation resulted in detection of 2.5%, 5.8%, 0.84% and 1.8% culture positive animals respectively. The obtained results were applied to our rates of positive and contaminated individual fecal cultures in the period 1998 - 2000. Not re-testing animals following contamination after more than 8 weeks of incubation would have resulted in an estimated reduction of the rate of re-testing of 25%, while the estimated relative sensitivity of the individual fecal culture was still 98.5% in comparison to re-testing all animals following contamination. It is concluded that re-testing cattle following contamination of a fecal culture is not cost efficient if contamination occurs after more than 8 weeks of incubation.

Title Rapid detection of viable *Mycobacterium paratuberculosis* in milk using phage amplification.

Author(s) Stanley EC[1*] , Mole RJ[2] , Rees CED[1] .

Institution [1] Division of Food Sciences, University of Nottingham, UK. [2] BIOTEC Laboratories Ltd., Ipswich, UK.

Abstract Detection of *Mycobacterium paratuberculosis* (MAP) is hampered by the slow growth of the organism and by the low numbers that they are often present in food, such as milk. Phage amplification is a novel bacteriophage-based test, which offers the opportunity to rapidly and specifically detect low numbers of MAP bacilli in milk. The test uses host specific bacteriophage to report the presence of viable MAP. Positive results are only seen if viable MAP is present and appear as plaques in lawns of a fast growing reporter helper bacteria strain that is also susceptible to the phage (such as *M. smegmatis*). It is expected that the MAP will be detected in 24-48 hours rather than the conventional 18 weeks taken by standard culture methods. Our study aims to develop an assay that is able to specifically and rapidly detect the presence of MAP in milk. Detection within 24 hours has been demonstrated with several culture collection strains of MAP using the phage amplification assay. We are currently exploring the effects of milk on the assay, including the identification of an inhibitor of phage attachment present in milk and the elimination of expected contaminating bacteria.

Title Evaluation of different organism based methods for the detection and identification of *Mycobacterium avium* subspecies *paratuberculosis* from bovine feces.

Author(s) Payeur JB.

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

Abstract USDA regulations have stated that an organism-based test (culture) 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 United States Voluntary Johne's Disease Herd Status Program (USVJDHSP) indicate multiple culture methods were being used in the United States. The yearly evaluations have indicated that there were a wide range of sensitivities associated with the different culture methods. The National Veterinary Services Laboratories (NVSL) have been requested to establish a standardized protocol for detecting *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in fecal samples 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 3 years of proficiency tests, several methods have been chosen for further evaluation. These methods included sedimentation, centrifugation and liquid culture procedures which have been used by multiple laboratories. Preliminary evaluation based on proficiency test results indicate that centrifugation methods are more sensitive than sedimentation methods and liquid culture methods are faster than either sedimentation or centrifugation methods using Herrold's Egg Yolk (HEY) media with mycobactin. Varied growth performances in the solid media used with different culture methods were also noted during the last 2 check tests. There are now 2 commercial sources of HEY media with mycobactin available in the United States which will be evaluated along with in-house media for growth performance.

Title Identification of genes up-regulated in vivo in *Mycobacterium avium* subspecies *paratuberculosis* using modified IVIAT screening of expression libraries.

Author(s) Doran T[1] , Kowalski M[1] , Vaughan J[1] , Stewart D[1] , Stiles P[1] , Cahill D[2] , Tizard M[1*] .

Institution [1] CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia. [2] School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria, Australia.

Abstract In vivo induced antigen technology (IVIAT) is a method that utilizes sera from infected animals (in vivo) absorbed versus unabsorbed with protein from in vitro grown bacteria to differentially screen expression libraries in E. coli. This characterizes antigens and potential virulence determinants expressed specifically by the pathogen in association with the disease process. A modification of this process has been used to differentially screen an arrayed expression library of *Mycobacterium avium* subspecies *paratuberculosis* (Mptb) and identify genes apparently up-regulated in vivo. Protein preparations were generated from Mptb organisms retrieved from infected tissues of Johne's disease affected animals and separately from Mptb grown in artificial culture medium. These protein preparations were used to raise hyper-immune sera in rabbits. Replicates of 384 well plate arrays of an E. coli expression library of Mptb were probed with the sera. A number of differences were observed the most striking of which were from four clones (pMK19, pMK22, pMK20, pMK40) which represented two independent copies each of two separate genes. Of these pMK19 and pMK22 each contained a major in-frame fragment of the gene katG, the catalase-peroxidase, which was only detected by anti-sera to the in vivo (disease) protein extracts. This confirmed independent evidence from RNA studies and support this approach as a useful method for identifying clearly up-regulated (or down-regulated) genes of Mptb in the in vivo environment. pMK20 and pMK40 each contained a substantial in-frame fragment of the same gene sequence, representing an as yet uncharacterised potential protein antigen associated with the in vivo disease state, which has no presently characterized protein homologues. Assessment of more extensive expression libraries continues.

Title Detection of in vivo mRNA from *M. avium* subspecies *paratuberculosis* extracted from infected tissues.

Author(s) Granger K[1] , Moore R[1] , Davies J[2,1] , Stewart DJ[1] , Vaughan J[1] , Tizard MLV[1*] .

Institution [1] CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia. [2] Monash University, Clayton, Victoria, Australia.

Abstract After several decades of study little is known of the response of *Mycobacterium avium* subspecies *paratuberculosis* (Mptb) to the hostile environment it encounters within the infected host. In order to address this issue a pilot study of gene expression in the in vivo environment was undertaken. Real Time polymerase chain reaction was used to analyse bacterial mRNA extracted from the infected tissue of animals clinically affected with Johne's disease. Small intestine was rapidly removed surgically, lumenal contents washed away and the mucosa and submucosa scraped off. Mycobacteria in this material were released from the intra-macrophage environment by gently isotonic lysis and recovered for RNA extraction by differential centrifugation. This RNA was compared with RNA extracted from bacteria grown in artificial culture media (so called in vitro sample). Genes selected for study included a number flanking IS900 insertion sites, and others were genes that could potentially be associated with response to the intra-macrophage environment. One gene in particular, katG encoding the catalase/peroxidase, seemed to show an on/off switch between the in vivo and in vitro environment. In addition differential expression of the gene 2 associated with the IS900 insertion locus 13, and gene 1 of locus 10, was observed. A comparison of mRNA samples from cultured Mptb with samples from *M. avium* subspecies avium indicated a general up-regulation for genes associated with IS900 insertion loci covered in this study. This approach to the identification of disease associated gene expression will be extended with partial (sub-set) genome microarrays.

Title A molecular diagnostic assay based on a novel target ssrA/tmRNA for the detection of

M. avium subsp. *paratuberculosis.*

Author(s) Hernon F[1*] , Bohane H[1] , Glennon M[1] , Barry T[2] , Smith T[1] , Maher M[1] .

Institution [1] National Diagnostics Centre, BioResearch Ireland, National University of Ireland, Galway, Ireland. [2] Department of Microbiology, National University of Ireland, Galway, Ireland.

Abstract

Introduction.

Reports have indicated that *M. avium* subsp. *paratuberculosis* (MAP) survives high-temperature short-time pasteurization. Therefore, to ensure the safety of milk and dairy products it is important to detect viable MAP. The aim of this study is to develop a rapid molecular diagnostic assay for detecting viable MAP cells using PCR and NASBA assays for the SsrA gene and it's RNA transcript, tmRNA.

Methods.

Based on the SsrA gene, primers and DNA probes were designed for the amplification and detection of PCR and NASBA products to distinguish between the species of the *M. avium* complex. PCR assays were performed on DNA from mycobacterial species and non-mycobacterial species. Southern blot analysis confirmed specificity and sensitivity of DNA primers and probes. NASBA assays were performed on RNA extracted from mycobacterial species using Hybaid ribolyser method with analysis performed using NucliSens technology.

Results.

Universal PCR primers amplified ~350bp of the SsrA gene from members of the mycobacteria genus. A specific DNA probe (PAV1) was designed and developed to detect members of the *M. avium* complex and a further DNA probe (PAV2) was developed to specifically detect MAP and *M. avium* only. The SsrA sequences for *M. avium* and MAP were found to be 100% homolgous and therefore indistinguishable. NASBA amplification of RNA from a range of mycobacteria with primers designed from tmRNA followed by detection with probe PAV1 detected M. intracellulare, *M. avium* and MAP. Whereas PAV2 detected only the latter two.

Discussion.

The application of the tmRNA NASBA assay for monitoring the presence of viable MAP cells in raw and pasteurised milk samples is currently under investigation. We propose that this assay could be used initially to identify the presence of viable MAP and/or *M. avium* in milk samples. The application of an IS900 molecular assay could then be applied to distinguish between MAP and *M. avium.*

Title Assembly & validation of a whole-genome DNA microarray for the *Mycobacterium avium* complex.

Author(s) Zhai G, Semret M[*] , Cleto C, Mostowy S, Behr M.

Institution McGill University Health Centre Research Institute.

Abstract

Background.

The biological differences among members of the *Mycobacterium avium* complex (MAC) are undoubtedly encoded in their genomic sequence. Newly-developed DNA microarray technology along with sequence data from the *M. avium* subsp. *paratuberculosis* (MAP) and *M. avium avium* (MAA) projects together provide the opportunity to assemble a MAC whole genome DNA microarray.

Design.

Exploiting the inherent gene homology between M. tuberculosis (MTB) and MAC, we have acquired a set of synthetic 70bp oligonucleotides that represent each of the 3924 open reading frames (ORFs) of MTB. To this set, we have added custom-designed oligonucleotides for 647 ORFs that are either unique to MAA (no homology to MTB) or present in MAP but absent from MAA. These combined oligonucleotide sets are printed together to create the first generation

MAC DNA microarray.

Validation.

In preliminary experiments, we have validated the use of oligonucleotide probes for the GC-rich MAC. Probes have been designed for homologues of MTB genes known to be induced by isoniazid. Gene expression profiling using this array yields results consistent with those seen by semi-quantitative RT-PCR. In ongoing experiments, MAA DNA is applied to the combined array in parallel with MTB DNA: for each gene representative, the intensity of the two signals is plotted against the degree of DNA homology of the 70 base-pair probe between the 2 species. A homology cut-off point below which hybridization intensity is inadequate is thus determined. For these genes, oligonucleotides specific to MAA are designed and used in the construction of the second generation array.

Conclusion.

The assembly and validation of a whole-genome oligonucleotide microarray is being carried out. This tool will enable comprehensive comparative and functional genomic studies of MAC.

Acknowledgment.

This work is supported by grants from the National Science and Engineering Research Council (NSERC) and Canadian Institute for Health Research (CIHR).

Title Application of a liquid phase proteomics approach to identification of secreted antigens derived from *Mycobacterium avium* and *Mycobacterium avium* subspecies *paratuberculosis* cultures.

Author(s) Beddome G, Shiell BJ, Vaughan JA, Stiles PL, Stewart DJ, Michalski WP* .

Institution CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Abstract Johne's disease (JD) is a chronic intestinal infection of ruminant livestock caused by infection with *Mycobacterium avium* subspecies *paratuberculosis*. JD is an economically significant disease that is widespread and worldwide. Current control measures are either diagnosis/management or vaccination. There is a need for better tools and reagents to effectively control the disease. A number of antigens within the protein/peptide complement of Johnin purified protein derivative (PPD) and cell free culture supernatants of *M. avium* subspecies *paratuberculosis* are capable of eliciting a strong interferon-g release response early in the course of disease. The aim of this project is to identify and characterise a variety of antigens derived from culture media suitable for improved diagnosis by cell mediated immunity assay (IFNg release assay, BOVIGAM™, CSL Animal Health Ltd). A strategy involving a liquid phase proteomics approach was employed for rapid identification of suitable secreted proteins of *M. avium* and *M. avium* subspecies *paratuberculosis*. This consisted of preparative isoelectric focusing separation followed by SDS-PAGE and whole gel protein elution. Liquid phase separation allowed continuous monitoring of IFNg release assay activity of fractionated protein samples. 15 proteins were identified in active fractions applying direct N-terminal sequencing. Subsequently genes encoding these identified antigens were expressed in various systems and the recombinant proteins tested for their suitability in the diagnostic test. This approach is being utilized in conjunction with recombinant antigen screening.

Title Isolation of high affinity peptides for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in milk.

Author(s) Stratmann J* , Gerlach GF.

Institution Institute for Microbiology and Infectious Diseases, School of Veterinary Medicine, Hannover, Germany.

Abstract In this study a highly sensitive detection method for *Mycobacterium avium* subspecies *paratuberculosis* (M. ptb) in milk and colostrum via a capture PCR using peptides linked to

paramagnetic beads was developed. Primers were derived from the M.ptb specific insertion sequence ISMav2. Using a library of filamentous phages expressing random 12-mer peptides on their surface, it was possible to initially select nine clones that bind to M. ptb. Selection of these clones was performed during five rounds of biopanning, with whole M. ptb as target, in the last round washing buffers containing 4M guanidine hydrochloride or 6M urea were used. The specificity of the clones derived was confirmed via fluorescence microscopy and in a plate binding assay. For the fluorescence microscopy the phages were conjugated with FITC to visualize binding to M. ptb. In the plate binding assay M. ptb was coated onto microtiter plates and bound phages were detected using streptavidin alkaline phosphatase as conjugate. This led to the selection of two specific phages. The amino acid sequences of the binding petides were deduced via DNA sequencing and the peptides expressed on these phages were synthesized with an N-terminal biotinylation using amino-hexa-carbone-acid as spacer. By linking the peptides to streptavidin coated paramagnetic beads it was possible to enrich M. ptb in milk. Subsequent processing envolved boiling and DNA purification with the Gene clean(r) Kit. The final PCR reproducibly allowed for the detection of 10^2 M. ptb per milliliter milk. Using this method it was possible to detect M. ptb in milk from naturally infected cows in the diagnostic laboratory.

Title Development of a PCR test to detect *Mycobacterium paratuberculosis* in bovine feces.

Author(s) Chevallier B*, Versmisse Y, Blanchard B.

Institution Adiagène SA, Saint Brieuc, FRANCE.

Abstract A PCR test based on IS900 oligonucleotide sequence has been developed to detect *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. An internal control has been developed for each PCR test to avoid false negative results often obtained with inhibitors contained in feces. The detection threshold of this test was determined by dilution of a suspension of *M. avium* subsp. *paratuberculosis* in bovine feces. The level of detection has been evaluated approximately to 50 CFU/g of feces. The specificity of the test has been assessed on 45 strains of *M. avium* subsp. *paratuberculosis* and 48 other mycobacteria (representing 13 species), 42 other bacteria (20 species) and 27 bovine feces fungi. None of bacteria, fungi and others mycobacteria tested gave a postitive signal. All the 45 M avium subsp. paratuberculosis strains gave a positive result. For field evaluation, between June 1999 and July 2000, 1041 bovine feces were collected from French herds suspected to be infected by *M. avium* subsp. *paratuberculosis* and were analyzed by culture or by PCR. Infected animals were found in 15 herds by culture and 17 herds were PCR positive. 72 feces samples were found positive by culture and 77 feces samples were found positive by PCR. Both methods show an equivalent sensitivity in term of herds but some discrepancy on individual results has been observed. These different results between the 2 methods could be due to the heterogeneity of the feces. This heterogeneity could give discrepant results, especially for low contaminated samples.

Title PCR-ELISA as the method for improving the diagnosis of paratuberculosis.

Author(s) Pislak M, Ocepek M*, Zabavnik Piano J, Pogacnik M.

Institution Veterinary Faculty, University of Ljubljana, Gerbiceva 60, Ljubljana, Slovenia.

Abstract

Introduction.

The separation of PCR-product using electrophoresis and ethidium bromide staining is the most usual method for detection of PCR-products. However, in the cases of low amount of PCR-product this method is not sensitive enough. In the article, the method of PCR-ELISA that can be used in the molecular diagnostics of paratuberculosis in order to increase the sensitivity is described.

Material and methods.

Suspensions of *Mycobacterium avium* subsp. *paratuberculosis* in 10 different concentrations were added to 10 faecal paratuberculosis-negative samples to compare the sensitivities of

rent methods: culture method, PCR with detection of PCR-products with electrophoresis and PCR-ELISA. For comparison of sensitivity of PCR with detection of PCR-product whit electrophoresis, nested PCR, dot-blot hybridisation and PCR-ELISA 20 samples of *Mycobacterium avium* subsp. *paratuberculosis* DNA isolated from intestines and lymph nodes tissue of serologically positive sheep and goats were used. For the PCR-ELISA procedure we have designed the 'ELISA 900' DNA-probe (5' CTC CGT AAC CGT CAT TGT CCA GAT CAA CCC AGC 3').

Results and discussion.

We have observed the lowest sensitivity in the culture test method, which was 20% positive when containing 300 bacteria/ml. The most sensitive method was PCR-ELISA method where 10% of samples with 6 bacteria/ml were positive. The comparison of the sensitivity of PCR with electrophoresis and ethidium bromide staining, nested PCR, dot-blot hybridisation and PCR-ELISA we have obtained the following results: in PCR 40% samples were positive, in dot-blot hybridisation 45%, in nested-PCR 50% and in PCR-ELISA 65% samples were positive.

Conclusions.

PCR-ELISA is highly sensitive and specific method for detection of *M. avium* subsp. *paratuberculosis* DNA in faecal and tissue samples. The method enables the processing of the numerous samples and automation of the procedure.

Title Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from dairy goats in Norway by immunomagnetic PCR.

Author(s) Djønne B[1*] , Jensen MR[1] , Grant IR[2] , Holstad G[1] .

Institution [1] National Veterinary Institute, Post Box 8156 Dep., N-0033 Oslo, Norway. [2] Department of Food Science (Microbiology), The Queen's University of Belfast, Belfast BT9 5PX, N. Ireland, UK.

Abstract

Introduction.

Paratuberculosis is common among goats in Norway, and the disease is controlled by vaccination. There is some concern that cheese manufactured from raw goats' milk might lead to transmission of *M. paratuberculosis* to humans. The aims of the present study were to determine if *M. paratuberculosis* could be detected in goats' milk in Norway and to elucidate the possible role of a live vaccine for the presence in milk.

Material and methods.

Milk samples from 340 goats were examined for *M. paratuberculosis* by culture and immunomagnetic separation combined with IS900 PCR (IMS-PCR). PCR products from positive samples were sequenced, and BLAST queries against public databases were performed.

Results.

Viable *M. paratuberculosis* were not detected by culture in any of the 340 milk samples, but 24 samples tested positive by IMS-PCR. The percentage of IMS-PCR positive milk samples from herds where paratuberculosis had previously been reported was lower (3,3%) than from herds where the infection had never been diagnosed (9.5%). Herds with a known paratuberculosis problem might have a herd management that prevent spread of the infection, while the infection is free to spread in herds with an unknown paratuberculosis status. Vaccination of goats with a live paratuberculosis vaccine did not seem to interfere with shedding in milk, since *M. paratuberculosis* was detected in almost the same percentage of milk samples from vaccinated and unvaccinated goats. There were a significantly higher proportion of positive samples among those collected in May (13,8%) than among those collected during February to April (2,9%). PCR products from five milk samples showed homologies of 97 to 100% with the IS900 sequence from *M. paratuberculosis*. This is important as IS900-like elements detected by PCR have been found in mycobacteria other than *M. paratuberculosis*, so detection by IS900 PCR alone might lead to false positive results.

Title Examination of in-line milk filters to detect *Mycobacterium avium* subsp. *paratuberculosis* infection at farm level.

Author(s) McKee R[1*] , Grant IR[1] , Rowe1 MT, Buckley HG[3] , Buckley JF[3] , Fanning S[4] .

Institution [1] Department of Food Microbiology, Queen's University Belfast. [2] Department of Agriculture and Rural Development for N. Ireland, Belfast, N.Ireland, UK. [3] Veterinary Department, Cork County Council. [4] Institute of Technology, Cork, Ireland.

Abstract *Mycobacterium avium* subsp. *paratuberculosis* (MAP) can be present in bulk tank milk at farm level as a consequence of direct excretion of the organism within the udder and/or indirect contamination with infected faeces during the milking process. In-line milk filters are generally sited between the milking equipment and the bulk tank to remove gross foreign material including faeces from the milk. Examination of these milk filters may provide a means of detecting MAP infected milk at farm level. A survey of in-line raw milk filters from dairy farms in Southern Ireland is currently in progress to assess this. To date 122 milk filters have been examined for the presence of MAP. Upon arrival at the laboratory each sample consists of a filter and some transit milk. The filter is removed and homogenised in a stomacher with 100ml Phosphate Buffered Saline containing Tween 20 (0.05%) for 2 min. The homogenised sample is recombined with the transit milk, which is divided into two 50ml portions. One portion is subjected to immunomagnetic separation followed by IS900 PCR (IMS-PCR), the other portion is decontaminated with 0.75% (w/v) hexadecylpyridinium chloride for 5 h before culture on Herrold's egg yolk medium slopes and in BACTEC 12B radiometric medium. Of the 122 in-line milk filters tested, 15 have tested positive for MAP by IMS-PCR and also a number of suspect culture positives have been obtained. An assessment will be made of the feasibility of this method to identify potentially Johne's infected herds.

Title False positive *Mycobacterium paratuberculosis* IS900 PCR.

Author(s) Bölske G[*] , Englund S, Johansson KE.

Institution National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden.

Abstract In order to make a fast identification of *M. paratuberculosis* in culture, a PCR based on the IS900 gene was introduced and evaluated on suspected colonies found in the routine diagnostic work. Except for one case, there has been agreement between the PCR identification and the conventional methods based on growth characteristics, acid-fastness and mycobactin dependence. The isolates of acid-fast bacilli obtained from routine cultures were compared for mycobactin dependence and IS900 PCR reaction with the p36/p11 primers. In one case, a single colony suspected to be *M. paratuberculosis* was detected from a faecal sample of a healthy dairy cow in Western Sweden. The isolate had acid-fast bacilli, a little longer than usual for *M. paratuberculosis* and some bacilli were bent. It tested positive with the PCR for IS900, but at subculture it turned out not to be mycobactin dependent. Genetic probe against *Mycobacterium avium* complex (Accuprobe, GenProbe, San Diego, USA) did not hybridise with this strain as should have been expected for *M. paratuberculosis*. By sequencing the 16S rRNA gene, this isolate was shown to be unrelated to *M. paratuberculosis*. Instead, it grouped with *M. cookii* and *Mycobacterium sp.* strain IMVS B76676. The similarity values for strain 2333 to *M. cookii* and *Mycobacterium sp.* strain IMVS B76676 were 98.3% and 98.8%, respectively. The similarity value to *M. paratuberculosis* was only 96.4%. Five other PCR systems targeting IS900 were used to test this isolate and they were all positive. Restriction endonuclease analysis of the IS900 PCR products according to Cousins et al. (1999) did not reveal any differences as compared to the IS900 PCR products from *M. paratuberculosis*. Our results clearly demonstrate that a positive IS900 PCR alone does not prove the presence of *M. paratuberculosis*. Further confirmation has to be made, for instance by isolation and fenotypic identification or by identification with an alternative PCR.

Title Analysis of an expression library to find novel antigens.

Author(s) Gioffré A[*] , Caimi K, Santangelo MP, Zumárraga M, Bigi F, Alito A, Paolicchi F, Romano MI,

Cataldi A.

Institution Instituto de Biotecnología-INTA, Los Reseros y Las Cabañas s/n, CP:1712, Castelar-Argentina.

Abstract The study of the interaction of the immune system with molecules from pathogens may lead valuable information of mechanisms involved during infection and potential tools for the diagnosis. The aim of the present study was to evaluate an expression library of *Mycobacterium avium* subsp. *paratuberculosis* (Mptb) constructed in lambda zap II to identify new antigens. Sera from naturally infected bovines or from mice inoculated with the whole bacteria or sonicated was used for the immunoscreening of the library. Sera were previously analysed by Western blot to determine the number and strength of band recognition. Bacterioferritin (antigen D) was identified with the bovine pooled sera. From the analysis of the library with mice serum several antigens were identified, some of them already described as antigens in Mptb and other mycobacteria: HSP70 and dnaK. Other potentially novel antigens were identified by sequence similarity with *M. tuberculosis* genome as serine/threonine protein kinase (99% of identities with pknB from *M. tuberculosis*, Rv 0014), isocitrate lyase (aceAa, 100% of identity with Rv 1915). In other hand, a sequence of 876bp which codes for a peptide of 32kDa was present in *M. avium* subsp. *avium* (99% of identity), but absent in the genome of *M. tuberculosis*. No previous description of this sequence was found (BLAST). The recombinant antigens fraction was recognised by all the bovine infected sera when were assayed by Western blot.

Title Optimization and application of extraction methods and a One Tube Nested PCR for detection of *M. avium* subsp. *paratuberculosis* (Map) in bovine feces.

Author(s) Antognoli MC[1] , Boegli-Stuber K[*2] , Jensen S[1] , Triantis J[1] , Jemmi T[2] , Salman MD[1] .

Institution [1] Animal Health Population Institute, College of Vet Med And Biomedical Sciences, Colorado State University, Fort Collins,CO, 80523-1676, USA. [2] Swiss Federal Veterinary Office, Microbiological Laboratories, 3003 Bern, Switzerland.

Abstract Confirmation of PTB infection has been hampered by both the lack of sensitivity of the currently available methods for Map isolation and the long time required for results. A promising method is the use of PCR in fecal samples having the advantage of providing rapid results and similar sensitivity as culture. Several PCR protocols based on the detection of the species-specific IS900 have been published. Sensitivity and specificity of PCR is increased by using nested PCRs. Additionally, cross-contamination between samples is prevented by a one tube nested (OTN) PCR since both amplification cycles take place in a single closed lid tube not opened until both amplification cycles are completed. In this study we optimized a OTN PCR and DNA extraction procedure from bovine feces for detection of Map in spiked fecal samples. This PCR will be run in parallel with traditional culture methods for confirmation of disease status in a study involving the evaluation of tests based on the detection of cellular and humoral immunity in young calves. Fecal samples were spiked with Map strain 19698 (final concentration: 1000, 250, 125, 62, and 31 cells/gram). DNA extraction was performed by combining freeze and thaw cycles with a commercial DNA extraction kit (Roche). Our OTN PCR utilizes a unique combination of those primers published by Englund et al. (1999) and IS900 is the target sequence. Extraction and OTN PCR were repeated 8 times/cell concentration. Obtained results of this study are: The OTN PCR detected as low as 62 cells/gram of feces in 87.5% of the spiked samples. Lower cell concentrations (31 cells/gram) in spiked feces were detected inconsistently, and in only 25% of the samples. The currently available culture methods provide a detection limit between 10 to 100 viable Map cells/gram of feces. Therefore, this extraction and PCR procedures demonstrate sensitivity levels in spiked fecal samples similar to culture.

Title Efficiency of polymerase chain reaction using a novel method of DNA preparation and amplification for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples.

Author(s) Kojima K[1*] , Nishimura N[1] , Mori Y[2] , Yokomizo Y[2] .

Institution [1] Shimadzu Corporation (Tsukuba Science city, Ibaragi, Japan). [2] National Institute of Animal health (Tsukuba Science city, Ibaragi, Japan).

Abstract Polymerase chain reaction (PCR) based on amplification of the IS900 sequence has been reported to be a rapid test for detecting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in feces. However, fecal culture is still recognized to be the most sensitive technique as the confirmative diagnostic test of MAP, because of the problems associated with the low numbers of MAP in feces during early infection and the resistance of the bacterial cell wall to lysis, together with PCR-inhibitory substances present in fecal samples. We have developed a novel method of DNA preparation from fecal samples, which is able to effectively extract bacterial DNA and eliminate inhibitory substances. In this method, DNA was extracted using micro beads in lysis buffer, then purified using organic solvents. We compared the usefulness of our DNA preparation method for MAP-PCR test with the classical heat extraction method. Fecal samples of known MAC status were obtained, which contained a lot of MAP organisms. DNA samples were prepared by both methods from fecal residues, which had previously been subjected to a single heat extraction. IS900-specific PCR products were detected from fecal DNA fraction prepared by our method, but not detected from samples prepared by additional heat treatment. These results show some MAP organisms are excreted into feces in the conditions exhibiting a highly resistance against the heat treatment alone. In addition, we have developed a reagent cocktail for PCR (Ampdirect(r)) that can neutralize the inhibitory effects of substances in biological samples on DNA amplification. Using our DNA preparation method and Ampdirect(r) reagent, IS900-specific PCR products were detected in all four fecal samples from experimentally infected calves, that showed none or a few formation of MAP colony in fecal culture. The combination of our DNA preparation method with PCR using Ampdirect(r) thus offers a detection method for MAP that is more sensitive than the fecal culture and much more rapid.

Title A comparison of six different protocols to extract *M. paratuberculosis* DNA from bovine faeces.

Author(s) Zecconi A[*] , Mosca A, Piccinini R, Robbi C.

Institution Dept.Animal Pathology- Infectious Diseases Lab-. Istituto Zooprofilattico Sperimentale delle Venezie - Verona.

Abstract The recovery and isolation of *M. paratuberculosis* in faeces represent one of the major limits in the development and application of control programs in dairy herds. Up to now, different protocols have been proposed to isolate the pathogen from the faeces, but none of them showed to be fully satisfactory. The aim of our work was to compare different extraction protocols and commercial kits suitable for further application of PCR technique. Composite samples of negative faeces were contaminated with a single strain of *M. paratuberculosis* taken from an agar-plate and suspended in 1 ml sterile water, positive samples were obtained mixing 1g of faeces and 250 ml of bacteria suspension and stored at -20°C. A nested PCR protocol based on primer suggested by Linsby et al. (1994), was applied to the extracts from the different protocols. They were both experimental protocols and commercially available kits, among these latter ones some suggested for DNA extraction from plant and mouse tail, the other specifically developed for detecting *M. paratuberculosis* by PCR from bovine faeces. The results showed that only this latter method gave a positive nested-PCR amplification. The preliminary sampling of faeces taken from seropositive cows confirmed these results and therefore suggests that this protocol could be applied in field in the programs aimed to eradicate the disease from dairy herds.

Title Usefulness of immunomagnetic separation for preparation *Mycobacterium avium* subsp. *paratuberculosis* template DNA in milk.

Author(s) Ocepek M[1*] , Gruntar I[1] , Pate M[1] , Krt B[1] , Cvetnic #[2] .

Institution [1] Veterinary Faculty, Gerbiceva 60, 1115 Ljubljana, Slovenia. [2] Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia.

Abstract

Introduction.

The aim of this study was to establish usefulness of the Immunomagnetic separation (IMS) for the selective isolation of *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) from milk samples for the IS900 PCR.

Material and methods.

The milk samples from 38 seropositive cows and milk samples spiked with known number of *M. paratuberculosis* cells (ATCC 43015, 1200, 600, 120, 60 and 12 bacterial cells per ml of milk) were tested. IMS was performed using Dynabeads-Protein A (Dynal, Norway) and *M. paratuberculosis*-specific goat hyperimmune serum, according to the manufacturer's instructions, followed by DNA extraction with High Pure PCR Template Preparation Kit (Boehringer-Mannheim, Germany) and IS900 PCR. The PCR detection level of the target bacterium in milk was determined also with the standard method (sample concentration and decontamination with 0.75% HPC).

Results and discussion.

The procedure incorporating IMS was found to be capable of detecting less than 12 *M. paratuberculosis* bacterial cells per ml of milk and was significantly more sensitive than the classical PCR procedure without IMS (detection limit: 600 *M. paratuberculosis* bacterial cells per ml). Among 38 milk samples of seropositive animals 6 (15.79%) samples were positive in IMS-PCR. 3 of them were also culture positive. Our results suggest that IMS considerably improves the sensitivity of PCR IS900 for the detection of *M. paratuberculosis* in milk samples and should consequently increase the sensitivity of other direct methods in the diagnostics of paratuberculosis.

Title Detection of *Mycobacterium paratuberculosis* (Mptb) in fecal samples using immunomagnetic capture and TaqMan PCR.

Author(s) Willemsen PTJ[*] , Ruuls R, Damman M, Bakker D.

Institution Central Institute Animal Disease Control (CIDC), Lelystad, The Netherlands.

Abstract PCR as diagnostic method for detecting Mptb offers an attractive alternative for the traditional cultural methods combining both rapidity and specificity. Allthough the Mptb specific IS900 sequence offers a specific PCR target, reports on false positive IS900 PCR results implicate the need for product confirmation by hybridization. The TaqMan technology combines the benefits of PCR with confirmation by hybridization. Furthermore the TaqMan technology enables quantitative PCR and high through-put screening. The aim of this study was to develop a rationalized approach for isolating and detecting Mptb from faecal samples taking into account the specificity of the primers and probe for IS900 and monitoring PCR inhibition by spiking samples with a mimic DNA template and subsequent detection with TaqMan technology. The extraction of DNA in our method avoids organic extraction by capturing Mptb cells from fecal samples using anti-Mptb directed monoclonal antibodies coated to magnetic beads. Faeces from non-infected cows (repeatedly tested by culturing) were spiked with Map cells and isolated by immunomagnetic capture. An average of 10 cells per gram of faeces could be detected by using this technique. Inhibition of the PCR reaction was very low, especially when compared to detection methods based on DNA binding to a silica-gel column. Furthermore, 34 faecal samples from a known cultural status (19 positive,15 negative) were tested using the PCR method showing a good agreement with the culturing data as is expressed by a kappa value for agreement of 0.76.

Title Alternative PCR for *Mycobacterium paratuberculosis*.

Author(s) Englund S, Heldtander M[*], Bölske G.

Institution National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden.

Abstract The PCR systems directed against the IS900 gene have proved very useful for the identification of *Mycobacterium paratuberculosis*. However, when IS900-like genes were found in other unrelated Mycobacterium species, it was revealed that these PCR systems are not completely specific for *M. paratuberculosis*. It is therefore important to investigate alternative PCR systems for confirmation of positive IS900 PCR tests. A duplex PCR system (Coetsier et al 2000) targeting the p34 gene and the f57 gene was applied to 91 strains of *M. paratuberculosis* and other mycobacteria from different animals and countries. Two fragments of expected sizes were detected from the 60 tested *M. paratuberculosis* strains and one fragment, from the amplified p34 gene, was found in the 16 *M. avium* strains. Strain 2333, with an IS900-like gene, gave no fragment in the p34/f57 PCR. One strain each of *M. tuberculosis* and *M. bovis* both resulted in a fragment from the p34 gene, slightly shorter than the corresponding fragments from *M. paratuberculosis* and *M. avium*. Fragments of the amplified p34 gene were found in 8 of the other mycobacterial strains tested. The p34/f57 PCR seems suitable for confirmation of samples tested positive for *M. paratuberculosis* in IS900 PCR-systems. A novel fingerprinting PCR system representing a reproducible randomly amplified polymorphic DNA (RAPD) was developed and applied to strains of *M. paratuberculosis* and other mycobacteria. The PCR is based on primers targeting the enterobacterial repetitive intergenic consensus (ERIC) elements and IS900. Sixty *M. paratuberculosis* strains, 16 *M. avium* and 15 other mycobacteria, including the IS900 positive strain 2333, were tested. Reproducible fingerprints were obtained for all mycobacteria analysed. The *M. paratuberculosis* strains all exhibited the same banding pattern, clearly different from the fingerprints of strain 2333 and other mycobacteria. This PCR-system offers an alternative to IS900 PCR for identification of *M. paratuberculosis*.

Title A real-time PCR assay suitable for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) using SYBR Green and the Light Cycler.

Author(s) O'Mahony J[*] , Hill C.

Institution Microbiology Department, National University of Ireland, Cork, Ireland.

Abstract Molecular based detection strategies for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) offer the benefit of speed over the traditional culturing methods. In this study we attempted to evaluate and optimise a real-time PCR approach which may be suitable for detecting and quantifying MAP in culture using SYBR green and the highly characterised P90 / P91 primers. All parameters of the PCR were carefully adjusted including Mg concentration, primer concentration and annealing temperature After optimising the assay, fluorescence readings for each sample per PCR cycle were recorded to determine the sensitivity of the reaction and sophisticated melting curve analysis was used to determine specificity. This method was tested using a variety of templates including purified PCR products, isolated MAP DNA, pure colonies or liquid culture sources. The sensitivity of this assay was as follows: purified template - 20 copies, isolated DNA - 50fg, MAP in broth - 25 cells. Furthermore, this assay was found to be specific for MAP even in the presence of heavily contaminated samples, as determined by the presence of an identifiable melting peak at 92.2ºC. Two critical factors in optimising the success of this assay were primer (0.05µM) and Mg. concentrations (3mM). By adapting this assay it was found that it could be used to accurately enumerate the numbers of MAP cells growing in broth when compared with pre-determined standards. This application is significant as it may present researchers with an alternative or confirmatory method to counting colonies on slopes, or measuring growth in radiological media. In conclusion we have optimised a sensitive and specific real-time PCR assay for MAP which is effective using a variety of templates, and may be useful to researchers as a rapid means of detecting and enumerating MAP in culture

Title Detection of *Mycobacterium avium* subsp. *paratuberculosis* by buoyant density centrifugation, sequence capture-PCR and dot blot hybridisation.

Author(s) Nilsen SF[1] , Halldórsdóttir S[1] , Englund S[2] , Djønne B[1] , Olsaker I[3] , Holstad G[1] .

Institution [1] National Veterinary Institute, P.O. Box 8156, Dep., NO-0033 Oslo, Norway. [2] National Veterinary Institute, SE-751 89 Uppsala, Sweden. [3] Norwegian School of Veterinary Science, P.O. Box 8146, Dep., NO-0033 Oslo, Norway.

Abstract

Introduction.

Cultivation of *M. paratuberculosis* is the golden standard of paratuberculosis diagnosis although the method is time consuming, laborious and shows low sensitivity in subclinically infected animals. Detection of *M. paratuberculosis* by PCR is often hampered by the lack of efficient methods for sample treatment. Faecal material contains an especially high content of PCR inhibitors, and different methods have been performed to avoid such factors. The aim of the present study was to achieve a sensitive method to detect *M. paratuberculosis* in fecal specimens.

Material and methods.

To assess the optimal sensitivity of the PCR and detection system chosen, serial dilutions of *M. paratuberculosis* genomic DNA were set up and analysed by PCR and dot blot hybridisation. In addition, serial dilutions of bacterial genomic DNA were analysed by sequence capture-PCR followed by dot blot hybridisation. Furthermore, known amounts of whole *M. paratuberculosis* bacteria, defined as colony-forming units (CFU), were analysed by sequence capture-PCR and dot blot hybridisation. Finally, faecal samples spiked with known amounts of bacteria, were subjected to buoyant density centrifugation in Percoll(r) prior to subsequent analysis by sequence capture-PCR and dot blot hybridisation. To measure the loss of bacteria by buoyant density centrifugation, known amounts of whole *M. paratuberculosis* bacteria were submitted to the density centrifugation procedure prior to sequence capture-PCR.

Results and discussion.

By using buoyant density centrifugation, sequence capture-PCR, and dot blot hybridisation, we achieved a sensitivity of 10^3 colony forming units (CFU) per g faeces. The detection limit by culture was assessed to 10^2 CFU per g of faeces. We conclude the described protocol to be a fast and sensitive alternative to bacterial culture of faecal samples.

Title Detection of *Mycobacterium avium* subsp. *paratuberculosis* DNA from formalin-fixed paraffin-embedded tissues.

Author(s) Pislak M, Ocepek M* , Juntes P, Zabavnik J, Pogacnik M.

Institution Veterinary Faculty of Ljubljana, Gerbiceva 60, Ljubljana, Slovenia.

Abstract

Introduction.

Detection of *Mycobacterium avium* subsp. *paratuberculosis* with routine histopathology examination and Ziehl-Neelsen staining is not specific enough for conclusive diagnosis of paratuberculosis if lesions are not well developed e.g. in the subclinical cases of the disease. In samples that already were formalin-fixed the culture examination is impossible. A rapid and specific method for the isolation and detection of *M. avium* subsp. *paratuberculosis* DNA from archival samples with IS900-based polymerase chain reaction (PCR) is described.

Material and methods.

In this study 70 archival samples of formalin-fixed paraffin-embedded tissue blocks of cattle, sheep and goat intestines and lymph nodes were used. For PCR procedure several paraffin tissue sections were transferred in microcentrifuge tube, deparaffinized with xylene, transferred to 100% ethanol and air dried. Isolation of genomic DNA was performed using High Pure PCR Template Preparation Kit (Boehringer, Mannheim). PCR was performed with IS900 specific primers in 40 cycles at 94ºC, 62ºC and 72ºC. PCR-products were separated on electrophoresis and analysed by scanning and visualisation system (BIO-Rad).

Results and discussion.

All 26 histologically positive samples were positive with IS900-based PCR, also 10 of 20 inconclusive samples, and one of 24 histologically negative samples. In this study an agreement between the results of histological examination and IS900-based PCR in formalin-fixed

paraffin-embedded tissues was obtained.

Conclusions.

These results show that the procedure used in our laboratory for the isolation of *M. avium* subsp. *paratuberculosis* DNA from formalin-fixed paraffin-embedded tissues is useful for the final diagnosis of paratuberculosis, especially in the inconclusive cases.

Title Identification of *M. avium* paratuberculosis from bovine fecal samples by PCR technique.

Author(s) Arrigoni N* , Cesena C, Belletti GL.

Institution Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia Romagna (Sezione di Piacenza).

Abstract Tests based on the PCR technique have the advantage to be rapid and highly specific, but, when applied on fecal samples, they lack sensitivity. This is probably due to different kind of problems: difficulty in lysing the cell wall, presence of inhibitory substances, not suitable conditions of the PCR reaction. We have taken into consideration the three kinds of problems, obtaining good results by the use of physical methods of lysys, in combination with a commercial DNA extraction kit (QIAamp stool mini-kit, Qiagen) and by the optimisation of the reaction conditions (MgCl2, BSA, Taq DNA Polymerase concentration and number of cycles).

Title Comparative study on detection of *Mycobacterium avium* subsp. *paratuberculosis* by PCR diagnosis and conventional culture on faeces of water buffalo herds in Latium region (Italy).

Author(s) Lillini E* , Scherm B, Gamberale F, Cersini A, Fagiolo A.

Institution Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy.

Abstract Breeding of water buffaloes is an important zootechnic reality in the Latium region due to the international level of their DOP products ("mozzarella di bufala campana"). The species *Bubalus bubalis* is genetically different from the buffalo species situated in our region and that is breeded for another productive target. In order to increase our knowledge on the susceptibility of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to this species, we carried out a study on 130 buffaloes of a herd in the province of Latina. In 1998 three animals presented the typical symptoms of paratuberculosis infection and serological and cultural tests showed positive results. Diagnostic controls have been periodically repeated during the following years and revealed another 3 positive animals which led to the establishment of a sanitary control program for this farm. 108 sera and 75 fecal samples have collected from all buffaloes older than 2 years without any clinical signs related to paratuberculosis. Both, AGID and ELISA, tests showed negative results, whereas the cultural approach on HEYM medium revealed 3 animals with positive fecal samples. All isolates were typed as MAP. These 75 fecal samples were analised with two different PCR approaches (home-made and the commercial kit ADIVET™ PARATUB) to test for other non- or low-shedding subjects. Parameters and conditions of the two PCR protocols were worked out using 30 previous tested bovine fecal samples of an international proficiency test and therefor used as positive and negative controls. The results of both PCR methods correlated completely with the controls so that we are now carrying out PCR tests on DNA extracted from the 75 buffalo fecal samples to value the sensitivity and specificity of our approach and to validate these PCR systems as rapid and reliable diagnostic tools for our purpose.

Title Detection of Mycobacterium bovis with LCx amplification assay.

Author(s) Valente C[1*] , Cuteri V[2] , Ausili E[3] , Piersimoni C[3] .

Institution [1] University of Perugia. [2] University of Camerino. [3] GeneralHospital, Ancona, Italy.

Abstract LCx amplification assay, usually employed to detect *Mycobacterium tuberculosis* complex in respiratory specimens, was evaluated by comparing the results it gave with those obtained using Lowenstein-Jensen solid medium and pathological findings on 55 lymph nodes from cattle with positive and 10 lymph nodes form cattle with negative skin tests for tuberculosis. Fifty-three cultures (51 and 2 respectively) were positive for *Mycobacterium bovis* while the results for the LCx assay and the histological method were positive in 48 (45 from skin test positive and 3 from skin test negative) and 24 (20 from skin test positive and 4 from skin test negative) samples, respectively. The results obtained with the LCx assay, compared with the bacteriology, regarded as the gold standard among the diagnosis methods, gave a specificity of 91.6% and sensitivity of 90.5%. Although the sensitivity of LCx was sub optimal, DNA of *M. bovis* was detected in 81.8% of the skin test-positive animals. Amplification assay could rovide a rapid and reliable tool for detecting tuberculosis in cattle.

Title Use of loop-mediated isothermal amplification for the rapid detection of *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Enosawa M* , Sawai K, Kageyama S, Watanabe K, Onoe S, Mori Y, Yokomizo Y.

Institution Hokkaido Animal Research Center, Shintoku, Japan.

Abstract Loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions have been developed by Notomi et al (Nucleic Acids Res.28, 2000). This method employs a DNA polymerase and a set of four specially designed primers that recognize total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. In the present investigation, we evaluated the usefulness of LAMP for the rapid identification and sensitive detection of *Mycobacterium avium* subsp. paratubeculosis (MAP). The primers for LAMP were designed to amplify the gene sequence of IS900, HspX, dnaJ and F57 fragments of MAP. DNA templates were prepared from the cultured bacterial suspension of MAP strain ATCC 19698. Amplification and detection of the genes to be amplified was completed in a single step, by incubating the mixture of gene sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (in the region of 65ºC). The amount of DNA fragments synthesized was assayed by gel electrophoresis and by real-time monitoring of the turbidity produced by white precipitate of magnesium pyrophosphate in the reaction mixture. As a result, two gene fragments of IS900 and HspX were effectively amplified by LAMP test, but other two gene fragments of dnaJ and F57 were not. Two MAP strains yielded positive results, but other bacterial strains including *M. avium* subsp. *avium* yielded negative results in the tests for IS900. The concentration of DNA templates of MAP amplified by LAMP test was 0.01 pg/ul in the case of IS900. The LAMP test had a sensitivity equal to or greater than that obtained by traditional PCR techniques and were much more rapid, taking only two hours compared with 4 hours for PCR test. These results show that LAMP test might have a potential usefulness as a rapid and sensitive method to detect MAP in faeces or tissues of animals infected with MAP.

Title INVITED SPEAKER: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and its relation to Crohn's disease.

Author(s) Hermon-Taylor J.

Institution Department of Surgery, St. George's Hospital Medical School, London SW17 0RE UK.

Abstract Crohn's disease (CD) is a systemic disorder whose principal clinicopathological manifestation is chronic inflammation of the intestine. Although present in human populations at a low background level for many years, CD really began to emerge in Western Europe (WE) and North America (NA) in the mid 1940s. Thereafter, with some geographical variations, the trend in the incidence has continued to climb. It is estimated that there are at least 500,000 CD sufferers in the USA rising by about 25,000 new cases each year. Estimates for WE are comparable. In some districts in Manitoba the incidence of CD has reached 26.2/100,000/yr the highest reported in the world. CD is therefore a major human healthcare problem in developed societies. Does MAP cause CD, as proposed by the Glasgow surgeon Thomas Kennedy Dalziel in 1913 ? It is known that MAP infection is widespread in domestic livestock in WE and NA and that there are wildlife reservoirs. It is known that live MAP is transmitted to humans in the UK (and by implication elsewhere) in retail pasteurized milk. Is MAP present in the inflamed gut in CD ? After uncertainty generated by the conflicting results of clinical research over the period 1994-99, the answer emerging from contemporary work in several laboratories using methods of proven validity, is an unequivocal yes; our own figure for MAP in CD gut is currently > 90%. It is known that there are 'bovine' and 'ovine' strains of MAP. Genotyping MAP from CD is now consistent with the emergence of a 'humanised' strain. The microscopical picture of paucimicrobial MAP enteritis in animals closely resembles that of CD in humans. What is MAP doing in CD gut ? It is known that MAP is a specific primary cause of chronic inflammation of the intestine affecting many species including primates. For MAP in CD gut merely to have a bystander role, it would have to be harmless to humans. That this is very unlikely is evidenced by four independent open clinical studies available so far, which show that treatment with anti-MAP drugs can heal CD. It is known that MAP in CD is present in a tough ZN-negative form. Pathogenic mechanisms and drug susceptibilities are not like tuberculosis. Intracellular MAP in CD minimises immune recognition. MAP in CD parasitises immunoregulatory cells in the gut of people with an inherited or acquired susceptibility, resulting in immune dysregulation and a leaky mucosal lining. The transmural inflammation itself occurs as a perturbed response to leakage into the gut wall of food residues and bacteria from the gut lumen. Treatment by immune modulation, elemental diets, and killing the invading enteric bacteria with ordinary antibiotics, can temporarily improve CD by antagonising disease mechanisms, but not disease causation. With MAP still present the disease returns. Therapeutic DNA vaccines for CD to assist in immune-mediated clearance of MAP are needed. The overwhelming balance of evidence favours causation of CD by MAP, a Public Health problem of tragic proportions. The list of remedial measures begins with the introduction of farm practices which minimise the transmission of MAP infection, the certification of MAP-free herds, and the incremental introduction of industrially applicable procedures which ensure that retail milk does not contain live MAP. Given the prevalence of MAP infection in domestic livestock together with wildlife reservoirs, and the increasing ability to identify individuals with an inherited susceptibility to CD, we also need preventative MAP vaccines for animals and for humans before we can expect progress towards a global resolution of this complex overall problem, to be achieved.

Title Impact of commercial HTST pasteurisation on *Mycobacterium avium* subsp. *paratuberculosis* in naturally infected cows' milk.

Author(s) Grant IR[1*] , Hitchings EIJ[1] , Ball HJ[2] , Rowe MT[1,2] .

Institution [1] Department of Food Microbiology, Queen's University of Belfast. [2] Department of Agriculture and Rural Development for N. Ireland, Belfast, N. Ireland, UK.

Abstract A large-scale survey of commercially pasteurised cows' milk, and pasteurisation trials using commercial-scale plant (2,000 l/h with turbulent flow) and naturally infected milk, were carried out to determine the efficacy of HTST pasteurisation applied to milk naturally infected with

Mycobacterium avium subsp. *paratuberculosis* (MAP). During the milk survey a total of 567 commercially pasteurised milk samples from approved dairy processing establishments throughout the UK were tested for the presence of viable MAP. In the pasteurisation trials, naturally infected milk from two local farms was subjected to four different heat treatments (73ºC for 15 and 25 s, with and without prior homogenisation) on twelve separate occasions. In both studies, milk samples were subjected to decontamination with 0.75% (w/v) cetylpyridinium chloride for 5 h at room temperature before culture on Herrold's egg yolk medium and in BACTEC 12B medium. Overall, viable MAP was cultured from 10 (1.8%) of 567 pasteurised milk samples tested during the UK milk survey and from 10 (6.7%) of 144 pasteurised milk samples tested during the commercial-scale pasteurisation experiments. Surviving MAP were isolated from pasteurised milk samples that had been heated at 72ºC for both 15 and 25 s. The results of these two studies provide clear evidence that MAP bacteria in naturally infected milk are capable of surviving commercial HTST pasteurisation on occasion. It should be noted that the above studies differ from previous laboratory-scale pasteurisation studies in several respects: no MAP were artificially added to milk prior to heat treatment, a larger volume (50 ml) of pasteurised milk was tested thereby increasing the chances of detecting survivors, and 24-72 h elapsed between heat treatment and milk testing potentially giving sub-lethally heat-injured MAP cells time to recover viability.

Title Survival of *M. avium* subsp. *paratuberculosis* in raw milk cheese.

Author(s) Spahr U[*] , Schafroth K.

Institution Swiss Federal Dairy Research Station Liebefeld, 3003 Bern, Switzerland.

Abstract A strength/weakness analysis of the current procedure was performed by a team of both experts and on the other hand strong analytical thinkers with no experience in the field of paratuberculosis. The project resulted in a number of recommendations with regard to likely risk factors. The sampling and transportation procedure was improved. A new and detailed instruction of this improved procedure was designed and communicated to veterinary practitioners. The handling procedure of the samples in the laboratory was improved at several points. Moreover a new charge of cycloheximide was used to prepare the antimicrobial stock solution and natamycine was added as antimicrobial to the modified Löwenstein-Jensen medium.

Title Reliable detection of MAP in human intestine by optimized PCR, MGIT culture and protozoan culture.

Author(s) McMinn EJ[1*] , Bull TJ[1] , Sidi-Boumedine K[1] , Skull A[1] , Rhodes G[2] , Pickup R[2] , Hermon-Taylor J[1] .

Institution [1] Department of Surgery, St Georges Hospital Medical School, London. [2] Centre for Ecology and Hydrology, Lake Windermere, UK.

Abstract Attempts to demonstrate *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease (CD) tissues by many laboratories 1994-1999 using a variety of sample processing and PCR conditions, lead to conflicting results. We have developed reliable procedures for the detection of MAP in fresh human intestinal tissues, incorporating mechanical disruption of the tissue lysate to access MAP DNA and PCR conditions uniquely specific for IS900. In an initial ongoing open-label clinical study, DNA is prepared from fresh ileocolonoscopic mucosal biopsies using SDS proteinase K, mechanical disruption in the Hybaid Ribolyser system, and phenol chloroform extraction. Five µl aliquots of DNA extract are amplified by nested IS900 PCR. A second mucosal biopsy is decontaminated for 20 mins in 500µl BBL Mycoprep, transferred directly to a BBL MGIT culture system containing the antibiotic cocktail PANTA and incubated at 37ºC. The cultures are tested for MAP at intervals. To date MAP has been identified in 17 of 18 (94%) of CD samples and 4 of 16 (25%) samples of uninflamed mucosa (p=0.0004). In a parallel double blinded study, fresh full thickness surgical resection gut samples from CD and control patients are cut with crossed scalpels, digested in trypsin collagenase, and lysed using BBL Mycoprep. The MAP-enriched centrifugal

pellet is divided into three portions and tested for MAP using nested IS900 PCR, incubation in the MGIT system, and by feeding to cultures of *Acanthamoeba polyphagia* maintained for many months and in which MAP can persist and replicate. To date 23 of 44 (52%) of surgical samples are MAP positive and 21 of 44 (48%) are MAP negative. These studies confirm that new and optimized methods can reliably detect MAP in human intestine. The highly significant association of these chronic enteric pathogens with inflamed CD tissues is consistent with a causative role for MAP in CD.

Title Genotyping of human isolates of MAP provides evidence for 'humanised' strains.

Author(s) Bull TJ[1*] , Sidi-Boumedine K[1] , Naser S[2] , McMinn EJ[1] , Skull A[1] , Hermon-Taylor J[1] .

Institution [1] Department of Surgery, St Georges Hospital Medical School, London. [2] Department of Biomolecular Sciences University of Central Florida, USA.

Abstract Previous studies using IS900 RFLP and MPIL typing of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) strains have shown distinct genomic differences between strains isolated from bovine and ovine origin suggesting that the clonal expansion of MAP in different species has led to the development of strain specificity for particular hosts. Seven strains of MAP isolated from 7 patients with Crohns disease (CD) were obtained from Dr S. Naser, University of Central Florida, USA. These were sub-cultured and checked for purity in MGIT (Becton Dickinson) liquid medium. 2 strains were isolated from patient breast milk and 5 from resected gut tissue. Growth was relatively rapid in 6/7 strains with good yields obtained in under 4 weeks. ZN staining of these cultures showed these strains all exhibited both ZN positive and ZN negative forms. The identity of each strain was confirmed as MAP by using PCR reactions specific for the MAP genome (gsd-mpa junction and MAP MIRU2 & 3 locus PCR). MPIL typing of each strain using PCR reactions specific for 14 different IS900 loci was performed to determine the number of loci filled with IS900 and any genomic re-arrangements associated with IS900. PCR products from IS900 Locus 5 were sequenced to determine SNP differences between strains. Results showed that all but one of the strains were MPIL type 3 (RFLP type B/C5) and were missing an IS900 insertion at Locus 5 resulting in the desA1 gene remaining intact. Strains could be further differentiated from bovine/ovine strains and a previous MPIL type 3 MAP strain BEN isolated from a human with CD by SNPs present in locus 5. These results suggest that the loss of IS900 from locus 5 in MAP and the presumed resumption of desA1 gene expression is associated with MAP strains isolated from within the inflamed gut wall of patients with CD.

Title Heat inactivation of *Mycobacterium paratuberculosis* in milk.

Author(s) McDonald WL, O'Riley K, Schroen CJ, Condron RJ[*] .

Institution Victorian Institute of Animal Science, 475 Mickleham Road Attwood 3049, Australia.

Abstract Effectiveness of heat inactivation during processing and the concentration of *Mycobacterium paratuberculosis* in raw milk have been identified in quantitative risk analysis as the most critical factors contributing to the possible presence of viable *M. paratuberculosis* in dairy products. Laboratory simulations of pasteurisation have suggested that increasing the withholding time would enhance the likelihood of killing *M. paratuberculosis* during processing. A quantitative assessment of the lethality of pasteurisation was undertaken using a sensitive culture technique with minimal decontamination treatment capable of detecting one organism per 10 ml. *M. paratuberculosis* was artificially added to raw whole milk which was then homogenised and pasteurised in a pilot industrial pasteuriser designed for research purposes to simulate a commercial continuous flow milk pasteuriser. The holding tubes were tested to ensure pasteurisation times of 15 s, 20 s and 25 s with a flow-rate of about 3000 l/hr and a Reynold's number of 62,112. The heat exchanger was tested prior to use to confirm there was no leakage and the inlet and outlet hold-tube temperatures and flow-rate were monitored every 10 s during operation of the plant. Twenty batches of milk containing 10^6 to 10^7 organisms/l were processed with various temperature and time combinations of 72-78°C and 15-25 s. Thirty 50 ml milk samples from each processed batch were cultured and the

c reduction in *M. paratuberculosis* was determined. In 17 of the 20 batches no viable *M. paratuberculosis* were detected representing greater than 6 log10 reductions. These experiments were conducted with very heavily artificially contaminated milk to facilitate the measurement of the logarithmic reduction. In 3 of the 20 batches of milk pasteurised at 72°C for 15 s, 75°C for 25 s and 78°C for 15 s, a few organisms were detected. Pasteurisation in these experiments was found to be very effective in killing *M. paratuberculosis* with a logarithmic reduction in all batches greater than 4 log10.

Title Heat inactivation of *Mycobacterium avium* subspecies *paratuberculosis* in milk.

Author(s) Rademaker JLW* , Giffel MC.

Institution NIZO food research, Department of Processing, Quality & Safety, PO Box 20, 6710 BA Ede, The Netherlands.

Abstract Heat treatment is the most frequently applied process to inactivate e.g. (pathogenic) micro-organisms and to ensure the quality and safety of milk and milk products. Several research groups have published data on inactivation of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in milk under various pasteurization conditions. Different conclusions about the heat resistance of MAP result from the differential experimental conditions such as the history of the MAP cultures, cell clump disruption, heat application, inactivation data acquisition. In the presentation, parameters critical for heat inactivation experiments will be discussed, in particular the influence of clumping of cells. Application of predictive models has confirmed that tailing of the inactivation curve of MAP is most likely due to the presence of cell clumps and not caused by a more heat resistant cell fraction. According to the model perdictions, the higher the fraction of cells in clumps and the larger the cell clumps, the more tailing will occur. The knowledge of critical factors influencing the heat inactivation experiments was used to set up new studies to ascertain the effectiveness of pasteurization for inactivation of MAP. Industrial pasteurization was simulated in a pilot plant using a turbulent flow at high temperatures and short holding times. Under various conditions, the heat resistance of MAP in milk was determined

Title Survival of *Mycobacterium avium* ssp. paratuberculosis at high temperature short time pasteurization in a pilot plant pasteurizer at elevated temperatures and extended holding time.

Author(s) Hammer P[1*] , Kiesner C[2] , Walte HG[1] , Teufel P[1] .

Institution Federal Dairy Research Centre, [1] Institute for Hygiene and Food Safety, [2] Institute for Dairy Chemistry and Technology, P.O. Box 6069, D-24121 Kiel, Germany.

Abstract

Introduction.

Own investigations and data from the literature strongly support the thesis, that small numbers of *M. paratuberculosis* survive high temperature short time pasteurization of milk. The aim of the studies reported here was to determine an end point for this type of heat treatment, at which surviving organisms are no longer detectable.

Materials and methods.

Inoculation of raw milk: three field-strains of *M. paratuberculosis* at 10^4 cfu/ml each Heat treatment: pilot plant pasteurizer, sample volume 10-20 l, holder spiral shaped, heating at 72-90°C, flow rate 30-100 l/h, holding time 40-60 s. Detection of heat injured *M. paratuberculosis*: centrifugation of heated milk samples at 14,000 x g for 10 min without decontamination, inoculation of pellets simultane-ously into modified Dubos medium for resuscitation and directly onto HEYM. Confirmation of cultures by acid-fast staining and IS900 based PCR, viability stainings with iodo-nitro-tetrazolium chloride.

Results and discussion.

A 5 to 6 log reduction of initial counts was achieved by temperature-time combinations of 72,

75, 80, 90°C for 40 and 60 s. However, in 45 of 48 experiments surviving *M. paratuberculosis* cells were detected. All positive results were obtained within 8-12 weeks of incubation, which is much faster than in experiments with holding times below 30 s (data not shown). These results were obtained even without resuscitation. According to these observations a possible heat activation of *M. paratuberculosis* is supposed. This thesis is supported by viability stainings: in bacterial clumps from a fresh culture a uniform distribution of 1-10% active cells can be seen. After heating of this culture only a few clumps show active cells concentrated in active spots.

Title The recovery of *Mycobacterium avium* subsp. *paratuberculosis* following heat treatment of inoculated milk in a turbulent-flow pasteuriser is not adversely affected by decontamination and antibiotic selection.

Author(s) Pearce LE[1*] , Crawford RA[1] , Truong HT[1] , Yates GF[2] , de Lisle GW[2] .

Institution [1] New Zealand Dairy Research Institute, Palmerston North, New Zealand. [2] AgResearch, Wallaceville, New Zealand.

Abstract Evaluation of the effect of milk pasteurisation on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) requires kinetic data on heat inactivation that reflect the conditions of the commercial process. Decontamination and antibiotic selection are essential for MAP isolation from raw milk. We examined whether these treatments affect the recovery of potentially heat-damaged bacteria in large-scale experiments. Bulked commercial supply raw milk was divided into two parts. The first was inoculated with ATCC 19698 at ~ 3x 10^5 cfu/ml and processed in a pilot plant heat exchanger at 62, 63, 64, 65, 66, 67, 68, 69, and 72°C for 15 sec. The second portion was sterilised by UHT treatment (140°C/4 sec.) and similarly inoculated and processed. MAP recovery was followed on HEYMM slopes and in BACTEC medium with and without decontamination and antibiotic selection. No survivors were detected from either raw or UHT milks after heating at 72°C /15 sec. The recovery from heated UHT milk on HEYMM was unexpectedly and consistently higher in runs with selection than without selection. The predicted kills (95% confidence) at 72°C/15 sec were ~7-log and ~10-log respectively. Statistical analysis of the BACTEC curves in the same comparison also predicted a higher recovery with selection. BACTEC, however, was the more sensitive recovery medium than HEYMM with 50% tubes showing survivors at 69°C and all at 68°C in each UHT treatment. No survivors were recovered on HEYMM at these two temperatures. BACTEC vials showing no initial growth were given an extended incubation without any evidence of growth. MAP was significantly more heat sensitive in UHT milk than in raw milk. The results confirm and extend our earlier work on MAP heat inactivation. Standard pasteurisation under commercial conditions gave effective inactivation of MAP independent of the milk type or recovery conditions.

Title Can *Mycobacterium avium* subsp. *paratuberculosis* exhibit cross-protection when subjected to stresses relevant to the water treatment industry?

Author(s) Whan LB[1*] , Ball HJ[2] , Scott R[3] , Rowe RMT[1,2] .

Institution [1] Department of Food Science (Food Microbiology), Queen s University Belfast. [2] Department of Agriculture and Rural Development for N. Ireland. [3] N. Ireland Drinking Water Inspectorate, Belfast, N. Ireland, U.K.

Abstract Water is a possible route of transmission of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from cattle and other ruminants to humans. This is compounded by the fact that Map has been shown to survive elements of the water treatment process, e.g. chlorination, as well as surviving for protracted periods in the environment. When some microbial populations are subjected to a non-lethal primary stress, which is then removed before a secondary stress is applied, the microbial populations can exhibit elevated resistance to the secondary stress. This phenomenon is termed cross-protection. This study was concerned with the effect of nutrient starvation, to which Map would be exposed in water, on chlorine resistance. Water is generally of low nutrient status, which could be considered as a primary stress, followed by a second stress of chlorination. In starvation studies, stationary phase cultures of Map (strain NCTC

8578) were added to duplicate tubes containing 50 ml of distilled water to give a final cell concentration of approximately 5.5 x 10^6 CFU/ml. Cultures were subjected to nutrient starvation for 24, 48, 72 and 96 hours. Starved and control (freshly inoculated) cultures were then subjected to chlorine disinfectant (0.5, 1.0 and 2.0(g/ml) for 15 and 30 minutes. The results showed a highly significant difference ($P < 0.001$) in chlorine resistance between the control and nutrient starved cultures. However, in respect of starvation time there was not found to be a significant difference in log10 survival until 96 h when the survival decreased significantly. Survival of nutrient starved cultures ranged from 86.4% after 24 h to 84.7% after 96 h. This work indicates that Map does not appear to exhibit cross-protection when subjected to at least 96h nutrient starvation.

Title Comparisons with leprosy, tuberculosis and Johne's disease: is Crohn's disease caused by a mycobacterium?

Author(s) Greenstein RJ.

Institution Veterans Affairs Medical Center Bronx NY USA.

Abstract Although Crohn's disease (CD) is considered to be autoimmune, there is increasing evidence that its etiology is infectious. The most plausible candidate is *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Compellingly, Koch's postulates have been fulfilled for MAP and CD, even though they still have not been met for *M. leprae* and leprosy. In animals MAP causes Johne's disease a chronic wasting intestinal diarrheal disease evocative of CD. Johne's disease occurs in wild, agricultural and domestic animals, including dairy herds. Viable MAP is found in human and cow milk, and is not reliable killed by standard pasteurization. Pathogenic in humans, MAP is found ubiquitously in the environment including potable water. Since cell wall deficient MAP cannot be identified by Ziehl-Neelsen staining, in man identifying MAP requires detection of its DNA, RNA or by culture. Reminiscent of H. pylori and stomach ulcers, if infectious CD should be curable with appropriate antibiotics. Multiple studies arguing against an etiological role for MAP in CD employed antibiotics that are inactive against MAP. Recent data, from trials including macrolides indicate that a cure of CD is possible. The necessary length of therapy remains to be determined. Mycobacterial diseases have protean clinical manifestations, as does CD. The necessity of stratifying CD into two clinical manifestations (perforating and non-perforating) when interpreting the results of antibiotic therapy is discussed. Apparent paradoxes, such as the effect of immune-modulation and the discovery of a "Crohn's related gene", are addressed. Rational studies to evaluate appropriate therapies to cure CD are proposed.

Title *Mycobacterium avium* subp. paratuberculosis in biopsies from patients with Crohn's disease by in situ hybridization.

Author(s) Sechi LA[*] , Manuela M, Francesco T, Amelia L, Giovanni F, Stefania Z.

Institution Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Università degli studi di Sassari, Viale S. Pietro 43/B, 07100 Sassari, Italy.

Abstract Crohn's disease is a chronic inflammatory disease of the gastro-intestinal tract, despite is first correlation with *Mycobacterium avium* subs. *paratuberculosis* since 1913, it is not yet established its role in Crohn's disease. In this study, we report the presence of cell wall deficient *M. paratuberculosis* in paraffin embedded tissue from patients with Crohn's disease by in situ hybridization. IS900 PCR was positive in 10% of tissues analyzed whereas Ziehl Neelsen stain was negative. We are currently trying to use policlonal and monoclonal antibodies to detect *M. paratuberculosis* within the same samples. These results suggest the possible use of in situ hybridization in the clinical practice to confirm the presence of *M. paratuberculosis* in Crohn's disease patients.

Title Regional case control study of Crohn's disease

Author(s) Hirst H[1] , Adams R[1] , McCluskey B[2] , Garry F[1] .

Institution [1] Department of Clinical Sciences, Colorado State University, Fort Collins, CO 80523. [2] Centers for Epidemiology and Animal Health, APHIS-VS, Fort Collins, CO 80521.

Abstract

Materials and methods

A survey was designed and mailed to 2,000 members of the Rocky Mountain Chapter of the Crohn's Colitis Foundation (CCF) with mailing addresses in Colorado, Utah and Wyoming. The survey was also sent to 1,000 members of the Colorado Multiple Sclerosis Society (MSS), which was selected as a source of controls because of the organizational similarity to CCF. Members of the MSS include people with and without MS, which is a chronic, but non-enteric disease. Case respondents provided age of onset, basis of diagnosis, and family members affected. For each life stage, information collected included types and numbers of animals on premises, commercial livestock proximity, occupational history, dairy and meat consumption, and drinking water supply. Univariate analysis of binomial variables was performed with results considered significant at $P<0.05$.

Results

Response rate was 32.6% and 19.4% for the CCF and MS Society, respectively. A total of 395 cases and 455 controls were available for analysis. Age of cases was 48.7 years + 50.6 (SD) and 46.8 years +16.4 for controls. Age at diagnosis was 34.2+15.9 years. Univariate analysis identified several significant variables. Logistic regression analysis will be used to identify the factors that best explain the occurrence of CD. A cumulative risk model will also be developed.

Conclusions and limitations

Historical information obtained from this study will be the basis for a model describing exposure to risk factors and/or protective factors that are associated with CD. A key feature of this study was the assessment of potential risk factors for development of CD where early life exposures may be important.

Title INVITED SPEAKER: Control of paratuberculosis by vaccination

Author(s) Juste RA* , Geijo MV, Sevilla I, Aduriz G, Garrido JM.

Institution NEIKER (Instituto Vasco de Investigación y Desarrollo Agrario), Dpto. de Sanidad Animal. Berreaga 1, 48160 Derio. Bizkaia. Spain.

Abstract Control of paratuberculosis is a very complex issue that has prompted many different approaches. Vaccination is an old control method for paratuberculosis which has been used worlwide, but that has won rather little recognition. Its use on cattle in France by Valleé and Rinjard in the early years of the 20th century reportedly was very successful. In sheep, the epidemic of paratuberculosis in Iceland prompted the development of a vaccine that according to Sigurdson in 1957 led to total disappearance of clinical cases. In the sixties, the Moredun group carried out a series of experiments on sheep that laid the grounds for all subsequent knowledge on paratuberculosis vaccine performance. Later on, work on goats in Norway again proved vaccination to be a highly efficient practical control method. There are no reports on vaccine failure, except one referring to unconventional administration (oral), another of reduced efficacy due to vaccine composition (sonicated cells), and a recent one reporting no effects on fecal shedding compared to other control measures. In addition to the technical aspects of vaccination, it should be born in mind that paratuberculosis has been considered largely as an economic problem. In this sense, vaccination has proven to be a highly advantageous strategy over other control approaches that are more difficult to evaluate. Although vaccination is generally applied to 2-4 weeks old animals, the disease progression being very slow, it is now clear that, at least in sheep, vaccination can modify the immunopathogenesis of paratuberculosis and stop the progression from early subclinical infection to open disease. In spite of all this evidence vaccination is not widely accepted as a choice for paratuberculosis control. One reason is that there is a self-injection risk for practitioners. The risk of some minor local damage was around 1/4000 doses in one study. Other complaint about vaccination is that it can interfere with tuberculosis diagnosis. Although this can be circumvented by the comparative tuberculin testing, it might still represent a commercial concern where avian reactions are rare and commercial considerations make any tuberculin reaction undesirable. Both problems might be overcome with improved vaccines. However, the main drawback for vaccination is that it does not completely protects from infection, and therefore that by itself cannot lead to Map eradication. This point has become even more important since more evidence has been produced on the human exposure Map as a potential risk for Crohn's disease. The most recent evidence on the dimensions of Map spread in wildlife and clinically healthy herds, as well as failures to eradicate the infection and evident difficulties to widely implement other measures, open a way for vaccine use re-assesment as a readily available means for limiting paratuberculosis transmission and economic costs. In conclusion, vaccination is not as clean as test and cull strategies, but can advantageously compete with it in implementation and economic readiness. Depending on the species, epidemiological circumstances and socio-economic constraints it could be the only difference between doing nothing and beginning to win the war against paratuberculosis.

Title A preliminary survey on the prevalence of paratuberculosis in dairy cattle in Spain by bulk milk PCR

Author(s) Sevilla I* , Aduriz G, Garrido JM, Geijo MV, Juste RA.

Institution NEIKER (Instituto Vasco de Investigación y Desarrollo Agrario), Dpto. de Sanidad Animal. Berreaga 1, 48160 Derio. Bizkaia. Spain.

Abstract Paratuberculosis was first reported in Spain in 1973 in sheep and in 1983 in cattle. Since then it has become evident that this infection is not rare, and that there is an increasing demand of laboratory testing. However, no estimate is available on the prevalence of the infecion in the whole country. The only detailed study on paratuberculosis prevalence covered only the north-central area of Spain comprising the Basque Country and adjacent provinces and reported a general prevalence of subclinical infection of 30% of individual cattle. In order to have at wider estimate, we carried out two studies based on the PCR detection of Map in bulk milk samples. In one the whole country was represented by a small number of samples (5x14)

randomly taken from 14 out of the 17 Spain's larger adminstrative divisions. In the other, the same type of sample and processing was used to verify the former results on a more restricted area roughly corresponding to the Bay of Biscay coastal strip. This area contains 69% of cattle farms in Spain. In this study, 200 bulk milk samples were processed. All samples were frozen and stored at -20ºC until used. An IS900 PCR protocol was carried out on the centrifugation pellet of 10 ml of milk, and the products separated in an agarose gel. A band of 389 bp was considered a positive result which was confirmed by sequencing in a few cases. In the first bulk milk survey the sample proportion of PCR positive samples was 10% whereas in the second the frequency was 8%. Given the small sample size these estimates had a large error and therefore only had a value in setting the maximum estimated prevalence which was 47.6% and 19.3%, respectively.

Title Seroprevalence of paratuberculosis in bull mothers herds in Slovenia.

Author(s) Ocepek M* , Krt B, Pogacnik M.

Institution Veterinary faculty, Gerbiceva 60, 1115 Ljubljana, Slovenia.

Abstract

Introduction.

Paratuberculosis is a common disease of ruminants in Slovenia. Since 1997 the percentage of paratuberculosis positive animals in Slovenia has increased and paratuberculosis is spreading among and within the herds. The aim of this work was to estimate the seroprevalence of paratuberculosis in bull mothers herds in Slovenia.

Material and methods.

Animals, older than 2 years in bull mothers herds in Slovenia were tested for the presence of antibodies to *M. avium* subsp. *paratuberculosis* in years 2000 and 2001. A total of 9388 cattle sera from 302 bull mother herds were examined using ELISA Paratuberculosis kit (Institut Pourquier).

Results and discussion.

A total of 41 (0.44%) animals from 35 (11.59%) herds were positive, 25 (0.27%) animals from 20 (6.62%) herds were doubtful and 9322 (99.30%) animals from 247 (81.79%) herds were negative. In Slovenia paratuberculosis was first found in 1961 in imported Jersey cows. Between 1964 and 1993 there were no new cases of disease. In 1993 paratuberculosis was found in a sheep flock and since then several outbreaks of diseases in cattle, goats and sheep have been reported. In 1995 the systematic screening test, ordered by the state, began. Between 5 and 20% of cattle in all herds were tested yearly in order to estimate prevalence and geographic distribution of paratuberculosis in Slovenia. In last two years we decided to test bull mothers herds, because they are significantly involved in animal trade. The prevalence of infected animals is rather low, whereas the prevalence of infected herds is high, so some precautions to decrease the prevalence of paratuberculosis would be necessary. The work was supported by Ministry of Agriculture, Forestry and Food of the Republic of Slovenia.

Title Dairy and beef cattle paratuberculosis survey in intensive and extensive farming conditions.

Author(s) Yayo Ayele W* , Fischer O, Svastova P, Alexa M, Machackova M, Pavlík I.

Institution Veterinary Research Institute, Department of Bacteriology, Mycobacteriology Unit, Veterinary Research Institute. Hudcova 70, 621 32 , Brno. Czech Republic.

Abstract In the Czech republic, prevalence of paratuberculosis was increased since 1990, when clinically healthy animals were imported. Prevalence of paratuberculosis was studied in 101 beef cattle herd kept on pasture, and 95 dairy cows confined in stable. The objective of this study was to determine which risk factors influenced the likelihood of the herds to became infected and to compare the extent to which animals are infected in these two separate management practices and design a suitable control programme. Following very high positive serological survey and

faecal culture, all animals were slaughtered. Tissue culture of 53 (52.5%) animals in beef herd and 52 (54.7%) animals in dairy herd were positive for paratuberculosis. Environmental samples (n=279) like faeces, wall scrapings, water, feed-leftovers, larvae and imagoes of dipterous flies in the barn and grass, soil and mud, pond water in the pasture were cultured, and 9.1 % and 2.0 % from the barn and pasture tested positive for *Mycobacterium avium* subsp. *paratuberculosis*, respectively. Prevalence of the disease in both herds was similar. Positive cows were compared against calves for the possible prenatal or neonatal infection of their daughters. Thirteen positive calves (68.4%) came from positive beef cows, whereas only 5 (17.2%) positive calves were born from positive dairy cows. This demonstrates the risk of direct mother-daughter infection on pasture and pooled milk feeding derived infection in dairy calves. The youngest calves with *M. paratuberculosis* infection were 7 months old. Similar rate of prevalence and identification of the same RFLP type in both herds demonstrate the absence of correlation between the breed type and susceptibility to paratuberculosis. For massive infection of both herds and contamination of the environment by *M. paratuberculosis*, a radical control programme was selected. Supported by the grants No. QLRT - 2000 - 00879 (Brussels, EC) and QD1191 (Min. of Agriculture, Czech Republic).

Title The paratuberculosis-attributable mortality rate in a f lock of Merino sheep in Australia.

Author(s) Abbott KA[1*] , McGregor H[2] , Windsor P[2] , Britton A[3] .

Institution [1] Royal Veterinary College. Hawkshead Lane. North Mymms Hatfield. HERTS AL9 7TA. UK. Ph 01707 666 467 (office hours). 01707 666 293 (after hours). Fax 1707 652 090. [2] The University of Sydney. [3] CSL Limited, Melbourne.

Abstract Paratuberculosis was first reported in sheep in Australia in the early 1980s. In the subsequent 20 years the number of infected flocks in the country has risen to over 500. While early reports suggested that the biological and economic impact of the disease might be small, in the late 1990s an increasing number of sheep farmers reported high mortality rates in their flocks which they attributed to paratuberculosis. In one of these flocks, which contained about 10,000 Merino sheep, we commenced a study in April 2000 with the objectives of estimating the paratuberculosis-attributable death rate and relating the death rate to the seroprevalence of paratuberculosis and the rate of faecal shedding of MAP. Paratuberculosis vaccination of the whole flock occurred towards the end of the first year of the study. Estimates of the paratuberculosis-attributable mortality rate prior to vaccination were made by performing necropsies of all sheep which died on 20 days of each year. Estimates of the crude mortality rate were made by deduction following annual counts. The paratuberculosis-attributable mortality rate in the adult sheep (12 months or greater)in the first year was 14.6%. We present also data for seroprevalence, faecal excretion rate, pathology and mortality rates for both years of the study.

Title Prevalence and risk factors for paratuberculosis among beef cattle in the state of Texas, USA.

Author(s) Roussel AJ[*] , Thompson JA, Libal MC, Stewart EM, Withlock RL, Barling KS, Hairgrove TB.

Institution Dept of Large Animal Medicine and Surgery, and TVMDL, Texas Vet Med Cntr, Texas A&M University, College Station, TX, USA and Johne's Research Laboratory, University of Pennsylvania, Kennett Square PA, USA.

Abstract

Introduction.

This study was conducted to estimate the seroprevalence and culture prevalence of paratuberculosis in purebred beef cattle in Texas and to estimate risks of ranch management practices associated with paratuberculosis.

Methods.

Approximately 7000 letters of invitation were mailed to beef cattle producers in Texas. Of 648

respondents, 115 were selected and completed the study. All cattle over 2 years old in a herd, up to 50 cattle, were sampled cattle. The IDEXX ELISA was used on serum, and cattle with SP values >0.25 were cultured for fecal MAP using a centrifugation technique and Herold s Egg Yolk media twice at 2 laboratories. Analyses of survey data included univariate analysis of management factors, backward stepwise regression analysis and Bayesian mapping of spatial risk.

Results.

For the ELISA test, 36/4609=2.95% of cattle and 50/115=43.5% of herds were positive. For culture 9/136=6.6% of samples or 9/4609=0.2% of cattle, and 9/50=18% of seropositve herds or 9/115=7.8% of total herds were positive. Breed was classified as: Brahman, Brahman-influence (Beefmaster, Brangus, Santa Gertrudis, Braford, Simbrah) and non-Brahman. The risk factors identified ($P < 0.05$) for seropositive herds were having: 1) Brahman, relative risk(RR)=16.0, or Brahman-influence, (RR)=4.0 vs. non-Brahman cattle, 2) a dairy-breed nurse cow for calves to suckle, RR=2.0, 3) the presence of running water sources, RR=0.47, 4) observed signs of Johne's Disease on the ranch, RR=2.68. The spatial analysis identified the region of greatest risk for seropositive herds as the central and southern parts of eastern Texas.

Discussion.

The increased likelihood for Brahman and Brahman-influence cattle to be seropositive may be due to increased susceptibility to infection or a difference in immune response to exposure. The difference in likelihood for seropositive herds by region may be due to an increased prevalence of infection or an increased prevalence of microorganisms which cross-react with the ELISA test.

Title Effects of paratuberculosis on lactation curves of Danish dairy cows.

Author(s) Kudahl A[1*] , Nielsen SS[2] , Tsørensen JT[1] .

Institution [1] Department of Animal Health and Welfare, Danish Institute of Agricultural Sciences, Foulum, Denmark. [2] Dept. of Animal Science and Animal Health; The Royal Veterinary and Agricultural University; Gronnegaardsvej 8; DK-1870 Frederiksberg C; Denmark.

Abstract Antibodies to *Mycobacterium avium* subsp. *paratuberculosis* in milk samples from 6955 cows in 108 Danish herds were tested with enzyme-linked immunosorbent assay (ELISA). Optical density (OD) recorded on a continuous scale were standardized according to parity and stage of lactation as previous studies on the same material show significant increase in ELISA response with increasing parity and in the first and last weeks of the lactation of infected cows. A hierarchical three-level test day model was set up with Energy Corrected Test Day Milk yield (ECTM) as response. Besides standardized OD-values (stOD) - seven fixed covariates were included in the model together with quadratic terms and first order interactions. Cow and cow nested in herd were included as random effects. The material was analysed in three separate parity groups: Parity one, parity two and higher parities. Additionally the total milk production 305 DIM was analysed with an equivalent two-level model with Herd-ID as random effect. The slope of lactation curves after peak yield was significantly steeper in first and second parity cows, where an increase of one stOD-unit resulted in a 3.8 kg depression of ECTM day 305 in first parity and 3.3 kg in second parity. The correlation was linear, so halving the increase in stOD resulted in half the loss. The peak yield was not significantly affected by stOD, though close to in second parity ($P<0.10$). In third and older parities there was a significant effect of the quadratic term of stOD of -3.2 kg indicating exponentially increasing losses with increasing OD-values. The total production loss 0-305 DIM due to an increase of one stOD-unit was estimated to be 496 kg Energy Corrected Milk (ECM) in primiparous cows, 1318 kg ECM in second parity and 625 kg ECM in later parities.

Title The effect of sub-clinical Johne's disease on milk production, fertility and milk quality in Israel.

Author(s) Chaffer M[1*] , Grinberg K[1] , Ezra E[2] , Elad D[1] .

Institution [1] Department of Bacteriology, Kimron Veterinary Institute, Bet Dagan 50250. [2] Israel Cattle Breeder's Association, Cessarea, Israel.

Abstract The economic impact of Johne's disease is difficult to quantify. Although, the adverse effects of the disease in the clinical stage are well documented, its specific impacts on fertility, mastitis and milk production during the sub-clinical stage are less well documented. The aim of this study was to address these questions by comparing anti- *M. paratuberculosis* antibodies, tested by commercial IDEXX ELISA test kit, to milk production, between partum interval and somatic cell counts obtained from the Dairy herd book of the Israeli Cattle Breeder's Association. Statistical analysis was conducted using SAS and a regression model that included independent variables such as, ELISA results, farm, lactation and days in milk. Blood was collected from all the milking cows (n=723) in three farms. Prevalence of *Mycobacterium paratuberculosis* test-positive animals in the three herds was 7%, while according to the age was 1.5% for the first lactation, 7% for the second, 14% for the third and 10% for the fourth lactation period and forth. Animals recorded negative in the ELISA test during one month of study produced significantly more milk than those tested positive (p=0.01). The comparison of the previous milk recordings among the positive cows, showed more production of milk during their first, second and third lactations and less throughout later periods in. None, of these differences, however, were statistically significant. Comparison of between partum interval and somatic cell counts among the two groups showed no significant differences. It is therefore concluded that the factors evaluated in this study did not pose a significant herd problem.

Title Paratuberculosis control programme in cattle in 1992-2001 by faecal culture and associated economic losses.

Author(s) Pavlík I[1*] , Parmova I[2] , Yayo Ayele W[1] , Machackova M[1] , Lamka J[1,3] , Svoboda J[4] , Pokorny J[5] , Bazant J[6] , Vitasek J[6] .

Institution [1] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic. [2] State Veterinary Diagnostic Institute, Prague, Czech Republic. [3] Charles University, Faculty of Pharmacy, Hradec Kralove, Czech Republic. [5] Veterinary and Pharmaceutical University, Faculty of Veterinary Medicine, Brno Czech Republic. [6] State Veterinary Administration, Prague, Czech Republic.

Abstract Faecal culture examination using the method 0.75% HPC paratuberculosis was controlled in 60 herds during 10 years (53 cattle herd, 1 sheep flock, 1 herd of Capricorn, 1 herd of antelope, 1 herd of moufflon, 1 herd of fallow deer and 2 herds of deer) comprising 9810 animals (9 140 heads of cattle, 670 other ruminants). Successful control was adopted in 8 (13.3 %) herds (7 herds of cattle and 1 herd of Capricorn). The main reason for the achievement of the successful outcome was repeated faecal culturing, removal of positive animals form 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 21 (35.0 %) herds (20 herds of cattle and 1 herd of deer). The cause of prolonged and unsuccessful control programme was failure to remove all progenies of infected animals and feeding of calves with mixed colostrum or unpasteurised milk. Introduction of infected animals form other farms was the main reason to fail in controlling the disease in one farm. A radical control programme was applied in 23 herds (18 cattle herd, 1 deer herd, 1 flock of goat, 1 herd of antelope, 1 fallow deer and 1 herd of moufflon). High prevalence of infection (with clinical cases in time of first diagnosis), rearing of calves with their mothers during the first 3 months, co-pasturing of young animals to the age of 18 months with adult animals were other factors contributed the failure of the control programme. For financial limitations the control programme was suspended in 8 (13.3 %) cattle herds. Economic losses were analysed in one cattle herd with 350 Holstein cows. Supported by the grants No. QLRT-2000-00879 (Brussels, EC) and QD1191 (MAgr., Czech Republic).

Title Deer as a model for natural and experimental Johne's disease.

Author(s) Griffin JFT[1*] , Chinn DN[1] , Rodgers CR[1] , Liggett S[1] , Spittle E[1] , Mackintosh CG[2] .

Institution [1] Disease Research Laboratory, Department of Microbiology, University of Otago,

Dunedin, New Zealand. [2] AgResearch, Invermay Research Centre, Mosgiel, New Zealand.

Abstract Infection caused by *M. paratuberculosis* has been diagnosed by microbiological isolates, histopathology and immunodiagnosis in wild and captive cervids in many countries. It has emerged as a significant clinical entity in a small proportion of New Zealand's 4000(+) farmed deer herds. The prevalence of infected herds has increased incrementally in the past decade. Unique aspects which characterise Johne's disease (Jd) in farmed deer, is the early onset of clinical disease with significant fatalities in red deer less than 1 year old. Deer can become infected with either 'bovine' or 'ovine' strains of *M. paratuberculosis*. Not only has cervine Jd become a production limiting disease, it is now a major cause of non-specific mycobacterial sensitisation in deer herds, causing false positive reactions to the tuberculin skin test. This has become a confounding factor in the implementation of deer whole herd testing as part of the National Tb eradication scheme. This paper will consider the changing patterns of sensitisation to Jd, seen from the New Zealand Tb testing database, over recent years. Diagnostic testing within herds with significant levels of Jd infection and clinical disease have been carried out. Patterns of immune reactivity (cellular and humoral) which provide optimal diagnostic precision in naturally infected deer will be outlined. Immune profiles seen in animals vaccinated with live or killed attenuated *M. paratuberculosis* (316F) vaccines will be discussed. We will attempt to define 'immunological signatures' that are compatible with protection and distinguishable from diagnostic patterns that typify natural infection or disease. Different vaccine formulations are currently being tested using measurements of protective efficacy against experimental infection. Deer may provide an unique model to study Jd infection, pathology, immunopathology and protective immunity, because of the acutely florid presentation of Jd in this group of ruminants.

Title The potential role of wildlife in the epidemiology of paratuberculosis in domestic animals.

Author(s) Greig A[1] , Beard P[2] , Daniels MJ[3] , Henderson D[1] , Hutchings MR[3] , Stevenson K[2] .

Institution [1] SAC Veterinary Science Division, Oakbank Road, Perth, PH1 1HF. [2] Moredun Research Institute, Edinburgh, UK. [3] Scottish Agricultural College, West Mains Road, Edinburgh EH 9 3JG.

Abstract

Introduction.

The isolation of *Mycobacterium avium* paratuberculosis from rabbits (*Oryctologus cuniculus*) on farms in Scotland exposed a major gap in our understanding of the epidemiology of paratuberculosis by indicating that non-ruminant wild life pose a threat to domestic livestock which has important implications for the control of paratuberculosis. Here we determine the potential for other wild life to harbour *M. a. paratuberculosis* and to play a role in the epidemiology of paratuberculosis in farmed livestock.

Methods.

Carcasses of mammals and birds were collected on four farms where clinical cases of paratuberculosis were regularly encountered in farmed livestock and rabbits were known to be infected. Carcasses were subjected to gross examination, before tissues (primarily intestine and associated lymph nodes) were collected for microbiology and histopathology. Isolates of *M. a. paratuberculosis* were confirmed by demonstrating IS9000 by PCR.

Results.

Mycobacterium avium paratuberculosis was isolated from foxes (23/27), stoats (17/37), weasels (2/4), rats (3/35), wood mouse (3/88), hare (1/6), crow (36/60), rook (3/53) and jackdaw (1/38) and in the cases of foxes (12/26), stoats (1/13), weasels (2/4) and crows (1/60) was accompanied by lesions consistent with paratuberculosis. Significant numbers of bank voles, house mouse, feral and wood pigeons and house sparrow were sampled with negative results on culture.

Discussion.

Animals and birds, which either predate on rabbits or scavenge rabbit carcasses infected with

M. a. paratuberculosis, are very likely to become infected with the organism. It is unlikely that the carnivores pose a threat to farmed livestock since their faeces are unlikely to be ingested by them. Bird faeces on the other hand could contaminate pasture and stored feedstuffs and be ingested by livestock on the home farm or adjacent premises. Based on the findings to date we consider rabbits to have the greatest potential for being involved in the epidemiology of paratuberculosis in farmed livestock. SAC receives funding from Scottish Executive Environment and Rural Affairs Department.

Title *Mycobacterium paratuberculosis* in wild ferrets-a potential wildlife reservoir of Johne's disease.

Author(s) de Lisle GW[1*] , Yates GF[1] , Cavaignac SM[1] , Collins DM[1] , Paterson BM[2] , Montgomery RH[2] .

Institution [1] AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand. [2] AgriQuality, Mosgiel, New Zealand.

Abstract Ferrets (*Mustela putorius furo*) were released in New Zealand in the 19th century for the control of rabbits. Currently they inhabit large portions of the North and South Islands, especially those areas with moderate to high numbers of rabbits. *Mycobacterium bovis* was first isolated from wild ferrets in 1982 and they have been extensively studied to determine their role in the maintenance and spread of bovine tuberculosis. Recently, ferrets from the North and South Islands were identified with lesions in mesenteric lymph nodes and livers that contained acid-fast staining bacteria. The histological picture of these cases was not typical of that seen in ferrets infected with *M. bovis* and mycobacteria were not isolated from them using non-mycobactin, supplemented media. However, IS900 was detected in these lesions by PCR. Subsequently, *M. paratuberculosis* was isolated from these animals using Bactec vials supplemented with mycobactin, egg yolk and antibiotics. Characterisation of the isolates by Southern blotting using a DNA probe from IS900 showed that these isolates were the "ovine" subtype of *M. paratuberculosis*. Sources of infection for ferrets of the "ovine" subtype in New Zealand include sheep, farmed deer and possibly rabbits. While rabbits are known to carry *M. paratuberculosis* in some countries, in New Zealand they have not yet been examined for this organism. A wildlife reservoir of infection has major implications for the control of paratuberculosis.

Title Role of the external environment, plants and non-vertebrates for the spread of *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Pavlík I[1*] , Yayo Ayele W[1] , Fischer O[1] , Matlova L[1] , Svastova P[1] , Bartos M[1] , Machackova M[1] , Alexa M[2] , Lamka J[1,3] .

Institution [1] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic. [2] Veterinary and Pharmaceutical University, Faculty of veterinary medicine, Brno Czech Republic. [3] Charles University, Faculty of Pharmacy, Hradec Kralove, Czech Republic.

Abstract From 20 cattle herds infected with paratuberculosis, 2 906 samples of the external environment were examined, and 57 (2.0%) tested positive for *M. avium* subsp. *paratuberculosis*. RFLP types of these strains were always the same to RFLP types isolated form animals residing in the same locality. In an infected farm a 2 years study was carried out to assess the persistent of *M. paratuberculosis* in slurry. The identified RFLP type was not changed in 10 months. *M. paratuberculosis* was isolated from non-vertebrates 1 (3.0%) from 33 samples of worms, 78 (22.2%) from 351 larvae of drone flies (Eristalis tenax) and 4 (2.0%) from 202 samples of dipterous flies of the family Scatophagidae (*Scatophaga sp.*) and *Calliphoridae* (*Calliphora vicina* and *Lucillia caesar*). RFLP types of isolated strains were identical with RFLP types from infected ruminants in the respected farms. For other possible spread of *M. paratuberculosis*, vegetables were cultivated (lettuce, radish, tomato) four weeks on soil artificially infected by suspension of *M. paratuberculosis* of RFLP type B-C1 usually isolated from ruminants and the environment. Identical strains of RFLP types were isolated from the root, stem, leaf, radish and tomato. Non-vertebrates (*Lumbricus terrestris, Blatta orientalis, Tenebrio molitor, Zophobas atratus*) were infected with *M. paratuberculosis* of RFLP type

B-C1 suspension isolated from cows' faeces. Shedding of the organism in faeces of non-vertebrates was detected until 72 hours after infection. Long term persistence of the infectious agent, however was not found. In view of possible significance of the diseases paratuberculosis control programme should respect a stringent hygiene and thorough sanitation practice including disinfection and disinfestation of stables and holding areas. Supported by the grants No. QLK2-CT-2000-00928 (Sacrohn, Brussels, EC) and QD1191 (MAgr., Czech Republic).

Title Potential wildlife to ruminant transmission routes for *M. a. paratuberculosis*.

Author(s) Hutchings MR* , Daniels MJ, Henderson D, Greig A.

Institution Scottish Agricultural College, West Mains Road, Edinburgh EH 9 3JG.

Abstract

Introduction.

The recent isolation of *M. a. paratuberculosis* from non-ruminant wildlife species opens up the possibility of wildlife to domestic ruminant transmission via the faecal oral route. Here we determine the level of contact between cattle and rabbit faeces in the grazing environment (Exp 1) and between cattle and rodent faeces via farm-stored concentrate feed (Exp 2).

Methods.

The rates of deposition of rodent faeces in farm stored feed and rabbit faeces on grazing pastures on four farms with a history of paratuberculosis in cattle and wildlife were estimated by random stratified quadrat sampling. Exp 1: Remote behaviour-monitoring systems in conjunction with stratified surveys of sward height were used to quantify the grazing behaviour of 57 cattle in relation to rabbit faeces-contaminated pasture. Prior to grazing each field, 4 pasture treatments were created by contaminating 40 plots (0.5x0.5m2) with 0, 10, 50 or 250 rabbit faecal-pellets. Exp 2: Ten cattle were presented individually with 3 repeats of 5 feed treatments: 3 levels of contamination of concentrate feed (none, 20 and 80 faecal-pellets/400g feed) x two rodent species (rat and mouse).

Results.

The mean number of faecal-pellets deposited by adult rabbits on pasture was 7357?2571 faeces/ha/day. Grazing cattle did not avoid rabbit faeces and 54/57 cattle were recorded grazing seven 250-faecal-pellet plots. The mean number of faecal-pellets deposited by rodents in stored feed was 79.9 (95% CI: 37.5-165.9) faeces/m2/month. Cattle ingested 42% and 82% of rat and mouse faecal-pellets during Exp 2, respectively.

Discussion.

Given that rabbit faeces contains up to $4x10^6$ cfu *M. a. paratuberculosis*/g and the experimental doses needed to produce disease in domestic ruminants range from 10^3 to 10^9 organisms, the ingestion of a few wildlife faecal-pellets may constitute an infective dose for cattle. Current evidence suggests that rabbits pose the greatest wildlife risk of paratuberculosis to cattle.

Title Evaluation of possible cross-infection of *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) between sheep, goats and cattle in Norway.

Author(s) Holstad G[1*] , Djønne B[1] , Sigurðardóttir Ó[1] , Pavlík I[2] , Ahrens P[3] , Tharaldsen J[1] , Schönheit J[1] , Storset A[4] , Nyberg O[1] .

Institution [1] National Veterinary Institute, Post Box 8156 Dep., N-0033 Oslo, Norway. [2] Veterinary Research Institute, Hudcova 70, Brno 621 32, Czech republic. [3] Danish Veterinary Laboratory, Bulowsvei 27 DK-1790, Copenhagen, Denmark. [4] Norwegian School of Veterinary Science, Post Box 8146 Dep., N-0033 Oslo, Norway.

Abstract

Introduction.

There has been uncertainty about the existence of a strictly goat-pathogenic *M. paratuberculosis* strain in Norway. The aim of the present study was to obtain information about cross-infection of *M. paratuberculosis* between ruminant species in Norway.

Materials and methods.

From 1966 to 1999 samples from 5152 cattle and 33100 goats were examined for *M. paratuberculosis* by bacterial culture. All goat and 97 cattle-samples were collected from animals suspected for paratuberculosis. Samples from 5055 cattle were examined in a national control and surveillance program. In one cattle herd (A), positive seroreactions were found in eight cows. Paratuberculosis had been diagnosed in 31 goats before 1985, but there had been no goats in the herd since 1992. Paratuberculosis was confirmed by culture from one of these cows, and the animals were slaughtered. Pathogical and bacteriological examinations were performed on 45 animals. Altogether 58 goat isolates of *M. paratuberculosis* from 58 herds and 5 cattle isolates from 4 herds were typed by RFLP and AFLP analyses. One goat and two cattle isolates originated from herd A.

Results and discussion.

M. paratuberculosis infections has been more common in goats than cattle in Norway. In a few farms infection was diagnosed both in goats and cattle. Cross-infection between the different ruminant species cannot be excluded. Paratuberculosis was diagnosed in two cows in herd A, and the bacterial isolates had the same RFLP and AFLP patterns (BC1/g) as the isolate detected from a goat in this herd. There was no information about import of infected cattle to this herd. The majority of the other Norwegian goat isolates examined, and the isolates detected from cattle imported from Sweden and Denmark had the same pattern. These findings might indicate that the same *M. paratuberculosis* strains infect goats and cattle in Norway, and cattle in the neighbouring countries.

Title Within-herd transmission of paratuberculosis and the possible role of infectious calves.

Author(s) van Roermund HJW* , de Jong MCM.

Institution Quantitative Veterinary Epidemiology, Institute for Animal Science and Health ID-Lelystad, P.O.Box 65, 8200 AB Lelystad, The Netherlands.

Abstract This study shows the results of an analysis of infection data of 21 Dutch dairy farms, where during 10 years each culled animal was tested for the presence of *Mycobacterium avium* subsp. *paratuberculosis*. Twenty of the farms were part of a vaccination study. By allocating animals of the herd according to the S-L-I concept (Susceptible, Latently infected and Infectious), the transmission parameter ß of the infection was estimated by generalised linear modelling. This parameter ß is the average number of new infections caused by one initial infection per unit of time. Through ß, the reproduction ratio R0 can be derived. The effect of management on the farm, time since start of vaccination, and several assumptions about the infectious period, were studied. The present analysis indeed showed that management had a significant effect on ß and R0, and the advised hygienic measures decreased the within-herd transmission on these 21 farms. Furthermore, the transmission decreased significantly with time since the start of vaccination, probably due to a combination of calf vaccination and improved management on the farms. When assuming that infected calves are infectious during a certain period immediately after infection, the model fits much better to the data. According to this model, the level of infectivity of (infected) calves is much higher than that of heifers or cows. This does not mean that calves shed more bacteria, because the effect can be explained by more contacts with the other calves on the farm. If the role of calves in transmitting the infection to other calves is indeed as important as this statistical study suggests, is now being studied in transmission experiments, in which calves are housed in-between faecal-culture positive cows during 3 months. After that period, these calves will be transferred and housed together with new calves during another 3 months.

Title Surveillance programmes for paratuberculosis-free dairy herds, based on quantification of between-herd transmission.

Author(s) van Roermund HJW* , Weber M, de Jong MCM.

Institution Quantitative Veterinary Epidemiology, Institute for Animal Science and Health ID-Lelystad, P.O.Box 65, 8200 AB Lelystad, The Netherlands.

Abstract A mathematical model was developed to calculate the between-herd transmission of paratuberculosis, expressed as the reproduction ratio Rh, which is the average number of new infected herds caused by one (initial) infected herd. In a surveillance programme herds are visited regularly and a certain number of animals is sampled and tested. The model developed here to design effective surveillance programmes consists of three parts: (a) within-herd dynamics of the infection after (accidentally) introducing the infection in a herd, (b) the detection probability of the infected herd, and (c) the final equation for Rh. The model is age structured. The most important input for part (a) is the within-herd reproduction ratio (R0) of the infection, assumptions about the infectious period, culling rate of cattle, herd size, and time between subsequent visits. Output is number of infected cattle at each visit. For part (b) input is number of infected animals in the herd at each visit, number (and age) of animals sampled, and the (infection-age dependent) sensitivity of the diagnostic test. For part (c) Rh is calculated by multiplying the detection probability at a certain visit by the cumulative number of infected cattle at that moment, accumulated for all visits of the herd in time, and then multiplied with the rate with which animals are sold from one herd to another. This yields the average number of infected animals sold from the infected herd (before detection) to other herds, i.e. Rh. The current surveillance protocol in the Netherlands for paratuberculosis-unsuspected herds (yearly pooled faecal culture of all cows>2 year) yields an Rh-value of 0.58, which is sufficiently below 1. Other effective alternatives will be shown. Testing all animals>1 year with the pooled faeces test every two years is an attractive, because cheaper, alternative for which Rh is sufficiently below 1 as well.

Title Absorption of IgG in calves fed colostrum replacer products derived from bovine serum.

Author(s) Quigley JD* , Kost CJ, Wolfe TA.

Institution APC Inc., Ames, IA, USA.

Abstract Maternal colostrum (MC) infected with *M. paratuberculosis* contributes to transmission of Johne's disease on dairy farms. Pasteurization is not a viable option, as it reduces colostral IgG and does not eliminate *M. paratuberculosis*. Therefore, alternatives to MC are required. Sources of exogenous IgG include milk, colostrum, blood and eggs, which are ingredients in colostrum supplements available in the market. However, current products provide insufficient IgG to replace MC. Blood derived IgG are inexpensive, readily available, and can be collected hygienically. Our objective was to develop a highly concentrated fraction from bovine blood for use in a colostrum replacer (CR). Bovine blood was collected from abattoirs under government supervision. Blood was passed fit for human consumption and processed to remove fibrin, lipids, and albumin. The resulting fraction (40 to 60% of DM as IgG) was spray-dried, mixed with ingredients including lactose, whey, whey protein concentrate, fats, vitamins and minerals to produce a CR. Five experiments were conducted, using a total of 238 calves fed MC or CR. Intake of IgG from MC or CR ranged from 100 to 253 g in one or two feedings at 1 and 8 to 12 hours of age. Mean plasma IgG at 24 hours of age ranged from 5.5 to 14.1 and 13.8 to 17.8 g/L in calves fed CR and MC, respectively. In experiments where calves consumed >120 g of IgG from CR, plasma IgG at 24 hours of age was >10 g/L in 75 to 88% of calves. Survival of calves to 56 days of age (determined in one study) did not differ from that of calves fed MC and was >95%. A CR containing Ig concentrate is a viable alternative to MC in Johne's control programs.

Title Various certification schemes for Johne's disease compared with a simulation model.

Author(s) Weber MF[1*] , Groenendaal H, van Roermund HJW[2] , Nielen M.

Institution [1] Animal Health Service, PO Box 9, 7400 AA Deventer, The Netherlands. [2]

Quantitative Veterinary Epidemiology, Institute for Animal Science and Health ID-Lelystad, P.O.Box 65, 8200 AB Lelystad, The Netherlands

Abstract Dutch dairy herds can obtain a '*Mycobacterium avium* subsp. *paratuberculosis* (Map) free' status after serial testing of all cattle >= 3 yr. with an ELISA and fecal culture, followed by four annual pooled fecal cultures of all cattle >= 2 yr. with negative results only. However, this certification scheme was felt to be too expensive, especially for closed herds. Therefore, alternative certification schemes were studied using a stochastic simulation model. The model, called JohneSSim, simulated the within-herd transmission and economic aspects of Map in closed Dutch dairy herds. The model was validated with field observations on Map unsuspected herds. The current Dutch certification scheme was compared with nine alternative test schemes in which the individual and pooled fecal culture, ELISA, Johnin intradermal test and gamma-interferon ELISA were employed, varying the test frequency, tested age group and number of tested animals. In the most attractive alternative certification scheme, the 'Map free' status was reached after four herd examinations, at two-year intervals, consisting of serial testing of all cattle >= 2 years of age with a pooled fecal culture and individual fecal culture of positive pools. This scheme resulted in lower total and annual discounted costs and a lower prevalence at reaching the 'Map free' status compared to the current scheme, assuming that there was no new introduction of the infection. However, with none of the certification schemes, all simulated herds were truly 'Map free' on reaching the 'Map free' status. The ensuing risk of transmission of Map between 'Map free' herds was studied separately. Results of the model were very sensitive to the assumed sensitivity of the fecal culture test and to management measures that prevent within-herd transmission of Map infections. If these preventive measures were taken, the probability of undetected Map infections in closed 'Map free' herds was substantially decreased.

Title Farm-level interpretation of a kinetics ELISA in New York State dairy herds.

Author(s) van Schaik G* , Jacobson RH, Schukken YH, Stehman SM, Shin SJ.

Institution Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853, USA.

Abstract Testing for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is typically done on a group of cows (e.g. in control programs), which changes the interpretation of the herd test results. In New York State, a kinetics ELISA (KELA) with multiple thresholds is used and the results of individual cows are reported to the farmers. The implication of the KELA results for the whole herd are not reported yet. In a previous study, the predictive value of the KELA for moderate or heavy shedding was obtained at cow level. The objective of the current study was to use probability theory to interpret KELA results at the herd-level. The likelihood of being a non-infected or an infected herd was determined based on the proportion of cows in the four KELA categories. Moreover, for infected herds the expected true prevalence was estimated. The herd-level interpretation of the test results was validated on real herd data. The probability that all cows test negative (KELA<65) decreases rapidly from 95% when a single cow is tested to 5% when 60 cows were sampled. When in a sample of 10 cows a single cow tests positive (65<KELA90%) to contain at least one truly infected cow. Herd-profiles for the probability of infection were developed based on the distribution of cows in the KELA categories. The probability for a herd to be infected is mostly dependent on the proportion of cows in the highest KELA category.

Title A longitudinal study to investigate variation in ELISA and fecal culture results for *M. paratuberculosis* in commercial dairy herds in New York State.

Author(s) van Schaik G* , Rossiter CR, Stehman SM, Shin SJ, Schukken YH.

Institution Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853, USA.

Abstract A longitudinal pilot study was designed to investigate variability of test results at commercial

dairy farms in New York State. ELISA and fecal culture results of individual cows seem to vary considerably over time, which complicates the determination of the infection status of a cow or a farm. However, it is of great importance, for example for eradication of the disease, to be able to correctly identify animals that might be shedding or are likely to develop clinical disease. The objective of the study was to determine the causes and amount of variation in ELISA and fecal culture results of individual cows. Sixteen cows in each of six herds were tested monthly with a kinetics ELISA (KELA) and fecal culture was done bimonthly during 2001. Cow- and herd-level data were collected at every sampling date from the management information system (DAIRYCOMP), which was used by all farms. The KELA results were modeled with the MIXED procedure in SAS with a random cow-nested-in-herd effect with a CS covariance structure. The hazard rate till a cow had a positive fecal culture result was investigated with a Bayesian model in WinBUGS 1.3. Random effects were included in the survival model to correct for repeated observations on cow-level and the potential risk factors were investigated. The KELA model showed that cows in second or higher lactation have increased KELA values compared with heifers. Cows that calved at most 15 days ago had the lowest KELA value that first rapidly increased and from 60 days in lactation decreased. Moderate and heavy shedders had significantly higher KELA values than fecal culture negative cows. In the second model for fecal culture, the hazard rate of becoming test positive was significantly affected by a random cow-effect and by season. KELA was not a significant factor in the model and had a limited predictive value for a fecal culture positive test result.

Title Predictive value of a kinetics ELISA to detect fecal shedding of *M. paratuberculosis* in New York State dairy herds.

Author(s) van Schaik G[*] , Jacobson RH, Schukken YH, Stehman SM, Shin SJ.

Institution Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853, USA.

Abstract In NYS a state-wise voluntary program is in place to reduce the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) prevalence on dairy farms. Farms are advised on the implementation of management that reduces the within and between farm spread of paratuberculosis. Moreover, a farmer can opt for (discounted) testing of the herd and taking specific measures such as culling of the test positive cows. The two standard tests that are used in the program, a kinetics ELISA (KELA) and fecal culture, are done at the Animal Health Diagnostic Laboratory at Cornell University. In the study we investigated the predictive value of KELA results for moderate or heavy fecal shedding, correcting for possible confounders such as different KELA batches and paratuberculosis prevalence on a farm. Optimal cutoff values for the KELA were distinguished based on the predictive value for fecal shedding. Sensitivity and specificity estimates were calculated for the different thresholds. The study provided additional information on the usefulness of KELA and fecal culture in MAP elimination programs. Moreover, the study resulted in herd-specific cutoffs based on the expected MAP prevalence in the herd. The correlation between KELA results and moderate or heavy fecal shedding was sufficiently large to be able to use KELA in screening programs. However, there were a fair number of moderate and heavy shedders with low KELA results. The KELA appeared not to be a good test for detection of low shedders. For the best possible predictive value of a KELA, the cutoff values should be determined based on the expected prevalence in a herd.

Title Optimisation of the sensitivity of ELISA and faecal culture for paratuberculosis: Selection of population or correction by population characteristics?

Author(s) Nielsen SS.

Institution Dept. of Animal Science and Animal Health, The Royal Veterinary and Agricultural University, Gronnegaardsvej 8, DK-1870 Frederiksberg C, Denmark.

Abstract Latency and chronicity of paratuberculosis makes diagnosis challenging even in adult cattle. Often, "optimisation" of sensitivity of diagnostic tests for chronic diseases is done by selection of a population containing many late-stage animals. Actually, random selection of animals with

subsequent correction through various population characteristics must be considered epidemiologically correct. The purpose of this study was to estimate sensitivity and specificity of faecal culture (FC) and ELISA for specific animal groups through stratification by cow characteristics and alternative disease definitions. First, the sensitivities and specificities of an ELISA and faecal culture were estimated using a no-gold standard method and the overall accuracy of the ELISA was described. Second, the probability of being ELISA-positive was determined for cows grouped by parity and stage of lactation. Information was then combined to increase the interpretability of the accuracy of both faecal culture and ELISA. The probability of ELISA-positivity varied significantly by parity and stage of lactation. In general, the probability of being ELISA positive was 2-4 times higher in second and higher lactation than in first lactation. For cows with low levels of antibodies, the sensitivity of FC was 0.20-0.30 in first parity cows. In higher parity cows the sensitivity of FC was 0.45-0.55. For cows with high levels of antibodies, the sensitivity of FC increased to >0.80. The likelihood of providing a correct diagnosis was on average 0.89 using an uncorrected ELISA-value. Correction by parity increased the likelihood to 0.92 for first parity cows and decreased the likelihood to 0.86 for second and higher parity cows. Thus, the probability of being ELISA-positive is higher in higher parities, but the ELISA response is apparently purer in the first parity. It is concluded that inclusion of cow and population characteristics can improve the interpretation of diagnostic tests.

Title Interpretation of the serum ELISA for detection of *Mycobacterium paratuberculosis* fecal shedding in dairy cattle herds.

Author(s) Wells SJ[1*] , Godden S[1] , Whitlock RH[2] , Collins J[1] .

Institution [1] University of Minnesota,College of Veterinary Medicine, University of Pennsylvania. [2] University of Pennsylvania, School of Veterinary Medicine.

Abstract Serologic and fecal culture assays for paratuberculosis are being used on an increasing basis to identify infected cattle herds and control Johne's disease. Despite this, the utility of these assays in cattle herds for these purposes is not well understood. The objective of this study was to compare results from a serum ELISA and fecal culture in infected and uninfected dairy cattle herds, in order to define optimal use of these tests in herd control programs. Fecal and serum samples from all adult cows in 49 infected herds and 7 uninfected herds were tested using fecal culture and a commercially available ELISA in Minnesota and Pennsylvania. Results from this study indicated that the specificity of the ELISA in uninfected dairy herds varied by herds selected, 97% in one group of 4 herds and 72% in another 3 herds. Within infected dairy cattle herds, 40% of culture-positive cows were ELISA-positive, an indication of the relative sensitivity of ELISA compared to detectable fecal shedding, and similar to the ELISA sensitivity estimates reported in the scientific literature. ELISA relative sensitivity, however, varied by fecal shedding prevalence from 57% in herds with <5% shedding prevalence to 33% in herds with greater than 15% shedding prevalence. This indicates different interpretation should be applied to test results from dairy herds with low compared to high fecal shedding prevalence. In infected dairy herds with <5% shedding prevalence, fecal shedding was detected in 33% of cows with ELISA S/P above 1.0, compared to 88% of cows with ELISA S/P above 1.0 in herds with greater than 15% shedding prevalence.

Title Infection rates in reactors to an absorbed ELISA used in a bovine Johne's disease test and cull program.

Author(s) Holmes IRL[1] , Jubb TF[2*] , Callinanb APL.

Institution [1] Department of Natural Resources and Environment, PO Box 441 Echuca, Victoria Australia 3564. [2] Department of Natural Resources and Environment, Box 2500 Bendigo Delivery Centre, Victoria Australia 3554.

Abstract The proportion of reactors to a commercial enzyme linked immunosorbent assay (ELISA) for bovine Johne's disease that were confirmed infected with Johne's disease by histology and tissue culture was determined. ELISA positive dairy cattle from the Echuca district of northern

Victoria, were slaughtered at an abattoir where a standard range of specimens (mesenteric and ileocaecal lymph nodes, sections of jejunum, ileum, ileocaecal valve and colon) were collected for histology and tissue culture. Only if samples were histologically negative, were further samples submitted for tissue culture. The diagnosis was positive if histologically there were acid fast organisms with the morphology of *M. paratuberculosis* in typical granulomatous lesions of Johne's disease or *M. paratuberculosis* was detected on BACTEC culture of the tissues. Confirmation rates increased from 70.4% in 1996 to 89.4% in 2001. This is mainly because more reactors were confirmed positive by tissue culture each year as laboratory techniques improved, the proportion increasing from 0% in 1997 to 27.5% in 2000 but reducing to 16.7% in 2001. There were no significant differences between the age groups in the proportion confirmed infected. Confirmation rates were high and supported a high specificity of >99% for the ELISA when used under field conditions. The high confirmation rates support the program policy of only following up ELISA reactors at slaughter when individual herds are thought to be approaching eradication.

Title Risk of introduction of *Mycobacterium paratuberculosis* into dairy herds: effects of prevalence and test sensitivity.

Author(s) Gardner IA[1*] , Carpenter TE[1] , Collins MT[2] .

Institution [1] Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, U.S.A. [2] Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, U.S.A.

Abstract A stochastic model was developed to assess the risk of introduction of *Mycobacterium paratuberculosis* infection into a dairy herd through purchase of replacement females. Effects of infection prevalence in the source herd(s) and use of ELISA alone or ELISA and fecal culture as risk mitigation strategies also were evaluated. A hypothetical dairy herd, free from *M. paratuberculosis*, which replaced 30 (1 lot) of its cows per year, was considered. Probability distributions were estimated for the sensitivities and specificities of ELISA and fecal culture, the proportion of infected herds and within-herd prevalence for a randomly-selected replacement source herds (high prevalence) and herds in level-3 of the Voluntary Johnes Disease Herd Status Program (low prevalence). For the initial model assumptions that the ELISA sensitivity was 45%, simulation results predicted that 1% to 10% of the *M. paratuberculosis*-infected lots would not be detected by the ELISA. The negative predictive value (NPV) (the probability that given the total lot-test result is negative, all the animals in the lot are truly not infected) ranged from 77% for ELISA results to 83% for fecal culture, if the lot was comprised of animals purchased from high prevalence (randomly-selected) herds. On the other hand, the NPVs were both 99%, for lots of cattle purchased from low-prevalence (level-3) herds. The benefit of testing introduced cattle with ELISA alone or in combination with fecal culture was minimal if cows were purchased from known, low-prevalence (level-3) herds. The value of testing by ELISA alone or in combination with fecal culture increased greatly in high-prevalence herds. Testing of random-source replacement females can partially mitigate the risk of introduction of *M. paratuberculosis*, but not as well as by using test-negative herds as the source, with or without testing.

Title Rapid eradication of paratuberculosis.

Author(s) Kalis CHJ[*] , Collins MT, Hesselink JW, Barkema HW.

Institution Animal Health Service. The Netherlands.

Abstract Appropriate management measures to prevent new infections in infected herds are emphasized in the Dutch paratuberculosis control program with the aim is to raise a new uninfected cow generation to replace the infected live stock. Alternative methods are pursued to answer to the demand of farmers wishing to eradicate paratuberculosis faster. One of the postulated alternatives is a rapid eradication program aiming to identify all infected cattle during a one year test period, using three different tests simultaneously. The outlines of this schedule were: serological test by ELISA in all animals > 24 months, fecal culture of all animals > 6 months, and a gamma interferon assay of all young stock > 6 months. At the same time It was advised to remove all test-positive animals promptly and to carry out alle required preventive management measures. This approach has been tested in 14 dairy herds with a low Mptb-prevalence (<10% culture-positives). The herds were tested twice during the first year and were followed another three years with yearly tests to estimate the results of the program. After 4 years only 3 of the 13 herds were repeatedly test-negative. In 4 herds culture-positive animals were detected in each herd test. In 4 herds culture-negative herd tests were alternated with culture-positive herd tests. From 3 herds results were incomplete because 2 farmers quitted the program due to their refusal to cull the test-positive animals and 1 farmer ended business. It can be concluded that, with the strategy as applied in this trial, not all infected animals are diagnosed during the one year test period. So, freedom of paratuberculosis can not be guaranteed in these herds afterwards. Until now removal of fecal shedders in combination with management measures remains the most cost-effective way to eradicate paratuberculosis from dairy herds in a shortest possible period of time.

Title Testing to control Johne's disease in dairy herds in Victoria.

Author(s) Jubb TF[*] , Galvin JW.

Institution Department of Natural Resources and Environment, Animal Health Operations Branch. Box 2500 Bendigo, Victoria Australia 3554.

Abstract In Victoria, in 1996, a voluntary JD test and control program was established by the cattle industry and government. Participating farmers are provided with advice on disease control and an annual test of their adult herd using an absorbed ELISA. The test is used annually on all animals two years and older and positive reactors are culled. Private veterinarians under contract with the government deliver the program. There are over 500 dairy herds enrolled in the program. The components of the program and the progress in 36 herds that had completed 4 or more annual herd tests were reported at the previous colloquium. There are now nearly 400 herds that have completed 5 or more annual herd tests. The evidence for progress in the program is the decline in ELISA reactor prevalence from 1.7% to 1.0% over 7 years and the decline in clinical case rates from about 0.4% to less than 0.1% However, an analysis of the sensitivity of the commercially available ELISA using reactor and clinical case rates in the program indicates a lower diagnostic sensitivity than previous estimates. In any test-round, the number of ELISA positive animals detected was between 20 and 30% of the total number of animals from that round that became ELISA positive or developed clinical disease at later test rounds. Taking into account the number of reactors that would have occurred in animals that were culled or died, the sensitivity is estimated to be between 10 and 15%. The test is read at a cut-point that yields a very high diagnostic specificity.

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

Author(s) Windsor P[1*] , Whittington R[1] , Eppleston J[2] , Jones S[3] , Britton A[3] .

Institution [1] NSW Agriculture, PMB 8, Camden, NSW, 2570 (present address: University of Sydney, PMB 3, Camden, NSW, 2567). [2] Central Tablelands Rural Lands Protection Board, PO Box 20, Bathurst, NSW, 2795. [3] CSL Animal Health, 45 Poplar Rd., Parkville, Vic, 3052, Australia.

Abstract Ovine Johne's disease (OJD) is a significant cause of mortality of adult sheep in some parts of Australia and vaccination for sheep with OJD with Gudair[TM], a killed *Mycobacterium*

paratuberculosis preparation, is being investigated as a disease control tool. This paper presents preliminary data on the impact of vaccination on faecal shedding of *M. paratuberculosis* (as assessed by pooled and individual faecal culture), mortality rate, lamb growth, condition score and wool productivity, vaccine injection site lesions and cellular (BOVIGAM™) and humoral (PARACHEK™) immunity. On each of three properties in New South Wales experiencing significant OJD losses (5 to 15% per annum), 200 Merino lambs (age 1-4 months) were vaccinated with Gudair™, and 200 lambs were sham vaccinated with saline (1 property in December 1999 and 2 in June 2000). Animal assessments and sample collections are being conducted twice yearly. Data to date indicates that Gudair™ significantly delays faecal shedding for the first year post-vaccination (p.v.) and when shedding commences, it is at a significantly lower rate than unvaccinated animals. There have been no OJD attributable deaths in vaccinated sheep compared to 19 confirmed OJD mortalities in control sheep. No significant differences have been noted in live weight, condition score and wool productivity. Vaccine injection site lesions were detected in almost 50% of sheep at 2 months p.v., reducing to 10-30% by 2 years p.v. The vaccine stimulates both CMI and ELISA responses in a high proportion of vaccinated lambs which tends to decline over time, accompanied by a significant increase in the proportion of unvaccinated animals with positive immune reactions, presumably reflecting an increasing prevalence of OJD in this group. This data has been used to seek registration of Gudair™ in Australia and it is expected that the vaccine will have an important role in OJD control in Australia.

Title Paratuberculosis in sheep in Iceland - Is eradication by vaccination possible?

Author(s) Fridriksdottir V* , Hjartardottir S, Poulsen S, Sigurdarson S, Gunnarsson E.

Institution Institute for Experimental Pathology, University of Iceland, Keldur v/Vesturlandsveg, IS-112 Reykjavik, Iceland.

Abstract Paratuberculosis was brought to Iceland in 1933 by the import of infected sheep from Germany. The infection in sheep spread rapidly throughout the main sheep breeding areas and attempts to eradicate the disease were unsuccessful. These included quarantine areas, widespread immunological testing, extensive culling of sheep in endemic areas and restocking with healthy sheep. Vaccination experiments on the other hand gave good results and showed that mortality could be reduced by 94%. The Icelandic strain of *M. avium* subsp. *partuberculosis* is a sheep strain and infects both sheep and cattle. Since 1966 vaccination of sheep has been compulsory in endemic areas and losses from paratuberculosis have been reduced considerably. The vaccine used is a heat killed vaccine produced in Iceland. The country is divided into 36 quarantine areas with boundaries made up of hundreds of kilometres of fences and natural geographic hindering. The quarantine areas are categorized as: 1) areas where paratuberculosis has never been diagnosed, 2) areas with compulsory vaccination, 3) areas where vaccination has been stopped. In 2001 paratuberculosis suddenly appeared on a farm in an area which had been declared free from paratuberculosis. This was a major drawback, as vaccination had been stopped in the whole quarantine area 3 years earlier. Sheep from all farms in the area were then tested serologically, using both ELISA test and Complement Fixation Test. Out of a total of 400 sheep on the affected farm, 50 were seropositive and/or had clinical symptoms. One seropositive sheep was found on another farm. All other farms in the area were seronegative. All positive animals on the two farms were culled and the rest vaccinated. Vaccination is now compulsory again in the whole quarantine area. The reason for the outbreak is still unclear and this raises the question whether eradication of paratuberculosis by vaccination is possible or not.

Title Improving Australian assurance programs and risk management for Johne's disease.

Author(s) Kennedy DJ* , Allworth MB, Mitchell R.

Institution Animal Health Australia, Suite 15, 26-28 Napier Close, Deakin, ACT, 2600, Australia.

Abstract The original National Johne's Disease Market Assurance Program (MAP) for cattle was in the vanguard of herd certification programs for paratuberculosis when launched in 1996. There are now 1,600 herds in the program, and MAPs have also been introduced for sheep, goats and

alpaca. Compliance with biosecurity requirements for introductions of cattle and movements of animals away from the farm, together with negative testing of the herd or flock, provide a pathway to three levels of herd or flock status, the highest level (Monitored Negative 3) being attainable in a minimum of 4 years. Improvements have been made to each MAP on one or more occasions. More flexibility has been introduced to testing schedules and management options based on risk assessment and management. These include options for maintaining a certain herd or flock status, introducing young cattle for rearing and small numbers of breeding animals. More flexibility has also been introduced to protect the status of animals from assessed herds and flocks at sales and exhibitions. The program manuals for the MAPs for cattle and sheep were upgraded in 2000 to full quality manuals that are consistent with other Australian on-farm quality assurance programs. These loose-leaf manuals contain explanations of the program components, codes of practice to comply with and a range of forms to help record that compliance. Annual updates are sent to owners and veterinarians. As well as assisting herd owners manage and record the program, the manuals facilitate external auditing. A lower assurance level (Beef Cattle - Trade Assurance Scheme) has also been developed for the commercial beef industry, based on biased testing of 50 adults. In the dairy industry, a calf rearing accreditation program has recently been launched and dairy processing companies are introducing calf rearing standards into their farm quality assurance programs.

Title Directions for the future control of Johne's disease caused by cattle types of *M. paratuberculosis* in Australia.

Author(s) Kennedy DJ* , Hood R, Allworth MB.

Institution Animal Health Australia, Suite 15, 26-28 Napier Close, Deakin, ACT, 2600, Australia.

Abstract The restricted distribution of Johne's disease in Australia resulted in States implementing regulatory controls for many years to try to stop the spread of infection to free areas and herds. This has contributed to the virtual absence of paratuberculosis in northern and western Australia. National zoning for Johne's disease, introduced in 1999, formally recognised the differential distribution of infection and should strengthen regional control. At the same time zoning permits more appropriate controls in infected areas and cattle enterprises than are required in protected and free zones. A new national approach to the control of cattle (C) types of *M. paratuberculosis* is being developed by Animal Health Australia in conjunction with industry and government stakeholders. Within the infected areas of south-eastern Australia the dairy industry is focussing more on reducing contamination of farm and product and improving management of replacement heifers. It appears that the beef industry in Australia has very little disease but some infected breeder herds have distributed the infection with sales of bulls and female breeders. Different risk-based strategies are being developed in both industries to allow trading of live cattle while still aiming to reduce the spread of paratuberculosis to non-infected herds and regions. The occurrence of C types of *M. paratuberculosis* in alpaca and goats in Australia has diminished but outbreaks in deer herds in the past 2 years are presenting new challenges.

Title Paratuberculosis control in The Netherlands.

Author(s) Franken P.

Institution Animal Health Service.

Abstract The current state of the paratuberculosis program will be presented. The approach in The Netherlands is based on results of epidemiologic modelling and risk assesment in which present knowledge is used. The framework and the current research will be presented.

Title Control programme for paratuberculosis in Swedish beef herds.

Author(s) Sternberg S* , Holmström A, Viske D, Robertsson JÅ, Bölske G, Larsson B.

Institution National Veterinary Institute.

Abstract

Background.

A voluntary control programme was initiated in 1998. The programme is financed by the Swedish Board of Agriculture and run by the Swedish Animal Health Service. The aims of the programme are to detect any paratuberculosis infection in breeding stock, and to prevent any spread of the infection within the cattle population. A stamping out policy is adopted in all infected herds.

Programme design.

In herds within the programme, yearly faecal samples are taken from all cattle older than 24 months. During the first year, all samples are cultured individually. In the second year of testing, samples are pooled three by three, except for samples from imported animals or animals purchased into the herd. From the third year, samples are pooled five by five, except for imported or purchased animals. Herds in the programme may only buy animals from other controlled herds in the same category. Three categories are included: C (having tested negative 1-2 times), B (having tested negative 3-4 times) and A (having tested negative 5 times).

Programme status

By the end of 2001, 774 herds were included in the programme. Most of these were pedigree beef herds, but some production herds and some dairy herds selling calves to production herds were also included. A total of 189 herds were in category C, and 485 herds had reached category B. So far, paratuberculosis has been found in one herd. All the animals in the infected herd were culled, all buildings were cleaned and disinfected, and restrictions were put on the farm.

Problems encountered in the programme.

Rules regarding contacts between farms in the programme and other farms are strict. Animals in the programme may not have contact with animals from animals in a lower category, or animals outside the programme. This causes practical problems with shows, auctions, common pastures etc.

Title Progress of the Australian National OJD Control and Evaluation Program.

Author(s) Allworth MB* , Hood R, Kennedy DJ.

Institution Animal Health Australia, Suite 15, 26-28 Napier Close, Deakin, ACT, 2600, Australia.

Abstract The National Ovine Johne's Disease Control and Evaluation Program (NOJDP) is a AUD40.1 million, six- year program aimed at delivering necessary information on the national distribution of the disease and the technological tools and information needed for an informed decision on the future management of OJD. At the same time, the Program set out to control the disease during this evaluation phase. The Program is jointly funded by Commonwealth and State governments and national and state industry bodies through levies on producers. The NOJDP is now in its fourth year and has made significant progress, including extensive surveillance that has enabled better definition of disease distribution, the implementation of 21 research projects (worth AUD5.6 million with a further AUD2.4 million planned) and the implementation of a communications program. A Mid-Term Review was undertaken to assess whether the Program was meeting its objectives and whether those funding the Program were still supportive of its aims and its operation. A revised three-year Plan has been adopted for the second half of the Program. In addition to the ongoing commitment to research and surveillance, the revised Plan incorporates a specific Control subprogram and a new national Financial Assistance subprogram to support the NOJDP. Despite its success, the Program continues to face a number of challenges. The identification of infected flocks through increased surveillance, together with trading restrictions placed on infected and suspect flocks and flocks in high risk areas has brought opposition from those affected. This is mainly from some sectors of the pedigree breeding industry and in States where, to date, there has been little or no financial assistance. The issue of financial assistance is currently being addressed through both national industry and State initiatives. Delivery of an effective assistance package is essential to ensure the successful completion of the Program.

Title A national surveillance program for ovine Johne's disease in Australia. Methods, improvements and results.

Author(s) Allworth MB[*] , Kennedy DJ, Sergeant ESG.

Institution Animal Health Australia, Suite 15, 26-28 Napier Close, Deakin, ACT, 2600, Australia.

Abstract The National Ovine Johne's Disease Control and Evaluation Program (NOJDP) is a AUD40.1 million, six-year program aimed at delivering necessary information on the national distribution of the sheep type of Johne's disease and the technological tools and information needed to make an informed decision on the future management of OJD. The Program is jointly funded by Commonwealth and State governments and national and state industry organisations through levies on producers. The NOJDP is now in its fourth year and has made significant progress, particularly as a result of the extensive surveillance program that has enabled better definition of disease distribution. During the first 3.5 years of the program, 5,400 investigations were carried out on-farm, involving the testing of 550,000 sheep by serology and 400,000 sheep by pooled faecal culture (PFC) and conducting 6,070 post mortem examinations. This resulted in the detection of 709 previously unknown infected flocks. In addition, four percent of 41,000 groups or "lines" (comprising 11.8 million sheep) have been found infected by post-mortem inspection at abattoirs. Initially surveillance was based largely on tracing from known infected flocks. On-farm investigations involved the serological assessment of 400-500 sheep using the AGID. The development of PFC and of abattoir surveillance have greatly improved the program. The PFC has a higher sensitivity than serology and is cheaper, resulting in a 3-5 fold reduction in costs per investigation. The development of abattoir monitoring, based on manual and visual screening together with histological examination of suspect intestines has allowed surveillance to be applied more uniformly across regions. Industry is currently introducing a sheep identification system which will further enhance the surveillance program, and reduce tracing costs.

Title Progress with Ovine Johne's Disease Control in New South Wales.

Author(s) Links IJ[1*] , Roth I[2] , Evers M[3] , Denholm L[2] .

Institution [1] NSW Agriculture, Pine Gully Rd, Wagga Wagga NSW 2650 Australia. [2] NSW Agriculture, Orange. 3 NSW Agriculture, Young, NSW, Australia.

Abstract Surveillance, based on abattoir monitoring, testing of traced flocks using the Pooled Faecal Culture (PFC) and investigation of clinical cases has confirmed that OJD remains restricted in NSW. A three-zone policy involving high, moderate and low prevalence regions is due to be implemented from 1st October 2002. Abattoir surveillance has proven cost-effective with 85% of adult sheep slaughtered in NSW currently monitored. The proposed introduction of national sheep identification will enable it to be used for assurance. Control of disease spread has centred on quarantine of infected or suspect flocks and regional zoning, however, transmission of infection between adjoining properties by mechanisms other than by intended movement of sheep has also been identified as a major cause of local spread, possibly involving faecal contamination of run-off water. Research on the epidemiology of OJD, including survival of the organism in the environment and age related susceptibility, has enabled development of Property Disease Management Programs (PDMPs). Research in NSW on the Gudair(r) Spanish vaccine has demonstrated significantly delayed bacterial shedding, with Australian registration of the vaccine anticipated in April 2002. Financial assistance (up to A$25,000) is available to implement PDMPs in infected flocks. PFC and abattoir monitoring are detecting infection at a low prevalence, thus reducing the risk of significant spread having occurred prior to detection. Infected Flock Profiling (IFP), which involves whole flock testing using the PFC, was developed to determine the prevalence and distribution of infection within sheep studs. Trace-forward rams from profiled flocks can often be defined as low-risk, thus avoiding need for quarantine of the destination flock. Traced rams can be tested by Serial Faecal Culture (SFC), using pooled faeces collected on three occasions at 10-14 day intervals to detect intermittent excretion. This is an alternative to autopsy and markedly reduces the loss of valuable genetics. Risk-based ram trading options have been developed for infected studs where IFP confirms continued low risk of disease spread.

Title The Victorian Ovine Johnes Disease Eradication Program- effects on sheep owners and industry.

Author(s) Tobin FM.

Institution Victorian OJD Action Group "Greenhills" 1842 Caramut Rd Winslow Victoria Australia 3281 3281.

Abstract In December 1996 the Department of Natural Resources and Enviroment with the support from the Victorian Farmers Federation (The states major farmer representative body) commenced an eradication program of Ovine Johnes Disease in the state of Victoria, Australia. The program was based on compulsory slaughter and destocking for a minimum of 2 summers of all sheep, goats, deer, and alpaca on properties where OJD was identified.The Victorian OJD Action Group a group of sheep owners directly affected by the program presented a paper to the sixth International Colloquium held in Melbourne Australia in 1998 highlighting the affect of an eradication program on sheep owners. This paper will update our 1998 paper, with specific reference to the circumstances which ultimately forced the Victorian Government and the states major farmer representative body to terminate eradication in favour of a voluntary control and management program. Our paper will also reflect on the status of an ever changing National OJD program, its prospects of success and its relationship to the OJD program in Victoria. It seems some natural justice has prevailed in Victoria where-in common sense has achieved a partial gain at the expense of closed door bureaucracy.

Title Diagnosis, epidemiology, and Program of Control of Paratuberculosis in bovine herds of Argentina.

Author(s) Paolicchi F[1*] , Morsella C[1] , Verna A[1] , Spath E[1] , Martinis D[3] , Zumarraga M[2] , Giofree A[2] , Cataldi A[2] , Romano M[2] .

Institution [1] Grupo de Sanidad Animal, Departamento de Producción Animal, EEA INTA-Facultad Ciencias Agrarias, Universidad Nacional Mar del Plata, CC 276, Balcarce (7620), Argentina. [2].-Instituto de Biotecnologia, CNIA INTA Castelar, Argentina. [3] Facultad Ciencias Veterinarias, UNNE, Corrientes, Argentina.

Abstract Since 1985 activities of diagnosis and investigation in Paratuberculosis in ruminants through serological, bacteriological, and immunopathological methods are carried out in our Bacteriology Laboratory from INTA - FCA UNMdP, Province of Buenos Aires, Argentina. Between 1992 and 2002 the serological technique used has been the absorbed indirect ELISA test with a sensibility of 66% and a specificity of 98%, adjusted by ROC - MedCalc Program. A total of 68,335 sera (bovine: 61,525, cervine: 6,670, and ovine: 140) has been processed, while a total of 9,123 samples from reports of 4-year-old or older cattle of the Provinces of: Buenos Aires (BA:n=3,160), La Pampa (LP:n=716), Corrientes (C:n=761), La Rioja (LR:n=101), Neuquén (N:n=74), and Río Negro (RN:n=385) has been studied. Apparent seroprevalences were adjusted to obtain the real seroprevalence, which was in BA: 26.5% (meat) and 56% (milk), LP: 2.4%, C: 1%, LR: 0,2%, N: 0%, RN: 7%. Since 1985 feces (F), organs (OR), or milk (M) samples have been processed. A total of 136 strains of *Mycobacterium avium* subsp. *paratuberculosis* was isolated from F (n=100: 66 meat cattle (mc), 28 dairy cattle (dc), 6 deer (de)), OR (n=34: 12 mc, 3 dc, 19 de), and M (n=2 dc). Of all the strains isolated and typified by P.C.R. to confirm IS900 insertion sequence, 61 strains have been typified by R.F.L.P. in 4 different patterns designated "A" (75%), "B" (10%), "C" (6,1%), and "E" (13%), and were compared with isolations from Europe. The more prevalent pattern in Argentina has been identical to the least frequent in Europe, R9(C17), while the other patterns were not found in Europe. Deer only have pattern "A". These results demonstrate the important prevalence of Paratuberculosis in Argentina and the relevance of identifying diseased herds to organize its control and eradication.

Title History of incidence of *Mycobacterium avium* subsp. *paratuberculosis* in domestic and wild ruminants in the Czech Republic.

Author(s) Pavlík I[1*] , Bazant J[2] , Vitasek J[2] , Machackova M[1] , Yayo Ayele W[1] , Pokorny J[3] , Parmova I[4] , Lamka J[1,5] .

Institution [1] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic. [2] State Veterinary Administration, Prague, Czech Republic. [3] Veterinary and Pharmaceutical University, Faculty of veterinary medicine, Brno Czech Republic. [4] State Veterinary Diagnostic Institute, Prague, Czech Republic. [5] Charles University, Faculty of Pharmacy, Hradec Kralove, Czech Republic.

Abstract Paratuberculosis was first diagnosed in 1962 in one cow imported from Denmark. Until 1989 the disease was diagnosed in 40 herds (20 cattle herd, 16 sheep and goat flock and in 4 herds of farmed deer and ruminants in zoological gardens). In 10 (25%) herds, animals were imported from the former Soviet Union, Great Britain, France, Hungary and Canada. Based on history and RFLP analysis, the distribution of paratuberculosis strains in different parts of the country was determined (RFLP type D-C12 in the West, B-C9 in the North and A-C10 central part of the country). Reason for the spread of the disease was mainly cow-calf weaning and common pasture for young and adult animals. Between 1990 and 1996, 30 000 heads of cattle and several hundreds of wild ruminants were imported. Until 1998 paratuberculosis was diagnosed in 12.2% of 428 imported groups of animals. During the period between 1990-2201, *M. paratuberculosis* was detected in 100 herds (71 herds imported), in 10 sheep and goat flocks (7 herds imported), and in 20 herds of domestic and wild ruminants. From these results it is evident that the spread of paratuberculosis started not only in domestic animals but also in wild ruminants. Thus control of paratuberculosis has taken place since 1998 with the provision of the District Veterinary Administration and the financial support funded by the ministry of Agriculture. Until 2001, the number of cattle population decreased from 1.2 mil nearly by half. This control programme relied on a twice per year faecal culture and removal of all positive animals which shed *M. paratuberculosis* in their faeces. Progenies of positive cows were also culled and the management practice applied with separate rearing of calves from their mother. Supported by the grants No. QLRT - 2000 - 00879 Brussels, EC and QD1191 Min. of Agriculture, Czech Republic.

Title Johne's disease serological prevalence in Uruguayan dairy cows.

Author(s) Piaggio J[*] , Nuñez A, Gil A.

Institution Universidad de la República - Facultad de Veterinaria. A. Lasplaces 1620 CP 11600. Montevideo - Uruguay.

Abstract A serologic study was conducted to determine the seroprevalence of *Mycobacterium paratuberculosis* in dairy herds and cows, in the most important dairy region of Uruguay. At 1945, Casamagnani isolated the agent and made the first report of clinical disease in Uruguay. At 1983, Errico made the last report of this disease, in the country. The objective of this study was to estimate the serologic prevalence and the spread of the disease among herds. The dairy population in the study region is 2000 herds with 180000 milking cows. A two step random sample was drawn from the population. In the first step 36 dairy herds were selected and in the second step 20 milking cows were selected in each farm by systematic sampling. The total number of studied cows was 720. Data were evaluated in the analysis weighted by the number of milking cows sampled using the routine of Intercooled Stata 7.0. Sera were tested with an ELISA kit from IDEXX Lab Inc. Apparent prevalence was 16.02% (2.58. A positive herd was defined as any herd with two or more ELISA positive cows. The proportion of positive herds was 72% (26/36). In conclusion, the disease is present in Uruguay with a high spread among herds. Therefore, it is recommend to start developing control programs.

Title Prevalence of paratuberculosis (Johne's disease) in dairy farms in northeastern Italy.

Author(s) Robbi C[1*] , Rossi I[1] , Nardelli S[2] , Marangon S[3] , Vincenzi G[4] , Vicenzoni G[1] .

Institution [1] Laboratorio di Verona - Area territoriale 1. [2] Laboratorio di Immunologia -Via Romea, 14/A, Legnaro (PD). [3] Centro Regionale di Epidemiologia Veterinaria - Via

Romea, 14/A, Legnaro (PD). [4] Dipartimento di Prevenzione - Regione Veneto - Venezia.

Abstract

From November 2000 to December 2001 a serological monitoring programme for Paratuberculosis was carried out in 419 dairy farms in the Veneto region. Blood samples were tested using a commercially available ELISA (Herdchek, IDEXX), with sensitivity and specificity values of respectively 45% and 99%. Out of 27135 tested animals 3,50% (CI 95% = 3,28-3,72) reacted positively. On average reactors were about two years older than the sampled population. If all farms with at least one positive animal were considered as positive, a high (65%) apparent farm prevalence would be obtained. However, the probability of false positive reactions, increasing with farm size, should be taken into account. These false positive results could determine a misclassification of some non-infected herds, as confirmed by following epidemiological and laboratory data:

- About 40% of positive farms showed only one positive animal ('singleton reactor').
- Frequency distribution of positive animals in herds with more than 100 heads showed a bimodal pattern.
- ELISA reaction on 'singleton reactors' gave low positive S/P values in about 80% of the animals, whereas higher values were obtained in positive animals of herds with more than 5 positive heads.
- Retesting of positive animals using another ELISA reaction (Serum Verification, Pourquier), confirmed only 6% of the'singleton reactors', whereas positive reactions were confirmed more frequently in farms with more than 5 positive heads.

In conclusion, the apparent farm prevalence should be reduced in order to get near to the true value. It appears reasonable firstly to define for each farm, according to the size of the herd, the cut-off value (number of positive animals) to classify the farm as positive, so that herd-level specificity does not drop below 95%. If the latter key was applied, the farm prevalence would be reduced from 65% to 27%. Secondly, it should be evaluated the reliability of a confirmatory test (particularly for 'singleton reactors') to be applied in series on positive samples.

Title Screening of Swedish dairy farms for paratuberculosis.

Author(s) Sternberg S, Bölske G, Robertsson JÅ, Olsson SO, Viske D.

Institution National Veterinary Institute.

Abstract

Introduction.

The overall prevalence of paratuberculosis in Sweden is very low. So far, all infected animals have been found within the beef production sector. Swedish dairy farms are generally believed to be free of paratuberculosis. To establish whether this view was well-founded, a survey was conducted on Swedish dairy farms.

Methods and material.

Faecal samples were taken from a total of 4000 animals in 200 herds and cultured for *M. paratuberculosis*. The herds were chosen so as to be representative of the geographical distribution of dairy herds in the country. In each herd, 20 of the older cows were randomly chosen for sampling. If any animal in the herd was in poor body condition, it would be included in the sampling. All samples were analysed by standard procedures at the National Veterinary Institute in Uppsala.

Results.

None of the 4000 faecal samples were culture positive. The number of tested animals should suffice to ensure a prevalence of paratuberculosis in Swedish dairy cattle below 0.1% (95% confidence).

Discussion.

This study supports the view that Swedish dairy cattle are free of paratuberculosis. However, the above calculations of prevalence does not take into account the low sensitivity of faecal culture.

This method was chosen because serology has proven to be even less sensitive when used in Swedish cattle, and because the Swedish legislation on paratuberculosis is too strict for farmers to want to risk any false positive results. Thus, culture remains the best screening method for Sweden. Further tests on fallen stock and culled cattle are planned to continue the surveillance of dairy cattle for paratuberculosis.

Title A survey of the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in bulk-tank milk samples all over Switzerland.

Author(s) Corti S, Stephan S[*] .

Institution Institute for food safety and hygiene of the University of Zurich. Winterthurerstr. 270. CH-8057 Zurich.

Abstract

Introduction.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of Johne's disease in ruminants. Some of subclinically and clinically infected dairy cattle shed this organism in their milk. Since it is presumed that the thermal resistance of *Mycobacterium avium* subsp. *paratuberculosis* may be increased and there is a possible implication in human Crohn's disease, MAP has become a special food-hygienic relevance. The aim of our study was to collect data about the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in bulk-tank milk samples of different regions all over Switzerland. Furthermore, we examined eventual correlation between the presence of MAP and the somatic cell counts, the total colony counts and the presence of Enterobacteriaceae.

Material and methods.

1384 bulk-tank milk samples were collected from 18 different regions all over Switzerland. An IS900 nested PCR method specific for MAP was used. The somatic cell counts were determined of 1212 bulk-tank milk samples by a fluorescence optic method, the total colony counts and the Enterobacteriaceae counts of 450 bulk-tank milk samples by the standard methods on plate count agar and violet red bile glucose agar.

Results.

273 (19.7%) of the 1384 milk samples were IS900 PCR-positive. In the 18 regions very different prevalence were found. The results of somatic cell counts ranged from 11000 cells ml-1 to 981000 cells ml-1, with mean value of 130670 cells ml-1. In 42 (3.5%) milk samples, the somatic cell counts were higher than the Swiss legal limit of 350000 cells ml-1. Total colony counts and Enterobacteriaceae counts generally show good milking-hygiene.

Discussion.

The prevalence of 19.7% IS900 PCR-positive bulk-tank milk samples shows a wide distribution of subclinical MAP infections in dairy stocks in Switzerland. A comparison of the somatic cell counts and the total colony counts of PCR-positive and PCR-negative milk samples shows no statistically significant differences ($p>0.05$). Enterobacteriaceae occur as often in IS900 PCR-positive as in PCR-negative milk samples.

Title Detection of Anti- *Mycobacterium paratuberculosis* antibodies in Brazilian herds.

Author(s) Ferreira R[1] , Fonseca LS[1] , Lilenbaum W[2*] .

Institution [1] Department of Medical Microbiology, Universidade Federal do Rio de Janeiro, Brazil. [2] Department of Microbiology, Universidade Federal Fluminense.

Abstract Paratuberculosis, or Johne's disease, is a chronic enteritis produced by *Mycobacterium avium* subsp. *paratuberculosis*, that affects ruminants. In the early stages, the disease is asymptomatic and the animal shed bacteria on feces, spreading the disease in the herds. Paratuberculosis has been detected frequently in United States, Australia, New Zealand, Japan and many countries in Europe. In Brazil, the *M. paratuberculosis* has been isolated, but there

are not statistic studies about Johne's disease. In this study, were evaluated 1004 bovines from 45 properties situated in Rio de Janeiro State. Serum samples were tested for the presence of antibodies against *M. paratuberculosis* using a commercial ELISA test (PARACHECK). Eighteen per cent of the animals were reactive, and 82% of the herds had at least one reactive animal. The percentage of reactive animals range from 4,7% to 46,5%/herd. These results suggests the presence of the agent in Brazilian herds and a need for a broad study on Paratuberculosis, to help a program of prevention and control of the disease in Brazil.

Title Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from an infected dairy herd in Southern Region - Brazil.

Author(s) Gomes MJP[1*] , Driemeier D[1] , Lanzon LF[2] , Asanome W[3] , Wunder Jr EA[3] , Ribeiro VR[4] .

Institution [1] School of Veterinary Medicine - University of Rio Grande do Sul Sate (UFRGS) Av. Bento Gonçalves, 9090, Porto Alegre, CEP 91540-000, RS - Brazil. [2] VMD 1059 El Paseo - Turlock. California 95380, USA. [3] Vet. Students of School of Vet Med UFRGS - Brazil. [4] School of Veterinary Medicine Rural University of Rio de Janeiro State (UFRRJ) - Brazil.

Abstract

Introduction.

There are a few reports about the Johne's disease in Brazil.

Objective.

The purpose of this study was to isolate and to identify the *Mycobacterium avium* subsp. *paratuberculosis* from clinical cases of Johne's disease and quantify the prevalence in this dairy herd of the Rio Grande do Sul State.

Material and methods.

Eight (3.33%) Holstein cows with 4-5 years old among 240 animals showed profuse and incoercible diarrhea, progressive weight loss, rapid decrease in their milk production and maintenance of appetite. All animals were sacrified and tissue samples were collected during the necropsy. Samples of terminal ileum, ileocecal valve and intestinal lymph nodes were inoculated in Herrold's Egg Yolk Medium (HEYM) to the *M. avium* subsp. *paratuberculosis* isolation. The identification of agent was based on its phenotypic properties of slow growth, acid-fast stain and mycobactin dependency. IDGA and ELISA tests were conducted essential as described by Lab instructions.

Results.

M. avium subsp. *paratuberculosis* was isolated in HEYM up to 16 weeks from terminal ileum, ileocecal valve and intestinal lymph nodes of animals with the clinical form of the disease. OIE Reference Laboratory in Argentine confirmed the strain like *M. a. paratuberculosis*. The main histopathological findings from 8 cases were granulomatous enteritis, lymphadenitis and lymphangitis. The inflammatory infiltrate was composed of macrophages, epithelioid cells and Langhan's giant cells containing large numbers of acid-fast bacilli. The attempts to isolate the agent from 221 fecal samples stored during 2 years were unsuccessful. The AGIDT used as a screening test detected 26 positive cows (11.4%) among 228 tested animals at the slaughterhouse. The absorbed ELISA test detected 125 (39.4%) positive animals to 314 serum samples and 47 (14.9%) suspect ones. Nonabsorbed ELISA test detected more 32 (10.1%) positive animals than the absorbed ELISA test.

Conclusion.

It is registered the occurrence of clinical and subclinical forms of Johne's disease in dairy cattle of Rio Grande do Sul state which calls for the need of control measures against bovine paratuberculosis in Brazil.

Title Dealing with incursions in a Johne's disease free zone: Western Australia.

Author(s) Cousins D[1*] , Morcombe P[1] , Moir D[2] , Butler R[3] , Young G[1] , Carson B[1] , Evans R[1] , Kalkhoven

M[1] .

Institution [1] Department of Agriculture 3 Baron-Hay Court South Perth WA 6151. [2] Department of Agriculture Narrogin 6312. [3] Department of Agriculture Merredin WA 6415.

Abstract Western Australia is considered a Free Zone for Johne's disease in respect of cattle, camelids, sheep and goats. A series of surveys using serological and culture tests demonstrated this status to the satisfaction of national Animal Health bodies within Australia from October 1999, and maintaining JD Freedom status is an advantage to the state in terms of trade. WA dealt with eight cases of BJD in cattle and one in an alpaca between 1952 and 1994. In 2000 the first case of OJD was found in a Cashmere goat and subsequently in 2001 a case was found in a Corriedale ram. With one exception, infection has been limited to a single animal and similarly, infection has been found in an imported animal with only one exception. The follow up work involved for in-contact animals can range from simple to complex operations. In the recent case where an infected goat was detected, follow up involved 42 properties, 19,503 animals and 7755 tests (1070 AGID, 5999 ELISA, 72 necropsies and 686 cultures, including pools) were performed. In all cases, infection has been detected by WA's policy of testing traceforward animals (and their cohorts) that have originated from infected flocks, information that may only become available after the animal has arrived in WA. In order to minimise the risk of spread, WA's policy is to destock all animals that may have been exposed and decontaminate land over a minimum of 15 months including two summers. Conditions of entry of livestock into WA have become increasingly stringent since 1994, thereby decreasing the risk of entry of infected animals. Modeling simulations have determined the probability of importing the disease, and these estimates have been used to determine changes under which livestock can move into WA. Currently the risk of importing an infected bovine into WA is estimated at one incursion every 250 years and for sheep the current risk from NSW is estimated at about once every 77-333 years.

Title Epidemiological study of paratuberculosis in ruminants in Alentejo Portugal.

Author(s) Ferreira A[1] , Mariano I[2] , Caetano MC[1] , Núncio P[1] , Carrilho E[1] , Sousa C[1] , Lopes S[2] , Almeida V[3] , Penha Gonçalves A[4] .

Institution [1] DRAAL - Évora. [2] LVM - COPRAPEC - Montemor-o-Novo. [3] FMV - Lisboa. [4] LNIV - Lisboa.

Abstract

Objectives.

1. To estimate the prevalence of paratuberculosis at herd/flock level in the Alentejo region.
2. Identification of vulnerability profiles according to management practices.

Material and methods.

- Cross-sectional observational study.
- Target population: 7.233 cattle herds and 13.040 sheep flocks.
- Epidemiological unit: Herd and sheep flock.
- Sample size: 365 herds and 374 sheep flocks.
- A stratified random sample of 3.960 blood samples were analyzed by ELISA. Proportional allocation was used to decide upon the number of sampling units in each stratum.
- A questionnaire was filled by veterinarians of the local Animal Health Defence Association (OPP) recording data about the production system, epidemiological scenario and disease profiles.

Results.

- 95% CI prevalence: [13-25%] for cattle herds (N = 167), [6-18%] for sheep flocks (N = 126).
- 95% CI prevalence: [4.8-7%] for bovine (N = 1840), [7.8-10.2%] for sheep (N =

2120).
- The infection with M. paratuberculosis was confirmed in 56% counties of the region (N=46). The infection could not be detected on 16% counties.
- Three vulnerability herd/flock profiles were identified according to the presence/persistence of M. paratuberculosis and management practices. These were used to propose guidelines for the future control of the disease as well as to design voluntary herd/flock certification programmes.

Title Prevalence of paratuberculosis and tuberculosis in caprine herds in Madrid.

Author(s) de Juan L* , Aranaz A, Montero N, Díez de Tejada P, Romero B, Vela AI, Mateos A, Domínguez L.

Institution Dpto. Patología Animal I, Facultad de Veterinaria, U.C.M. Spain.

Abstract The real prevalence of paratuberculosis and tuberculosis in caprine farms in Spain is almost unknown because there has not been exhaustive epidemiological studies focus on this animal species. The main objective of this study was to know the prevalence of these mycobacterial diseases in goats from Madrid (centre of Spain). This study included the slaughter of 102 animals from 28 farms located in 16 different areas of Madrid. Detailed post-mortem analysis was carried out and samples were obtained for serological and microbiological analysis. *Mycobacterium avium* subsp. *paratuberculosis* was isolated from 16 goats (39.2% of the studied farms). There was no agreement between post-mortem lesions, Ziehl-Neelsen stain, and microbiological analysis, i.e. only one of the culture-positive samples was also ZN positive and came from an animal with macroscopic lesions, *M. a. paratuberculosis* was isolated from 5 animals negative to both stained smear and lesions, furthermore, isolation was not possible in 28 animals with a positive ZN stain and/or lesions. These poor results prompted us to apply a complementary diagnostic test to determine the real status of the animal population. This test is based on a direct DNA extraction method from tissue samples and PCR amplification with primers aimed at the IS900. Negative controls underwent all the protocol to test the specificity of the method. By this new technique, we detected 13 out of the 16 culture-positive animals, plus 18 culture-negative animals. *Mycobacterium bovis* was isolated in 26% and 32% of the animals and farms, respectively. Four out of 28 farms had a mixed infection with both diseases, this fact should be taken into account in future paratuberculosis and tuberculosis eradication programs in goats. This study was supported by funds from the Spanish Ministerio de Agricultura, and by the Comunidad de Madrid. Attendance to this Congress was sponsored by the EU-funded project FAIR6-CT98-4373.

Title An investigation of wildlife roe deer as possible source of introduction of paratuberculosis into a certified-free dairy herd.

Author(s) van Weering H, Kalis CHJ* , Overduin P, Hesselink JW.

Institution Animal Health Service. The Netherlands.

Abstract In the Dutch paratuberculosis program certified herds are monitored yearly by fecal culture. Despite closed herd management, positive test results occurred up to 4 years after the enrolment in the program. In positive fecal cultures of certified herds, strains of *Mycobacterium paratuberculosis* (Mptb) are further investigated by determining the IS900 RFLP patterns. In 1 certified dairy herd on Terschelling, a West Frisian island, a pattern, different from Mptb strains normally found in cattle, was determined. This pattern R27, is considered to be restricted to wildlife. Terschelling was not inhabited by roe deer until last decade. Just in 1992 roe deer was imported on the island: 4 roes and 1 roe buck. Actually the estimated size of the roe population is about 200. These animals lived in the forest adjacent to the farm. After the exclusion of other possible ways of introduction of Mptb into the dairy herd, the presence of Mptb in the roe population was considered as a possible source of infection. To further investigate this hypothesis, the intestines of the roes, shot by members of the Roe Control Association, were cultured with the modified Jørgensen method. One of 18 intestinal convolutes was Mptb

culture-positive. However, the pattern of this strain was IS900 RFLP strain R9, as frequently isolated from infected cattle. Further inquiries into the origin of the roe deer population were performed. The imported roe buck, obtained from a wild park, was coming from a roe calf rearing center, where orphan calves of roes killed by car accidents, were raised by feeding them raw cow's milk. This finding leads to the hypothesis that the roe buck might be infected by the uptake of raw milk from an infected cow. However, the origin of the "deer" strain in the infected cow remains unexplained.

Title Survey on paratuberculosis in roe deer (*Capreolus capreolus*) and small ruminants in North-Western Italy

Author(s) Robino P[*] , Nebbia P, Meneguz PG, De Meneghi D.

Institution Department of Animal Production, Epidemiology and Ecology, University of Turin, Italy.

Abstract The presence of Paratuberculosis in roe-deer and in small ruminants -grazed on the same pastures- was investigated in NW Apennines (Alessandria province). Serum samples (N=94) and mesenteric lymph nodes (N=47) were collected from 94 roe deer culled in game reserves (GRs) within the study area. No paratuberculosis clinical and post-mortem signs were observed. Sera from 109 small ruminants (56 goats, 53 sheep), bred on 7 farms located within the above GRs, were also collected. Antibodies against *Mycobacterium paratuberculosis* (M.p) were detected (ELISA test) in 13 roe-deer (13.8%), 7 goats and 1 sheep (7.3%) sera. Sero-positive small ruminants were from 3 flocks: in particular, 4 of 6 positive goats (from a 19 head flock, with high seroprevalence) showed specific clinical symptoms. Difference in sero-prevalence amongst small ruminants flocks -characterised by presence or absence of clinical signs, but with sero-reactors- was statistically significant (P= 0.004, Fisher's exact text), while the difference in sero-prevalence between domestic and wild ungulates was not significant. As regards lymph nodes analysis, acid-fast organisms (Ziehl-Neelsen staining: Z.N.) were detected in 23 samples (49%), and M. p. DNA (Nested PCR, IS900) was found in 17 samples (36.2%). Agreement between results of Z.N. and Nested PCR was evaluated by Kappa coefficient (K=0,40). Moreover, ongoing bacteriological tests allowed to isolate M.p. in 5 out of the 15 roe deer samples so far tested, and in one sick sero-positive goat slaughtered on purpose. Although clinical and gross pathological signs of paratuberculosis in roe deer have not yet been observed, the results of this research suggest that M.p. is widely present within the studied roe deer population. Small ruminant sero-reactors seem to be clustered at flock/farm level, while sero-positive roe deer are present throughout the whole area. Epidemiological patterns of paratuberculosis infection in sympatric roe deer and small ruminant populations are still being investigated.

Title Mortalities due to Mycobacterial Infections in Wild Red Deer (*Cervus elaphus*) in Belgium.

Author(s) Godfroid J[1*] , Pirard M[2] , Fonteyn PA[3] , Bughin J[1] , Walravens K[1] , Portaels F[2] , Gala JL[2] .

Institution [1] Veterinary and Agrochemical Research Centre, Brussels. [2] Laboratory of Applied Molecular Technology, Université catholique de Louvain, Brussels. [3] Mycobacteriology laboratory, Institute of Tropical Medicine, Antwerp, BELGIUM.

Abstract Paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* has recently been described in farmed red deer in Belgium. The infected animals were found in apparently healthy deer. Discrete gross pathology were limited to the gut associated lymphnodes. Histopathological changes compatible with mycobacterial infections, were seen in the lymphnodes as well as in the ileal tissues. Ziehl-Neelsen staining gave consistently negative results in Mpt culture positive samples. All isolates were IS900 positive. Mortalities occured since then in the wild deer population, both in adults and young animals. The animals were severely emaciated, showing bloody diorrhea. Erosion and ulcers of the gastric and enteric mucosa were seen. All the animal were BVD negative. Ziehl-Neelsen staining on faeces and on mucosal smears gave consistently positive results, i.e. high numbers of acid-fast bacilli. Cultures on HEYM media

with and without Mycobactin yielded mycobacterial growth either in the absence and/or in the presence of Mycobactin. A duplex PCR for Differential Identification of *Mycobacterium bovis*, *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* was applied on those bacterial cultures and gave always negative results for *M. bovis*. In line with the culture results, different types of duplex PCR results were obtained for *M. avium* subspecies: *M. a. paratuberculosis* positive and/or *M. a. avium* negative. Finally these results were validated by PCR results based on the IS900 and IS901 (IS900+, IS901-, IS900-, IS901+, IS900+, IS901+). All together these results suggest that in wild deer, probably due to winterfeeding or to possible exposition to contaminated water or feedstuff, mortalities due to *M. a. avium*, *M. a. paratuberculosis* or dual infections may occur. This work was partially funded by grants from the Belgian Ministry of Agriculture (DG6).

Title Control of Paratuberculosis in an farm of red deer in captivity in Argentina.

Author(s) Soler P[1] , Verna A[1] , Morsella C[1] , Casaro A[1] , Paolicchi F[1,2*] .

Institution [1] Laboratorio de Bacteriología, Departamento de Producción Animal, EEA- INTA. [2] Facultad de Ciencias Agrarias, Universidad Nacional Mar del Plata, CC 276, Balcarce (7620), Argentina.

Abstract Red deer in captivity clinically shows Paratuberculosis at an early age, with a high percentage of mortality. The identification of positive animals facilitates the removal of young deer from the herd, which reduces the infection rate within the herd. We applied a control model to improve the production in an establishment dedicated to breed red deer in captivity in Argentina, characterizing the disease by the absorbed indirect ELISA test and the culture of feces, together with the application of management measures during the 1997-2002 period. An annual bleeding was made on a population of 950 animals, from 15 up to 84 months of age. A total of 5717 sera were analyzed during 6 years. The prevalence of seropositive animals found in the first year of work was of 18.9% but it decreased significantly the following year until reaching 7.8%, generating a great impact at the beginning of the control of the disease. In successive years the prevalence stabilized between 7.5% and 8.5%. This apparent stabilization of the prevalence was determined by the lack of removal of all seropositive animals, many of these of 15 months of age. Since 2000 control measures were adjusted and the removal of 100% of positive and diseased animals reduced prevalence up to 4.0%. The high quantity of diseased young animals positive to ELISA indicates a high contagion and infection rate in red deer under captivity conditions. High prevalences hinder the control of Paratuberculosis in populations of deer, therefore it is necessary to implement rigorous measures of removal of positive animals during the control of this disease, which contributes to diminish the population of infected animals and the rate of contagion with Paratuberculosis.

Title A survey on Paratuberculosis in red deer (*Cervus elaphus*) in hunting areas of Extremadura (Western Spain).

Author(s) Hermoso de Mendoza J[1*] , Parra A[1] , Tato A[2] , Alonso JM[1] , Peña J[1] , Teixidó J[2] , Rey JM[1] , García A[1] , Larrasa J[1] , Marcos G[2] , Cerrato R[1] , Fernández-Llario P[1] , Casas F[1] , Hermoso de Mendoza M[1] .

Institution [1] Cátedra de Patología Infecciosa, Depto. de Medicina y Sanidad Animal, Facultad de veterinaria, Universidad de Extremadura, 10071-Cáceres (Spain). [2] Consejería de Salud y Consumo, Junta de Extremadura.

Abstract

Introduction.

Although wide studies on the different domestic ruminants species have not been carried out, it is known and assumed since years that paratuberculosis is an important and prevalent disease in Extremadura Region. Nevertheless, no evidence of wildlife infections has been found up till now. Thus, the importance of wild reservoirs has not been evaluated in those areas where wildlife is abundant. For this reason we have just started a survey looking for *M. avium* subsp. *paratuberculosis* in the most likely potential wild reservoir: the red deer.

Material and methods.

Sampling from a statistically significant number of hunted red deer, we obtained ileal valves and neighbour mesenteric lymph nodes, that were microbiologically processed for detection of the agent. Also red deer serum samples from the main hunting areas were obtained, incubated with *Mycobacterium phlei* to bind unspecific antibodies, and tested using commercial Protein G-based indirect ELISA to detect Map specific antibodies.

Results and discussion.

The results suggest the convenience to test more animals, to evaluate the actual prevalence on domestic ruminants and to look specifically for wild animals in bad condition, as the present sampling has been done on hunted animals supposed to be healthy, a random system that seems good for prevalence studies but not to look for Map faecal shedders nor diseases that would be in a very low clinical prevalence.

Title Evidence of paratuberculosis in wild red deer (Cervus elaphus) in Belgium.

Author(s) Linden A[1*] , Canivet P[1] , Mousset B[1] , de Rijk P[2] , Rigouts L[2] , Portaels F[2] .

Institution [1] Dptmt of Infectious and Parasitic diseases, Faculty of Veterinary Medicine, University of Liege, Belgium. [2] Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium.

Abstract A survey of paratuberculosis was conducted in 237 hunter-killed free-living cervids (*Cervus elaphus*, n = 191; *Capreolus capreolus*, n = 46) from 8 different geographic areas in southern Belgium. A complete necropsy was conducted on each animal, which allowed sampling of serum (198) and mesenteric lymph nodes (237). Serum samples were tested by use of a commercial ELISA (HerdChek, IDEXX) and, if suggestive of lymphadenitis (hypertrophy and/or purulent discharge after cross-section), smears of lymph nodes were examined by Ziehl-Neelsen. Finally, an IS900-based PCR was performed on all cases that were found positive by one or both diagnostic approaches. Macroscopic examination of roe deer viscera never revealed any change compatible with paratuberculosis and the 31 sera tested remained negative. Similarly, no macroscopic lesions were observed in young (< 2 yrs) red deer (n=102), but 1 tested positive by ELISA and was confirmed positive by PCR. Among the remaining 89 adult (> 2 yrs) red deer, 5 (5.6 %) displayed significant modification of the volume and/or content of mesenteric lymph nodes, but no signs of chronic diarrhoea was noticed. They all tested positive by PCR. The serological survey revealed 13 positive cases out of 167 red deer sera tested (7.8 %) among which 11 had no macroscopic lesions. Among these latter, only 1 remained negative by PCR. This first and ongoing survey of paratuberculosis in Belgium thus establishes the presence of *Mycobacterium avium paratuberculosis* among wild red deer in the country, whereas roe deer don't seem affected. The preliminary data gathered here indicate that 5.6 % adult red deer display macroscopically visible pathologic mesenteric lymph nodes due to paratuberculosis and that 7.8 % are seropositive. Additional data will be collected in the future to strengthen the epidemiologic picture.

Title Preliminary results of a survey for Johne's diseases in the Antwerp Zoo.

Author(s) Vansnick E[1*] , Vercammen F[2] , Bauwens L[2] , Haese ED[3] , Nelis H[3] , Geysen D[1] .

Institution [1] Institute of Tropical Medicine, Antwerp, Belgium. [2] Royal Zoological Society of Antwerp, Belgium. [3] Laboratory for Pharmaceutical Microbiology, Ghent University, Belgium.

Abstract In this study, the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in the animal collection of the Royal Zoological Society of Antwerp was investigated. The specific situation of zoo facilities (limited space for different animals) is a cause of concern. Up to one third of zoos accredited by the American Zoo and Aquarium assocation have reported the occurrence of at least one infection since 1995. Faecal and post mortem samples of a total of 38 ruminants were tested using bacteriological and molecular methods. Samples were cultured on

Löwenstein-Jensen (+ mycobactine J) solid medium and in BACTEC 12B radiometric medium after decontamination with the double incubation method described by Whitlock and Rosenberger. DNA templates of all samples were also subjected to PCR using primers for IS 900. A modified Boom-extraction was used for the post mortem samples whereas faecal samples were extracted using a newly developed protocol based on sequence-capture. No evidence of paratuberculosis was found in the animal collection using both detection tests. One sample in culture became positive for *Mycobacterium avium*. Accurate diagnosis of MAP is very difficult by current tests. Negative isolation of MAP from animals may be due to the limited distributions of focal lesions, sporadic excretion of the organism or the detection limit of the faecal culture methods. Faeces are a lot easier to sample in a zoo than blood or biopsy material. For these practical reasons, a faecal DNA extraction was developed and used in this study. Faeces contain a lot of PCR-inhibitors and difficulties are experienced in recovering DNA from such complex matrix. Negative PCR results may be due to inhibition or the low detection limit of the PCR.

Conclusion.

We couldn't detect a positive animal in the RZSA with the specific diagnostic tests used.

Title Paratuberculosis in wild ruminants in the Czech Republic in the years 1997-2001.

Author(s) Machackova M[1*] , Lamka J[1,2] , Parmova I[3] , Yayo Ayele W[1] , Rozsypalova Z[1] , Svastova P[1] , Bartos M[1] , Straka M[4] , Ludvik V[4] , Pavlík I[1] .

Institution [1] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic. [2] Charles University, Faculty of Pharmacy, Hradec Kralove, Czech Republic. [3] State Veterinary Diagnostic Institute, Prague, Czech Republic. [4] District Veterinary Administration, Rokycany, Czech Republic.

Abstract Due to the occurrence of infection of *Mycobacterium avium* subsp. *paratuberculosis* among cattle and sheep, survey of paratuberculosis in wild ruminants was carried out. The survey was based on cultivation of faeces of 1373 animals and tissue samples of the small intestine and corresponding lymph nodes of 1774 animals. Wild ruminants (n=3 147) comprised 968 red deer, 1145 roe deer, 564 fallow deer, 470 moufflons in 71 of 77 districts in the Czech Republic, 58 game parks (736 animals) and 23 deer and moufflon farms (832 animals). *M. paratuberculosis* has been isolated from 150 animals so far. Paratuberculosis was detected in 12 animals from the wild in 6 districts, 21 from 9 game parks and 117 from 5 deer and moufflon farms. The prevalence of paratuberculosis in the wild nature was very low 1579 (0.7%) animals, in game parks 2.8% of 736 animals, but in deer farms higher 14% of 832. Three RFLP types B-C1, M-C16, and D-C12 were revealed in this study (predominated B-C1 type). In infected claw-hoofed game from the wild nature RFLP type B-C1 was detected, which source is considered as infected cattle sharing the same pasture. On the contrary the source of infection of RFLP type M-C16 in 2 roe deer and D-C12 in one fallow deer in the wild were not been discovered. Also cross-infection by RFLP types M-C16 and B-C1 was found out in 3 deer in one farm. At the same red deer farm *M. paratuberculosis* of RFLP type B-C1, was isolated from amnion fluid, which supports intrauterine transmission and from the milk, which support the postnatal infection. Any relationship between the RFLP types and the wild ruminant species was not found. Supported by the grants No. QLRT - 2000 - 00879 Brussels, EC and QD1191 Min. of Agriculture, Czech Republic.

Title Influence of ultraviolet-B (UV-B) on viability of *Mycobacterium avium* subsp. *paratuberculosis*.

Author(s) Katayama N[*] , Kamata GS, Yokomizo GY.

Institution Shizuoka Toubu Livestock Hygiene Service Center, Nippon Veterinary and Animal Science University, National Institute of Animal Health.

Abstract The transmission of *Mycobacterium avium* subsp. *paratuberculosis*(MAP) occurs mainly through grazing in pastures contaminated with faeces of paratuberculous animals. The

ultraviolet rays included in sunlight have been considered an effective disinfectant against pathogens in pastures, but few data have been published on its relative effectiveness against MAP. In the present study we investigated the influence of UV-B irradiation on the viability of MAP cells suspended in distilled water (DW) and diluted slurry under moist or dried conditions. BBM2201 and ATCC19851 strains were used in this experiment. One hundred micro litter of inoculum adjusted to 10^7cells/ml with distilled water, or diluted slurry was dropped into a 15mm circle on a slide glass. Irradiation energy was set to 0, 265, 530, 1, 060, 2119, 4239, 8477, 16954, 33909, 135635, 271270, 542540, and 1085081J (W/m2). The dried inoculum which was dried at 37 degrees for 6hrs, and a moist one which was dropped just before irradiation were prepared. Irrespective of moisture conditions, the number of viable cells in DW was reduced starting at irradiation levels of 1.5-2.0kJ/m2, and fell to below the detectable number at about 8.5kJ/m2. When Y and X indicated the log (10) of viable MAP cells in DW and the amount of UV-B irradiation respectively, calculations were made for Y=-0.0007X+6.0881 (R2=0.9717) under moist conditions and Y=-0.0008X+6.4918 (R2=0.9622) under dried conditions. However, MAP in slurry survived under irradiation of 1,085,000J/m2, which is equivalent to sunlight of several months. The result indicates that MAP residing inside the slurry or behind of grass leaves may survive for a long term even under exposure to sunlight.

Title Factors affecting the herd level of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle.

Author(s) Fredriksen B[*] , Jarp J.

Institution National Veterinary Institute, Oslo, Norway.

Abstract A case control study of Norwegian dairy herds with high and low herd level, respectively, of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* was performed. Even if the prevalence of paratuberculosis in Norwegian dairy herds was expected to be very low, based on the fact that clinical cases of paratuberculosis have been almost absent the last decades, a high proportion of randomly selected herds had shown to have a considerable number of seropositive cows (the National Surveillance and Control Program 1999). Spatial, environmental and management factors were examined for possible association to the antibody level. Farmers were interviewed by the District Veterinary Officers using a specific questionnaire. Data from 51 case herds and 77 control herds, leaving an answering percentage of 99.2, were analysed using logistic regression. The herds were located within eleven of the 19 counties in Norway, and the final multiple regression model was adjusted for geographic location. The most important factors connected with high serological values of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in Norwegian dairy herds seemed to be, geographic location, red deer (Cervus elaphus) accessing the cattle pastures, the presence of wild birds in the feed storage and sharing common pasture. The association between the high seroprevalence for paratuberculosis and the density of red deer in the area was confirmed by analysis of data from hunting statistics. From existing results it is not possible to exclude the possibility that wild animals and birds represent a reservoir of the bacterium, but cross reactions to related bacteria seem more likely. There were large regional differences, with the northwestern and central part of the country having most of the case herds. The explanation(s) for this should be investigated closer.

Title Economic importance of paratuberculosis in a breeding of dairy cows in Croatia.

Author(s) Cvetnic Z[1*] , Brlek K[2] , Trstenjak J[2] , Ocepek M[3] , Spicic S[1] , Mitak M[1] , Krt B[3] .

Institution [1] Croatian veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia. [2] «Varazdinka», a Farm of Dairy Cows Krizevljan Grad, Croatia. [3] Veterinary Faculty, Gerbiceva 60, 1115 Ljubljana, Slovenia.

Abstract Paratuberculosis is a disease which cause great economic losses in breeding of ruminants and particularly in breeding dairy cows. Today paratuberculosis is one of the most prevailing and one of the most expensive disease of dairy cattle. Besides diarrhoea, weight loss and death, which are the final consequences of clinical paratuberculosis, the infected cattle suffer from

decreased production, infertility and increased susceptibility to other diseases. In the paper losses on a farm of dairy cows caused by eliminating the animals due to clinical paratuberculosis, registered during the period from 1994 to 1999 are presented: in 1994 out of 175 cows on the farm 13 were eliminated (7.4%), in 1995 out of 185 cows on the farm 9 were eliminated (4.9%), in 1996 out of 155 cows on the farm 9 were eliminated (5.8%), in 1997 out of 171 cows on the farm 11 were eliminated (6.4%), in 1998 out of 185 cows on the farm 5 were eliminated (2.7%), in 1999 out of 185 cows on the farm 8 were eliminated (4.3%). During the period monitored in total 55 animals were eliminated or annually 9.16 cows (5.2%) with 2.3 final lactation. Together with profuse diarrhoea sudden weight loss occurs with dehydrationand edema (sumaxillary edema). It should be mentioned that a clinical picture appears in two stages. The first stage usually shows up in the middle of previous lactation with milder symptoms and the second clinical stage of paratuberculosis shows up at the very beginning of next lactation when a cow should promptly be eliminated due to sudden weight loss. Parturition is usually normal, but the mass of fetus in 30 % less. The assumption that paratuberculosis is transmited diaplacentally and through colostrum can be supported also by our findings, where in 20 cows (36.6%) out of 55 eliminated due to paratuberculosis, we managed to demonstrate that they were the progeny of mother also having paratuberculosis.

Title Estimating the spread of paratuberculosis within dairy cattle using a deterministic mathematical model.

Author(s) Valente C[1*] , Nucci MC[1] , Cuteri V[2] , Marenzoni M[1] .

Institution [1] University of Perugia, 06126 Perugia, Italy. [2] University of Camerino, 62024 Matelica (MC), Italy.

Abstract A mathematical deterministic model is used to describe the spread of paratuberculosis within dairy cattle during a 9 weeks period. The number of susceptible and exposed calves, and that of susceptible and infective cows are calculated over time. Either a small ((A) 190 animals) or a large ((B) 570 animals) dairy cattle is taken into consideration, assuming the initial presence of one infective cow. After 9 weeks, one can infer that: (A) there will be about 183 animals left in cattle (A), of which 25 cows and 26 calves will be infected, and 76 cows and 56 calves still susceptible, (B) there will be about 551 animals in cattle (B), of which 301 cows and 245 calves will be infected and only 2 cows and 3 calves still susceptible

Title Comparison of blood PCR and ELISA for detection of Map infection in sheep and cattle.

Author(s) Juste RA[*] , Garrido JM, Geijo MV, Aduriz G, Sevilla I.

Institution Instituto Vasco de Investigación y Desarrollo Agrario (NEIKER) Berreaga 1, 48160 DERIO. Bizkaia. Spain

Abstract The main drawback of the ELISA test is that it misses many infected animals in the pre-clinical stage. Since there have been some reports on the use of PCR in blood samples in cattle, we wanted to know whether it was possible to define some conditions where PCR could improve ELISA performance. For this purpose we have used samples arrived to our laboratory for different studies, both in sheep and cattle. For sheep, we have used a set of 404 single blood samples of which 105 corresponded to dam/offspring pairs from 50 dairy flocks in the Basque Country. For cattle we have used a set of 278 samples from a single farm, where all ELISA positive animals were included (70) as well as a matched ELISA negative set. About half of the set were heifers, and half were 2 to 5 years old cows. The overall concordance for sheep was low (kappa= 0.1929) but statistically significant ($p<0.05$). The complementary sensitivity of PCR was 181% for a combined frequency of positives of 15.3% (PCR: 12.1%; ELISA: 5.4%). It was composed of a slightly better concordance for ewes (kappa=0.2135, $p<0.05$), and a non-significant weaker concordance for lambs (kappa=0.1667). There was also a highly unlikely proportion of dam/offspring positive results ($p<0.001$, kappa=0.6269, $p<0.05$). Four out of 6 lambs that were submitted to post-mortem examination one year after testing had paratuberculosis local microscopic lesions and a PCR positive result in the ileocecal valve. In

cattle the overall analysis showed a weak but significant discrepancy (kappa=-0.1665, p<0.05), composed of a moderate concordance among heifers (kappa=0.4471, p<0.05), and a moderate discrepancy among cows (kappa=-0.3670, p<0.05). The complementary sensitivity of PCR was 70% for a combined proportion of positives of 48.9% (PCR: 23.7%; ELISA: 28.8%). These results are consistent with an immunologically silent bacteriemia in the early phases of infection especially affecting young animals, and indicate that blood PCR can pick up infected animals missed by the ELISA test.

Title Diagnosis of *Mycobacterium paratuberculosis* in Europe.

Author(s) Thorel MF.

Institution AFSSA, Laboratoire de Recherches en Pathologie Animale et Zoonoses.

Abstract Paratuberculosis is a chronic enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis*. Diagnosis of paratuberculosis is divided into two parts, detection of subclinicalinfection and diagnosis of clinical disease, which is essential for control of the disease at farm, national or international level. To diagnose the presence of *M. paratuberculosis* in an individual clinically suspect animal, a number of laboratory tests can be used including necropsy, histology, faecal smears, faecal culture, DNA probes, PCR and serology. As a part of a "Concerted Action for the setting up of an European Veterinary Network on Diagnosis, Epidemiology and Research of Mycobacterial Diseases", we compared the methods used for bacteriological analysis of *M. paratuberculosis* from faecal samples and organs (mesenteric lymph nodes, ileocaecal valve or intestine) and those used for serological analysis. We also compared PCR used for identification of strains and/or detection of DNA of *M. paratuberculosis* from faecal samples, organs and milk. Fourteen European Laboratories took part in this scientific investigation : IVR Greece, LNIV Portugal, NVFRI Finland, IZS Roma Italy, IZS Teramo Italy, SVA Sweden, DVL Denmark, INRV Belgium, NEIKER Spain, DLO Netherlands, AFSSA France, VRI Czech Republic, MRI Scotland and CVRL Ireland.

Title Can a test-and-slaughter approach contribute to the control of ovine Johne's disease?

Author(s) Michel AL[1*] , Gous T[2] , Terblanche A[3] , Rudolp R[1] .

Institution [1] ARC-Onderstepoort Veterinary Institute, Private Bag x5, Onderstepoort 0110, South Africa. [2] Pathcare Veterinary Laboratory, 1070 Louis Luipold Medical Center,Broadway Road, Belville 7530,South Africa. [3] Provincial Veterinary Laboratory, Helshoogte Road, Stellenbosch 7600, South Africa.

Abstract

Introduction.

More than two decades after its introduction to South Africa in 1967 ovine Johne's disease (JD) spread to the sheep farming areas of the Western Cape Province and subsequently to two other provinces. Although JD is a controlled disease in South Africa, no surveillance is currently done to determine the prevalence in sheep flocks on provincial or national level and no measures are implemented to eradicate outbreaks of JD on infected farms. For this reason and because of the low sensitivity of the AGID test used in previous surveys, it must be assumed that ovine JD is more widely spread than currently known.

Materials and methods.

Between April 2000 and March 2002 a field study was carried out in sub-populations of two naturally infected Merino flocks in the Western Cape. Seventy-five adult sheep were identified per farm and tested for JD every four months. The diagnostic tests used included the Parachek™ Johne's Absorbed EIA (Commonwealth Serum Laboratories), HerdChek™ *Mycobacterium paratuberculosis* Antibody ELISA (IDEXX Scandinavia), Bovigam™ gamma interferon assay (CSL) and the AGID. A certain number of sheep with one or more positive test results were slaughtered and subjected to macroscopic and histopathological examination of the ileum and the ileo-caecal valve area, followed by bacterial culture on Herrold's egg medium. Culture isolates were identified by IS900 PCR. At the end of the study all remaining sheep were

slaughtered and examined accordingly.

Preliminary Results.

To date, preliminary data analysis indicates a JD prevalence in the two sub-populations of at least 38.4% and 40%, respectively. Only 7/150 sheep (4.6%) remained test negative for the entire study period. A total of 126 sheep (84%) showed reactions to more than one test. Despite repeated, multiple test reactivity typical microscopic lesions were often absent and bacterial culture was unsuccessful. On completion of data collection the sensitivity and specificity values for the different tests applied and their potential value in a test-and-slaughter approach to control ovine JD in sheep flocks will be evaluated.

Title Control strategies to obtain a paratuberculosis-free caprine herd.

Author(s) de Juan L* , Santos A, Aranaz A, Montero N, Álvarez J, Vela AI, Las Heras A, Mateos A, Domínguez L.

Institution Dpto. Patología Animal I. Fac. Veterinaria. U.C.M., Madrid, Spain.

Abstract Caprine paratuberculosis causes decrease in milk production, loss of weight and consequently significant economic losses. Eradication of the disease should involve strict control and prevention tools. This study demonstrates the usefulness of suitable control measures in a goat farm to obtain a parallel paratuberculosis free-herd. This still ongoing study was carried out in a herd of 600 goats in Toledo (centre of Spain) with a previously known history of paratuberculosis. The established program combined management practices and microbiological analysis of a representative number of animals. The control measures were based on 1) separation of neonates immediately at birth to avoid direct contact with the mother, 2) administration of artificial calostrum to neonates, 3) division of the kids in close groups according to dates of birth, keeping them separated from the rest of the infected herd, 4) use of disinfections measures and 5) no introduction of new animals in the herd to prevent new infection. A group of kids that remained with the goats after birth and were fed natural milk from their mothers was used as control. To test the effectiveness of these management practices, 150 kids randomly selected from each of the two test groups (n=100) and the control group (n=50) were analysed microbiologically by culture of pooled faecal samples. Serological tests have also been performed in all the whole flock. The preliminary results indicate the efficiency of this control program in the goat flock. Because the excretion of *Mycobacterium avium* subsp. *paratuberculosis* occurs 2-3 years post-infection further microbiological and serological tests will be done to corroborate the success of this program. Attendance to this Congress was sponsored by the EU-funded project FAIR6-CT98-4373.

Title Paratuberculosis vaccination of adult animals in two flocks of dairy sheep.

Author(s) García-Pariente C* , González J, Ferreras M C, Fuertes M, Benavides J, Reyes LE, Moreno O, García-Marín JF, Pérez V.

Institution Dpt. Patología Animal: Medicina Animal (Anatomía Patológica). Facultad de Veterinaria. Universidad de León. 24071 León. Spain.

Abstract Vaccination of paratuberculosis has been shown to be an efficient method for controlling the disease in sheep. Although usually animals are vaccinated when they are younger than 6 months, several experiences have been carried out immunizing adult sheep with positive results. In this work, we have studied two flocks (approximately 250 adult animals each) of dairy sheep (Assaf breed) in which paratuberculosis had been diagnosed. A 70% of adult sheep from each flock were vaccinated with a commercial killed vaccine (Gudair(, Schering-Plough), regardless their age. The remaining 30% were kept as controls. Milk production from each ewe was also recorded monthly 12 months before and the year after vaccination. In a total of 70 animals (50 vaccinated and 20 controls) from each flock, peripheral humoral immune responses were measured by ELISA and AGID and cellular responses by means of a g-IFN commercial assay at 30, 75, 150 and 365 dpv. In flock A, all the culled and dead sheep during the 12 months after vaccination were also registered. Vaccinated animals mounted high cellular and humoral

immune responses at 30 dpv, the 100% of immunized sheep offering positive results until 150 dpv, when the percentage of positive animals began to decline. During the 12 months after vaccination, a total of 56 sheep (40 -20.6%- vaccinated and 16 -30.7%- controls) were culled in flock A. In a total of 28 of those sheep, complete necropsy could be done. During the first 6 months, 17 sheep were studied (9 having diffuse lesions) and 11 between 6-12 mpv (3 having diffuse lesions). Vaccination in adult sheep has induced strong both humoral and cellular immune responses and has reduced the number of clinical cases of paratuberculosis, as seen by the decrease in culled sheep from the vaccinated group and the number of animals having diffuse lesions. Supported by grant 1FD1997-224 from MCYT.

Title Evaluation of different adjuvants in the vaccination against paratuberculosis in sheep.

Author(s) Reyes LE* , González J, Ferreras MC, García-Pariente C, Benavides J, Fuertes M, García-Marín JF, Pérez V.

Institution Dpt. Patología Animal: Medicina Animal (Anatomía Patológica). Facultad de Veterinaria. Universidad de León. 24071 León. Spain.

Abstract Vaccines against paratuberculosis, both live or killed have used different adjuvants that have been regarded as responsible of the adverse effects of the subcutaneous administration of the vaccines, such as nodule formation. In a first experiment, we have evaluated three different adjuvants in a killed vaccine made with the strain 316F of *M. a. paratuberculosis*. At four groups (A, B, C, D) of ten 1-month old lambs vaccines were administered subcutaneously. Group A was vaccinated with a commercial killed vaccine (Gudair®) made with mineral oil in a stable emulsion. In group B, adjuvant (Montanide Isa 266®) was a mixture (50%) of mineral oil-degradable oil and a purified surfactant. In group C the adjuvant was aqueous (Montanide Ims 1312®) and formed by immunogenic organic compounds. Group D was kept as unvaccinated control. Immune responses were assessed. Nodules induced by vaccination were examined by histological methods after serial euthanasia of lambs at 15, 30 and 75 dpv. Lambs from group B mounted the strongest cellular immune response and had the best defined nodules (round, mobile and well demarcated by fibrous tissue), with a better distribution, and probably presentation, of mycobacterial antigen to the inflammatory cells. In a second experiment, efficiency of adjuvants A and B was assessed in an experimental infection carried out in 18 lambs 1-month old (divided in 6 groups) that were vaccinated with either A or B vaccine, and at 1-month post-infection, infected orally with $2.14x10^{10}$ cfu of *M. a. ptb.*, depending on the group. Immune responses and nodule conformation were registered at different intervals between 15 and 240 dpv, when animals were euthanasied. The best cellular response was also found in lambs vaccinated with adjuvant B whereas no lesion characteristic of paratuberculosis was observed in any of the vaccinated groups. However, lesions appeared in all the infected and unvaccinated lambs. Both vaccines showed a good efficacy with less adverse effects in the case of adjuvant B. Supported by grant LE31/01 from JCYL.

Title Current progress of the National Program to control bovine paratuberculosis in Japan.

Author(s) Yokomizo Y[1] , Mori Y[1] , Fujimori N[2] , Tachibana S[3] .

Institution [1] Department of Immunology, National Institute of Animal Health, Japan. [2] Soya Livestock Hygiene Center.

Abstract In 1998, the National Control Program was improved with the addition of compulsory surveillance using *M. phlei* - absorbed ELISA test on all adult cattle at five year intervals. Cattle showing double ELISA-positive in dual tests at 2 week intervals or fecal culture-positive results have been subjected to compensated slaughter. In the renewed program, an infected herd must comply with regular 3 to 6 monthly testing using both ELISA and fecal culture until all animals give negative results. The total number of paratuberculosis cattle slaughtered under the National Control Program has been recorded to be as many as 2,469 (dairy 1500,beef 969) during the period of 1998-2000. Moreover, the animals with a high infection risk, which are mainly the progeny of infected animals, and their cohort are also subject to subsidized culling, resulting in 3,181 head having been slaughtered under the eradication campaign during this 2

year period. In Hokkaido district, the major cattle farm area in Japan, 0.09% (675/701,692 animals) tested by ELISA and 0.53% (772/143,250 animals) tested by fecal culture were diagnosed as having paratuberculosis during 1998-1999. The results revealed that 1.6% (177/10,944) of dairy and 2.6 % (76/2,926) of beef farms were infected with *M. paratuberculosis* (MP). Currently, the diagnostic standard of paratuberculosis is going to be reviewed by means of an evaluation of fecal PCR test as a suitable alternative of culture test as well as with a renewal of the ELISA kit. Meat inspection service activities do not allow fecal culture-positive animals to be brought into the abattoir, and cattle diagnosed as having paratuberculosis during meat inspection are not allowed to be utilized as food products. All dairy products supplied by milking plants are subject to pasteurization above the level of heat treatment necessary for the complete inactivation of MP (73ºC/15sec for cheese, 95ºC/60 sec for yogurt, 130ºC/2 sec for fresh milk) . This indicates that our country has assigned very strict control measures against MP, resulting the guarantee of the highest level of cleanliness of dairy and beef products in regard to the regulation of MP contamination.

Title Evaluation of the impact of the Minnesota Johne's Disease Control Program.

Author(s) Raizman E* , Wells S, Godden S.

Institution College of Veterinary Medicine, University of Minnesota.

Abstract The University of Minnesota, College of Veterinary Medicine in collaboration with the Minnesota Board of Animal Health has recruited 6 dairies across Minnesota as a part of an educational Johne's disease demonstration control program. The objectives of this program are to characterize the herds fecal shedding and ELISA test prevalence, and to evaluate the change in test results through time after implementation of management changes. This information will serve as a guide for veterinarians working with infected herds using test information and management changes. All cows were sampled in each herd at the beginning of the program in 2000, using serum ELISA and fecal culture tests to establish the baseline prevalence. Subsequently cows from 90-120 days in gestation have been tested monthly at the Minnesota Veterinary Diagnostic Laboratory. Of a total of 1200 dairy cows tested, the overall fecal shedding and ELISA baseline prevalence were 16% and 12% respectively. The sensitivity and specificity of ELISA relative to culture were 39% and 94% respectively. The ELISA prevalence was 10%, 16% and 10% among lactation one, two and three and above respectively. Fecal culture prevalence among the different lactations were 23%, 19% and 20% for lactation one to three and above respectively. Sixty five percent of the culture positive cows were low shedders (1-10 colonies /tube), 13% moderate shedders (11-50 c/t), and 20% high shedders (>51 c/t). Thirty percent of the culled cows in one-year period (1999-2000) were due to Johne's disease. Most of the cows culled due to the disease were from first and second lactation (32% and 25% respectively). The results emphasize that the control of Johne's disease in Minnesota will require a concerted, long term program of education, testing, preventive and control measures, and continued research.

Title The National Paratuberculosis Program in Norway.

Author(s) Djønne B, Fredriksen B, Nyberg O* , Sigurðardóttir ÓM, Tharaldsen J.

Institution National Veterinary Institute, Box 8156 Dep., Oslo, Norway.

Abstract

Introduction.

In Norway, bovine paratuberculosis has been diagnosed only sporadically since 1960, and the two last cases were seen in the same area in 1978 and 1979. In goats the infection has been more common and the disease has been controlled by vaccination.

Materials and methods.

The National Paratuberculosis Program started in cattle in 1997, and until 2000 the survey was based on an ELISA test, followed by histopathology and bacteriology of seropositive animals. Four categories were tested, cattle that had been imported before 1995, herds with both cattle

and goats, herds with a high average age and herds from the area where paratuberculosis had been diagnosed in cattle in 1978-79. In 1999 the survey was extended to include randomly selected beef and dairy herds. From 2000, a case-control study was performed on herds with seropositive animals, and the program was changed to bacteriological examination of faecal samples from cattle in randomly selected herds. In 2001, goats were included in the program, both bacteriological examination of faecal samples and a complement fixation test have been used. All animals clinically suspected for paratuberculosis have been examined by pathological and bacte-riological methods during the whole period.

Results.

About 8 % of the nearly 12 000 samples examined between 1997 and 1999 were positive for antibodies to *M. paratuberculosis*, but the infection could only be verified in 11 animals from 5 different herds. At the beginning of the program, serology was a useful tool to detect infected animals, but there were so many seemingly false-positive reactions that it was not considered to be suitable to estimate the prevalence of paratuberculosis in Norwegian cattle. In 2000, no positive animal was found by culture, but in 2001 *M. paratuberculosis* was isolated from two cattle in two different herds, and from 12 goats in 5 herds.

Title OIE Reference Laboratory for Paratuberculosis in Veterinary Research Institute in Brno (Czech Republic).

Author(s) Pavlík I[*] , Bartos M, Hruska K.

Institution Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic.

Abstract The OIE Standards Commission had recommended in September 2001 the authorization of the Veterinary Research Institute in Brno as a Reference laboratory for paratuberculosis. The mycobacteriology laboratory has a long experience in research and diagnosis of mycobacterial infections of animals. As paratuberculosis is the main subject of engagement in the department, complexes of methods are used: clinical, microscopic (Ziehl-Neelsen), histological, culture and serological (CF, AGID, ELISA) examinations. Further identification of isolated strains is performed by molecular biology methods (PCR, RFLP). In uncultivable strains of *M. paratuberculosis* and specimens with paucibacilar infection, nested PCR is used. Specimen comprise blood, milk, semen, faeces and tissue of susceptible animal species including intestinal tissue of Crohn's disease patients. Further samples are collected from larvae and imagoes of dipterous flies, worms, rodents and other small vertebrates for their possible role of being reservoirs or vectors. The laboratory also deals with the exchange of experiences with other laboratories by offering long and short term stay of peer experts and their students from around the world. We have a joint co-operation with other national laboratories for comparative identification and differentiation of strains obtained mainly from different countries of Europe, USA and Australia, by the molecular biology methods. The mycobacteriology laboratory is a Consulting centre for the Czech State Veterinary Administration. Mycobacteriology laboratory solve the projects dealing with paratuberculosis in domestic (cattle, sheep etc.) and wild animals (deer, moufflon etc.) and develop control programs. On an international level the laboratory shares in research projects studying the role of causal agent of paratuberculosis in Crohn's disease patients and in wild animals. The mycobacteriology laboratory participated in the development of electronic information system for FAO Established Veterinary Biotechnology and Epidemiology Network for Central and Eastern Europe (CENTAUR). Supported by the grant No. MZE-M03-99-01 of the Ministry of Agriculture (Czech Republic).