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The International Association for Paratuberculosis and Other Mycobacterioses, Inc. ("IAP") was founded in 1989. The Association is a non-profit organisation devoted to the advancement of knowledge and scientific achievement toward the eradication of paratuberculosis.

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EDITORS' PREFACE

These proceedings comprise 67 papers /extended abstracts and 117 abstracts, which were presented at the 9th International Colloquium for Paratuberculosis held in Tsukuba, Japan from October 28 to November 2, 2007.

The variation in contents of studies reported is interesting and shows that paratuberculosis research can truly challenge the imagination of authors, when studies are to be designed. The progress in research on paratuberculosis continues, which these proceedings document.

It is a pleasure to provide the scientific community with the proceedings.

Characterization of *Mycobacterium avium* subsp. *paratuberculosis* isolates by Large Sequence Polymorphisms (LSP) detection and hsp65 sequencing

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INTRODUCTION

Paratuberculosis or Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis (Map)*, affects ruminant and non-ruminant species, and causes reduced production, higher susceptibility to acquire other diseases and infertility (Kennedy & Benedictus, 2001). In Spain there are no official data regarding the prevalence of paratuberculosis, but the studies performed so far indicate it is present in all domestic ruminant species with variable prevalences (de Juan et al., 2006, Reviriego et al., 2000).

The complete genomes of two members of the *Mycobacterium avium* complex (MAC), *Map* strain k10 and *Mycobacterium avium* subsp. *hominissuis* strain 104, have been recently sequenced (Li et al., 2005). Comparison between these two genomes of closely related bacteria has revealed that, in spite of the identity between both genomes (over 97%) at the nucleotide level (Bannantine et al., 2003), there are large fragments of DNA present in one but not in the other, called Large Sequence Polymorphisms (LSP) that might be responsible of their divergent phenotypes (Semret et al., 2004, Wu et al., 2006).

Some of those LSPs are characteristic of bacterial subspecies within MAC, and can be used as diagnostic targets to correctly identify an isolate. Besides, some other LSPs can be used to distinguish between different types within one particular subspecies. In particular, three of those LSPs have been shown to distinguish *Map* isolates belonging to Types I and II: LSPa4-II, LSPa18 and LSPa20 (Semret et al., 2006). However, the LSP-profile of the Type III isolates of *Map* has not been determined yet.

In the present study a selection of Spanish *Map* isolates recovered from goats and cattle from different geographic areas were selected, in order to determine the LSPs profile of the these strains, particularly of the Type III isolates.

MATERIAL AND METHODS

Isolates were selected based on their host species, geographical origin and type of strain. The type of the isolates was determined using Pulsed-Field Gel Electrophoresis and PCR-REA analysis aimed to the IS*1311* element (Marsh et al., 1999). Most of the isolates belonged to Type III; however, some strains previously characterized as Type II recovered from cattle and goats were also included in the panel as controls (Table 1).

All these isolates were analyzed using three-primer PCRs in order to demonstrate the presence/absence of LSPa4-II, LSPa20 and LSPa18. Primers and PCR conditions were performed according to Semret *et al.* (2006).

All isolates were further characterized by sequencing of the 65-kDa heat-shock protein gene (*hsp65*) according to Turenne *et al.* (2006).

RESULTS

Based on the PCR results, all Type III isolates presented the LSPa4-II and the LSPa18 in their genomes, but did not harbor the LSPa20. Moreover, all of them also contained the same sequevar for the *hsp65*, code 6 (Table 1).

On the other hand, and as could be anticipated, the analyzed Type II Spanish *Map* strains yielded the expected results in the PCRs for the LSPs and contained the code 6 *hsp65* sequevar, the one found in all Type II strains worldwide.

Table 1. PCR results of all Map strains analyzed

Host species	*Map* Type	LSPa4-II	LSPa18	LSPa20	*hsp65*
Cattle	II	-	-	+	Code 5
Cattle	II	-	-	+	Code 5
Cattle	II	-	-	+	Code 5
Cattle	II	-	-	+	Code 5
Cattle	II	-	-	+	Code 5
Goat	II	-	-	+	Code 5
Goat	II	-	-	+	Code 5
Goat	II	-	-	+	Code 5
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Cattle	III	+	+	-	Code 6
Goat	III	+	+	-	Code 6
Goat	III	+	+	-	Code 6
Goat	III	+	+	-	Code 6
Goat	III	+	+	-	Code 6
Goat	III	+	+	-	Code 6
Goat	III	+	+	-	Code 6

DISCUSSION

LSPs are major genetic events that can be sources of the genetic variability found within MAC. These LSPs could be responsible, at least in part, of the different phenotypes that the MAC species can adopt, from widespread environmental mycobacteria (*M. avium* subsp. *hominissuis*) to obligate pathogens (*Map*).

Among *Map* strains, three types have been also described based on phenotypic and genotypic differences. The presence or absence of a panel of LSPs was evaluated in Type I and II strains, and some important differences were observed that could account for phenotypic divergence (Semret et al., 2006). In the present study, we analyzed a panel of Spanish strains, including Type III isolates, not examined before for the presence of LSPs.

All the Spanish Type II isolates showed the same *hsp65* sequence and LSP pattern that were previously described in isolates from different countries of Europe, America and Oceania (Semret et al., 2006), therefore confirming the homogeneity of this cluster regardless of the host species or the growth rate.

Regarding the Type III isolates, we found that all showed the same LSP-profile and *hsp65* sequevar than those described for Type I strains, thus indicating a close relatedness between these two types. These findings may indicate that Type I and Type III strains have followed similar evolution processes. However, more studies are needed to detect if other LSPs can discriminate between these 2 types. Moreover, we have demonstrated that all Type III strains showed the same LSP profile, thus confirming this type is able to infect both goat and cattle.

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

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INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (*M. a. paratuberculosis*) is responsible for paratuberculosis (Johne's disease), a chronic inflammation of the gastrointestinal tract that affects mainly livestock and wild ruminants. *M .a. paratuberculosis* isolates have been classified into three major groups by using traditional molecular techniques such as IS*900* restriction fragment length polymorphism (RFLP) (Pavlik et al., 1999) and pulsed-field gel electrophoresis (PFGE) (Stevenson et al., 2002).

Single Nucleotide Polymorphisms (SNPs) have been widely used to study genomic diversity in a broad range of microorganisms, but only a few studies have been reported in *M. a. paratuberculosis*. In this study, enoyl-(acyl carrier protein) reductase (*inh*-A) gene, an important gene in fatty acid biosynthesis, and previously classified as polymorphic at position 228 (Marsh et al 2006) was analyzed for the presence of SNPs.

MATERIAL AND METHODS

A panel of 20 *M. a. paratuberculosis* isolates from different types (I, II and III), different hosts (cattle, sheep and goat) and geographical origins (Spain, Scotland and Denmark) was selected (Table 1).

Table1. List of isolates used in our study.

No. of isolates	PFGE classification	Herd/flock	Geographic origin	Host
8	Type III	4 different herds	5 Central Spain 3 Central-south Spain	6 goats 2 cattle
7	Type II	5 different herds	5 Central Spain 2 Central-north Spain	5 goats 2 cattle
5	Type I	3 different flocks	3 Central Scotland 1 North Scotland, 1 Northwest Scotland	5 sheep

These strains were tested with a PCR-sequencing method directed to the full length *inh*-A gene. The forward and reverse sequences were analyzed with Biological Sequence Alignment Editor version 5.0.9.1, and then submitted to nucleotide-nucleotide BLAST against the *M. a. paratuberculosis* K10 (GenBank accesion no. NC_002944) to identify the location of the SNPs.

RESULTS

The sequencing of the *inh*-A revealed two SNP (Fig.1): one of them had not been previously described (position 224), and was Type III-specific. The SNP described by Marsh et al. 2006 at position 228 was also observed in the isolates tested, thus indicating the genomic diversity between *M. a. paratuberculosis* isolates. Moreover, the results are in agreement with previous studies where these strains were also classified by sequencing of *gyrA* and *gyrB* genes (Castellanos et al., 2007).

The enzyme SinI was used to differentiate between the strains Type I and III, by using the SNP located at position 224: a two-band pattern was obtained in the case of Type I and II strains (699 bp and 65 bp); while Type III strains yielded a three-band pattern (477 bp, 222 bp and 65 bp).

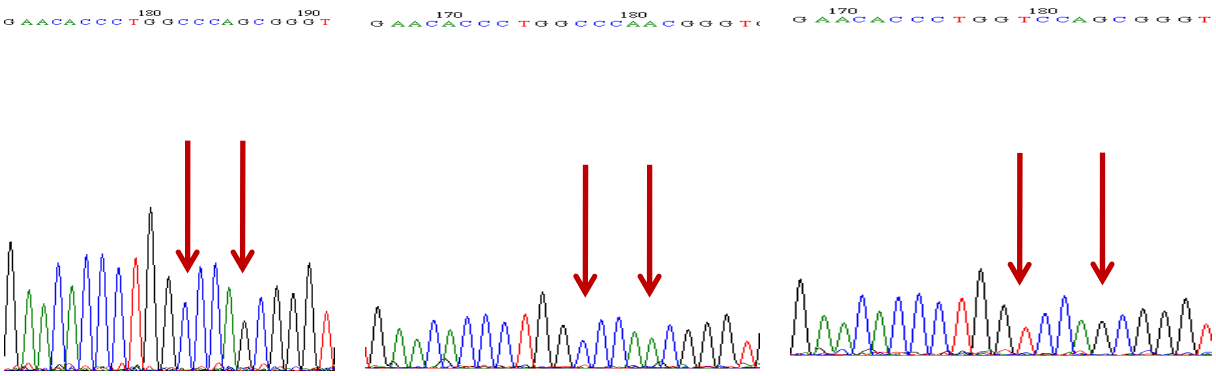

Fig.1. Chromatograms of the three *M. a. paratuberculosis* Types and the SNPs observed at nucleotides 224 and 228

DISCUSSION

From the two SNPs that were observed in our strains, the one that interested us the most was the one located at position 224, a Type III-specific non synonimous transition (Table 2). This SNP was able to distinguish between the two different types of sheep-related isolates (I and III), with similar culture characteristics and requirements and very difficult to grow to perform PFGE or RFLP on them.

Table 2. Location of SNPs in the *inhA* gene in each *M. a. paratuberculosis* Type

***M. a. paratuberculosis* Type**		**I**	**II**	**III**
Nucleotide change	224 bp	G**C**C-A	G**C**C-A	G**T**C-V
	228 bp*	CA**G**-Q	CA**A**-Q	CA**G**-Q

* Previously described SNP by Marsh et al. 2006.

Moreover, Type III-specific SNP at nucleotide 224 was susceptible to PCR-REA. This result, in combination with *gyrB* Type III specific SNP (Castellanos et al., 2007), permits the rapid differentiation of these Type III strains for diagnostic purposes.

This finding indicates there are some genomic divergences between Type III strains while compared to Types I and II. The results described in this study also match previous reports (Stevenson et al., 2002; Castellanos et al., 2007). These point mutations can be a small scale representation of the large genomic polymorphisms that may be present within our isolates.

However, more studies from different geographical areas and a wider range of hosts are needed to verify our results. Also, it would be of great interest to perform a whole genome scale approach between the three Types of *M. a. paratuberculosis* strains to gain an insight into the genetic divergences and its relation with these strains pathogenesis and phenotype.

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Amplified Fragment Length Polymorphism to investigate MAP in Italy

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INTRODUCTION

To investigate the molecular variability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Italy, different strains were analysed with the Amplified Fragment Length Polymorphism (AFLP; Vos *et al.*, 1995) technique. This technique produces a large number of molecular markers through selective PCR amplification, with no need for prior sequence information or probe isolation. The data obtained from this analysis will improve knowledge of MAP molecular variability, for which information on genomic sequence polymorphism is poor, and can be used a tool for epidemiological studies.

MATERIAL AND METHODS

Eight different MAP strains, collected in different regions and provinces of Italy, were used for the analysis. ATCC strain 19698, isolated in the USA, was added as reference and was used as an "outgroup" in the subsequent estimation of the UPGMA tree.

Genetic diversity among the strains was investigated using 8 AFLP primer combinations carrying two or three "selective" nucleotides (Table 1). Statistical analyses were carried out using PHYLIP software package (Felsenstein, 1989) and UPGMA cluster analysis was performed using the Nei-Li (1979) model. To obtain a robust tree, bootstrap was set at 1000 and the CONSENSE option selected.

Table 1. AFLP primer combinations used and number of polymorphic fragments per experiment

Restriction enzymes	Selective nucleotides enzyme 1	Selective nucleotides enzyme 2	N. of fragments produced	N. of polymorphic fragments
EcoRI-MseI	+A	+C	3	1
EcoRI-MseI	-	-	36	27
EcoRI-MseI	+G	+A	33	29
EcoRI-MseI	+G	+GG	13	10
PstI-MseI	+A	+C	9	5
PstI-MseI	+C	+TC	14	11
PstI-MseI	+C	+TT	25	24
PstI-MseI	+G	+CT	86	85
Total			219	192

RESULTS

The AFLP analyses produced a total of 192 polymorphic fragments (88%) with an average of 27.37 fragments per primer pair and a range between 1 and 85 (Table 1). Data were analysed and a UPGMA tree produced, which showed three different clusters of strains: the major cluster included five strains, the second grouped two strains and the third comprised an Italian strain, isolated in Cuneo and the ATCC 19698 strain (Fig. 1).

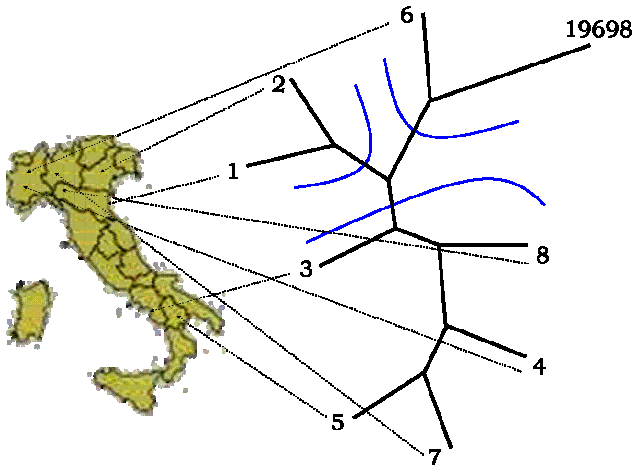

Fig. 1. UPGMA tree from AFLP data; blue lines separate three different strain clusters
19698= ATCC strain, U.S.A.; 1 – 8 = strains from Italy

DISCUSSION

Out of the eight AFLP experiments, “PstG-MseCT” primer combination produced 86 fragments, 85 of which were polymorphic (99%), and was able to differentiate all 8 strains in a single experiment.

As showed by the UPGMA tree, no apparent relationship was observed between clusters and geographic locations where isolates were obtained (Fig. 1).

CONCLUSIONS

These preliminary data indicate that the AFLP approach is able to produce a sufficient number of polymorphic markers to distinguish different MAP strains which can be used to track movements of strains and evolution of the bacterium. However to interpret the UPGMA data obtained in the current study, additional epidemiology information identifying contacts between farms may be necessary to explain the UPGMA data. Ongoing work is examining the variability of MAP strains within a restricted geographical region.

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Short Sequence Repeat typing of Indian *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' isolates

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ABSRACT

The knowledge of the strain diversity is important to have better understanding of epidemiology of infection and guiding disease control programs. The objective of the present study was to study the degree of diversity within Indian type II MAP isolates.

INTRODUCTION

In India, countrywide surveys on paratuberculosis prevalence have never been undertaken and economic losses have never been estimated or realized, but disease is endemic livestock species (Singh et al., 2007; Sharma et al., 2007; Kumar et al., 2007).

Mycobacterium avium subspecies *paratuberculosis* (MAP) strain typing studies has only been carried for the isolates involved in overt paratuberculosis cases in different host species of CIRG, Makhdoom and its premises. Preliminary typing of isolates from different host species including cattle, goats, buffaloes, sheep and blue bull with the help of Spain and Australian labs revealed that all these isolates from different host species belong to 'Bison type' genotype (Sevilla et al., 2005; 2007; Singh et al., 2005a; 2005b).

Since the Bison type genotype also belong to the type II group of MAP strains and type II strains have been divided into various subtypes using tools with more discriminatory power, the objective of the present study was to study the degree of diversity of some of these MAP Bison type isolates recovered from different host species (cattle, buffalo, goat, sheep and blue bull) with overt paratuberculosis using more discriminatory genotyping tool.

Short sequence repeat (SSR) typing has helped to decide that certain MAP genotypes are involved overt paratuberculosis and others result in sub-clinical infection, also SSR typing revealed that certain MAP genotypes are host restricted and others are shared (Ghadiali et al., 2004; Motiwala et al., 2003; 2004; 2005). Results of SSR analysis by Amonsin et al. (2004) indicated that mononucleotide G repeat (AAK46234) and tri-nucleotide GGT repeat (CAB06859) are most discriminatory of the 11 SSRs analyzed. Hence these 2 loci were selected for fingerprinting of Indian MAP Bison type isolates.

MATERIALS AND METHODS

Isolates in the study were from domestic and wild ruminants (cattle, buffalo, goat, sheep and blue bull) of CIRG, Makhdoom and its premises (Table 1). These ruminant populations (wild and domestic) share pasture, grass lands, water etc resources with each other and paratuberculosis is one of the major health and production problems reported in these livestock species. Preliminary typing of isolates recovered from these species has shown that MAP Bison type is the only genotype involved in clinical paratuberculosis in all these species (Sevilla et al., 2005; Singh et al., 2005a; 2005b).

Isolates were cultured as per method of Singh et al. (1996) on HEYM medium supplemented with mycobactin. MAP colonies were characterized on the basis of slow growth, fatness, mycobactin dependency and IS*900* PCR. Bison type genotype was determined using the IS*1311* PCR-REA (Sevilla et al., 2005). Amplification of SSR loci was carried out as per conditions described by Amonsin et al. (2004). PCR products were purified by plate purification and sequencing was done using BDT chemistry. Sequence was analyzed using automated DNA sequencer ABI prisms 3700 DNA analyzer (ABS). Quality of

sequencing data was analyzed using ABS sequence scanner v1.0 software and number of repeats at each locus for every isolate was determined.

RESULTS AND DISCUSSION

All isolates yielded detectable PCR product for G and GGT repeat loci. Sequence analysis of these 2 loci revealed that all isolates recovered from different host species were having same profile of SSR repeats, 7 G and 4 GGT (7g4ggt). The accession numbers are in footnotes of Table 1. No allelic variation among the isolates may be an indicative of interspecies transmission of Indian MAP Bison type between, cattle, goat, sheep, buffaloes and blue bulls. One thing that needs special mention is that paratuberculosis in sheep is caused by distinct group of MAP strains. But based on the results of present investigation same Bison type strain with same SSR profile are resulting in overt paratuberculosis in sheep as well as other host species.

Table 1. MAP Bison type isolates

S. No.	MAP Isolate	Host Species
1	MAP S4	Goat
2	MAP S12	Sheep
3	MAP A35	Goat
4	MAP C43	Cattle
5	MAP C42	Buffalo
6	MAP B42	Blue Bull
7	MAP 120	Goat
8	MAP 121	Goat

Accession Numbers: EF514848, EF514849 EF514851, EF514852, EF514853, EF514856, EF514857, EF514858, EF514859, EF514860, EF514861

Studies on pathogenicity trials of Bison type isolates recovered from wild bison animals had shown that these isolates were more pathogenic to cattle comparer to wild animal bison, the species from which they were initially isolated (Stabel et al., 2003). Clinical data from our study indicate that Indian MAP Bison type is highly pathogenic for both domestic and wild ruminants. There have been enduring efforts of controlling JD in goats of CIRG, Makhdoom due to huge production losses and test and cull policy is in use since the last 15 years to reduce the risk of transmission to susceptible animals, but the efforts have gone in vain and incidence rates continue to increase. The higher incidence rates despite of control programs may be a result of continuous infection transmission to goats from wild animals (blue bulls) that are also infected with same MAP strain and share common pasture, grass land water resources etc. In India there is no movement restriction of animals (disease control programs) and livestock species like cattle, buffaloes, sheep and goat graze together or share other resources even if the infection status of particular species is known for particular disease (including paratuberculosis). The same occurs under farm conditions, although different species are not kept together but share resources of feed and water. At individual owner level, all the species are kept together at same place. Thus, there is every possible chance of transmission of infection from one to other species.

Molecular epidemiological studies indicated that MAP strains have a level of host preference but the host adaptation is not absolute. Our results provide strong evidences that interspecies transmission of MAP occurs among different host species. Since JD is not a priority disease in India despite the disease is endemic, results of the present study may guide future paratuberculosis control programs in India.

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Molecular Characterisation of Indian strains of *Mycobacterium avium* subspecies *paratuberculosis* by pulsed-field gel electrophoresis

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ABSTRACT
In the present study, Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from cattle (5) and goats (4) were characterised by pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR), and profiles were compared with standard strains. All isolates were found to have IS*900* and F57 genes by PCR. PFGE analysis with SnaBI revealed that the profiles of the cattle and goat isolates were identical and had a unique profile when compared with profiles currently in the PFGE database. PFGE analysis with SpeI demonstrated that all of the isolates were identical to SpeI profile 1 in the database. It was concluded that Indian isolates from cattle and goats were genetically similar but different from European strains. This study provides useful data that ultimately will facilitate control measures for the disease in India.

INTRODUCTION
Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Johne's disease), a chronic granulomatous infection of domestic and wild ruminants (Tripathi et al., 2002). The disease is prevalent in most countries including India (Tripathi et al., 2002) and causes significant economic losses to the cattle, sheep and goat industries.

Research has long been hampered by the lack of sensitive methods for the systematic differentiation of MAP. Phenotypic and genetic properties such as colony morphology, growth characteristics, pigmentation, biochemical reactions, antigen and cellular fatty acid compositions, and restriction endonuclease fragment patterns, which are reliable criteria for sub typing many other micro-organisms, were inadequate for sub typing MAP. Three groups of isolates have been described: C type, isolated from cattle, sheep, goats and deer; S type isolated from sheep; and I (intermediate) type isolated from sheep and goats based on IS*900*-RFLP (Whittington et al., 2000; Cousins et al., 2000). PFGE is another technique, which has been used for typing MAP from different origins (Stevenson et al., 2002; de Juan et al., 2005; Sevilla et al., 2007). The purpose of this study was to characterise by PFGE MAP isolates from cattle and goat originating from different geographical regions in India.

MATERIALS AND METHODS
Nine mycobactin dependent isolates of MAP, from cattle (5), goats (4), a vaccine strain (316 F, maintained at IVRI, Izatnagar) and 4 reference strains (NCTC8578, ATCC19698, 99 PW and 806R, maintained at MRI, Edinburgh) were used in the study.

Cultures of MAP isolates
Primary isolation and maintenance of MAP isolates were carried out on HEYM (Herrold's egg yolk medium) and/or Middlebrook 7H11 (MB7H11) slopes supplemented with 2 mgl^{-1} mycobactin J (Allied monitor, Fayette, MO, USA) and incubated at 37^0C until good growth was obtained (6-12 weeks). MAP isolates were cultured in broth media for PFGE analysis as described previously (Hughes et al., 2001). Plugs were prepared when the optical density (McFarland units) was between 2 and 4 as determined by a densimat (Biomerieux, Marcy-1, Eloile, France).

Preparation of plugs and DNA

Plugs were poured according to the procedure described by Hughes et al. (2001). Briefly, bacterial pellets obtained from the culture were resuspended in spheroplasting buffer at a concentration of 6 x 10^9 cells ml^{-1}, prewarmed to 55^oC in 0.5 ml aliquots in a water bath and mixed with an equal volume of 1.5% low melting point agarose (Incert, FMC, Flowgen, Staffs, UK) prepared in 0.05 M EDTA. The mixed suspension was then poured into reusable plug moulds (Bio-Rad) and then allowed to set for at least 30 min at room temperature. DNA was prepared and its release and quality was checked (Hughes et al., 2001).

PFGE analysis

Electrophoresis was carried out in 1% agarose gel prepared in 0.5 x TBE using a CHEF mapper (Bio-Rad, Herts, UK). The electrophoretic conditions chosen to separate fragments digested by SpeI were 30-400 Kb, gradient 6 V cm^{-1}, at included angle of 120^0 at 14^{0}C for 23 hrs, initial and final switch times of 2.16 and 35.38 S, respectively, with switch times ramped linearly . For SnaBI digest, the running conditions were selected for fragment sizes between 50 and 300 Kb and initial and final switch times of 6.75 and 26.29 S, respectively. Other conditions remained the same. For better resolution of the higher molecular weight fragments for SnaBI analysis, the parameters were changed for a fragment size range of 150 to 500 Kb. The other conditions were automatically adjusted by CHEF mapper. Gels were analysed and photographed.

PCR analysis of MAP strains

DNA from all the MAP isolates prepared in plugs for PFGE was analysed for the presence of IS*900* and F57 genes as previously described (Sanderson et al., 1992; Poupart et al., 1993).

RESULTS

PFGE analysis

Cattle isolates grew considerably faster than goat isolates. Colonies were rough, creamy and non-sticky and were acid-fast as determined by ZN staining. All the isolates were confirmed to be MAP by mycobactin dependency, acid-fastness and the presence of IS*900* and F57 genes by PCR.

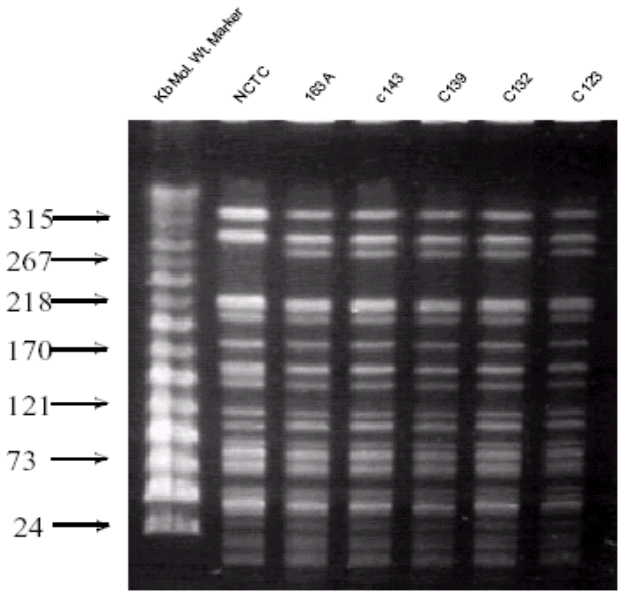

Fig. 1. PFGE profiles of Indian isolates of MAP generated with SnaBI (40 hrs run) as compared with NCTC 8578 standard *(*SnaBI profile 6). Solid arrows depict lanes showing polymorphisms. Numbers above the lanes are designations of isolates. PFGE running conditions are described in the text.

PFGE analysis with SnaB1 with a run time of 23 hrs showed that the cattle and goat profiles were identical but were different from the NCTC 8578 isolate. The higher molecular weight fragments could not be further resolved with longer run time (40 hrs) or even after modifying molecular weight parameters (150 – 500 Kb) and run time. The PFGE profiles of the Indian isolates showed the greatest similarity to the NCTC 8578 isolate (Type II, SnaBI profile 6), when compared with other profiles in the PFGE database at the time of study. The Indian isolates had a distinct profile with polymorphisms observed at approximately 160 and 280 Kb (Fig.1). The new profile was assigned profile number 25. One of the 9 isolates from Nepal showed additional bands, which were faint and presumably due to partial enzymatic digestion. This needs further repetition to confirm the SnaBI profile of this isolate. When all the 9 isolates were typed with SpeI, only one profile was obtained, which was similar to profile number 1 in the database represented by reference strains 99 PW, 806R and ATCC 19698.

DISCUSSION

Despite known limited genetic heterogeneity among isolates of MAP from cattle, sheep and goats in European countries, there have been only two molecular studies involving a small number of MAP isolates of Indian origin. India is a vast country with at least 4 major geographical regions (east, west, north and south zones) having different environmental and climatic conditions.

To date all the isolates or MAP characterised by PFGE can be broadly categorised into three clusters, referred to as types I, II and III. These clusters further contain several PFGE profiles (Stevenson et al., 2002; de Juan et al. 2005). In the present study, all cattle and goat isolates were of type II but had a different profile in comparison to those reported to date. As expected PFGE – SnaBI and SpeI profiles for cattle and goats were similar and support the convention that type II isolates show a broad host range. This is despite the fact that these isolates were from two different geographic and climatic regions (2500 km apart) i.e. cattle isolates were from South India and goat isolates were from North India. A few Indian sheep and goat MAP isolates from Mathura region have been characterized by PCR-REA (IS*1311*gene) and found to be bison type strains (Sevilla et al., 2005). Subsequent analysis of these isolates by PFGE (Sevilla and Heron, unpublished) using more discriminatory SpeI conditions (optimized for 20-250Kb fragments) has shown that at least one of the isolates in this study has a different SpeI profile from the isolates in the Sevilla study. This SpeI profile has not been reported previously and has been assigned profile 33. It appears that sheep and goat MAP isolates from Mathura and Bareilly, both in North India and just 200 km apart, and cattle isolates from South India are genetically very similar.

Although, the number of isolates analysed is too small to make any firm conclusion, it can be speculated that cattle and goats are naturally infected with a genetically similar strain. The findings of the present study will have a considerable epidemiological impact in deciding strategies for the diagnosis and vaccination for the control of the disease. Thus, a common diagnostic reagent and vaccine can be used at least for cattle and goats. Greater number of strains should be characterised from different parts of India to get a broader view of strain diversity. The strain similarity also suggests that cattle and goats should not be allowed to share the grazing pastures as interspecies transmission may occur.

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Note from the editor: This paper was presented only as an abstract at the 9ICP. It was not presented as a poster or an oral presentation. Therefore, the 9ICP delegates had no possibility of discussing the study.

Large Sequence Polymorphisms in *Mycobacterium avium* subspecies *paratuberculosis* (MAP) vaccine strains revealed by MAPAC microarray analysis

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

Techniques for allelic exchange in *Mycobacterium paratuberculosis*

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

A molecular biological approach to producing a live vaccine for paratuberculosis

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Vaccination has for many years been identified as a potentially cost-effective method for the control of paratuberculosis. However, current vaccines based on whole *Mycobacterium paratuberculosis* bacilli mixed with an oil adjuvant have major side effects, including large lesions at the injection sites and interference with diagnostic tests for bovine tuberculosis. In this presentation we outline the steps being undertaken using molecular biological methods, to produce a new, live vaccine based on the deletion of specific genes of the paratuberculosis organism. This strategy has been successfully followed for producing new tuberculosis vaccines. Two different approaches have been used to produce avirulent mutants. The first, was to make a library of mutants in virulent *M. paratuberculosis* through the use of the conditionally replicating shuttle phasmid phAE94 which contains the Tn*5367* transposon. The mutant library was screened using *in vitro* culture systems including inability to multiply in minimal media, increased temperature sensitivity, carbon source preference and altered colonial morphology, all phenotypes that have been associated with loss of virulence in other pathogenic species. The library was also screened by looking for mutants with reduced ability to survive in cultures of bovine peripheral-blood macrophages. The second approach was to inactivate specific genes through homologous recombination. The genes selected for inactivation were those which have homologues in *Mycobacterium bovis* and whose inactivation in *M. bovis* produced mutants with good vaccine efficacy against bovine tuberculosis. A crucial step in developing live vaccines is to determine the virulence of potential vaccine strains in an animal model. The loss of virulence of selected mutants has been determined by the intravenous inoculation of 10^8 bacilli into recently weaned goats. Initial studies showed that a range of different gut tissues were colonized with moderate numbers of *M. paratuberculosis* one year after being infected with virulent strains. In contrast, no or very low numbers of bacilli were isolated with mutant strains. Subsequent trials have shown that virulence can be determined by examining goats after a six month infection. Immune responses were monitored by measuring gamma interferon release to Johnin PPD and will be used for selecting strains for determining their vaccine efficacy. Vaccine efficacy will be determined in a goat vaccination / challenge model. Recently weaned BALB/c mice are being investigated as a possible alternative animal for the initial screening of mutants for loss of virulence. The reduction in the number of *M. paratuberculosis* bacilli in spleen and liver is associated with reduced virulence.

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

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We have reported the simple detection system of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) based on LAMP method, which was sensitive and specifically identify MAP DNA by means of white turbidity (J. Clin. Microbiol. 41:4359-65. 2003). Here we present the improved LAMP system with modified primer sets and additional denaturing step of DNA, which allows for a rapid detection of MAP in cattle feces. The new primer sets were designed from first one third region of IS900 sequence. As the additional step, the extracted DNA was denatured by heat-treatment and added to a reaction mixture.

Using the new system, 10 copies of cloned IS*900* DNA or 0.005pg of genomic DNA from cultured MAP were detected within 30 minutes. It specifically distinguished MAP from *M. avium* or *M. scroflaceum*. Non-specific amplification was not observed within 120 minutes in the LAMP assay with 10 fecal samples which were obtained from Johne's disease free herd. Thus, the improved LAMP system showed high sensitivity and specificity for detecting MAP in fecal samples.

In order to compare with the culture method using Herrold's egg yolk medium with mycobactin, LAMP system was evaluated using 40 cattle fecal samples obtained from MAP infected cattle herds. The samples were collected when the cattle were slaughtered, and were stored at 4°C for less than 3 days before conducting the culture test. Nineteen samples, including 9 culture-positive samples, were positive with LAMP system. On the other hand, twenty one LAMP negative samples were all culture-negative. The LAMP's higher sensitivity would allow for the detection of tiny amount of DNA of dead or dormant MAP cells potentially present in the culture-negative but LAMP positive samples. Additionally, real-time monitoring data of LAMP indicates that there is some correlation between a number of colonies on culture media and amplification time to reach the threshold turbidity. These results suggest that LAMP system is robust and useful method for the detection of MAP DNA, and will contribute to rapid and reliable diagnosis of MAP infected cattle.

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

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BACKGROUND
In the last two decades a variety of different molecular typing methods have been developed to differentiate strains of the genomically highly conserved *Mycobacterium avium* subsp*. paratuberculosis* (Map). The most successful techniques are based on insertion sequences, repetitive loci, comparative genomics or single nucleotide polymorphisms. In the latter class, AFLP, RAPD and PFGE have been applied with variable success. In this study a single Map gene was targeted that is coding for a member of the polymorphic PPE protein family for which roles in immune evasion, antigenic variation and virulence have been suggested. AIM: To examine whether polymorphic PPE proteins can serve as a means of differentiation of Map isolates.

METHODS
We have identified at least four Map PPE proteins to be surface exposed on the cell wall of Map by enzymatic trimming of viable Map bacteria in specific in vitro conditions. One of the corresponding genes, Map1506 was sequenced from a collection of 60 isolates from different sources, hosts and typing profiles, including 7 isolates of bovine (C) and 6 ovine (S and I) variants representing most predominant IS900-RFLP profiles. IS1311 PCR-REA was performed on all isolates to determine their bovine (C) or ovine (S) subtype. Sequence data for the entire Map1506 gene was collected and compared. These sequencing results were used to select polymorphic regions in the gene to amplify for analysis by denaturing gradient gel electrophoresis (DGGE).

RESULTS
Grouping based on the sequencing data corresponded in general to the C versus S subtyping by IS1311 PCR-REA. One of the Map1506 gene sequences differed substantially from the sequenced K10 strain with only 79% identity. Polymorphic regions of Map1506 were selected for analysis by denaturing gradient gel electrophoresis allowing visual discrimination of bovine and ovine *M. avium* subsp. *paratuberculosis* isolates as well as separation of ovine isolates into two subgroups (S and I).

CONCLUSION
The Map1506 gene encodes a surface exposed polymorphic PPE protein with putative roles that are relevant to Map pathogenicity. Sequence polymorphisms in this gene are readily detectable by DGGE, and allow distinction of isolates correlating with the known C, S and I variants.

Survival, dormancy and the proteome of *Mycobacterium avium* subsp. *paratuberculosis* during the stress response to hypoxia and nutrient starvation

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Few data exist on physiological adaptation of *M*. *paratuberculosis (Mptb)* in either the host or the environment. The responses of the two distinct strains of *Mptb* (C and S) to hypoxia and starvation were studied *in vitro* in this study. The growth pattern of *Mptb* during stress appeared similar to the dormancy response of other mycobacteria. The C strain was more resistant to starvation stress than the S strain. A total of 66 protein spots differentially expressed in response to starvation and/or hypoxic stress were selected and identified, providing the first functional assessment of the genomic differences known to exist between these strains. Differentially expressed proteins were classified based on biological function and 13 categories were identified including antioxidant enzymes, amino acid metabolism, fatty acid metabolism, ATP and purine biosynthesis, proteolysis, cell wall synthesis, oxidoreducatse enzymes, protein synthesis, signal recognition, hypothetical proteins with putative function, hypothetical proteins with unknown function, cyanate hydrolysis, phosphate metabolism and cell division. These differentially expressed proteins are potential screening targets for future diagnosis, prevention and control of *M*. *paratuberculosis* infection and their identification will assist understanding the pathogenesis of the diseases caused by this organism.

Targeting differential expression to identify subspecies specific proteins for the diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infections

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Global screening of genomes and proteomes provides a powerful tool for identifying differences between two or more closely related organisms. In this study we have used comparative proteomics to identify proteins responsible for the phenotypic variations in the two genetically similar subspecies IS901^{+} *Mycobacterium avium* and *Mycobacterium avium* subspecies *paratuberculosis.* The advantage of this approach was that comparison of the proteomes of the two organisms would identify subspecies-specific proteins including the products of differential gene regulation that would not be detected by a comparative genomics approach. Comparisons were made between the proteomes of the two organisms grown *in vitro* at both log and stationary phases of growth. Differentially expressed proteins were identified in both organisms and those found to be upregulated in *M.a.paratuberculosis* were further investigated by mass spectroscopy and Mascot analyses. The proteomes were compared from different strains of the organisms to ensure that the proteins identified were representative of the subspecies. Comparison with the *in vivo* proteome of *M.a.paratuberculosis* confirmed that the proteins were expressed during natural infection of the target species. The genes encoding the proteins of interest were cloned and expressed in *Escherichia coli* and the immunogenicity of the recombinant proteins determined to assess their potential as specific immunological reagents for diagnosis and epidemiological studies.

Draft genome sequence of an ovine *Mycobacterium avium* subspecies *paratuberculosis* isolate

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

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

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

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

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A total number of 192 faecal and 10 tissue samples were collected in suspected cattle, sheep and goats from organized farms in Tamil Nadu, India for isolation using Herrold's egg yolk medium (HEYM). Two sheep isolates, two goat isolates and one cattle isolate were isolated. Biochemical characterization of the isolates revealed positivity for heat stable catalase at 68^0C, pyrazinamidase and Thiophene Carboxylic inhibition test and negative for nitrate reduction, potassium tellurite reduction, tween 80 hydrolysis and urease test. Cultural characterization showed medium growth at 33^0C and 37^0C, no pigment formation, no growth in the presence of 5% NaCl and in MacConkey agar. The sheep isolates formed moist glistening colonies in 9-10 months and cattle and goat isolates in 3-4 months. Molecular characterization was carried out by isolation of DNA using QIAGEN kit from HEYM and subjected to IS900 PCR. An amplicon size of 277bp was formed on amplification and restriction enzyme analysis using *MboI* showed similar restriction patterns among the isolates. Single strand conformational polymorphism done with the PCR amplicons showed variation in the mobility pattern of the sheep isolates from that of cattle and goat. Sequence analysis of the PCR amplicons of the isolates did not show much significant variation between the isolates. The allotted accession numbers for sheep 1, sheep 2, cattle and goat strains were DQ366928, DQ366929, DQ986325 and DQ986326 respectively. In conclusion, single strand conformational polymorphism can be a simple and effective method in differentiating isolates and could be further exploited by subjecting more isolates for characterization.

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

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Mycobacterium avium subsp. *paratuberculosis* (Map) causes paratuberculosis in animals and is suspected of causing Crohn's Disease in humans. Previous investigations have revealed a relative lack of genetic diversity amongst isolates. Combined with the slow growth of the organism in pure culture, strain differentiation among Map isolates has proved to be difficult and has limited the study of the molecular epidemiology of the disease. We here compare a set of 268 isolates from different hosts (cattle, sheep, goats, bison, deer and wild boar) that have been previously characterized for IS*1311* PCR-restriction endonuclease analysis and *Sna*BI-*Spe*I pulsed-field gel electrophoresis patterns with the more recently described short sequence repeat (SSR) analysis of locus 1 (G residue) and locus 8 (GGT residue).

The results show that a total of nineteen different multi-locus SSR (SSR1_SSR8) types were identified amongst the 268 isolates. In terms of host species distribution, there were 13 SSR types identified from cattle, 6 from sheep and 3 from goat isolates. Cluster analysis with both PFGE and SSR based typing methods confirmed that Map isolates are genetically divided into two main groups, the cattle type and sheep type groups. Amongst isolates recovered from Spain, SSR type type 7_4 accounted for the 54% of cattle isolates, while types 7_3 and 14_3 together accounted for the 29% of sheep isolates. Interestingly, amongst isolates recovered from goats, approximately the same proportion (43%) of isolates were typed as either cattle type (7_4) or sheep type (14_3). While the overall discriminatory power of both methods as calculated by Simpson´s index of diversity (D) was almost the same (0.693 for PFGE and 0.691 for SSR) for both methods, comparative analysis revealed that the most abundant PFGE 1-1, 2-1 and 23-16 profiles were subdivided into 11, 7 and 4 different types, respectively. Similarly, isolates representing the most abundant SSR type (7_4) could be subdivided into 19 different PFGE profiles. Amongst isolates recovered from sheep, there was a slightly higher discrimination with PFGE (D = 0.865) than with SSR (D = 0.775).

Taken together, the results of our studies confirm the utility of the SSR approach as an easy and rapid method based on PCR and sequence analysis that requires only small amounts of sample to perform. The results also suggest that the addition of a third locus to SSR typing may help in increasing the discriminatory power of this method. Overall, the results of our comparative analyses suggest that, based on current methodologies available, a combined approach that includes IS*1311* PCR-REA, SSR and PFGE provide the highest level of discrimination for Map strain typing.

Genetic characterization novel Indian *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' isolate (S5) used to prepare vaccine and diagnostic kits

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Mycobacterium avium subspecies *paratuberculosis* (MAP) the cause of Johne's disease in animals is distributed world over and has also been associated with inflammatory bowel disease (IBD or Crohn's disease) of human beings. Despite low productivity (1/6 in Asian countries), huge animal (>450 millions) and human population (>1.3 billion), has not been extensively screened for the presence of MAP. Limited studies have shown that MAP was endemic in animal herds and flocks in the country. Preliminary characterization of MAP showed that native strain of MAP prevalent in domestic ruminants was ' Bison type' (Sevilla et al., 2005).

Presently India lacks a referral laboratory to look into different aspects of research on *Mycobacterium avium* subspecies *paratuberculosis* in the huge population of domestic ruminants and human beings. Johnin has been used for last 40-50 years and was the only diagnostic reagent available to screen animals against Johne's disease. Johnin production was based on a strain of MAP imported from UK. Johnin production was discontinued due loss of strain in serial passages and has been restarted by importing a new strain of MAP, as there is no standard Indian MAP strain. Limited studies on diagnostics and vaccines developed utilizing antigens from strains prevalent outside the country were not as efficacious as to that indigenously developed utilizing antigens from native MAP 'Bison type' strain of goat origin. Therefore genetic contents including different markers (IS elements, short sequence repeats and large sequence polymorphisms) of native MAP strains were analyzed and compared with MAP K10 genome to have inference of differences in native MAP 'Bison type' isolate and to study the phylogeny of native isolates. Results of present study confirmed that MAP 'Bison type' (S5) genome has genetic differences in terms of SNPs, locus polymorphisms and genomic duplications and there is need to extensively analyze Indian MAP isolates to have insights in to their evolution and pathogenicity. In the present study different methods of the strain typing were also optimized for the first time in the country to guide geno-typing and futuristic molecular epidemiological studies.

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

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Paratuberculosis (Johne's disease) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is the most costly production disease of animals, has zoonotic concerns and invites trade restrictions. Johne's disease is endemic in Indian domestic livestock population (cattle, buffalo, goat and sheep) and wild animals. MAP has been recovered from cases of Crohn's disease, normal and in-contact animal attendance. India possesses huge population of animals however, per animal productivity is very low as compared to other countries. Despite test and cull policy, incidence of disease continues to increase, resulting in heavy economic losses that have never been estimated or realized.

Lack of effective vaccine has increased the demand of devising preventive measures based on epidemiological information. In India work on the characterization of strain was started from 1999 with the help of Australian and Spanish laboratories (Sevilla et al., 2005, Whittington, 2001, personal communication) giving first indication that Indian MAP strains may be different from other known strains of MAP in the world. In the present study, based on genome sequence of International Reference isolate (MAP K10), assays were designed to have insights of various molecular markers including IS elements, LSPs and SSRs of MAP 'Bison type' 9S5) of goat origin. In some of IS1311 loci a novel polymorphism was observed in MAP 'Bison type' S5, not seen in MAP K10 genome and MAP isolates from outside India. This is the first report of this polymorphism and so far these polymorphisms have not been observed in Indian and non-India strains. In the next stage presence of this polymorphism was tested in a panel of Indian MAP isolates (from different host species and diverse geographical regions) and non-Indian MAP isolates. Novel polymorphism was consistently present in all Indian MAP isolates tested despite differences in host species and diverse geographical regions and this polymorphism was not seen for non-Indian MAP isolates. Non-Indian MAP isolates were identical to International Reference isolate (MAP K10). Study provided unique characteristic molecular signature that can distinguish Indian MAP from other isolates. Study indicated the need for analyzing large number of Indian MAP isolates in order to confirm this polymorphism as a characteristic of Indian MAP isolates. This effort will provide new dimensions to paratuberculosis research in the country.

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

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Recognition of microbial pathogens by the innate immune system is essential to development of an adaptive immune response and is dependent on signaling by Toll-like receptors (TLR). TLR are Pattern Recognition receptors (PRR) that recognize and respond rapidly to invading bacteria leading to the production of pro-inflammatory cytokines, radical oxygen species, nitric oxide and the induction of adaptive immunity. In parallel to this microbes have co-evolved mechanisms to avoid host responses, and may even exploit TLR induced innate responses to their own advantage. Elucidation of the mechanisms of pathogen recognition and host immune evasion by the TLR pathways is central to our understanding of how these factors contribute to pathogenesis in the susceptible host and for developing improved approaches to control, treatment and immunoprophylaxis of this important disease.

In this study we have analysed the expression of eight ovine TLR genes in peripheral blood and several intestinal sites by comparative qPCR to determine if the levels of TLR expression is modulated in these tissues by disease. We have found significant changes in the expression of several TLRs at various sites. Additionally there appears to be a substantial difference in gene expression between lesion grades for some genes. The implications for diagnosis and pathogenesis of disease will be discussed.

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

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ABSTRACT

The objective of this study was to evaluate cytokine gene expression and populations of lymphocyte subsets in periparturient dairy cows naturally infected with My*cobacterium avium* subsp*. paratuberculosis* (MAP).

Blood was collected from noninfected, subclinical, and clinical MAP-infected dairy cows for 3 wks pre- to 4 wk post-calving. Expression of IFN-γ, IL-4, and IL-10 declined at calving compared with prepartum values in both control and infected cows. PBMCs isolated from infected cows had higher secretion of IFN-γ, IL-10, and TGF-β in the postpartum period compared with control cows. Flow cytometric analysis revealed that subclinical cows expressed a greater percentage of both $CD8^+$ and γδ T-cells compared with the clinical cows. The percentage of $CD4^+$ T-cells increased in clinical cows as parturition approached. Clinical cows expressed lower percentages of $CD4^+CD5^{bright}$ and $CD8^+CD5^{bright}$ compared with control cows, but greater percentages of $CD5^{dim}$ cells for all lymphocyte subsets.

These data suggest that parturition is a very dynamic time period for host immunity, with potential for altered immunity to hinder the ability of dairy cows to thwart infectious diseases.

INTRODUCTION

On-farm observations suggest that dairy cows infected with MAP may demonstrate increased signs of clinical disease during the weeks following parturition. Parturition has a major impact on the number of lymphocytes and monocytes in the peripheral blood of healthy cows and alterations in these percentages play a significant role in the ability of the animal to respond to infection. The transition from the subclinical to clinical stage of MAP infection is characterized by a shift from cell-mediated (Th1) immunity to an antibody-mediated (Th2) humoral response. To date, limited research is available characterizing detailed aspects of periparturient immunosuppression in the dairy cow. Further, it is not clear what impact this time period and its associated stressors may have on host immunity in cows with paratuberculosis. Therefore, the objectives of this study were 1) to characterize cytokine gene expression and secretion, and 2) to determine percentages of lymphocyte subsets in periparturient dairy cows naturally infected with MAP.

MATERIALS AND METHODS

Twenty-three Holstein dairy cows were grouped according to infection status. These three groups consisted of 5 non-infected healthy cows, 14 cows naturally infected with MAP, but asymptomatic, and 4 naturally infected cows with clinical Johne's disease. Non-infected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3-5 year period and were negative on any serological assays (ELISA, IFN-γ) performed during this period.

Blood was collected from the jugular vein in 2x acid-citrate-dextrose (1:10) at -21, -14, -7, +1, +7, +14, +21, and +28 days relative to parturition. Peripheral blood mononuclear cells (PBMCs) were isolated and cells were cultured at 1.4 x 10^6/mL in 48-well flat-bottomed plates with either medium alone (non-stimulated, NS), with concanavalin A (ConA; 10 µg/mL) or with MAP whole cell sonicate (MPS; 10 µg/mL) added to designated wells. Plates were incubated for 24 h at 39℃ in 5% CO_2 in a humidified atmosphere. After 24 h plates were removed and centrifuged at 400 x g for 5 min. Supernatants were removed and stored at -

20℃ prior to cytokine measurement. The secretion o f IFN-γ, IL-10, and TGF-β by PBMCs in the cell culture supernatant was determined by ELISA assay.

For flow cytometric analysis, PBMCs were resuspended in complete medium and 50 µL of the cell suspension was added to wells containing 50 µL of primary monoclonal antibody to $CD4^+$, $CD8^+$, γδ T-cells, B cells, and CD5. Analysis was conducted by gating on mononuclear cells based on forward and side scatter characteristics.

RNA was extracted from NS and ConA-stimulated PBMCs using the standard protocol for Trizol reagent. Total RNA was converted to first strand cDNA. For RT-PCR analysis, SYBR Green PCR master mixture, template cDNA, and gene-specific primers for IFN-γ, IL-12p35, IL-4, IL-10, and TGF-β were combined in a 20 µL reaction mixture . The β-actin gene was used as the control for calculation of dCt. RT-PCR was analyzed by using the 2^{-ddCt} method. The mean +1 DRTC dCt value within treatment was used as the reference expression point.

RESULTS AND DISCUSSION

Due to sampling error, we were not able to evaluate the cytokine gene expression data for the clinical cows in this study. When comparing control and subclinically infected cows, subclinical MAP infection did not have an effect on the gene expression of IFN-γ or IL-12. However, ConA-stimulated PBMCs from subclinical cows (14.36 ng/ml ± 1.6) tended ($P < 0.06$) to secrete more IFN-γ compared with the control cows (8.30 ± 2.2). Previous work in our laboratory showed that subclinical JD cows had greater IFN-γ expression compared with clinical cows (Stabel, 2000). Parturition had a significant effect on IFN-γ expression with declines in IFN-γ expression by NS ($P < 0.05$) (Fig. 1A) and ConA-stimulated ($P < 0.01$) PBMCs from both infection groups as parturition approached. This is in agreement with previous studies reporting a decline in Th1 cytokines at parturition (Shafer-Weaver et al., 1999). Interleukin-12 expression was not effected by infection or parturition (data not shown).

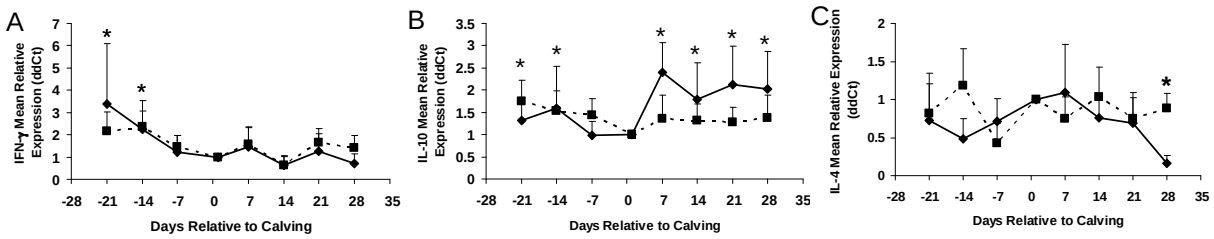

Fig. 1. Expression of cytokines by NS PBMCs from healthy control cows (♦) and cows naturally infected with MAP (■). Significant differences on a given day relative to day 1 are represented by asterisks ($P < 0.05$).

In addition to understanding the effects of parturition and MAP on the Th1 cytokines, we also sought to determine the role of the classical Th2 cytokines. In the current study, there was no effect of parturition or MAP infection on gene expression of Th2 or Th3 cytokines. Despite minimal differences in TGF-β expression, NS (Fig. 2A) and MPS-stimulated PBMCs isolated from clinical cows secreted ($P < 0.05$) more TGF-β during the immediate postpartum period compared with subclinical and control cows. In NS, PBMCs, control cows had greater ($P < 0.01$) IL-10 expression during the postpartum period (Fig. 1B). MPS-stimulated PBMCs from infected cows tended ($P < 0.09$) to have secreted more IL-10 than did the control cows at calving and during the postpartum period (Fig. 2B). Others have reported upregulation of IL-4 during the postpartum period, but this effect was not observed in the present study (Fig. 1C) (Shafer-Weaver and Sordillo, 1997).

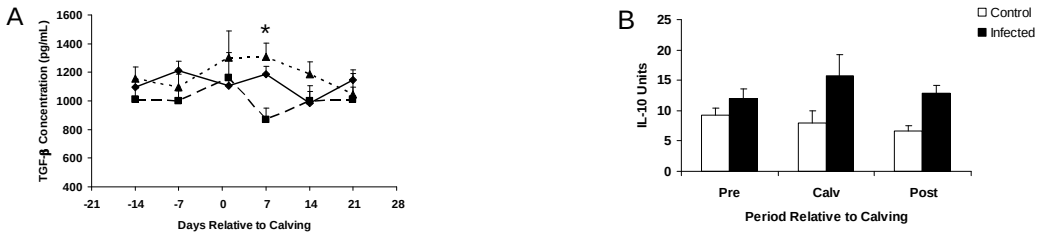

Fig. 2. TGF-β and IL-10 secretion by PBMCs isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with MAP. A) TGF-β NS PBMCs. B) MPS-stimulated PBMCs. Significant differences between infection groups on a given day relative to calving are represented by asterisks ($P < 0.05$).

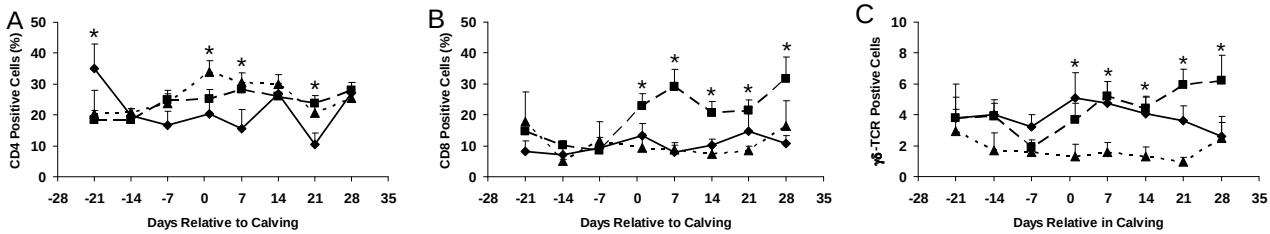

Fig. 3. Figure 3. Percentage of positive mononuclear cells from 24 h PBMCs isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

The number of $CD4^+$ T-cells in the peripheral blood averaged 21.6% ± 2.1, 24.3% ± 1.3, and 25.7% ± 2.4 for control, subclinical, and clinical cows, respectively, with no differences noted between infection groups (Fig. 3A). The percentage of $CD8^+$ T-cells (Fig. 3B) ranged from 10-22% which is equivalent to published values for adult dairy cattle (Meglia et al., 2005). An interactive effect of infection group and parturition ($P < 0.01$) was observed with subclinically infected cows expressing 2-fold higher percentages of $CD8^+$ cells in the postpartum period. The percentage of γδ T-cells is greatest in the calf (40%) and gradually declines to approximately 5% of adult PBMCs (Hein and MacKay, 1991). In our study, clinical cows had much lower ($P < 0.01$) percentage of γδ T-cells compared with subclinical and control cows (Fig. 3C). An increase in this cell population was noted as parturition approached. In advanced stages of JD, antibody production by B-cells does little to protect the host from progressive MAP infection. Overall, neither infection status of the cows nor parturition had an effect on the overall percentages of B cells (data not shown).

CD5 is expressed on all bovine T cells and a subset of B cells. Both CD5 bright and dim populations were examined as a percentage of marker positive lymphocyte subsets. For $CD4^+$, $CD8^+$, and B cell subsets, clinical cows expressed the lowest percentage of $CD5^{bright}$ cells ($P < 0.01$) and the greatest percentage of $CD5^{dim}$ cells compared to the control ($P < 0.05$). B-cells expressing CD5 are capable of producing the Th2 cytokine, IL-10 (Gieni et al., 1997). The increased proliferation of $CD5^{dim}$ B cells by clinical Johne's cows may directly inhibit the cell-mediated immune response by altering the cytokine microenvironment in favor of a Th2 response.

CONCLUSION

Results of this study indicate that parturition modulates IFN-γ and IL-10 expression in dairy cows. Furthermore, the percentages of both lymphocyte subsets and mononuclear cells are modulated by natural infection with MAP and by the periparturient period.

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Association between two polymorphisms in the bovine CARD15/NOD2 gene and paratuberculosis infection in Florida dairy and beef Cattle

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Paratuberculosis has been suspected to have a genetic component and estimations of heritability of about 0.15 have been reported. Caspase recruitment domain 15 (CARD15/NOD2) is a gene codifying for a cytosolic protein implicated in bacterial recognition by cells involved in innate immunity. This protein modifies inflammatory responses to bacterial triggers through activation of the nuclear factor-kB. Crohn's disease (CD) is an idiopathic inflammatory bowel disease in humans similar in many features to bovine paratuberculosis and involves an aberrant mucosal immune response in genetically susceptible individuals. The association between mutations in the CARD15/NOD2 gene and increased risk of CD has been described.

The objective of this candidate gene case-control study was to characterize the distribution of two polymorphisms in the bovine CARD15/NOD2 gene and test their association with paratuberculosis infection in Florida dairy and beef cattle.

The study population consisted of 432 adult cows composed of Holstein, Jersey and Brahman-Angus crosses distributed in four herds. The infection status for cases and controls was determined using five diagnostic tests (serum ELISA, milk/blood/fecal nested PCR, and fecal culture). Parallel interpretation of the results was used to compensate for limitations in sensitivity of available diagnostic tools.

Two previously reported single nucleotide polymorphisms (SNP1; C733R and SNP2; Q1007L) in the bovine CARD15/NOD2 gene, responsible for two amino acid substitutions, were established for the study population by the TaqMan® genotyping assay. The statistical analysis was based on Chi-square and Fisher's Exact Test and different models were proposed for the logistic regression analysis.

It was our central hypothesis that a combination of particular alleles in our candidate gene would be present in higher frequency in controls compared to cases, suggesting a role in resistance to infection. The resulting ratio of cases to controls was 1:2.5. Frequencies for the major allele in SNP1 and SNP2 were 0.957 and 0.543, respectively. The population was in Hardy-Weinberg equilibrium for SNP2 but not for SNP1. Values for coefficient of linkage disequilibrium (LD), the normalized LD, and the correlation between the two SNPs were -0.027, 1.0, and -0.23. Chi square test indicated that SNP1 and SNP2 are in linkage disequilibrium.

The statistical analysis resulted in significant differences in allelic frequencies between cases and controls for SNP1 ($p<0.001$) indicating a significant association between infection and mutant allele. In the analysis of genotypes a significant association was found between SNP1 and infection status ($P<0.0001$). A significant association between allele combinations and infection status was found ($P<0.0001$) when both SNPs were considered in the genotype. The low representation of the variant allele for SNP1 in Holstein and Jersey breeds raises the prospect of a potential confounding role of breed for its connection with infection. However, a significant association between SNP1 and infection was confirmed when tested within the Brahman-Angus sub-population ($P=0.02$). Preliminary results suggest a role for CARD15/NOD2 gene in the susceptibility of cattle to paratuberculosis infection. Amino acid substitution C733R (SNP1) appears to be associated to paratuberculosis infection in Florida cattle. These results could be the basis for further research to create a rapid method to select for more resistant individuals, genetically contributing to the control of Johne's disease.

Proteomic evaluation of sera and milk from healthy and paratuberculosis infected cows

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INTRODUCTION

Mycobacteria, and particularly *Mycobacterium avium* complex (MAP), are a closely related group of microorganisms that do not readily lend themselves to identification or differentiation. The *M. avium* complex includes the closely related species *M. avium* subsp. *avium* (*M. avium*), *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*), *M. intracellulare*, as well as the wood pigeon bacillus (*M. silvaticum*). This complex is characterized by over 90% similarity at the nucleotide level, but its members differ widely in terms of their host tropisms, microbiological and disease phenotypes, and pathogenicity. Members of the *M. avium* complex are associated with animal and human diseases including infections of the lung, lymph nodes, skin, bones, and gastrointestinal and genitourinary tracts (Karakousis et al., 2004; Katoch, 2004; Lauzi et al., 2000).

In recent years, the *M. avium* complex has assumed greater importance in human medicine, largely because it represents often intractable infections in AIDS patients and also because of the possible association of Crohn's disease with *M. paratuberculosis* (Karakousis et al., 2004; Lauzi et al, 2000). *M. paratuberculosis* is the causative organism of Johne's disease (or paratuberculosis), a debilitating chronic gastroenteritis in ruminants (Harris and Barletta, 2001; Lauzi et al, 2000). Crohn's disease is also a chronic inflammation of intestines and exhibits pathology similar to that of Johne's disease in ruminants. Several studies have associated *M. paratuberculosis* with some Crohn's disease cases (Bull et al., 2003; Schwartz et al., 2000). Although strain sharing has been documented (Ghadiali et al., 2004), the evidence for a link remains controversial as a causal role of *M. paratuberculosis* has not been demonstrated (Greenstein, 2003; Harris and Lammerding, 2001).

Johne's disease is now recognized to be of serious economic and animal health consequences in domesticated ruminant species (primarily dairy and beef cattle, sheep and goats) throughout the world (Lauzi et al., 2000; Whittington and Sergeant, 2001). Johne's disease has the greatest economic impact in dairy cattle, where premature culling, reduced carcass value, decreased weight gain and milk production can result from infection.

The advent of diagnostic probes based on specific bacterial DNA sequences has allowed fastidious microorganisms, such as *M. paratuberculosis*, to be rapidly identified. This strategy requires the identification of a DNA sequence present only in *M. paratuberculosis* but not in *M. avium* or other closely related mycobacteria. Initially, this approach was based on Southern hybridization analysis, but soon thereafter PCR tests based on these principles were developed (Vary et al., 1990). The unique regions of the *M. paratuberculosis* genome most widely used for PCR are the 16S rRNA gene and the insertion element IS*900* (Ellingson et al., 2000). In general, PCR analysis has been unable to match the sensitivity of faecal culture for identifying small numbers of bacteria but attempts to increase the sensitivity of diagnostic tests have yielded promising results.

The field of proteomics was established over a decade ago with the development of simply mass spectrometry-based protein sequencing methods coupled with methods to resolve protein mixtures using a variety of gel-based and chromatographic techniques. The critical parameter that has dictated the success or failure of this field is the ability to resolve individual proteins in complex mixtures. One of the most effective ways to separate proteins in a complex mixture is by the use of two-dimensional (2D) gel-based electrophoretic separations (Pandey and Mann, 2000). Using these methods proteins are separated

according to net charge in the first dimension (isoelectric focusing, IEF) and by molecular weight in the second dimension (SDS-PAGE) (O'Farrel, 1975). 2DE has the ability to resolve a large number of proteins including those with post-translational modifications (which often cause a change in charge and/or apparent molecular weight) as well as unique forms of proteins that result from differential mRNA splicing or proteolysis (Anderson and Anderson, 1998; Cordwell, 2001). Following separation on 2D gels, proteins are stained to allow visualization, excised as gel pieces and digested in-gel with trypsin. The resulting tryptic peptides can then be extracted from the gel slice and sequenced using mass spectrometry-based sequencing methods followed by identification of proteins by database searching. In general, 2DE-MS methods are used for two primary purposes: reference mapping and protein expression profiling.

In this study, 2D PAGE was used as a tool to investigate either the sera then milk from infected and healthy animals to evidence potential biomarkers of diseases at early state.

MATERIALS AND METHODS

Blood samples were collected in a red-top BD vacutainer tubes containing no preservatives or anticoagulant. The samples were allowed to clot or to sediment at room temperature for 1 h, and centrifuged at 4℃, 1500 x g for 15 minute s, divided into three aliquots of 100 µL each one and immediately stored at -80℃ until use. After being unfrozen, the samples were centrifuged (15 minutes, 4℃ at 14000g) and the res ulting supernatant was diluted in a solution containing 8M urea, 4% CHAPS, 1% DTT, 15mM Tris, 2% Ampholine pH 3.5-10 and left to incubate under stirring for 2 hours at room temperature. Subsequently, 300µL of the same solution for sample dilution was used to rehydrate IPG-Strips pH 3-10NL 18 cm long (Amersham Biosciences). After 9 hours of rehydration, isoelectrophocusing was performed on an IPGPhor II (Amersham Biosciences) up to a total of 80 kV/h. Following equilibration, IPG-Strips were transferred on a SDS-PAGE gel (acrylamide gradient 9-16%) for second dimension separation through a Protean II xi 2-D Cell (Biorad). Finally, gels were stained by silver nitrate (analytical gels) and by colloidal Coomassie G-250 (mass compatible). After acquisition of the digitalized gel images, image analysis has been performed by Image Master 2D Platinum (Amersham Biosciences) or PD quest (BIORAD) and the different expression proteins will be identified by mass spectrometry (MALDI-TOF and MS/MS). For milk samples, fresh milk, after a brief centrifuged (10000g, 4℃, 5 min) was incubated in a solution contain 7 M urea, 2M thiourea, 4%CHAPS, 2% Ampholites, 1%DTT. Following analysis had been the same of the blood samples (D'Auria et al, 2005).

RESULTS AND DISCUSSION

Serum proteomics

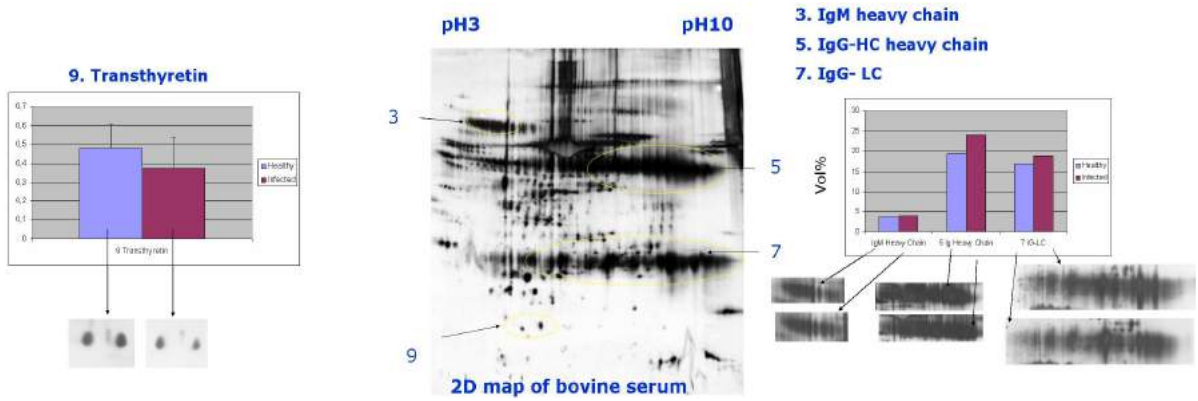
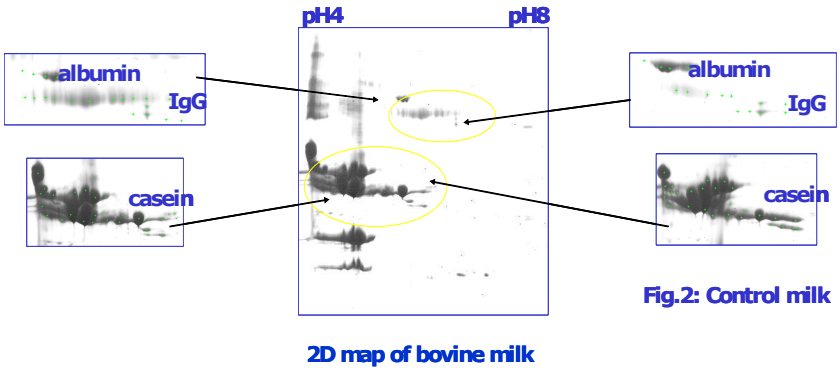


Image analysis of serum proteome from healthy and paratuberculosis infected bovine showed an increased expression of immunoglobulin IgM, IgG light chain, IgG heavy chain,

and a transthyretin decrease. In both gels about 435 spot proteins were detected and only 35 did not match each other. Blu: healthy; Violet: affected.

Milk proteomics



2D map of bovine milk

Fig.2: Control milk

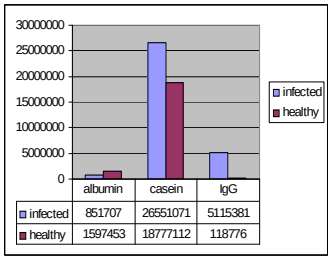


Fig.3

Quantitative analysis of coomassie-stained gels of milk from infected (left) and healthy (right) bovine has shown major differences in the area with pH 4.5. Our results (table) have shown that caseins and IgG are most abundant in milk from infected bovines that in controls milk while albumin is most represented in healthy milk.

So, proteomic analysis revealed unexpected results in term of total quantity of caseins; in serum transtyretyn increases in concentration in affected animals; this parameter will be further explorer to evaluate post translational modifications. PT modifications of proteins may be a useful early diagnostic tools.

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Innate resistance of mice to *M. avium* subsp. *paratuberculosis* is controlled by Slc11a1

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ABSTRACT

A luminescent *M. paratuberculosis* (MAP) expressing the luxAB genes of *Vibrio harveyi* has been recently developed to avoid fastidious and costly enumeration of CFU by plating. Here we have re-evaluated the effect of Slc11a1 (formerly Nramp1) polymorphism on susceptibility against MAP, using this luminometric method. A series of inbred mouse strains were infected intravenously with luminescent MAP S-23 and monitored for bacterial replication in spleen, lungs and liver for 12 weeks.

The results indicate that, as for *M. avium* subsp. *avium*, innate resistance to infection is genetically controlled by Slc11a1. In BALB/c and C57BL/6 mice (both Slc11a1^{s}) bacterial numbers in spleen remained stable during the infection, whereas in BALB/c congenic C.D2 mice (Slc11a1^{r}) and in (C57BL/6xDBA/2) F_1 (Slc11a1$^{s/r}$) the bacterial number had decreased more than tenfold during the first month. At later time points, additional differences in bacterial replication were observed among the susceptible mouse strains. Whereas bacterial numbers in the liver gradually decreased in C57BL/6 mice between week 4 and week 12, bacterial numbers remained more or less constant in liver from BALB/c mice.

INTRODUCTION

Natural resistance of mice to nontuberculous mycobacterial infections caused by *M. simiae*, *M. intracellulare*, *M. avium subsp. avium* and the attenuated tuberculosis vaccine strain *M. bovis* BCG is controlled by the natural resistance-associated macrophage protein (NRAMP1), now called SLC11A1. *Slc11a1* gene is expressed in late endosomes of macrophages derived from spleen and liver in which it regulates their antimicrobial activity. In mice, this gene presents two allelic forms, one encoding an aspartic acid at position 169 (Slc11a1^{s}, conferring susceptibility) and the other encoding glycine at this position (Slc11a1^{r}, conferring resistance). This integral membrane protein is highly conserved. Several studies have demonstrated that SLC11A1 protein is involved directly or indirectly in the maturation process of the phagosome by transport of bivalent cations (such as Fe^{2+}, Mn^{2+} or Mg^{2+}) (Vidal et al., 1993). It is still not completely elucidated how SLC11A1 controls the replication of intracellular parasites.

The role of Slc11a1 in susceptibility to MAP, the etiological agent of bovine paratuberculosis or Johne's disease, is less clear and has been studied mostly in the context of Crohn's disease. Mice are generally considered to be resistant to MAP and unsuitable for the study of this intestinal pathogen of cattle, goat, sheep and wild-life ruminants (Harris and Barletta, 2001). Some authors have reported on genetic variations in susceptibility of mice to MAP infection, but in these studies bacterial replication was analyzed by measuring hepato- or splenomegaly (Frelier et al., 1990; Tanaka et al., 194) not by actual enumeration of bacteria. The latter technique is seriously hampered by the fact that MAP requires 6-8 weeks of culture before colonies can be counted visually.

We have recently reported on the development of a luminescent *M. paratuberculosis* isolate that expresses the luxAB genes of *Vibrio harveyi* introduced by transformation with the shuttle plasmid pSMT1 (Rosseels et al., 2006). In this study, we have used this luminescent MAP isolate to re-evaluate the role of Slc11a1 in the susceptibility of a series of inbred mouse strains to intravenous MAP infection.

MATERIALS AND METHODS

Mice

Male BALB/c and C57BL/6 mice (both strains expressing the susceptible Slc11a1^s allele), BALB/c.DBA/2 (C.D2) congenic mice (strain expressing the resistant Slc11a1^r allele from DBA/2 origin on a BALB/c background) and heterozygous (C57BL/6xDBA/2)F1 mice were bred at the Pasteur Institute Animal Facilities. C.D2 mice were bred from breeding couples kindly given to us by Dr. E. Skamene (McGill University, Montreal, Canada). All mice were 2-3 months old at the time of infection.

Luminescence assay

The number of bioluminescent bacteria was determined using a bioluminescence assay with a Lumat LB 9507 luminometer (Berthold Technologies) and 1% n-decanal (Sigma) in ethanol as substrate (Rosseels et al., 2006). In this assay, only live bacteria are enumerated, because emission of light is dependent on the presence of reduced flavin mononucleotide (FMNH2), co-factor which is only found in living cells. The ratio CFU/ Relative Light Unit (RLU) for exponentially growing axenic MAP cultures was determined to be 1.2.

For statistical analysis (one way ANOVA, Tukey's Multiple Comparison Test), results obtained in RLU were converted to log10 RLU per organ.

Infection of mice

Luminescent MAP S-23 (Rosseels et al., 2006) was grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J (Allied Laboratories Inc, Synbiotics Europe, 2µg/ml) and hygromycin (100 µg/ml), to an O.D. between 0.6 and 0.8. Bacteria were centrifuged for 30 minutes at 2000 rpm, suspended in PBS to a concentration of 14 x106 RLU/ml and mice were infected intravenously in a lateral tail vein with 0.1 ml of bacteria.

RESULTS

Replication of luminescent M. paratuberculosis S-23 in mice carrying the slc11a1^s susceptibility or the slc11a1^r resistance allele.

In order to determine the influence of *slc11a1* gene on the susceptibility to MAP infection, animals from four inbred mouse strains were infected intravenously with $1.4x10^6$ RLU of luminescent *M. paratuberculosis* S-23 and monitored during 12 weeks for bacteria replication in spleen, lungs and liver. As shown in Fig 1, heterozygous (C57BL/6xDBA/2) F_1 mice and C.D2 congenic mice (expressing the resistant allele of DBA/2 origin on a BALB/c background) eliminated MAP rapidly, whereas BALB/c and C57BL/6 mice expressing the susceptible allele showed a persistent infection. In liver and spleen, higher RLU counts were measured than in lungs. At week 4 post-infection, SLC11a1^s mice showed ten- to one hundred fold higher RLU numbers in the liver and spleen than mice expressing the resistant slc11a1^r allele ($p < 0.001$).

Between week 8 and week 12 further differences between strains expressing the susceptible phenotype were detected. In the spleen of BALB/c mice, bacterial number decreased slightly but increased again up till week 12, whereas in B6, splenic bacterial number remained stable. In the liver of B6 mice, RLU counts gradually declined by week 12 to levels almost comparable to levels in resistant mice. In contrast, the number of bacteria in liver decreased much more slowly in BALB/c mice. Finally, Slc11a1^s mice showed similar (very low) RLU counts in the lungs as resistant Slc11a1^r mice.

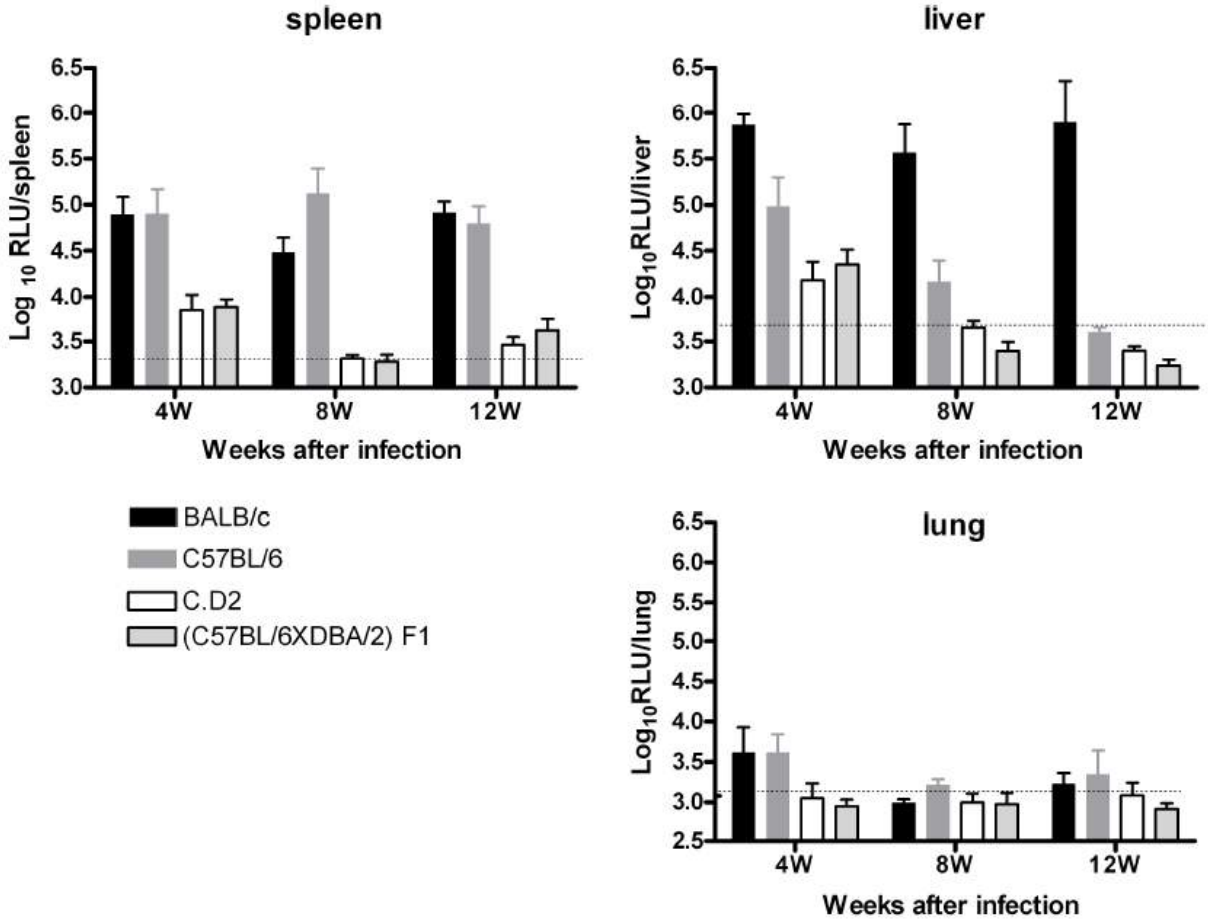

Fig. 1. Susceptibility of four inbred mouse strains to intravenous infection with luminescent *M. paratuberculosis* S-23. Mice were sacrificed at week 4, 8 and 12 weeks after infection and the number of mycobacteria in spleen, liver and lungs was determined by luminometry. Results are reported as log_{10} RLU values/organ (mean ± SD of five to six mice/group tested individually). Cut-off value (dotted line) has been evaluated by quenching quantification using organs from non infected mice.

DISCUSSION

Although genetic variation in susceptibility to MAP has been reported in cattle and red deer (Hines et al., 2004), the actual role of Slc11a1 polymorphisms in target species of Johne's disease is not clear. Some studies in sheep have shown associations of particular polymorphisms in this gene with susceptibility or resistance to infection (Reddacliff et al., 2005).The role of Slc11a1 in susceptibility of mice to MAP infection is not fully elucidated either. Chiodini and Buergelt (1993) compared three susceptible Slc11a1^s mouse strains (BALB/c, C57BL/6 and C57BL/10) using the LINDA strain isolated from a Crohn's disease patient. This study indicated that the reduction in bacterial burden was associated with the development of caseous necrotic lesions. Later, Veazey et al. (1995) compared actual colony forming units (CFU) counts in MAP infected C57BL/6 and C3H mice, which express the susceptible and resistant Slc11a1 alleles respectively, but which also differ at numerous other loci.

Here we have extended these studies and demonstrated by an easy luminometric method that Slc11a1 polymorphism indeed exerts a strong genetic influence on the innate susceptibility of mice to intravenous infection with MAP S-23, as indicated by a clear difference in bacterial replication in the spleen and liver between Slc11a1^s and Slc11a1^r mice.

Whereas initial resistance of mice to *M. paratuberculosis* appears to be controlled exclusively by Slc11a1, at later stages additional factors play a role in mice expressing the susceptible Slc11a1^s allele, particularly at the level of liver replication. Additional work is

needed to define the precise immune mechanisms involved at these later stages of *M. paratuberculosis* infection in susceptible BALB/c mice.

CONCLUSION

Experimental MAP infection and vaccination studies are seriously hampered by technical problems. Indeed, *M. paratuberculosis* is a slow growing mycobacterial species and determining the number of colony forming units (CFU) in organ homogenates has so far consisted of labourintensive plating experiments on expensive mycobactin-supplemented Middlebrook agar.

Here we have shown that a luminescent *M. paratuberculosis* isolate can facilitate these studies and could be of great benefit for future experimental *M. paratuberculosis* studies *in vivo*.

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Genetic association between bovine NRAMP1 and CARD15 genes and infection by *Mycobacterium avium* subsp. *paratuberculosis*

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INTRODUCTION

Classical methods such as treatment and vaccination can scarcely control Johne's disease, so another control strategy is necessary. The implications of environmental factors in infection development are widely acknowledged, but to achieve optimal disease prevention, we also need to take genetic factors into account, since some genetic variants provide animals with a resistance or susceptibility to infection.

Our approach to eradicating paratuberculosis, or at least to reducing its presence, is to increase this genetic resistance among animals. To that end, genes implicated in disease development must be identified. Then, animals can be selected based on their genetic resistance, so as to obtain yet more resistant animals and spread this resistance to the rest of the population through breeding.

Our main objective is to identify genetic resistance markers to paratuberculosis disease in cattle so as to determine genetic resistance patterns within improvement programs. In that sense, recognising the polygenic nature of disease is fundamental. Different common alleles with low or moderate effect on the infection could be responsible for the resistance or susceptibility of an animal to infection.

In recent years, studies in animal models, humans and livestock have indicated the role played by the genes involved in innate immunity against pathogens in resistance to infectious diseases. Of those genes, we selected Nramp1 and Card15.

Nramp1 is located in the BTA2 and comprises 15 exons. It codifies a membrane protein that works as an ion channel in phagosomes of macrophages and in neutrophils. Its function in relation to the innate immune system is to impair bacterial growth in the host. There is a considerable bibliography on genetic association between Nramp1 and intracellular infections in mammals (Adams and Templeton, 1998; Bellamy et al., 1998; Vidal et al., 1995), but not so much regarding cattle. In our previous work focusing on Nramp1, a microsatellite in the 3'UTR region called HORIN was analysed in a case-control study. The results suggested a genetic association between this polymorphism and resistance to paratuberculosis in Friesian cattle (Estonba et al., 2005).

Bovine Card15 is located in BTA18 and comprises 12 exons. It encodes an intracellular receptor whose function lies in recognising pathogen components and subsequently activating NF-kB in the cell, linked to the apoptotic pathway in response to the pathogen. The human Card15 gene has been identified as the first susceptibility gene-and indeed an important one- for Crohn's disease in humans (Hugot et al., 2001; Ogura et al., 2001). But there is hardly any information about the putative relationship between Card15 and Johne's disease.

OBJETIVES

Our objectives in the present work were to confirm the previously observed association between the Nramp1 gene and resistance to infection by MAP in an independent sample and new genetic markers, Single Nucleotide Polymorphisms (SNPs), and to analyze another putatively associated gene, Card15, with the same type of markers.

MATERIALS AND METHODS

This work was performed in two steps: first several SNPs were selected from both genes, and then a genetic association study was conducted on them. DNA from all animals was obtained using "QIAamp Mini Kit" (Qiagen). Genetic Analyzer 3100 Avant and Genetic

Analyzer 3130xl instruments (Applied Biosystems) were used to detect sequences for SNP discovery and individual genotypes for genetic association study.

SNP selection

Various tools were used for SNP selection: searches in literature and various databases (IBISS, NCBI, Ensemble, Animal Genome), and also new SNP discovery by comparative sequencing for Nramp1. For new SNP detection DNA samples from 14 different cattle breeds were used, almost all of them from *Bos taurus* species (Blonde, Limousin, Holstein Friesian, Gelbrieh, Red Angus, Jernsey, Guernsey, Salers, Pirenaica, Terreña, Betizu, and Monchina), except two crossbred with *Bos indicus* (Beefmaster and Brangus).

The sequences obtained from different breeds were compared with Nramp1 sequence from Ensemble database (Btau_3.1) using SeqScape shoftware (Applied Biosystems).

Case-control genetic association study

For the genetic association study all animals were selected from several naturally infected herds of friesian cattle. Their classification as susceptible or resistant to infection by MAP was determined by the ELISA method. A total of 136 control or resistant animals and 137 case or susceptible animals were analysed.

SNPlex technology and GeneMapper software were used to genotype the SNPs for all animals. Statistical analyses were performed first within herds, and secondly taking all the case and control animals together. They were conducted by comparing allele frequencies at two levels: individual SNPs and haplotypes, both of them with Haploview statistical software (Barrett et al., 2005), as well as the calculation of D' and r^2 parameters to analyse the linkage disequilibrium (LD) between different polymorphisms. The confidence intervals method was used to define blocks (Gabriel et al., 2002).

RESULTS

SNP selection

For SNP selection a total of 52 SNPs were studied on Nramp1. Twentyeigth of them were newly discovered in this gene. These new SNPs are mainly distributed along non codificant regions of the gene: 8 in the promotor region, 8 in different intronic regions and 9 in the 3'UTR region. Only 3 of the 28 are located in codificant regions. In Card15 a total of 17 SNPs were studied, all of them resulting from bibliographical (Taylor et al., 2006) and database searches.

Based on different criteria such as SNP frequency (MAF>0.01), Hardy Weinberg equilibrium and some methodological issues, 13 polymorphic SNPs were selected for the subsequent genetic association study in Nramp1 and 5 polymorphic SNPs for the same study in Card15.

Genetic association study

In Nramp1, SNP N23 was found to be genetically associated with resistance to the infection (p=0.0478), considering all the herds together. The frequency of the minor allele, T, was significantly higher among control animals than among case animals, so it could be considered as a "resistance allele".

For the LD analyses data concerning the HORIN microsatellite previously analysed were included. The results showed strong LD between 7 genetic markers (Fig. 1). D' and r^2 parameters were calculated: D'=1 for all the polymorphism pairs with an average of r^2=0.40**.**

These linked polymorphisms define a haplotype block. In this haplotype block, 5 different haplotypes were detected in our population and their frequencies were compared between cases and controls. Haplotype 4 showed a significantly higher presence among control animals than among case animals, suggesting a genetic association between this haplotype in Nramp1 and resistance to the infection (Table 1).

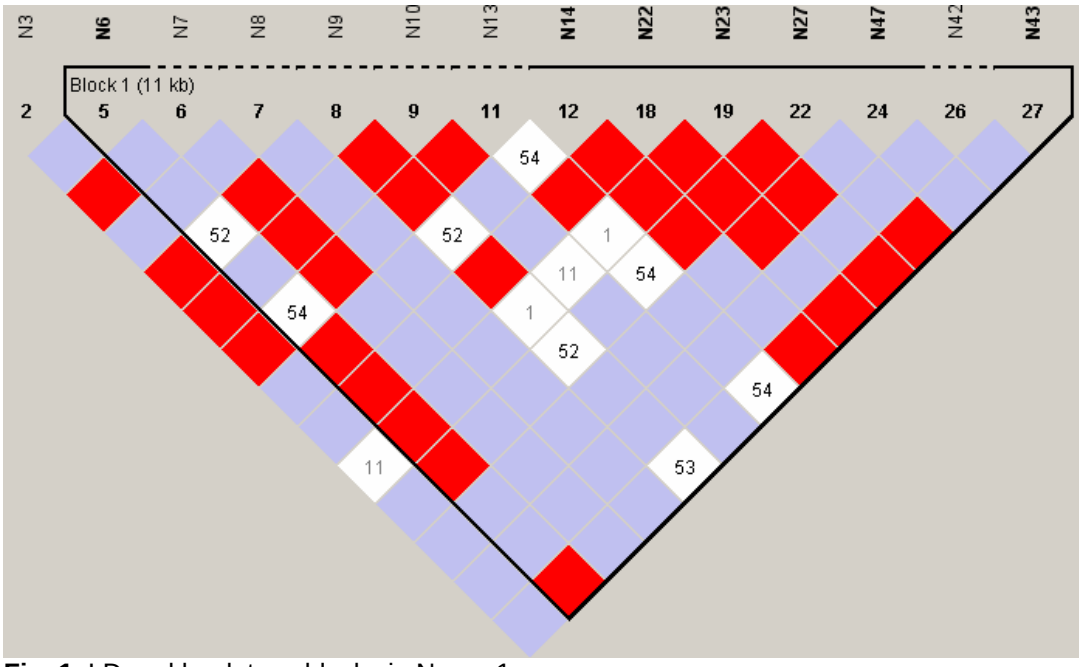

Fig. 1. LD and haplotype blocks in Nramp1

***Table 1**. Different haplotypes of the Nramp1 gene, their frequencies in the population in general, cases and controls, and p value for the genetic association.*

Haplotype	Frequency in population	Case/control frequencies	*p*-value
Haplotype 1	0.707	0.738, 0.661	0.6201
Haplotype 2	0.107	0.124, 0.082	0.8155
Haplotype 3	0.085	0.083, 0.089	1.0000
Haplotype 4	0.075	0.028, 0.146	**0.0002**
Haplotype 5	0.025	0.028, 0.022	1.0000

A genetic association was also detected between SNP C12 of Card15 and resistance to infection in one of the herds (p=0.0085). For this SNP, the frequency of the G allele was significantly higher in control animals than in case animals. Taking all the herds together SNP C25 was also found to be genetically associated with resistance to paratuberculosis (p=0.0017). The minor allele, T, is significantly more frequent among control animals.
A linkage disequilibrium analysis showed 4 SNPs linked in Card15 (Fig. 2). The D' value was 1 with an average of r^2=0.26. These linked SNPs form a haplotype block in the gene that shows 5 different haplotypes in our population. Haplotype 3 of Card15 has a significantly higher frequency in control population (Table 2), suggesting a genetic association with resistance to infection.

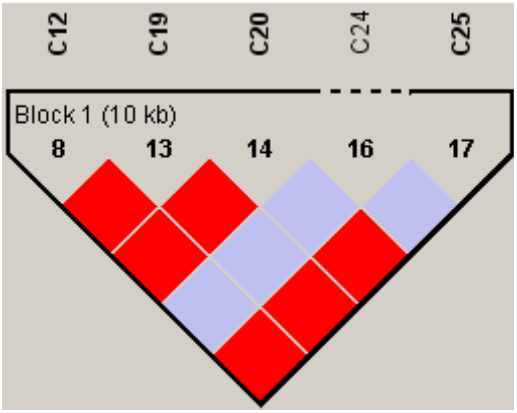

Fig. 2. LD and haplotype blocks in Card15

Table 2. Different haplotypes of the Card15 gene, their frequencies in the population in general, cases and controls, and *p* value for the genetic association.

Haplotype	Frequency in population	Case/control frequencies	*p*-value
Haplotype 1	0.410	0.433, 0.386	0.9039
Haplotype 2	0.236	0.277, 0.195	0.1642
Haplotype 3	0.145	0.096, 0.196	**0.0063**
Haplotype 4	0.141	0.137, 0.145	1.0000
Haplotype 5	0.067	0.057, 0.078	0.9750

CONCLUSION AND FUTURE STEPS

It is widely known that Nramp1 and Card15 genes work as a first defense barrier of host against pathogens such as mycobacteria, so mutations in their structure or function may result in a higher resistance to infection. Our results confirm that these two genes play a role in the resistance mechanism against Johne`s disease.

That being so, we must next direct our efforts toward understanding what function these polymorphisms may have in gene functionality or genetic expression, or even in protein structure. Furthermore, due to the polygenic nature of the disease, further candidate genes will also be analysed for their putative genetic association with resistance to infection by MAP.

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

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ABSTRACT

There is a critical need for a ruminant infection model that can reproduce the progression from subclinical to clinical disease, thereby following a pattern of infection commonly recognized in the field.

The objective of this study was to evaluate different methods of infection (oral, oral with dexamethasone pre-treatment, and intraperitoneal) of neonatal calves with either a laboratory-adapted K-10 strain or a clinical isolate of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on the pathogenesis of infection.

Calves given the clinical isolate of MAP shed higher numbers of bacteria in their feces for up to 4 months post-inoculation with only sporadic shedding noted for calves given the K-10 strain. Fecal PCR results mirrored the culture results with infrequent positive reactions after the first 4 weeks of infection, regardless of infection group. Colonization was present in a number of intestinal tissues and lymph nodes with the lowest number of affected tissues in the IP calves and the highest for calves orally dosed with the clinical isolate. Similarly, histopathologic lesions were predominantly found in the ileal and jejunal sections and their associated lymph nodes. Strain typing of the 2 isolates before and after calf infection showed that neither strain of MAP changed genotype in the host but that the genotypes were different for the laboratory-adapted and clinical strains.

These data suggest that oral inoculation remains the most effective method of experimental infection for MAP and that inoculation with a recent clinical isolate of MAP may induce more clinical signs.

INTRODUCTION

Animal models are useful to gain in-depth knowledge of the host immune response after infection with pathogens in a controlled environment. In addition, a successful animal model that mimics the natural disease state provides a testing platform for vaccine candidates and therapeutic agents. Various animal models for paratuberculosis have been developed and studied, including cattle, sheep, goats, deer and other wildlife, as well as rodents such as mice, rats, and rabbits. Attempts to develop a calf model of infection that will transition from subclinical to clinical infection in a more appropriate period of time (12 months or less) have failed (Stabel et al., 2003; Uzonna et al., 2003; Waters et al., 2003). The majority of these studies have achieved infection by oral inoculation to simulate uptake of the bacteria in the field via fecal-oral transmission. Oral inoculation of calves has successfully resulted in a subclinical infection model but has rarely resulted in the transition to clinical disease. This has dictated that we approach the development of an experimental infection model by incorporating alternate approaches to meet this need.

MATERIALS AND METHODS

Treatment groups consisted of: 1) Control, n = 4; 2) Oral, n = 4; 3) Oral/dexamethasone (Oral/DXM), n = 4; 4) Intraperitoneal (IP), n = 4; and 5) Oral/mucosal (Oral/M), n = 3. The Oral and Oral/DXM groups were fed milk replacer containing 1 x 10^{10} live *M. avium* subsp. *paratuberculosis*, strain K-10, 2x per day for 14 consecutive days. The Oral/DXM group were treated exactly the same as the oral group except the calves were administered 0.25 mg/kg BW dexamethasone IV for 3 days prior to bacterial challenge and on days 28 and 56 post-challenge. Intraperitoneal inoculation of calves was performed days 0, 7, 14, and 21 of the study. The Oral/M calves were inoculated by feeding milk replacer containing live *M. avium*

subsp. *paratuberculosis* obtained by scraping the ileal mucosa from a clinically infected cow on days 0, 7, and 14 of the study.

Fecal samples (2 g) were processed by the centrifugation and double-decontamination method previously described (Stabel, 1997). Culture of tissue samples was performed on HEYM by standard method. DNA was extracted from fecal samples using a MoBio kit and tissue DNA was extracted using a modification of the Qiagen DNA/RNA kit. Fecal and tissue DNA was subjected to 2-step nested real-time PCR with ISMap02 gene as the target. Sections of tissue were fixed in formalin, paraffin-embedded, stained with hematoxylin and eosin and Ziehl-Neelsen, and examined by light microscopy for lesions and cellular infiltrates. To determine if host-pathogen interactions affect the stability of MAP short sequence repeat loci (SSRs), individual colonies of MAP were analyzed by a method previously described (Harris et al., 2006).

RESULTS

Fecal culture and PCR data demonstrated that calves in the oral inoculation groups experienced shedding on days 7, 14, 21, and 28, indicative of “pass-through” shedding that is typically observed after large oral boluses of bacteria are administered (Table 1). Shedding was minimal and infrequent over the course of the study for calves in the Oral, Oral/DXM, and IP treatment groups. Calves in the Oral/M group shed high numbers of bacteria up to 4 months post-inoculation (TNTC). By 4 months post-infection, shedding was significant only in 1 of the 3 calves (79 cfu/slant), followed by sporadic shedding thereafter.

Fecal PCR results mirrored the culture results with infrequent positive reactions after the first 4 weeks of infection, regardless of infection group. Tissue culture results showed that calves exposed to MAP by either oral or IP methods became infected with colonization in multiple tissue sites. Colonization was present in a number of intestinal tissues and lymph nodes with the lowest number of affected tissues in the IP calves and the highest for calves in the Oral/M group. Recovery of viable MAP was low in tissues regardless of treatment group with the exception of one calf in the Oral/M whose tissues had very high numbers of MAP.

Table 1. Results of HEYM culture of tissues obtained from calves at necropsy following oral or intraperitoneal challenge with live *Mycobacterium avium* subsp. *paratuberculosis*.

		Treatment			
	Total	**Oral**	**IP**	**Oral/DX M**	**Oral /M**
Jejunum + LN	41	13	6	10	12
Ileum + LN	23	4	6	4	9
Duodenum + LN	14	4	4	3	3
Spiral colon + LN	13	5	2	3	3
Ileocecal valve	10	2	4	2	2
Spleen	9	2	4	2	1
Iliac LN	8	3	2	2	1
Transcending colon + LN	7	1	1		3
Ileoceca LN	4	1	2		1
Descending colon + LN	4	2		1	1
Mean positive tissues		10.3	8.5	9.5	15

Histopathologic lesions were predominantly found in the ileal and jejunal sections and their associated lymph nodes, as well as the ileocecal valve and node. Lesions were characterized by multifocal small infiltrates in the submucosa of intestinal tissues and small aggregates of macrophages with and without granuloma formation within the lymph nodes. Lesions were most predominant within the tissues from Oral/DXM calves and secondarily for the Oral group. Few lesions were found in the tissues of IP and Oral/M calves.

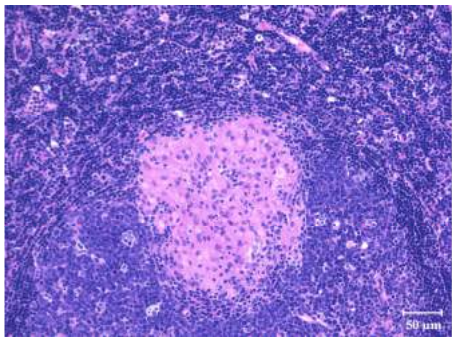

Figure 1. Histogram of lymph node section showing a typical lesion observed in infected calves

Analysis of the MAP isolates recovered from the infected calves indicated that there was no variation in the MLSSR types as compared to the original inoculum. However, strain K-10 demonstrated the genotype of >14-10-5-5, consistent with previous reports, but the clinical cow isolate yielded two similar MLSSR types; 7-9-4-3 and 7-10-4-3, suggesting that 2 strains of MAP were present in the ileum of the infected cow.

DISCUSSION

Minimal fecal shedding and level of tissue colonization were noted in this study regardless of the mode of inoculation utilized to infect the calves. Colonization was present in a number of intestinal tissues and lymph nodes with the lowest number of affected tissues in the IP calves and the highest for calves in the Oral/M group. Although many tissues were positive for the presence of MAP by either culture or PCR, the amount of viable MAP recovered was low with the exception of one calf in the Oral/M group that had very high numbers in many tissues. The predominance of lesions within the tissues of Oral/DXM calves suggests that experimental immunosuppression prior to infection may result in the inability of the host to retard localized damage due to colonization. The subsequent cellular infiltrate would result in increased levels of pro-inflammatory cytokines resulting in tissue damage. The experimental methods of infection did not result in clinical signs of disease within the 12-month period of the study, yet there were a significant number of affected tissues, either by culture, PCR, or histopathologic analyses. Although the IP method did result in infection, the oral method was the most consistent and, perhaps, natural approach of experimental exposure to MAP.

CONCLUSIONS

These data suggest that oral inoculation remains the most effective method of experimental infection for MAP in calves, and that inoculation with a low passage clinical strain of MAP may induce more clinical signs.

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Immunologic responses to *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves after oral or intraperitoneal experimental infection

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ABSTRACT

The current study was designed to compare experimental oral and intraperitoneal inoculation on early host immune responses to MAP infection. Blood samples were obtained on d -5 and -4, 7, 14, 21, 28, and monthly thereafter for the 12 month term of the study. Isolation of peripheral blood mononuclear cells (PBMC) was performed, followed by incubation with medium only (nonstimulated), concanavalin A (ConA), a whole cell sonicate of MAP (MpS), and johnin purified protein derivative (JPPD) for 24, 48, 72, or 144 hr for determination of cytokine secretion, lymphocyte proliferation, and flow cytometric analyses. Results demonstrated that oral inoculation of calves significantly increased lymphocyte proliferative responses to MpS at 12 months. Secretion of MPS-stimulated iNOS by PBMC was higher for oral infection groups at both 6 and 12 months post-infection compared to control calves. IP calves had the earliest antigen-specific IFN-γ responses at 7 d post-infection, preceding responses noted for other infection groups that followed between 90 and 120 d. Average IL-10 responses to ConA and MPS were higher at 1 and 6 months and declined significantly by 12 months post-infection. At 1 month, Oral and Oral/M calves had higher MPS-stimulated IL-10 than other treatment groups. By 12 months only the Oral/M calves had higher IL-10 secretion than control calves. Intracellular IFN-γ and IL-10 levels were measured for CD4+, CD8+, and γδ T cell subpopulations. At 3 months post-infection, there was significantly higher IFN-γ in CD4+ cells stimulated with MPS in the Oral treatment. Intracellular IL-10 was higher in CD4+ and CD8+ T cells in Oral and IP calves compared to the other treatments. Results demonstrated that oral inoculation of calves increased lymphocyte proliferative responses and iNOS secretion by PBMC stimulated with MpS. Antigen-specific IFN-γ responses were apparent for all infected calves by 90 d post-inoculation and remained elevated throughout the study. At 1 month, Oral and Oral/M calves had higher MPS-stimulated IL-10 than other treatment groups but IL-10 secretion declined by 12 months for all calves. Intracellular IFN-γ and IL-10 in T cell subpopulations stimulated with MPS was higher for calves in the Oral infection groups. T cell activation markers such as CD25, CD26, CD5, and CD45RO were upregulated in infected calves compared to noninfected controls. These results demonstrate that exposure and infection to MAP will invoke early immunologic responses characterized by IFN-γ, IL-10, and iNOS secretion.

INTRODUCTION

Infection models are useful for studying host responses to infection to provide further aid in the development of diagnostic tools and vaccines. Various animal models for paratuberculosis have been developed and studied, including cattle, sheep, goats, deer and other wildlife, as well as rodents such as mice, rats, and rabbits. The majority of experimental models for ruminants have utilized an oral inoculation of live *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in order to establish infection, thereby mimicking the fecal-oral route of transmission generally observed in the field. Attempts to develop a calf model of infection that will transition from subclinical to clinical infection in a more appropriate period of time (12 months or less) have failed (Stabel et al., 2003; Uzonna et al., 2003; Waters et al., 2003). The majority of these studies have utilized laboratory-adapted strains of MAP in the inocula, while a few studies have demonstrated more effective results with recent clinical isolates of MAP (Stewart et al., 2007). Investigators have not previously investigated the potential usefulness of dexamethasone to invoke host immunosuppression prior to oral inoculation with MAP to determine if this would cause more severe infection. In addition, although

intraperitoneal inoculation is common for the MAP mouse model it has not been attempted in larger species as a means of experimental infection. The current study was designed to compare experimental oral and intraperitoneal inoculation on early host immune responses to MAP infection.

MATERIALS AND METHODS

Treatment groups

Treatment groups consisted of: 1) Control, n = 4; 2) Oral, n = 4; 3) Oral/dexamethasone (Oral/DXM), n = 4; 4) Intraperitoneal (IP), n = 4; and 5) Oral/mucosal (Oral/M), n = 3. The Oral and Oral/DXM groups were fed milk replacer containing 1 x 10^{10} live *M. avium* subsp. *paratuberculosis*, strain K-10, 2x per d for 14 consecutive days. The Oral/DXM group were treated exactly the same as the oral group except the calves were administered 0.25 mg/kg BW dexamethasone IV for 3 day prior to bacterial challenge and on d 28 and 56 post-challenge. Intraperitoneal inoculation of calves was performed d 0, 7, 14, and 21 of the study. The Oral/M calves were inoculated by feeding milk replacer containing live *M. avium* subsp. *paratuberculosis* obtained by scraping the ileal mucosa from a clinically infected cow on d 0, 7, and 14 of the study. Throughout the study calves were monitored for lethargy, refusal to eat or drink, respiratory distress, diarrhea, weight loss.

Assays

Cells were cultured at 1.4 x 10^6/mL in 48-well flat-bottomed plates with either medium alone (nonstimulated, NS), with concanavalin A (ConA; 10 µg/ml), pokeweed mitogen (PWM; 10 µg/ml) or with MAP whole cell sonicate (MPS;10 µg/ml) for 24, 48, 72 or 144 hr (depending upon the assay) at 39℃ in 5% CO_2 in a humidified atmosphere. Cell supernatants were harvested after 24 and 48 hr and frozen at -20℃ un til analyzed for cytokine production. Cells were harvested and processed after 72 hr for lymphocyte blastogenesis and after 6 d for flow cytometric analyses. Additional blood samples were collected at each time point into vacutainer tubes and serum was harvested and stored at -20℃ for antibody titer.

Bovine IFN-γ and IL-10 production were measured in cell-free supernatants by ELISA after stimulation of PBMC with NS, ConA, and MPS for 24 and 48 hr. Similarly, bovine iNOS was measured using a colorimetric assay. Cells were stained for intracellular IFN-γ and IL-10 within CD4+, CD8+, and IFN-γ T cell subpopulations after 48 hr of incubation. Lymphocyte proliferative responses to NS, ConA, PWM, and MPS were assessed by incorporation of ^{3}H-thymidine by PBMC after stimulation of cells for 72 hr. After 6 days of incubation, PBMC were stained for CD4+, CD8+, γδ T cells, B cells, and activation markers, CD25, CD26, CD5, and CD45RO within those cell populations.

RESULTS

Measures of T cell activation such as lymphocyte proliferation and iNOS secretion were upregulated for PBMC isolated from infected calves after stimulation with MPS. Antigen-specific IFN-γ responses were apparent for all infected calves by 90 days post-inoculation and remained elevated throughout the study (Fig. 1).

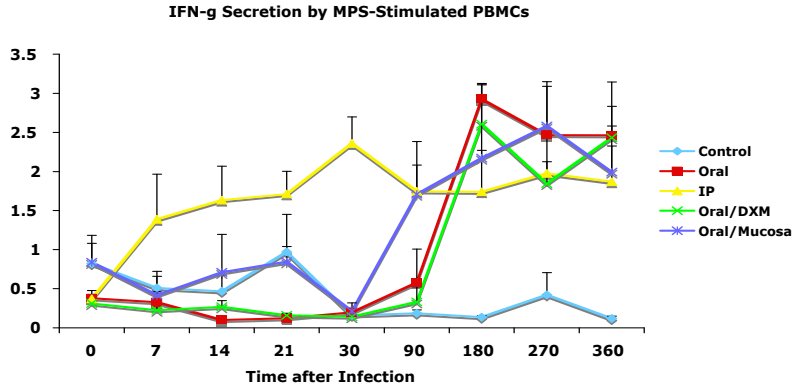

Fig. 1. IFN-γ secretion by MPS-stimulated PBMCs

Interestingly, IP calves had very robust IFN-γ responses by d 7 post-inoculation that remained steady over the 12-month period. Average IL-10 responses to ConA and MPS were higher at 1 and 6 months and declined significantly by 12 months post-infection, regardless of treatment group (Figure 2). At 1 month, Oral/M calves had significantly higher MPS-stimulated IL-10 compared to other treatment groups. By 12 months, only the Oral/M calves had higher IL-10 secretion than control calves. Intracellular IFN-γ and IL-10 levels were measured for CD4+, CD8+, and γδ T cell subpopulations. At 3 months post-infection, there was significantly higher IFN-γ in CD4+ cells stimulated with MPS in the orally inoculated calves. Early in the study (< 3 months) intracellular IL-10 was higher in CD4+ and CD8+ T cells for Oral/M and IP calves compared to the other treatments. By 12 months, intracellular IL-10 was higher in CD4+, CD8+, and γδ T cells from Oral/M calves after stimulation of cells with MPS.

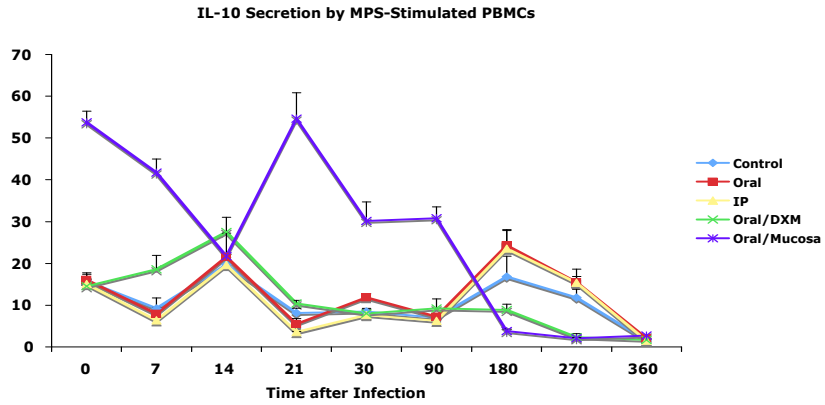

Fig. 2. IL-10 secretion by MPS-stimulated PBMCs

Experimental infection of calves resulted in the upregulation of activation markers on T cell subpopulations with similar expression patterns noted for CD4+, CD8+, and γδ T cell subsets. Figure 3 represents the increased expression of CD25 within the CD4+ subpopulation after stimulation of PBMC with MPS for 6 days. Infection of calves resulted in significant increases in CD25 expression for CD8+ and γδ T cells as well (data not shown). Similar upregulation of CD26, CD45RO, and CD5 activation markers was also noted on total PBMC populations and T cell subpopulations for infected calves throughout the study (data not shown).

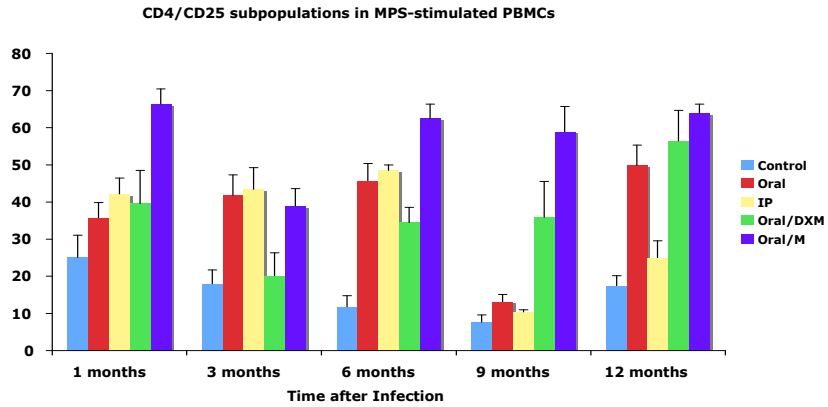

Fig.3. CD4/CD25 subpopulations in MPS-stimulated PBMCs

DISCUSSION

Experimental infection with MAP, by either oral or intraperitoneal routes, resulted in the upregulation of early cell-mediated immune responses by the host as demonstrated by the

robust antigen-specific IFN-γ responses observed for infected calves. IP calves responded by d 7 post-inoculation but calves in the oral infection groups did not respond consistently until d 90 of the study. The recall response to MAP antigen was observed in infected calves by increased expression of activation markers CD25, CD26, CD45RO, and CD5, regardless of T cell subpopulation. IL-10 secretion was higher in the early months of the study and declined steadily thereafter. This does not fit the paradigm of the Th1-Th2 shift in immunity during MAP infection but calves in this study did not demonstrate signs of advanced infection as antibody titers were negligible (data not shown), fecal shedding was minimal, and no clinical signs were observed.

CONCLUSIONS

Oral inoculation with a laboratory-adapted or clinical strain of MAP evoked strong cell-meditated immune responses in the host. IP inoculation may prove useful in the evaluation of acute immunologic responses to MAP infection as responses were similar to those for oral infection methods in this study, particularly in the first months post-inoculation. Useful immunologic markers of subclinical or early MAP infection for cattle as observed in this study were IFN-γ, iNOS, and T cell activation markers, CD25, CD26, CD45RO, and CD5.

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Presence and characterization of *Mycobacterium avium* subspecies *paratuberculosis* from vaginal secretions of post-parturient farm goats, using culture, IS*900* PCR and ELISA kit

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INTRODUCTION

Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is most serious infection of animals and human beings worldwide. Disease has significant impact on livestock economy and losses exceed $1.5 billion to US cattle industry every year. Despite low per animal productivity, in India these losses have not been estimated in the 120 millions goats. Johne's disease is endemic in farm goat herds in Mathura region and high prevalence has been reported from farm goats (Singh et al. 1996, Singh et al. 2007). These studies reported high prevalence of MAP in fecal samples. Principally disease is confined to the small intestine near ileo-caecal junction (ICJ) and associated mesenteric lymph nodes (MLN). But MAP disseminate to other tissues and organs, as evidenced by the isolation of MAP from milk, fetus, lung, semen, male accessory genital organs and semen in bulls (Larsen and Kopecky, 1970), semen in rams (Eppleston and Whittington, 2001), tissue fluid from fetus (Buergelt et al. 2006). However, information on distribution of MAP to other tissues, organs in goats is limited and in vaginal secretions are non-existent. Presence and distribution of MAP to different organs and vaginal discharges is crucial to endemicity of infection within a population. Study is maiden attempt to estimate comparative presence and recovery of MAP in vaginal secretions of post parturient goats by culture and IS*900* PCR tests *vis a vis* status of Johne's disease in these goats by ELISA.

MATERIALS AND METHODS

Animals and samples

Simultaneously, vaginal secretions (VS) and serum of 29 post-parturient goats of farm herds (CIRG, Mathura) were collected aseptically. VS were screened by culture and IS*900* PCR and serum by indigenous ELISA kit.

Culture of Vaginal Secretions

Culture method of Singh et al. (1996) was adopted for screening of VS. MAP colonies were primarily identified on the basis of mycobactin J dependency, acid fastness and slow growth.

DNA isolation and IS900 PCR (decontaminated pellets/cultures)

A modified method of isolation of DNA was used; wherein the decontaminated sediment (<1.0 ml) left after inoculation of HEYM medium was pelleted by centrifugation at 10,000 rpm for 10 minutes. Pellet was washed in PBS and used for isolation of DNA. Pellets and cultures were processed for DNA isolation as per van Soolingen et al. (1991). DNA was amplified by PCR using specific IS*900* primers (Vary et al. 1990). Presence and yield of PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis.

Screening of serum samples by ELISA kit

Serum samples were screened using indigenous ELISA kit developed for screening of goats (Singh et al., 2007), using semi-purified soluble protoplasmic antigen (PPA) from native MAP 'Bison type' genotype of goat origin. OD values were transformed to S/P ratios. Animals in strong positive and positive groups (S/P ratio) were considered positive for Johne's disease. An animal was categorized as positive for JD if any of vaginal swab) was positive in culture or PCR.

RESULTS

Screening of VS from 29 post-parturient goats showed that 37.9% were positive in culture. Comparatively, MAP colonies were isolated from VS in 13.3, 75.0 and 50.0%, of Barbari, Jamunapari and Sirohi breeds of goats, respectively. However, in S/P ratios (ELISA kit), considering goats in strong positive and positive categories, as positives, 55.1% goats were positives. Individually, 6 (40.0%), 7 (87.3%) and 3 (50.0%) goats of Barbari, Jamunapari and Sirohi breeds were positives, respectively (Table 1). The 37.9% goats each were positive in culture and ELISA. In S/P ratio, 11, 5, 2, 8 and 3, were strong positive, positive, low positives, suspected and negatives, respectively.

There was poor correlation between 11 (37.9%) positives in ELISA and VS culture (18.1%). However, of 15 (51.7%) serum in positive, low positive and suspected categories, 9 (60.0%), were positive in culture (Table 2). Culture and ELISA together detected 20.6% of 29 goats positive. The 17.2 and 34.4% goats were detected independently in culture and ELISA, respectively (Table 3). Of 29 decontaminated pellets of VS, DNA was obtained from 10 and 4 (13.7%) were amplified in IS*900* PCR. Comparison of 10 goats (VS and serum), by culture, ELISA and PCR revealed none was positive in 3 tests. One each was positive in combination of culture and ELISA and culture and PCR. Independently, 1 and 3 samples were positive in ELISA and PCR, respectively.

Table 1. Distribution of MAP in vaginal secretions (culture) and serum (ELISA kit) of goats from farm herds

Goat Breeds	Goats Screened	Positives	
		Culture of VS	ELISA kit
Barbari	15	2 (13.3)	6 (40.0)
Jamunapari	8	6 (75.0)	7 (87.5)
Sirohi	6	3 (50.0)	3 (50.0)
Total	29	11 (37.9)	16 (55.1)

Numbers in parenthesis are percents, VS – Vaginal Secretions

Table 2. S/P ratios and culture of vaginal secretion of post parturient goats

Disease Status	S/P ratio range	Number of goats	Positive in culture
Negative	00.00 – 00.09	3	-
Suspected	00.10 – 00.24	8	4
Low positive	00.25 – 00.39	2	1
Positive	00.40 – 00.99	5*	4
Strong positive	01.00 – 10.00	11*	2
Total		29	11

* Positive

Table 3. Comparison of culture (vaginal swabs) and ELISA for the presence of MAP infection in goats

Tests	Combinations			
	1	2	3	4
Culture	+	-	+	-
ELISA	+	-	-	+
Total (29)	6 (20.6%)	8 (27.5%)	5 (17.2%)	10 (34.4%)

DISCUSSION

MAP has been reported from supra-mammary lymph nodes (SMLN), milk samples, mammary glands, udder, uterus, testis, semen, of cows, bulls, deer, goats (Buergelt et al. 2006 and Sweeney et al. 1992). MAP is endemic in farm goatherds (Central Institute for Research on Goats, Mathura) using sensitive tests (Singh et al. 1996). Prevalence of MAP was high in kids as compared to adult animals (Kumar et al. 2007). High presence of MAP was reported in non-target (uterus and udder) than in target organs (intestine and MLN) and SMLN tissues of farm goats. These studies showed that MAP was widely distributed in different tissues and organs of animals correlated with high prevalence and endemicity of MAP in these herds (Singh et al. 1996).

Presence and distribution of MAP in tissues of kids (<6 months) showed that infection of animals occurred in fetal life. Hines et al. (1987) reported dissemination of MAP, from enteric sites to other organs. Study reported high presence of MAP in vaginal secretions by culture. In ELISA kit 55.1% goats were detected positive. There was poor correlation (37.5%) between culture of VS and ELISA kit (18.1% in strong positives) but was 80.0% in case of positives in kit. Similarly, correlation was high (50.0%) in weak positives and culture and suspected and culture (Table 2). This may be due to pregnancy (immunosupressive) and endemic JD (a protein loosing enteropathy damages immune system). Immune suppression has not been reported in goats with respect to MAP infection. The study reports suppression of immune response in goats due to JD. Singh et al. (2007) reported no animal in strong positive category in ELISA in advance clinical cases of JD in an outbreak. Wherein, correlation was 71.4% (high) in positive and cultures and 80.9% (very high) in low positives, suspected and negatives with culture. This may be due to severe loss of protein in diarrhoea and damage to immune system by prolonged endemicity of MAP infection and severe nutritional stress. However, in normal conditions of health and nutrition the correlation between strong positive and culture was high and with other categories (positives, low positives, suspected and negatives) and culture was low (Kumar et al. 2007). Recovery of MAP from low positives, suspected and negative goats showed the irreparable damage to the immune system of goats due to JD. Presence of highly enlarged mesenteric lymph nodes at necropsy of kids from farmer's (Kumar et al. 2007) and farm herds was common.

Information was based on limited data but presence of MAP was high in vaginal secretions of 3 important goat breeds from herds' endemic for JD (Singh et al. 1996). Positivity in culture and ELISA was highest in Jamunapari followed by Sirohi and Barbari breeds of goats. Study showed that vaginal secretions after parturitions in endemic goat herds are potential source of contamination to newborn kids, animal handlers and environment. Due to lack of symptoms infection by MAP usually goes un-noticed.

Comparison between culture of vaginal secretions and ELISA showed 48.2% correlation (positive–20.6% and negative-27.5%), whereas, 17.2 and 34.4% goats were detected independently in culture and ELISA, respectively (Table 3). However, comparison of 3 tests (culture, ELISA and PCR) showed that PCR performed better in comparison to culture of vaginal secretions. This is the first report confirming the MAP using IS*900* PCR in vaginal secretions. Recently, MAP has been recovered from *in-utero* infection of pregnant cow and from amniotic fluid (Buergelt et al. 2006).

Positive reaction in IS*900* PCR from vaginal secretions indicated chances of infection of fetus in pregnancy (Buergelt et al. 2006) and at parturition. Sweeney et al. (1992) reported that distribution takes place in clinically infected animals up to 35.0% into milk and up to 40.0% *in utero* to fetus. Vaginal secretion was convenient material for screening of goats and good yield of DNA was obtained using a swab. Sensitivity of PCR was lower than culture and could be improved by amplifying, all DNA samples, irrespective of quality and also by diluting DNA. Screening by PCR was important since it detected many VS missed by culture. In this study DNA isolation was standardized from very small quantities ($<10^{1-3}$ colonies per gram of tissue). Amplification of DNA from MAP colonies confirmed identity of cultures as MAP.

Detection of MAP DNA in vaginal secretions showed transmission by routes other than fecal–oral. Extra-intestinal pathway (vaginal secretions) should be addressed when planning control of disease.

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

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INTRODUCTION

Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a serious infection of animals and human beings worldwide. The disease is commonly reported both from farm and farmers' stocks in Mathura region of North India (Singh et al. 1996, Singh et al. 2007a; 2007b). A high prevalence was reported in faecal samples. Before faecal shedding or an immune response is present, tissue culture can detect MAP in multiple organs. Traditionally, the intestine near the ileo-caecal junction (ICJ) and associated mesenteric lymph nodes (MLN) are examined for the presence of MAP (Tripathi and Parihar, 1999). Information on distribution of MAP to other tissues (non-target) is scarce in India. The distribution of MAP to different tissues was crucial to endemicity of infection within herds.

The current study estimated presence of MAP in target (intestine and mesenteric lymph nodes) and non-target tissues (supra mammary lymph nodes (SMLN), udder, uterus and testes) of young and adult goats and sheep belonging to farm and farmer's stocks using culture and IS*900* PCR.

MATERIALS AND METHODS

Animals and samples

Sixty one goats and sheep from farm stocks (44 necropsied at Central Institute for Research on Goats CIRG), Mathura and farmers' stocks (17 sacrificed at slaughter house Mathura) between 29.01.05 to 01.04.05 were sampled. The 215 tissues (intestines near ileo-caecal junction, MLN, SMLN, udder, uterus and testes) were collected from 41 goats and 20 sheep. Of 61 animals, 16 goats, 17 kids, 10 sheep and 1 lamb were from farm stock and 8 goats and 9 sheep from farmer's stocks. Of the 44 animals necropsied at CIRG (farm stock), 40, 39, 22, 19, 19, 14, and 11 tissues were collected from intestines, mesenteric lymph nodes (MLN), left supra mammary lymph node (L-SMLN), right SMLN (R-SMLN), uterus, udder and testes, respectively. The 16, 17, 9, and 9 tissues from MLN, intestine, L-SMLN and R-SMLN, respectively were from 17 sacrificed animals (Mathura).

Culture of tissues, DNA isolation and IS900 PCR

The culture method of Singh et al. (1996) was followed for screening of tissues. Colonies were primarily identified on the basis of mycobactin J dependency, acid fastness and slow growth. A modified method of isolation of DNA was used. Decontaminated sediment (<1.0 ml) left after inoculation of solid medium was pelleted after centrifugation at 10,000 rpm for 10 minutes. Pellets were washed in PBS and used for isolation of DNA. Pellets and colonies were processed for DNA isolation as per van Soolingen et al. (1991). DNA was amplified by PCR using specific IS*900* primers. Presence and yield of specific PCR product (229 bp) was analysed by 1.8% agarose ethidium bromide gel electrophoresis. An animal was considered positive for JD if any of tissues (intestine, MLN, SMLN, udder, uterus and testes and vaginal swab) was positive in culture or PCR.

RESULTS

Of the 215 target and non-target tissues processed from farm and farmer's stocks, live cultivable MAP was recovered from 48% tissues (46% target and 50% non-target tissues). The prevalences of MAP were 51, and 42% in tissues of goats and sheep, respectively. MAP was isolated from 49 and 43% tissues from farms and farmers' animals, respectively. Screening of tissues of young and adult animals, 60 and 44% yielded MAP, respectively.

Species-wise prevalences of MAP were 55 and 42% in goats and sheep population (farm and farmer's stocks), respectively. The prevalences of MAP were 53 and 43% in animals from farm and farmer's stocks, respectively.

Target tissues
MAP was isolated from 46% target tissues (48% farm and 39% farmer's stocks).

Farm Animals
MAP was present in 48% tissues of animals (goats: 51%; sheep: 40%). In goats, MAP was recovered from 48% adults and 54% young animals. From MLN and Intestine, MAP was isolated from 44 and 53% goats and 43 and 64% kids, respectively. In sheep; MAP was present in 33 and 100%, each of MLN and Intestine, in case of adults and lambs, respectively. Comparative distribution of MAP in MLN and intestine (target tissues) was evaluated using culture. MAP was recovered from 69% farm animals of the total 39 farm animals screened. MAP was recovered from both the tissues in 28% animals and independently in MLN and intestine of 15 and 26% animals, respectively.

Farmer's Animals
MAP was recovered from 39% tissues of farmer's animals (goats: 34% and sheep: 47%). From MLN and Intestine, MAP was present in 38 and 25% tissues of goats and 38 and 56% tissues of sheep, respectively. Comparatively MAP was distributed in the 59% target tissues (MLN and intestine) of animals. MAP was recovered from both the tissues in 17.6% animals and separately in MLN and Intestine of 18 and 24% animals, respectively.

Non-target tissues
Of the 103 non-target tissues processed, MAP was recovered from 50% tissues (51% farm and 50% farmer's animals.

Farm goats
Of the 39 tissues screened from goats, 49% were positive for MAP in culture. MAP was recovered from 36, 36, 67, 56% and none, tissues of L-SMLN, R-SMLN, uterus, udder and testes, respectively. In kids, of 19 tissues screened, 63% were positive for MAP. MAP was recovered from 50, none, 100, 50 and 60% tissues of L-SMLN, R-SMLN, uterus, udder and testes, respectively.

Farm sheep
Of 26 tissues screened from sheep, 42% were positive. MAP was recovered from 33, 33, 33, 44, 60, 33% and none tissues of MLN, intestine, L-SMLN, R-SMLN, uterus, udder and testes, respectively. Of the 1 lamb screened, the intestine, MLN and uterus were positive for MAP in culture.

Farmer's animals
MAP was isolated from 75 and 30% tissues of goats and sheep, respectively. In goats, MAP was recovered from 38, 25 and 75% tissues of MLN, intestine and SMLN, respectively. In sheep, MAP was recovered from 36, 56 and 30% tissues of MLN, intestine and SMLN, respectively.

Gross lesions and culture (Target tissues, Farmer's stocks)
In Young animals prevalence of MAP was 45 and 33% in normal and inflamed MLN, respectively. The 62% tissues were positive from normal and one tissue from inflamed intestine. In adult animals, MAP was recovered from 44% normal and 38%, inflamed MLN tissues. The 14% normal and 60% inflamed intestinal tissues were positive.

Non-target tissues (Supra-mammary lymph nodes)
In Farm stock, the 37% of 43 SMLNs screened were positive for MAP. Recovery of MAP was 65 and 32 % from inflamed and normal SMLN, respectively. In Farmer's stock, MAP was isolated from 50% of 18 SMLN screened (Normal SMLN: 71 % and inflamed: 36%).

Uterus, Udder and Testes
In farm stock, MAP was cultured from 74, 50, and 55% of uterus, udder and testes tissues, respectively. Prevalences of MAP were 77, 73 and 55% in uterus, udder and testes of adult goats, respectively. In kids, prevalences were 100, 100 and 60 % in uterus, udder and testes, respectively. MAP was recovered from 67 and 33% of uterus and udder, respectively. Uterus of a lamb was positive.

Fig. 1. Supra mammary lymph nodes, where right supra mammary lymph node (right) is swollen and positive for MAP in culture and PCR

Screening of tissues by IS900 PCR (Target tissues)
In intestines, of 50 decontaminated pellets, DNA was recovered from 12, and out of these 3 were amplified by IS*900* PCR. Two MAP cultures from intestines also amplified in IS*900* PCR. In MLN of 56, decontaminated pellets, DNA was isolated from 12 and 8 (67%) were amplified in IS*900* PCR and of the 4 cultures, 1 was amplified.

Non-target tissues
In SMLN of 61 decontaminated pellets, DNA was isolated from 16 and 12 (75%) were amplified. Of the 5 MAP cultures, 1 (pauci-bacillary) was amplified. In udder of 14 decontaminated pellets, DNA was isolated from 5 and 3 (60%) were amplified. From cultures, none of the DNA was amplified. In uterus of 19 decontaminated pellets, DNA was isolated from 8 and 3 (38%) were amplified. In testes of 11 decontaminated pellets, DNA were recovered from 6 and 1 were amplified. From culture none was amplified.

DISCUSSION
Screening of target and non-target tissues by culture revealed high prevalence of MAP in goats and sheep in Agra region including Mathura (North India). Species-wise, the prevalence was higher in goat tissues and as compared to sheep. Goats and sheep belonging to farm stocks had higher susceptibility to MAP infection as compared to farmer's stocks. The high prevalence of MAP in tissues showed high pathogenicity. Farm stocks were reported to be endemic for MAP (Kumar et al 2007). Tripathi and Parihar (1999) reported a low prevalence of MAP in goats. The prevalence of MAP was high in kids and lambs as compared to adult animals (Kumar *et al* 2007). Screening of both MLN and intestinal tissues provided better estimates of MAP prevalence. In goats (both adults and young) recovery of MAP was higher in intestinal tissues as compared to MLN. The distribution of MAP in non-target tissues of farm and farmer's stocks were either comparable or were higher than in target tissues. The prevalence of MAP was higher in uterus and udder compared to target tissues and supra mammary lymph nodes (SMLN). Sweeney et al. (1992), detected MAP in culture of SMLN and milk from cows. Disseminated infections have been documented in sheep (Carrigan and Seaman, 1990). MAP was isolated both from normal and inflamed tissues. Buergelt et al. (2004) reported that unlike intestinal tissues, other organs did not elicit typical inflammatory response to presence of MAP. Inflammatory lesions were seen in MLN, SMLN (left or right or both and intestinal tissues in this study. SMLN was comparable tissue to estimate JD in female animals. Presence of MAP in testes of young kids showed need for screening of semen of in breeding age (Buergelt et al., 2004). MAP in udder tissues correlated with excretion of MAP in milk. The presence of MAP in tissues of young kids (<6

months) confirmed infection of kids in foetal life, and distribution of MAP in different tissues. Hines et al. (1987) reported dissemination of MAP from enteric sites to other organs. Despite long growth period and higher requirement of MAP shed into faeces (10^6 cfu/gram) culture from tissues has been largely recommended. Comparatively, colonies from sheep grew more slowly than from goats. MAP has been recovered from in-utero infection of pregnant cow and amniotic fluid of cattle (Buergelt et al., 2006). This study showed that decontaminated tissues left after inoculation of solid medium (0.5-1.0ml) was good starting material for isolation of DNA. Positive reaction in IS*900* PCR from all types of tissues indicated wide distribution of MAP in animals, as was also seen in culture. Though in this study the sensitivity of PCR was lower than culture but it identified few new animals. PCR performed better in MLN and SMLN tissues than in other tissues. It may be due to less contamination of DNA with inhibitory ions from these tissues as compared to intestine, uterus and udder. Poor sensitivity might have been due to presence of fewer bacilli in tissues or insufficient samples (pellet) volume.

Contamination of DNA by PCR inhibitors interferes with amplification processes (Gowzdz et al. 1997). The sensitivity of PCR could be improved by amplifying all the DNA samples, irrespective of quality of DNA and also by diluting template DNA. In this study DNA isolation was standardized for very small quantities ($<10^{1-3}$ colonies per gram of tissue). Specific amplification of DNA from MAP cultures using IS*900* PCR confirmed the identity of these cultures as MAP. Detection of MAP DNA by PCR in target and non-target tissues and vaginal secretions showed transmission by routes other than traditional fecal-oral. Extra-intestinal pathway such as milk (udder and SMLN), in-uterus (semen and vaginal secretions) should be addressed when considering control of JD.

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

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Crohn's disease (CD) is a clinically defined syndrome of unknown etiology. Although the etiology of CD is not fully understood, genetic and epidemiological studies indicate individuals with one or more allelic variants of genes involved in regulation of the immune response may be at higher risk for developing CD. The factors that induce persistent immune mediated inflammation of the bowel in these individuals are thought to include exposure to specific pathogens, bacteria present in normal microflora of the intestine, or other undefined factors. The mechanisms of induction and persistence of inflammation appear to include modulation of the immune response by regulatory T cells and activation of the IL-23-IL-17 pathway that promotes chronic inflammation. *Mycobacterium avium* subsp. *paratuberculosis (Map)* is the pathogen most frequently implicated in playing a role in the pathogenesis of CD. The implications are due to similarities in pathogenesis between CD and Johne's disease in cattle and the increased frequency of finding *Map* in CD patients over patients of other inflammatory bowel diseases. These findings could indicate *Map* plays a role in pathogenesis in a subset of genetically susceptible individuals with CD or that, individuals with CD are serving as sentinels that reveal the prevalence of *Map* in the environment.

Elucidation of the mechanisms of pathogenesis mediated by *Map* in its natural host could provide an opportunity to clarify the role of *Map* in CD pathogenesis and also insight into the sequence of events leading to erosion of protective immunity and development of clinical disease. To explore this possibility, we developed a bovine cannulated ileum model to analyze the immune response to bovine and human isolates of *Map* and determine how *Map* modulates the immune response during the early and late stages of disease. Comparative studies have revealed no significant differences in the capacity of bovine and human isolates of *Map* to infect calves. Both types of isolates elicit a prominent CD4 memory T cell response to PPD and soluble *Map* antigens detectable by flow cytometry 3 months post infection (PI). Morphologic changes in the ileum consistent with disease progression are detectable by 8 – 10 months PI. Although difficult to culture from tissue biopsies, presence of *Map* is detectable by PCR. Quantitative RT-PCR has revealed a complex pattern of expression of genes encoding IFN-γ, IL-17, and granulysin in experimentally infected animals 10 – 12 months PI indicating the presence of CD4 memory T cells associated with a Type I immune response and Th17 CD4 T cells associated with development of a proinflammatory response. The increase in expression of granulysin suggests the presence of effector memory T cells with bactericidal activity. The findings indicate a detailed analysis of immunopathogenesis of JD will facilitate understanding the role of *Map* in the pathogenesis of CD.

Signal transduction in *Mycobacterium avium* spp. *paratuberculosis*

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Signal transduction is a ubiquitous mechanism responsible for cell adaptation to environmental changes in both prokaryotes and eukaryotes. Cellular responses to the dynamic changes in the environment are mediated by a cascade of events involving protein kinases, which activate protein substrates by ATP-dependent phosphorylation on specific residues such as histidine and aspartate in prokaryotes (two-component systems) and serine/threonine, or tyrosine in eukaryotic or eukaryotic-like protein kinases. The reverse regulation of kinases (dephosphorylation) is mediated by protein phosphatases. Protein phosphatases participate in modulating a variety of cellular events such as metabolism, gene transcription, cell cycle control, immune response, and cell growth, etc. In addition, protein phosphatases have also been associated with virulence contributing to the intracellular survival of pathogens. For example, the tyrosine phosphatase YopH of *Yersinia pseudotuberculosis* dephosphorylates host proteins, the tyrosine phosphatase SptP from *Salmonella typhimurium,* which is translocated into the host, causes a disorganization of the actin cytoskeleton, while Stp, a serine/threonine phosphatase from *Listeria monocytogenes* dephosphorylates the host elongation factor EF-Tu.

Signal transduction in *Mycobacterium avium* spp*. paratuberculosis* (Map) is regulated according to the annotated genome by twelve two-component systems based on signal-transducing histidine kinases, nine serine/threonine protein kinases, five proteins containing serine/threonine kinase catalytic domains, and two tyrosine phosphatases. Interestingly, the annotated genome does not possess a defined tyrosine kinase, suggesting that both proteins might act in the host upon infection. Recently, we have reported that map1985 is a functional low-molecular tyrosine phosphatase, which is secreted intracellularly upon macrophage infection. Then, interfering with the host signal transduction could contribute to the pathogen survival in macrophages.

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

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

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

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Paratuberculosis is a chronic granulomatous inflammation of the small intestine of cattle and other ruminants, caused by infection with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). The disease can be found in ruminant herds worldwide, causing substantial economic losses at farm level due to premature culling and production losses. We have documented previously that mycobacterial heat-shock proteins (Hsp) are dominant antigens in various stages of bovine paratuberculosis. Especially the 70 kD Hsp (Hsp70) induces cell mediated responses in natural infection. Furthermore, recombinant MAP Hsp70 has been shown to be a successful subunit vaccine against bovine paratuberculosis. Surprisingly the main hallmark of vaccination induced immunity was antibody production rather than T cell immunity.

To explore the immunological mechanisms of induction of early cellular responses at the local draining lymphnodes within days after vaccination we adopted a murine model. Balb/c mice were vaccinated with Hsp70 and DDA adjuvant (Hsp70/DDA), comparable to the cattle vaccine. OVA/DDA was used as a control treatment. BrdU incorporation was measured by flowcytometry 4 and 7 days after vaccination with either vaccine. In addition lymph node cells and splenocytes were restimulated in vitro to address the functional differentiation of the immune response as measured by in vitro restimulation and antibody production.

Enhanced BrdU incorporation was observed in draining lymphnodes of mice that were immunized with Hsp70 compared to OVA treated mice 7 days after immunization. No differences in BrdU incorporation were observed in non-draining lymphnodes or at day 4 after immunization. Cellular proliferation following in vitro restimulation at 7 days after vaccination indicated equal responses against OVA and Hsp70. In addition, in vitro B cell restimulation showed an enhanced antigen specific B cells response in the draining lymph nodes only after Hsp70 vaccination at day 7, whereas B cells isolated from OVA treated mice did not produce significant amounts of antibodies in an antigen specific fashion.

Similar to the immunization outcome in cattle in the murine model there is a preferential activation of B cell activity following subcutaneous Hsp70/DDA vaccination. Therefore, the murine model presented in this study offers a convenient means to study the mechanism leading to this immuneresponse bias which is opposite to Hsp70 immune responses in natural infection and yet confers protective immunity to paratuberculosis.

Apoptosis of mononuclear cells in experimental and natural ovine Johne's disease

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Cell death by apoptosis is a part of normal development. Apoptosis also controls the immune response and is involved in the cytotoxic killing of infected cells. Cell-mediated immune responses play an important role in mycobacterial disease. Increasing our current knowledge of the immunological mechanisms involved in disease progression, including apoptotic responses, may allow advancements in the area of early diagnosis, identification of resistant animals and disease control.

For experimental infection, Merino lambs (randomly drafted into groups of 20) were challenged with either $3.36x10^8$ CFU of a clonal inoculum (Telford) or $3.76x10^8$ CFU of a gut homogenate from an animal with clinical ovine Johne's disease (OJD). One group received no treatment. Sequential blood samples were taken over a period of 12 months. Faecal shedding and presence of *Mycobacterium* avium subsp. *paratuberculosis* (Mptb) in tissue samples were determined by culture in a radiometric system (Bactec). Diseased sheep were categorised based on histological lesion type. In addition, samples were taken from sheep sourced from OJD-infected and disease-free farms.

Mononuclear cells were isolated from peripheral blood using density gradient centrifugation. Isolated cells were incubated with medium alone, Mptb antigen (10 μg/ml) or Con A (10 μg/ml) for up to 6 days. Caspase activity, a marker of apoptosis, was determined by flow cytometry on day 6 of culture. Phenotype markers were also used to identify specific apoptotic lymphocyte subpopulations. Apoptosis in intestinal tissue was studied by TUNEL assay and by the expression of apoptosis-related genes by Q-PCR.

Mptb antigen-driven apoptosis of mononuclear cells in experimentally challenged animals varies as disease progresses. There were also significant differences in the presence of apoptotic cells, as well as expression of apoptosis-related genes, in intestinal tissues from disease-free sheep and those with histological lesions.

DNA Cocktail Vaccination Induces Th1 Response and Protects Mice against *Mycobacterium avium* subsp. *paratuberculosis* Challenge

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Several novel antigens of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), have been studied as vaccine components and their immunogenicity has been evaluated. 85 antigen complex (85A, 85B, and 85C), Superoxide dismutase (SOD), and 35kDa protein of MAP has been found to induce significant lymphocyte proliferation as well as Th1-associated cytokine response. Based on these results, we cloned and expressed 85A, 85B, and 85C, SOD, and 35kDa protein genes into eukaryotic expression plasmid pVR1020. C57BL/6 mice were immunized three times intramuscularly with the recombinants as a DNA cocktail and pVR1020 vector DNA alone as control. A significant reduction has been detected in the bacterial burden of the spleen and liver of mice immunized with DNA cocktail in contrast to the control group. Also, the relative liver and spleen histopathology data paralleled with the MAP culture results and showed more multifocal granuloma and acid-fast bacilli in the control animals. Moreover, mice immunized with DNA cocktail developed both $CD4^+$ and $CD8^+$ T cell responses to the recombinant antigens and showed significant lymphocyte proliferation. The Th1 response related cyokine (IL-12, IFN-γ, TNF-α) gene expression levels increased in immunized animals. The results of ELISPOT assay also revealed an increase in the number of IFN-γ secreting cells in the immunized group than the control group, indicating a Th1 type of response.These results indicate that the use of recombinant DNA vaccine cocktail can induce protective immune response against MAP infection with a predominant Th1 response.

Proliferation of lymphocyte subsets in experimental ovine Johne's disease

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Protection against mycobacterial disease relies on T-cell dependent immune responses. In Johne's disease, cell-mediated immune responses predominate in subclinical disease. As disease progresses these responses diminish and can be undetectable while there is a concurrent strong humoral response, demonstrated by elevated serum anti-Mptb (*Mycobacterium avium* subsp. *paratuberculosis*) antibodies. If the infection is not eliminated by the initial T-cell response then Mptb are able to persist and proliferate resulting in lesion formation and chronic infection. Much work has been published on cellular responses throughout the course of bovine Johne's disease but knowledge of similar responses in sheep is lacking. The aim of this study was to identify changes in antigen-driven proliferation of lymphocyte subsets during the course of early ovine Johne's disease (OJD).

Merino lambs aged 4 months were randomly assigned into three groups, n = 20 per group. Two groups were orally challenged with either a clonal inoculum of Mptb (Group 1) or a gut homogenate prepared from an animal with clinical OJD (Group 2) while the third group was left unchallenged (Group 3). Blood samples were collected at 4 monthly intervals and prior to necropsy at 13 months post-challenge. Lymphocytes isolated from blood and lymph nodes (ileal, posterior jejunal and prescapular) were labelled with CFDA-SE and cultured in the presence or absence of Mptb antigen for 5 days. Cells were labelled for phenotype – CD5, CD4, CD8αβ, 86D (γδ T cells) and WC4 (B cells) – prior to data collection by flow cytometry. Faecal shedding and presence of Mptb in tissue samples were determined by culture in a radiometric system (Bactec). Diseased sheep were categorised based on histological lesion type.

Distinct differences were seen in the antigen-induced proliferative response in both blood and lymph node cells from sheep challenged with Mptb compared to the unchallenged controls. While 30% of Group 1 and 50% of Group 2 animals responded to Mptb antigen stimulation in the proliferation assay as early as 4 months post-challenge, none in Group 3 responded. The number of responders in Groups 1 and 2 continued to be higher than in Group 3 throughout the study. Differences in the phenotype of proliferating subsets in relation to infection status will be discussed.

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

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

New proposed immunopathological model for paratuberculosis in ruminants

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

Immune correlations with paucibacillary and multibacillary disease in sheep naturally infected with *Mycobacterium avium* subspecies *paratuberculosis*

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There are two possible disease outcomes in ovine Johne's Disease. Paucibacillary (PB) disease is defined by lymphocyte infiltration, a Type 1 immune response and few acid fast organisms present within lesions. Multibacillary (MB) disease is defined by macrophage infiltration, a Type 2 immune response and many acid fast organisms. Sheep with severe Johne's Disease were identified from within several small herds, necropsied, and blood and mesenteric lymph node samples harvested. Culture and histology results were used to define two groups; sheep with varying severity PB disease, and sheep with severe MB disease. Samples were further analysed by IFNγ ELISA, IgG1 antibody ELISA, flow cytometry and real-time PCR. This data was correlated with both disease status and disease severity to determine whether these naturally infected sheep have classical PB or MB immune profiles. This data revealed a strong johnin specific proliferative response in the PB diseased group and a less responsive antibody-based response in the MB diseased group. This was demonstrated by high levels of IFNγ protein and mRNA accompanied by increases in all cell populations monitored after stimulation with johnin in PB diseased animals. There were also higher levels of inherent IFNγ mRNA in the blood but not the posterior jejunal lymph node. These observations were in contrast to MB diseased animals which exhibited low levels of IFNγ protein and mRNA, no change in the cell populations monitored after stimulation with johnin, but high IgG1 antibody levels, high circulating levels of BCR+ cells, and high levels of inherent IL-10 mRNA in the posterior jejunal lymph node but not the blood. Interestingly both diseased groups had lower levels of inherent IL-4 mRNA in the blood and posterior jejunal lymph node than the control non-diseased animals. This data will also be compared to data from a current vaccine trial to investigate the effects of vaccination on disease development.

Susceptibility to paratuberculosis is associated with functionally relevant single nucleotide polymorphisms in bovine Toll-Like Receptor 2

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Paratuberculosis is a chronic intestinal infection in ruminants, caused by *Mycobacterium avium subspecies paratuberculosis (Map*). To study the role of host genetics in disease susceptibility 9 candidate genes (Toll-like receptors 2 and 6, Interleukin-10, Interleukin-12p35, Interleukin-12p40, Interleukin-12 receptor β1, Interferon-γ, Interferon-γ receptor 1, and NOD2/CARD15) selected for their potential role in immunity to mycobacterial infections were analysed for single nucleotide polymorphisms (SNP) and disease association.

For SNP discovery and disease association, a case-control study including 24 cows from farms with paratuberculosis was conducted. Sequence analysis of the 9 candidate genes from 12 paratuberculosis infected animals, and 12 age-matched healthy herd-mates, revealed 35 different SNP. The TLR2-1903T/C SNP was significantly associated with resistance to *Map*. This and 11 additional SNP were studied in a subsequent cohort study with 553 cows from farms with paratuberculosis. The allelic distribution of the TLR2-1903 T/C SNP was confirmed, and the TLR2-385 T/G SNP was also found to be significantly different between the infected and non-infected animals. In *in vitro* functional assays, ligand binding by the TLR2 of the resistant haplotype induced higher *in vitro* NFkB production as compared to the TLR2 of the susceptible haplotype. These findings suggest that higher activity may contribute to enhanced cell activation and a lower susceptibility to paratuberculosis.

In conclusion these data support previous work indicating a role for host genetics in susceptibility to bovine paratuberculosis, and the current study specifically identified the diversity in the TLR2 gene in the cattle population to be involved in resistance to bovine paratuberculosis.

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

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Paratuberculosis, also called as Johne's Disease (JD) is a chronic infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and development of improved vaccines is urgently required considering its economic importance. Here, we report the cloning and expression of a 74kDa recombinant fusion protein (Map 74F) and evaluation of its protective efficacy against MAP challenge in mice. Map74F was generated by the sequential linkage in tandem of the ORFs of the~17.6-kDa C-terminal fragment of Map3527 to the full-length ORF of Map1519, followed at the C terminus with ~14.6-kDa N-terminal portion of Map3527. C57BL/6 mice immunized with Map74F along with MPL+TDM emulsion had a significant IgG1 response but not IgG2a. In vaccinated animals, the IgG1/IgG2$_a$ ratio which increased until 4 wk after MAP challenge, decreased gradually from 8 wk indicating a possible shift to a Th1 response. Antigen specific IFN-γ responses measured by IFN-γ ELISA and ELISPOT assay were significantly higher in mice immunized with Map 74F than the control animals. The results revealed that IL-4 and IL-10 mRNA expression by spleen cells from immunized animals were higher than that of control animals, whereas no significant differences were detected in the expression levels of other cytokine genes including IL-2, IL-12, TNF-α and IFN-γ. Antigen specific $CD3^+$ and $CD4^+$ T cell populations increased significantly in mice immunized with Map 74F, whereas, no significant differences were detected in the $CD8^+$ T cell populations between the immunized and control animals. Following challenge, MAP load was significantly lower in spleen, liver and mesenteric lymph nodes of immunized animals compared to the control animals indicating protection against MAP infection. This was further evident by the improved spleen and liver pathology of the immunized animals which had fewer granulomas and lesser numbers of acid-fast bacilli. Results of our study indicated that immunization of mice with Map74F protected mice against MAP infection.

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

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

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

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

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

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

Comparison of the cell-mediated immune responses to reduced doses *of Mycobacterium avium* ssp *paratuberculosis* vaccine in cattle

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The effectiveness of *Mycobacterium avium ssp paratuberculosis* (*MAP*) killed-cell in oil vaccine at full, half, and quarter doses for inducing T cell mediated immune responses was evaluated using three groups of 10 age-matched calves (1-35 days of age). Another group of 10 calves were mock vaccinated and served as a control group. At 2 and 4 months after vaccination, blood samples were collected for cell-mediated immunity assays. *MAP*-PPD was used as recall antigen in the whole blood (WB) IFN-γ assay to detect the extracellular interferon gamma (IFN-ɣ expression and in the multi-parameter flow cytometry assay to detect the expression of high affinity IL-2 receptor (CD25), intracellular IFN and intracellular interleukin-4 (IL-4) expression by CD4+, CD8+, and γδ TCR+ T cells. At 2 months after vaccination, the WB IFN assay and the multi-parameter flow cytometry assay showed significant ($P<0.05$) increases in CD25, IFN-γ, and IL-4 expression in all T cell subsets of the full dose vaccinated group compared to the control group (except that the γδ TCR+ T cells did not express IL-4). Among the 3 vaccinated groups, the responses were not significantly different. At 4 months after vaccination, the WB IFN assay responses of the full and quarter dose vaccinated groups were still significantly higher than the control group ($P<0.05$). By 4 months after vaccination, at least 2 of the 3 vaccinated groups had significant increases in CD4+ cell expression of CD25, IFN-γ, and IL-4. The CD8+ T cells did not have detectable increases in expression of either CD25, IFN-γ, or IL-4, and the γδ TCR+ T cells had increased expression of CD25 and IFN-γ but not IL-4. There were no significant differences between the vaccinated groups. The results demonstrate that the WB IFN-γ and the multi-parameter flow cytometry assays could detect significant T cell specific responses to *MAP*-PPD at 2 months after vaccination but the responses decreased when detected at 4 months after vaccination. All three doses of vaccine were able to induce similar significant responses to *MAP*-PPD with both assays.

Immune profile differences in red deer following experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis*

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The differences between immune responses in red deer with either low-grade or high-grade histopathology were profiled following vaccination with a killed whole cell *Map* vaccine with mineral oil adjuvant and experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Immune responses generated were monitored using a *Map*-specific IgG1 antibody ELISA, an interferon gamma ELISA and flow cytometry. The initial 24 animals in the study were divided into 6 outcome groups on the basis of histological and culture data available post-mortem. Animals mounted a mixed immune response to both vaccination and experimental challenge characterised by high levels of interferon gamma and significant antibody responses. The profile of the *Map*-specific IgG1 response in the unvaccinated and challenged group showed marked differences between animals with low-grade and high-grade histopathology. Unvaccinated animals with low-grade histopathology tended to show cross-reactive interferon gamma responses. No significant differences were observed in the circulating cell populations. Vaccination eliminated immunological differences between the low and high-grade histopathology groups and affected Johne's disease diagnosis. These profiles provide information on the different immune processes that affect Johne's disease progression in red deer.

Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis

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Three forms of Johne's disease have been described in sheep – multibacillary, paucibacillary and asymptomatic. We used real-time RT-PCR to compare the expression of thirteen cytokine and cytokine-related genes in ileal tissue from sheep with these three forms of the disease to try to understand the immune responses underpinning these three pathologies.

Three pathological forms of sheep paratuberculosis were defined on the basis of histopathology, cytochemistry (Ziehl-Neelsen) and IS*900* PCR. Paucibacillary lesions have lymphocytic infiltration and are ZN negative; multibacillary lesions have macrophage infiltration and large numbers of acid-fast bacteria. The pauci- and multibacillary forms are linked to the differential expression of IFNγ and IL-10 respectively. In addition the increased levels of the proinflammatory cytokines (IL-1β and TNFα), IL-8, IL-18 and TRAF-1 in both diseased forms is indicative of persistent inflammatory lesions. No changes were seen in IL-1α in any sheep ileum tissues. Asymptomatic animals are IS*900*+ with normal histology but have significantly decreased levels of IL-18 and increased levels of TNFα and thus can be distinguished, in terms of cytokine expression profile, from uninfected controls.

Mechanisms used by MAP to impair CD40-mediated IL-12p40 and iNOS gene expression in bovine monocyte-derived macrophages

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Mycobacterium avium ssp. *paratuberculosis* (MAP) is a facultative intracellular pathogen, residing in subepithelial macrophages. Clearance of MAP critically depends upon an appropriate pro-inflammatory and cytotoxic Th-1 immune response leading to activation and/or lysis of persistently infected macrophages to promote bacterial killing. However, work in vivo has shown that the appropriate Th-1 immune response occurring early in MAP infection is lost, followed by an ineffective, antibody-mediated Th-2 response. Our overall hypothesis is that once MAP persists within naïve macrophages, it reduces the ability of infected macrophages to react to normal T cell signaling thereby macrophages fail to be activated and destroy MAP, and fail to properly signal T cells to respond. To test this hypothesis, we investigated the effect of MAP infection on CD40 signaling, a main pathway used by T cells to activate macrophages. Using Q-RT-PCR analysis, we recently demonstrated that once bovine monocyte-derived macrophages (MDM) are infected with MAP, they fail to up-regulate the expression of iNOS and IL-12p40 encoding genes in response to CD40 ligand (CD40L). Our recent studies investigate possible mechanisms that are responsible for MAP-specific changes in CD40L-mediated gene expression. Using flow cytometric analysis we demonstrated that failure of infected macrophages to respond to CD40L was not due to down-regulation of CD40 on the cell surface of MAP-infected MDM. Studies with specific inhibitors revealed that the CD40L-mediated increase in IL-12p40 and iNOS gene expression is dependent upon activation of p38. However, western blot analysis revealed that interference with CD40L-mediated increases in gene expression does not appear to be due to prevention of early and rapid p38 activation. Paradoxically, while p38 activation in CD40L-stimulated MDM cells is only transient, we observed a sustained p38 activity in MAP-infected MDM cells upon CD40L stimulation, suggesting p38 plays a role in MAP interference with CD40 signaling. Additionally, our data revealed the possibility of a third potential mechanism. We observed a dramatic increase in IL-10 gene expression in MAP-infected MDM upon CD40L stimulation relative to uninfected cells. IL-10 has been shown to negatively regulate IL-12p40 gene expression. Therefore, we hypothesize that MAP-induced IL-10 expression at early times following CD40 signaling interferes with subsequent IL-12p40 and iNOS expression. Continuing studies are underway to uncover the role of sustained p38 activity and enhanced IL-10 expression for MAP interference with CD40 signaling in infected macrophages.

Mycobacterium paratuberculosis under stress: What can the bacteria do?

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

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

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

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

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INTRODUCTION

Serological ELISA test on individual milk has given promising results in paratuberculosis diagnosis, showing a sensitivity only slightly lower than the same test on blood samples (K=0.81-0.91) (Arrigoni et al., 2004; van Weering et al., 2007).

The reported specificity of Pourquier ELISA test on milk varies between 99.8-100% on individual milk and 100% on bulk milk (van Weering et al., 2007; Arrigoni et al., 2004).

The objective of this study was to investigate the relative sensitivity of a commercial ELISA test in detecting antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in bulk milk of infected herds, compared with the results of ELISA individual test on blood.

MATERIALS AND METHODS

This study was carried out on 52 dairy herds, previously classified as positive for Map infection by an ELISA test (Institut Pourquier), performed on individual blood samples of cows over one year of age. When the number of seropositive animals was below a statistical threshold, the positivity was confirmed by faecal culture (Arrigoni et al., 2006).

On the basis of the prevalence of seropositive animals, the herds were classified as:

- "low prevalence" herds (<5%);
- "moderate prevalence" herds (5-15%);
- "high prevalence" herds (>15%).

The bulk milk of the infected herds was repeatedly sampled, each farm being submitted to an average of 3.5 samples, at a frequency interval of at least 30 days, with the aim of sampling all the lactating cows, resulting in a total of 183 bulk milk samples being collected.

The ELISA test on milk was performed by *Mycobacterium paratuberculosis* ELISA kit (Institut Pourquier), following the manufacturer's instructions.

RESULTS

In the statistical analysis, all the positive and inconclusive samples were classified as "reactive", following the manufacturer's instructions.

Fifty five samples (30.1%) of 183 samples assessed tested positive and were distributed as follows: 8 samples (11.1%) out of 72 coming from "low prevalence" herds, 32 samples (33.7%) out of 95 coming from "moderate prevalence" herds, and 15 samples (93.8%) out of 16 coming from "high prevalence" herds.

On the whole, taking into consideration the herds, 21 herds out of 52 controlled (40.4%) registered at least one positive sample, and were distributed as follows: 4/21 (19.0%) coming from "low prevalence" herds, 13/27 (48.1%) coming from "moderate prevalence" herds, and 4/4 (100.0%) coming from "high prevalence" herds.

The overall relative sensitivity of ELISA test on bulk milk resulted 30.1% (95% C.I.: 27.5-37.3%).

The repeated sampling enhanced the sensitivity value of the test; in fact, testing 3.5 times the bulk milk of the same herd, it was possible to reveal 40.4% (95% C.I.: 27.0-54.9%) of the infected herds.

DISCUSSION AND CONCLUSIONS

The ELISA test is a rapid, economic and cost-effective diagnostic tool, with a high-throughput for the laboratory; furthermore, the ELISA paratuberculosis test can be applied on bulk milk samples collected for Brucellosis and Leukosis diagnosis, lowering the sampling costs.

The data shown in this paper agrees with the results of analogous studies carried out in Holland, where the sensitivity was 24-30% (van Maanen et al., 2005; van Weering et al., 2007).

The overall sensitivity of the ELISA test appeared strongly correlated with the herd sero-prevalence; in particular, the test was very sensitive (93.8% on a single sample, 100% on repeated samples) in herds where the sero-prevalence was over 15%; for this reason the use of this test could be recommended to detect high prevalence herds, more frequently producing Map contaminated milk.

On the other hand, for its low overall sensitivity (30.1% on a single sample, 40.4% on repeated samples) the ELISA test on bulk milk is not a suitable tool in paratuberculosis certification programmes.

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Nested Polymerase Chain Reaction for detection of *Mycobacterium avium* subspecies *paratuberculosis* in bovine allantoic fluid and fetuses

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INTRODUCTION

Paratuberculosis (Johne's disease), an insidious infectious disease of ruminants, occurs worldwide. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), a facultative intracellular pathogen. It is currently believed that the principal pathway of transmission of Map is the fecal-oral route and that the calf is the most susceptible to such transmission.

Culture studies have shown that between 20% and 40% of fetuses from cows with advanced Johne's disease were infected in –utero, compared with 9% of fetuses being culture positive from subclinical cows (Sweeney et al., 1992). Because presently there is no possibility to identify such in-utero infected calves prenatally, the decision to keep a calf born to a proven infected dam as replacement is difficult after infection detection in the dam regardless of the diagnostic method chosen.

Here we report the feasibility of the nested PCR technique for potential application as prenatal test in the cow via placental fluid collection during late pregnancy.

MATERIALS AND METHODS

Animals

The 12 pregnant cows investigated were all Holsteins and were obtained from 4 dairy herds with a proven history of paratuberculosis. Pregnant animals were selected for studies when they developed clinical signs of paratuberculosis, had positive Map serology results or had positive Map results on blood nested PCR or milk nested PCR when still lactating.

Preparation of Animals for Percutaneous Allantoiscentesis

Animals were tied into a headstand. Rectal examination was performed to determine the site of pregnancy in the uterus (right or left uterine horn). Once the site was determined, the hair of the ventral flank of that site above the udder was shaven, and the skin was cleaned with warm water and soap. The prepared area was sterilized with alcohol and iodine. Lidocaine (2%) was applied into the subcutis with a syringe to locally sedate the skin. Fetal fluid was collected by inserting dorso-cranially a 18-gauge spinal needle through the abdominal wall and into the allantoic cavity. Fluid was collected by free flow or aspiration with a 12 mL syringe. Fluid collection was attempted on the standing animals every second day up to five times all together.

Specimen Handling

Tissues from fetal liver, spleen, lung and brain as well as tissues from the placentome were prepared for nPCR by touchpressing on glass slides. After air-drying, 200 µL of 0.2N NaOH were dispersed over tissues and a sterile razor blade was used to scrape samples from the glass slides. Samples were placed into 1.5 mL centrifuge tubes and boiled at 110^0C for 20 minutes. For the PCR reaction, 1 mL of supernatant was chosen. Liquid samples from the allantoic cavity (50 mL) and fetal abomasum(25 mL) were centrifuged for 60 minutes at 1,000 g. Pellets were washed 3x with PBS, suspended in 0.2 NaOH and boiled.

PCR

A nested PCR developed in our laboratory and previously described (Buergelt and Williams, 2003) was used for identifying Map in allantoic fluid and fetal tissue samples. A volume of 10 µL of the PCR reaction products was run on 1.5% agarose gel by electrophoresis in TAE running buffer (Continental Lab Products, San Diego,CA). DNA extracted from *Mycobacterium avium* subspecies *paratuberculosis* laboratory strain No. 295 was used as

positive control for primers P90, P91 and J1, J2(nested control). Sterile water was used as negative control for the PCR assay. Gel inspection was performed using ultraviolet light and recorded with a computerized digital camera (UVP Transilluminator System, Upland,CA).

ELISA

The ELISA originally developed by W.D. Richard (Allied Laboratories, Ames, IA) was performed with crude, soluble protoplasmic antigen (Allied Monitor, Fayette,MO). The test sera were preabsorbed with *Mycobacterium phlei*. Results were calculated for wavelength readings (OD at 405 nm) of triplicates and recorded as negative (<1.5 OD), suspicious (1.5 to 1.9 OD), and positive (>2.0 OD).

AGID

The same crude antigen that was used for the ELISA was selected for the agar-gel immunodiffusion test. Final readings were performed after 48 hours.

Histopathology

Tissue sections collected after a complete necropsy were fixed in 10 % buffered formalin, paraffinized, cut on a microtome, stained with hematoxylin-eosin (H & E) and acid fast stains (Fite's) and examined under a lightmicroscope to qualitatively determine the extent of the granulomatous inflammation and the number of acid-fast bacilli.

RESULTS

The average age of the cows was 5.6 years. Necropsy confirmed that all cows had paratuberculosis characterized by granulomatous enteritis and mesenteric lymphadenitis and the presence of intralesional acid-fast bacilli. All were pregnant with cow #10 having twins. The average gestational stage was 6.4 months. The sex of the fetuses was male in 7 and female in 5 animals. All but one animal had a positive ELISA reading and 8 of 12 animals were positive on agar gel immunodiffusion. Blood nPCR showed 4 of 12 cows positive and 2 of 10 cows had a positive milk nPCR reading. Fecal culture was not attempted. The placentome when subjected to nPCR was positive in 2 of 4 tissues examined. In cow # 9 the placentome showed an individual cluster of acid-fast bacilli on touch preparation examined under the light-microscope. There was no evidence of a granulomatous inflammatory response in the placentome tissue. Allantoic fluid was positive on nPCR in 1 of 12 animals. Fetal tissue had positive nPCR reaction products in 3 of the spleens tested, in 2 of the livers tested, in 1 of the 2 brains tested and in the abomasal fluid of one of 10 samples tested. When the fetal tissues were examined microscopically, they showed no evidence of an inflammatory response or evidence of acid-fast bacilli.

DISCUSSION

The detection of Map DNA by nested PCR in fetal fluids and various fetal tissues supports the concept that Johne's disease is a disseminated infectious disease with in-utero transmission as a possibility to be considered in addition to the traditional fecal-oral route. This additional pathway of transmission should be considered in the epidemiology and control of the disease through testing and culling. The management is often reluctant to remove the offspring together with infected dams from the herd. The diagnosis of prenatal infection via allantoiscentesis is considered a possibility to help along this decision process.

The results from a small population of 12 pregnant infected cows demonstrate that Map is transmitted transplacentally, even though bacterial DNA may not appear very often in placental fluid. This may be the result of a low number of bacilli in comparison with the large volume of placental fluid during late pregnancy or the low number of phagocytes in the fluid to be used by the bacilli for replication. The successful isolation of Map DNA in the allantoic fluid at necropsy in a previous cow at 2 months of gestation might reflect this volume disadvantage (Buergelt and Williams, 2003). While the technique of percutaneous allantoiscentesis on the standing animal in late gestation can be easily performed technically (Callan et al., 2002), it may have the disadvantage to dilute the few bacilli shed from the fetus

within the fluid at the time when this technique is attempted during late pregnancy. The presence of PCR inhibitory substances in the allantoic fluid might have also influenced the DNA amplification outcome. The identification of acid-fast bacilli in one randomly collected placentome by lightmicroscopy is additional proof of transplacental transmission of Map.

Cultural isolation of Map from fetuses has been reported form the UK and US (Sweeney et al., 1992). A tissue reaction such as inflammation to the presence of organisms was not noticed in all fetuses of various gestations upon microscopic examination. It appears that fetuses are immuno-tolerant to the virulence of Map bacilli, regardless of gestational age.

CONCLUSION

It is technically possible to obtain allantoic fluid for prenatal Map infection testing and easily to be performed aseptically in late pregnancy (> 7months) without inducing peritonitis or abortion. The positive nested PCR in the allantoic fluid of only 1/12 animals is disappointing. More late term pregnant animals should be tested to yield a data base. The detection of Map DNA in various fetal tissues (50%) supports the concept of trans-uterine transmission of Map in addition to the oral-fecal or milk route of transmission and should be of concern to the management of disease control by test and cull of only infected adult animals.

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Paratuberculosis in sheep: diagnostic methods and genetic susceptibility

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INTRODUCTION

While many features of infection caused by *Mycobacterium avium subspecies paratuberculosis* are similar across ruminant species, the clinical presentation, and the availability, performance, and interpretation of diagnostic tests differ between cattle and other ruminants such as sheep and goats. Variations among the *M. avium paratuberculosis* strains, isolated from different host species, may contribute to some of the differences. Because of these variations, diagnostic tests validated in cattle should be fully validated in sheep. Moreover, the clinical signs in affected sheep are sometimes not so evident, the faecal culture needs a long incubation time, many ovine strains do not grow well on artificial media and the cost of analysis is high related to the value of a single animal. The diagnosis of paratuberculosis in sheep requires additional testing. The present study also attempted to assess whether different genotypes may lead to different susceptibility in developing paratuberculosis in sheep, as reported for human Crohn's disease. Two genes, *NRAMP1 (natural resistance-associated macrophage protein)* and CARD15/NOD2 (*caspase recruitment domain1)*,which are associated with susceptibility to Crohn's disease and Blau Syndrome in man and are likely to influence the outcome of mycobacterial infection in many species, have been analysed.

The objectives of the study were:

1) to compare different diagnostic tests in sheep;
2) to identify polymorphisms in *NRAMP1* and CARD15/NOD2 sheep genes that could be associated with the development of paratuberculosis.

MATERIALS AND METHODS

Serological data

1720 adult sheep from 4 flocks were analysed. We performed a first ELISA screening on serum (Table 1). A second ELISA verification was performed on serum and milk of positive individuals to confirm results (Table 2). A commercial kit (Institut Pourquier) was used to diagnose paratuberculosis with the ELISA test. Sera were treated according to the enclosed protocol. PCR on the specific insertion sequence IS*900* was carried out on faeces from positive sheep. The results are reported in Table 3. A commercial kit (Institut Pourquier) was used to diagnose paratuberculosis with the ELISA test. Sera and milk were treated according to the enclosed protocol.

Table 1. Serum ELISA test: Screening and Verification

Sheep flock	Total sheep	+ screening	+ verification	% verif. vs. screen	% Pos.
1	502	33	20	60%	3.9%
2	703	86	41	47%	5.8%
3	286	7	2	28%	0.7%
4	229	41	27	65%	11.0%
Total and Mean	1720	167	90	54%	5.2%

Table 2. ELISA: Milk vs. Serum

Flock	+ ELISA verification	+ ELISA milk	% + milk vs. + serum
1	20	8	40%
2	41	22	54%
3	2	2	100%
4	27	6	22%
Total and mean	90	38	42%

PCR

DNA extraction from tissues and faeces was conducted by means of QIAmp DNA minikit (Qiagen) according to the enclosed protocol. A qualitative IS*900* specific PCR was carried out on the total extracted DNA to detect *M. avium paratuberculosis*. A commercial Adiavet paratube (Adiagene) kit was used on purpose.

Table 3. PCR vs. ELISA

Flock	+ ELISA	+ PCR	% PCR vs. ELISA
1	20	4	20%
2	41	7	17%
3	2	0	0%
4	27	2	7%
Total and mean	90	13	14%

Genetic analysis

DNA extracted from a total of 31 individuals (18 infected and 13 controls) using standard methods was screened for polymorphisms. Primers for sequencing CARD15, unpublished in sheep, were designed on the basis of *Bos taurus* sequences available in GenBank. Fragments of exon 1 (901bp) and exon 2 (486bp) of NRAMP1 and of exon 1 (270pb), exon 2 (500pb), exon 3 (235 pb), exon 4 (2000 bp), and intron 1-2 (1008 pb) intron 5-6 (300 pb) and the promoter region (541 pb) of CARD15 were amplified and sequenced. Each polymerase chain reaction (PCR) was performed in volume of 30 µl containing 30 ng of genomic DNA, 1.6 pMol of each primer, 200 µM dNTPs, 1X PCR buffer and 0.2 units of Taq DNA polymerase (Promega) on a PCR Express cycler (Hybaid). Polymerase chain reaction products were purified through ExoSap-IT (USB Corporation) to remove residual primers and dNTPs and used as templates for two sequencing reactions. Sequencing was performed using a ceq8800 sequencer using DTCS QuickStart Kit (Beckman Coulter) and purifying with Agencourt CleanSEQ 96 (Beckman Coulter), according to manufacturers instructions. To identify SNPs, sequences of at least six individuals were analysed and aligned with Bioedit software (T.A. Hall 1999, Nucleic Acids Symp. Ser. 41: 95).

RESULTS AND CONCLUSIONS

Serological data

From our results the sero-prevalence among flocks were from 0.7% to 11%, with an average prevalence of 5.23, only 42% of the sero-positive animals were also positive in milk ELISA showing a sensitivity, which is very low if we consider that the sensitivity of the ELISA serum test is already low at 45%. Individuals showing a higher value of S/P are more frequently to be positive in both tests. So we can confirm that the ELISA S/P value is a useful tool in the diagnosis of paratuberculosis, to assess the level of infection, particularly in subclinical cases. ELISA in milk could only be used to find the infection in a flock in individual milk samples.

PCR

PCR analysis confirms as positive only 14% of the samples. Difficulties are experienced in recovering DNA from the small number of organisms in clinical specimens, especially in complex samples such as faeces. The low sensitivity could be attributed to the presence of low shedding of the MAP in faeces or PCR inhibition by faecal constituents.
We believe that the low sensitivity was most likely due to the extraction method used, which was not adequate to recover small amounts of *M. paratuberculosis*. An extraction protocol for the concentration and purification of *M. avium* subsp. *paratuberculosis* DNA from faecal samples is needed in the future.

Genetic Analysis

Sequences of sheep CARD15 are published in Gene Bank (accession number EF141018). Exon 1 and exon 2 of NRAMP1 and exons 1, 2 and intron 1-2 of CARD 15 were monomorphic on the analysed samples. Exon 4 and intron 5-6 resulted polymorphic, showing different frequency estimates. A chi square test showed no significant associations between SNPs in CARD15 and disease, even if the number of analysed samples should be increased. A further step of the study will be to investigate a large cohort of infected animal and controls. The results are shown in Tables 4 to 6.

Table 4. Results of genetic analysis

Gene	Exon monomorphic	Intron monomorphic	Exon polymorphic	Intron polymorphic
NRAMP1	1-2			
CARD15	1-2-3	1-2	4	5-6

Table 5. SNPs and Frequency in exon 4 of CARD15

Alleles	Control	Infected	Frequency
C	9	10	61%
C/T	2	7	29%
T	1	2	10%
Frequency	39%	61%	
P-value			0.43
Contingency coefficient			0.23
Chi-square			1.668
DF			2

Table 6. SNPs and Frequency in intron 5-6 of CARD15

Alleles	Control	Infected	Frequency
A	2	8	33%
A/G	7	9	53%
G	2	2	14%
Frequency	37%	63%	
P-value			0.40
Contingency coefficient			0.24
Chi-square			1.848
DF			2

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Cross-pooled faecal culture and individual faecal PCR for paratuberculosis herd level monitoring

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INTRODUCTION

Isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map) by faecal culture is the gold standard test for in vivo diagnosis of paratuberculosis in infected animals. Culture detects around 50-100 cells/g of faeces. But Map shedding varies according to the immuno-pathological status of animals. The presence of sub-clinically infected animals and other factors including infection with strains difficult to grow reduce considerably the sensitivity of this method. In addition, Map culture is expensive, slow and laborious. Pooled faecal culture reduces the number of cultures needed to diagnose paratuberculosis in farms (Kalis et al., 2000; Whittington et al., 2000). But the samples in a positive pool must be re-cultured to identify the animals excreting Map. Consequently, shedder animals remain unidentified in the farm due to this delay in the time to diagnosis.

Thus, the aims of the present study were to evaluate a cross-pooled faecal culture system and to evaluate the usefulness of the culture method described in combination with the individual faecal PCR to reduce costs and the time to diagnosis.

MATERIALS AND METHODS

Phases

Phase 1: setting up of the cross-pooled culture method

Fifty faecal samples from a paratuberculosis affected Holstein herd in active production were used. The calculated within-herd sero-prevalence was 5.45%. The samples were submitted to individual and cross-pooled culture (described below) and the results obtained with both methods were compared.

Phase 2: usefulness of a strategy combining the cross-pooled culture method with the individual faecal PCR of positive pools. Comparison to results of general individual PCR.

Once the cross-pooled culture method was adopted faecal samples from 699 dairy cattle from herds under paratuberculosis follow-up programs were tested. The samples were cultured using the cross-pooled method. When the cross-pooled culture strategy could not directly determine the final result of samples (inconclusive pooled culture), PCR of individual faeces in the pool was carried out instead of using re-culture in order to quicken the diagnosis. The results of pooled culture combined with those from PCR of inconclusive pools (when needed) were summarized as “final result of culture”. Then a general PCR was performed on all 699 individual faeces and the results were compared to those assigned by “final result of culture”.

A variable called complementary sensitivity (CS) was calculated as the ratio of positive results in only individual PCR method to the total number of positive results in the “final result of culture” method (cross-pooled culture + PCR of inconclusive samples). This variable was meant to represent the additional detection efficacy of one method over the other, assuming both were highly specific.

Costs including both the materials and the labour for each diagnostic strategy were calculated.

Individual faecal culture

The sedimentation culture technique described for Herrold´s egg yolk (HEY) and Lowenstein-Jensen (LJ) media by Aduriz et al. (1985) was used. Briefly, 38 ml of 0.75% HPC were added to sterile filter-bags containing 2 g of faeces and the mixture was homogenized in a lab blender. Bags were allowed to settle for 15 min and then 15 ml were aspirated drawing a

perpendicular line with the pipette to collect material from different sites of the suspension, and transferred to sterile centrifuge tubes. The tubes were left in a standing position for 18 h. Finally 0.2 ml of the suspension at the bottom of the tube were used to inoculate 2 HEY and 2 LJ slants.

Cross-pooled faecal culture

The cross-pooled culture method lies in crossing pools of 5 samples in 5x5 squares of samples (see Fig. 1). The number of cultures (pools in this case) to be done for each 5x5 square is 10. The advantage of the method is that positive samples can be directly identified by crossing the culture result of pools. However, when the prevalence within the herd is 4% or higher the likelihood of direct identification of positives is reduced as indicated a preliminary statistic approach.

1	6	11	16	21	pool A
2	7	12	17	22	pool B
3	8	13	18	23	pool C
4	9	14	19	24	pool D
5	10	15	20	25	pool E
pool F	pool G	pool H	pool I	pool J	

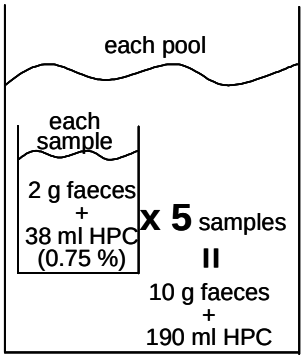

Fig. 1. Graphic representation of 5x5 squares designed for cross-pooled culture. Numbers in the square represent individual samples. Each pool contains 5 samples (2 g/sample). Ten cultures are needed for every 25 samples (one 5x5 group).

Decontamination and concentration steps for pooled cultures were exactly the same as in individual samples but with 10 g of faeces and 190 ml of HPC. The final volume transferred from each pool bag after settling for 15 min was 50 ml.

DNA extraction and PCR

DNA from individual faecal samples was performed with ADIAPURE® extraction kit. Two µl of purified DNA were used for PCR amplification with ADIAVET® and ADIAVET RealTime PCR kits as described by the manufacturer (conventional ADIAVET PCR at the beginning and RealTime PCR later).

RESULTS

Phase 1

According to the individual faecal culture 5 of the 50 cows analyzed (10%) were shedding Map, a situation not interesting for the cross-pooled method. Due to this unfavourable situation 11 samples could not be directly classified as positive or negative with this culture method. In other words, the number of cultures needed to detect the same 5 shedder cows with the new method was 31 (10 per 5x5 group=20 + 11 inconclusive). The reduction achieved in the number of cultures needed using the pooled culture method was of 38% compared to the individual culture, and the calculated costs were reduced by 27.1%.

Phase 2

Six-hundred and ten of the 699 samples were directly classified by the cross-pooled culture method. The results obtained when the “final result of culture” strategy was applied are shown in Table 1. For pooled culture samples were included in 28 groups of 5x5. In addition to the 280 cultures 89 individual PCR tests were needed for the definitive diagnosis, since 89 samples were classified as inconclusive. A reduction of 52.8% was observed in the number

of tests (cultures and PCRs) required. The reduction in the costs for diagnosis when "final result of culture" was compared to the general individual culture was of 43.5%.

Table 1. Sample classification according to the cross-pooled culture result and PCR of individual samples in inconclusive pools.

		final result of culture[1]		Total
		+	–	
cross-pooled culture result	+	21		21
	–		589	589
	? (inconclusive)	19	70	89
Total		40	659	699

[1]cross-pooled culture result + PCR result of samples in inconclusive pools

The general individual PCR classified 86 samples as positive and 613 as negative (Table 2). Six-hundred and thirty-nine samples obtained the same result in both categories, 33 positive and 606 negative. Seven positive samples were detected only by cross-pooled culture but 53 samples that were negative in culture were classified as positive by the individual PCR. Taking the "final result of culture" as the reference method the general individual PCR showed a complementary sensitivity (CS) of 132.5%.

The costs for the "final result of culture" strategy were reduced by 14.14% in comparison to the costs calculated for the general individual PCR system.

Table 2. "Final result of culture" against the general individual PCR results of all 699 samples tested in the 2nd phase of the study.

		result of general individual PCR		Total
		+	–	
Final result of culture[1]	+	33	7	40
	–	53	606	659
Total		86	613	699

[1]cross-pooled culture result + PCR result of samples in inconclusive pools

DISCUSSION AND CONCLUSIONS

Cross-pooled culture method directly classified most samples (87.26 %) as positive or negative, without repeating individual culture of positive pool samples. Thus, noticeable reduction in costs and time to diagnosis can be achieved by the use of this system.

The reduction in the culture number needed using cross-pooled culture compared to individual culture was of 59.9%. But it should be considered that PCR was needed to confirm the final result of culture in 12.7% of samples. Collectively, the reduction in the number of tests needed was of 52.8 %.

Costs of individual culture were interestingly reduced (43.5%) by the use of cross-pooled culture with PCR confirmation of samples in inconclusive pools ("final result of culture"). Nevertheless, the reduction was not so high when costs of "final result of culture" strategy were compared to the individual faecal PCR of all 699 samples (14.14%).

The use of individual PCR instead of re-culture for classification of samples in inconclusive pools is effective and reduces the time to diagnosis.

Although a good agreement between "final result of culture" and individual PCR was observed (91.4% of the samples gave the same result in both strategies), our results indicate that individual PCR alone can perform better than the cross-pooled culture in herd monitoring strategies. These results are encouraging to carry out further studies using a cross-pooled faecal PCR strategy.

Nevertheless, the cross-pooled culture method is still interesting when isolation of Map is required for confirmation of its presence in the herd and/or culture is the selected test for paratuberculosis screening of herds.

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Evaluation of serological diagnosis capacity for paratuberculosis using proficiency testing by interlaboratory comparison at the level of veterinary laboratories from Romania in 2006

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INTRODUCTION

Paratuberculosis (Johne's disease) is a disease of domestic and wild ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis*, culminating with chronic enteritis (Anon., 2004b). In 2006 year, Immunology Department from Institute for Diagnosis and Animal Health, organised a paratuberculosis serological diagnosis capacity testing through proficiency testing by inter-laboratory comparisons of veterinary laboratories in Romania.

The aim of this testing was the improvement of quality system for testing and evaluation of laboratories by statistic analyses, studying new classification models of the veterinary laboratories depending on the obtained results in testing (Anon., 2004a).

MATERIALS AND METHODS

The 39 participant laboratories received each a set with 6 bovine sera: 2 with positive (E3, E4), 2 doubtful (E1, E2) and 2 negative (E5, E6) results.

The samples were analysed by ELISA (Enyzme-linked immunosorbent assay) with absorption step. Each sample had to be worked for 8 times on the same plate, in the same day and the average of the 8 results was considered the final result.

The interpretation of results was made according to the criteria established by the kit producer and statistical analyses were made after the reference models described by ISO/IEC GUIDE 43-1 and 43-2 (Anon., 1997a; 1997b). Grubbs Test was used for identifying aberrant results and for elimination of laboratories with unsuitable results (Anon., 1993). Thus for each laboratory the standard deviation of the results repeatability and coefficient of variation then standard deviation of the reproducibility results and Z - scores were calculated using the result of Immunology Laboratory of IDAH, as a reference value.

RESULTS

The final report contains comparative results of laboratories (Table 1, Fig.1) and statistic analyses (Table 2, Fig. 2).

Table 1. The synthesis of qualitative interpretation of results samples

Number of E **Result of reference**	**Results of samples (E1, E2, E3, E4, E5, E6)** **No. of laboratories + IDAH (%)**		
	Positive	**Doubtful**	**Negative**
E1-Doubtful	4 (10)	24 (60)	12 (30)
E2-Doubtful	11 (28)	21 (53)	8 (20)
E3-Positive	37 (93)	1 (3)	2 (5)
E4-Positive	38 (95)	2 (5)	0 (0)
E5-Negative	1 (3)	0 (0)	39 (98)
E6-Negative	1 (3)	0 (0)	39 (98)

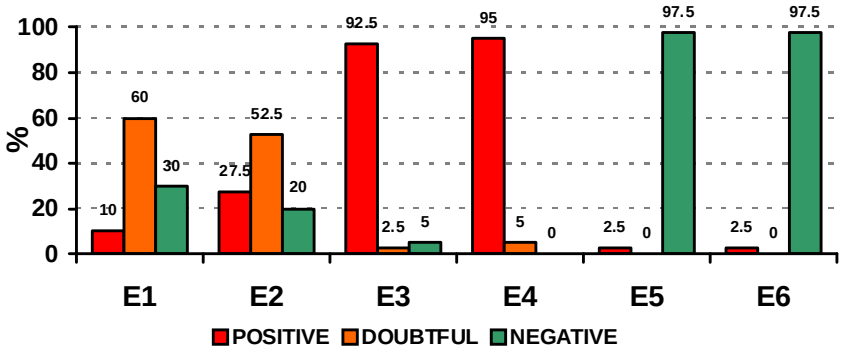

Fig. 1. Distribution of qualitative results of samples E1 to E6

Table 2. The synthesis of CV expressed in qualifying terms

Number of E Result of reference	Qualifying terms /coefficients of variation No. of laboratories + IDAH (%)		
	Very well 0.04-6.00%	Well 6.1-20%	Unsatisfactory >20%
E1-Doubtful	22 (55%)	15 (38%)	3 (8%)
E2-Doubtful	20 (50%)	18 (45%)	2 (5%)
E3-Positive	21 (53%)	18 (45%)	1 (3%)
E4-Positive	23 (58%)	16 (40%)	1 (3%)
E5-Negative	10 (25%)	25 (63%)	5 (13%)
E6-Negative	13 (33%)	22 (55%)	5 (13%)
Mean	(45%)	(45%)	(7%)

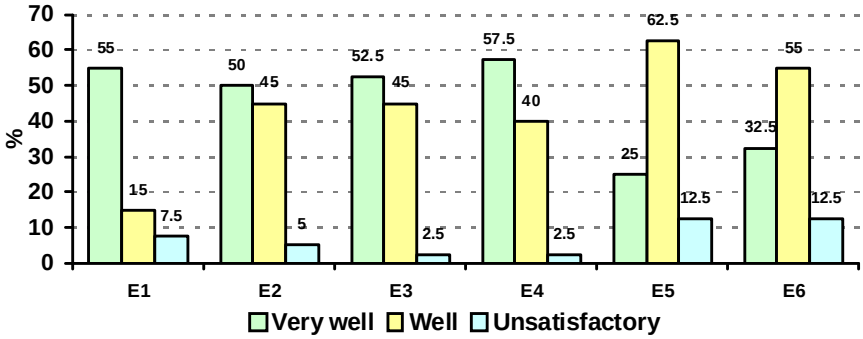

Fig. 2. Distributions of coefficients of variation

The results of Z–scores (|Z| ≤ 2: satisfactory, 2 < |Z| 2 ≤2: questionable and |Z|>3: unsatisfactory) results were presented in diagrams (Figs. 3, 4, 5, 6, 7, 8).

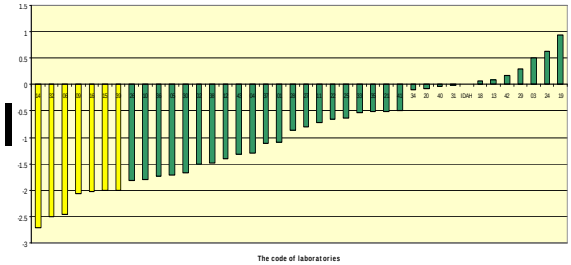

Fig. 3. Distribution of Z-score values of participating laboratories for sample E1

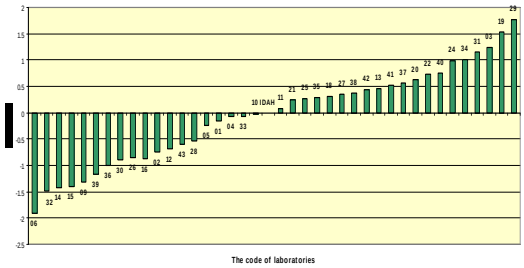

Fig. 4. Distribution of Z-score values of participating laboratories for sample E2

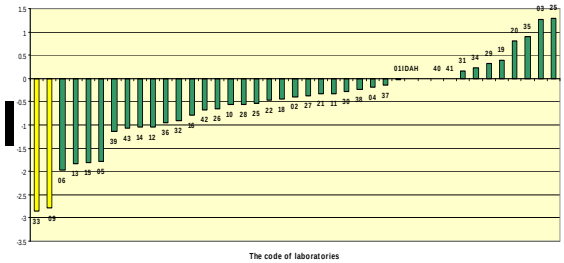

Fig. 5. Distribution of Z-score values of participating laboratories for sample E3

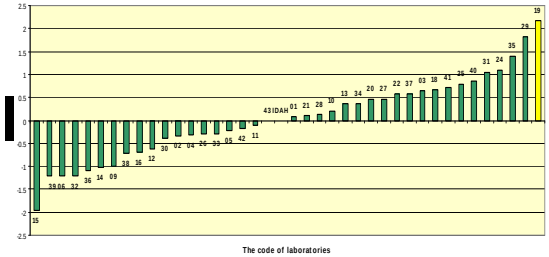

Fig. 6. Distribution of Z-score values of participating laboratories for sample E4

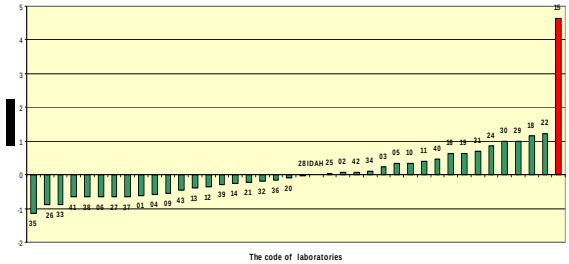

Fig. 7. Distribution of Z-score values of participating laboratories for sample E5

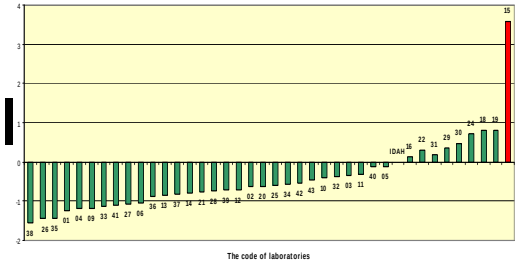

Fig. 8. Distribution of Z-score values of participating laboratories for sample E6

The sum of Z - scores (Fig. 9) was used for the classification of laboratories which had the same result at the quality interpretation test. Finally, a correlation between the obtained results of Z–scores and those of qualitative interpretation was made. Thus the correlation for negative samples E5 and E6 was 100%, for positive samples E4 of 97.5%, and E3 of 95%, and for doubtful samples it was as follows: E1: 78% and E2: 53% (Fig.11). Based on the results obtained, 31 laboratories had a satisfactory result, 6 had questionable results and 2 had unsatisfactory results (Fig.12).

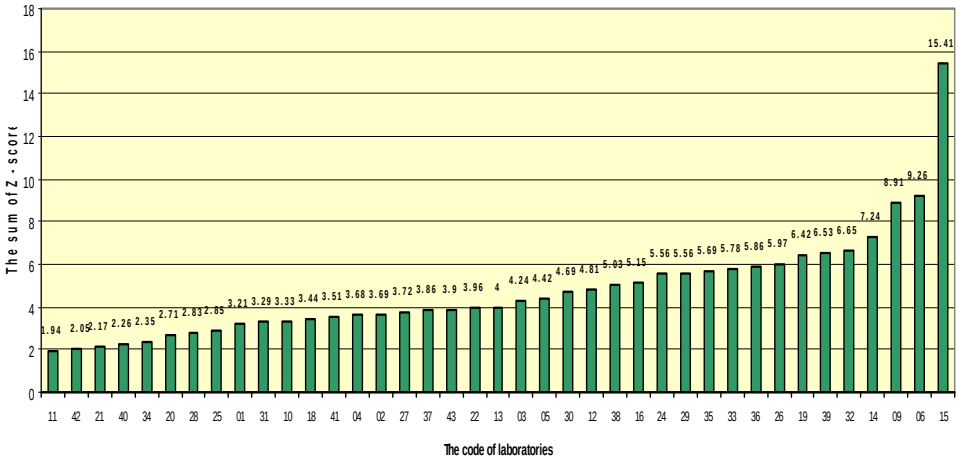

Fig. 9. Classification of laboratories according to sum of Z-scores

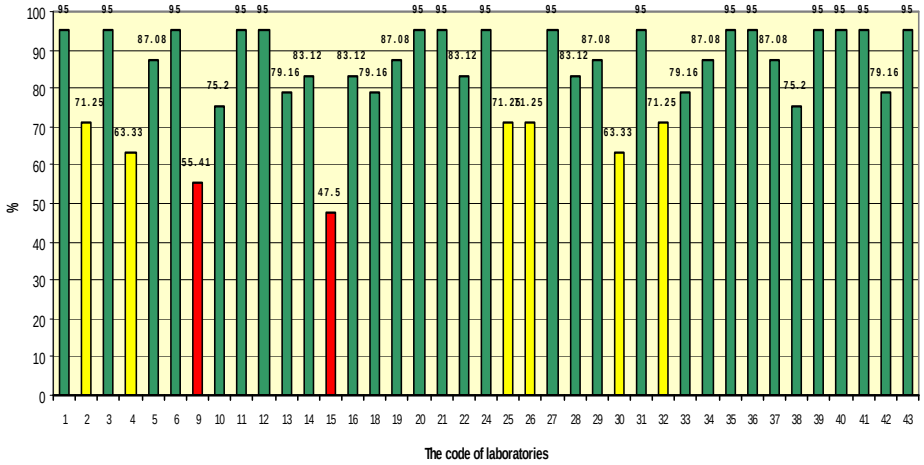

Fig. 10. Diagram based on evaluation of laboratories after qualitative interpretation of results of all samples.

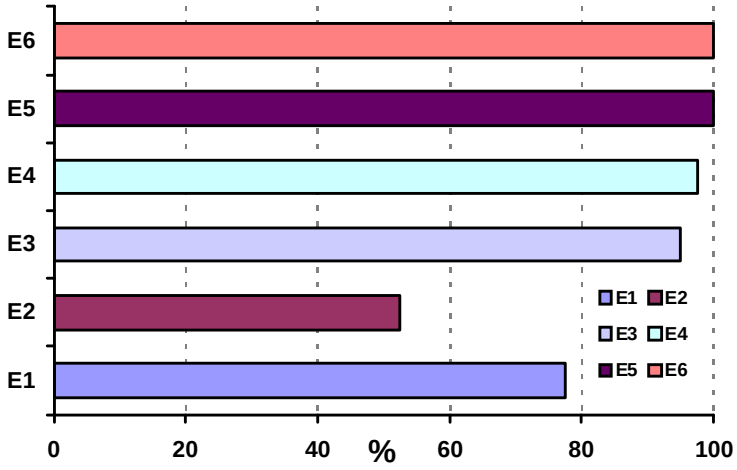

Fig. 11. Correlation between obtained results of Z-score and qualitative interpretation

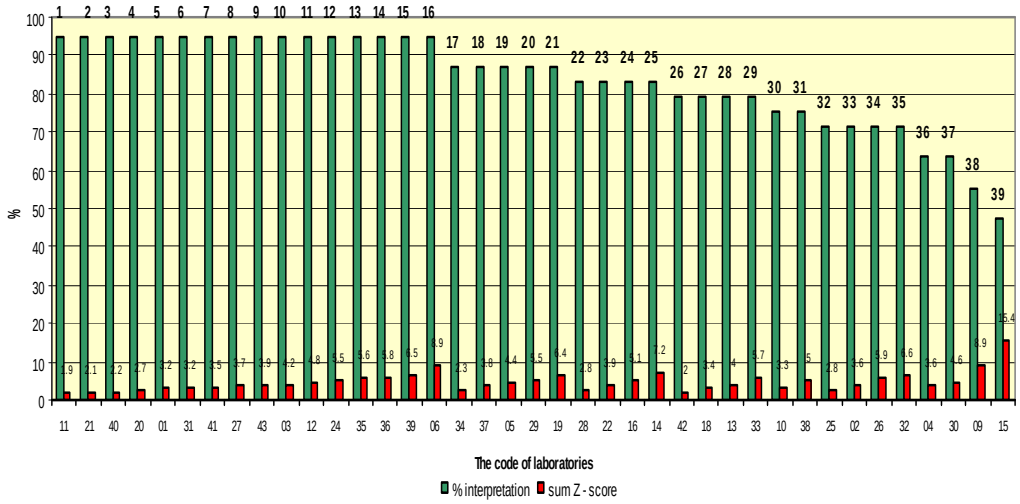

Fig. 12. Classification of laboratories after qualitative interpretation of the test and the sum of Z score at the proficiency testing

DISCUSSION AND CONCLUSIONS

Thirty seven laboratories (95%) of the participating laboratories used ELISA kits made by the same producer within the same series, which showed uniformity in the use of reagents, and which in the current case reduced the batch variation.

After the qualitative interpretation of ELISA, the following results were correctly identified: E5 and E6 (negative): 98%; E4 (positive): 95%, E3 (positive): 92%, E1 (doubtful): 60%, E2 (doubtful): 53%. The highest number of doubtful results, 16 for E1 and 19 for E2, were recorded for the samples which had doubtful results. The result of these samples may be elucidated if the laboratory has calculated extended measurement uncertainty.

The results showed that a classification as “Very well” regarding coefficients of variation (CV) were obtained on samples that came from E4 (23 laboratories) and E3 (21 laboratories) for sera with a high concentration of antibodies.

The correlation of Z - scores with the results obtained after the interpretation of the qualitative test, leads us to the conclusion that, when sera have a constant homogeneity and high constancy as in case of positive and negative samples, the differences between the two criteria of estimation were insignificant.

The interpretation of the results at 95% certainty of the test together with statistic analyses (coefficient of variation, Z - scores) permitted a more efficient evaluation of laboratories.

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

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INTRODUCTION

Paratuberculosis is endemic in the dairy cattle population of Germany. However, the actual prevalence on the individual animal as well as on the herd level is not known. About 3561 paratuberculosis cases have been recorded between 1995 and 2006 (Fig. 1).

A national paratuberculosis guideline came into force in February 2005 giving recommendations for paratuberculosis control on a voluntary basis. In Germany, because of the intensive cattle husbandry, paratuberculosis control in dairy cattle can only be successful if strict hygienic measures within the herds are combined with immediate removal of already detected faecal shedders. Up to now, faecal culture is still the most sensitive method for the identification of faecal shedders, although it is expensive and time consuming. Improvement of the effectiveness of the identification of shedders is urgently needed.

It was the objective of the present study to clarify, whether the detection rate of faecal MAP shedders can be increased by optimising the time point of faecal sampling. Therefore, the influence of individual host factors (age, lactation period, milk yield) on faecal shedding was investigated.

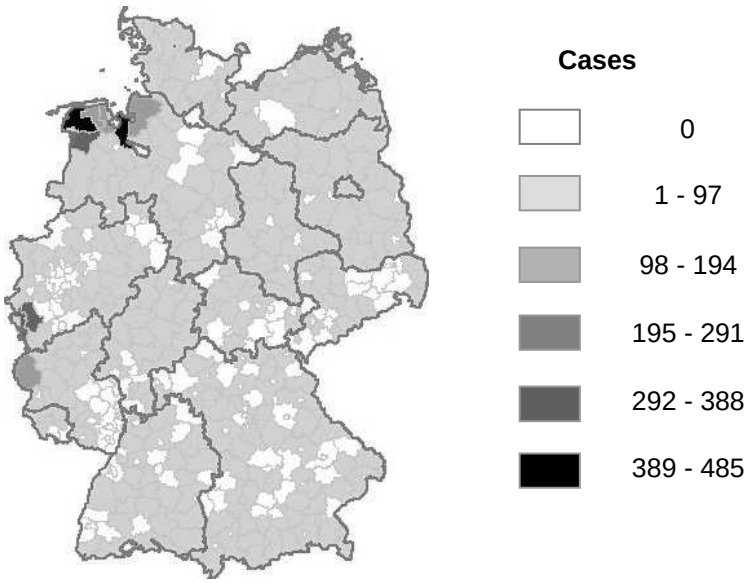


Fig. 1. Regional distribution of paratuberculosis cases in Germany, 1995 – 2006

MATERIALS AND METHODS

Herds

Two paratuberculosis positive dairy herds with an average of 245 (herd A) and 390 (herd B) lactating cattle, respectively, were included in the study. The milk yield in 2006 was about 7,212 kg/animal in herd A and 11,404 kg/animal in herd B. Culling of faecal shedders was not systematically performed in herd A while in herd B shedders were removed regularly. The proportion of faecal culture positive animals amounted to 30.3 % in herd A and 12.6 % in herd B, respectively.

Faecal samples were collected 4 times every 5 – 7 months in both herds (n=2,779). At the time of sample collection, individual data of each animal included in the study were obtained from the herd records (age, lactation stage [LS], daily milk yield [DMY]).

Faecal Culture

From each sample 3 grams of faeces were put into a screw cap tube containing 30 ml of 0.75 % hexadecyl pyridinium chloride (HPC). After vigorous shaking, the samples were kept upright for 5 minutes in order to allow sedimentation of coarse material. Then, the supernatant was transferred into a new screw cap tube. These samples were agitated for 30 min at 200 rpm on a shaker followed by incubation in upright position in the dark for 48 hours. The supernatant was carefully discarded to leave about 2 ml of sediment. 200 µl of sediment were inoculated onto each of two slants of Herrold´s Egg Yolk Agar with Mycobactin and ANV (BD, Heidelberg, Germany). The slants were incubated for up to 14 weeks and examined every second week for mycobacterial growth. Characteristic colonies were checked for the presence of acid fast bacilli by Ziehl-Neelsen staining, the presence of MAP was confirmed by PCR with the primers s204 and s749 according to Englund et al. (1999).

Statistics

Data analysis was performed using SPSS 12.0 for Windows and R 2.4.0 (R Development Core Team, 2006). Fisher´s Exact Test was applied for the comparison of frequency counts.

RESULTS

In herd A, a higher proportion of faecal shedders were detected in older animals and in animals in the third trimester of lactation. In herd B, however, the influence of age and lactation stage on faecal shedding was not obvious (Fig. 2 a, b). In both herds, there was no clear relation between milk yield and the risk of faecal shedding (Fig. 2 c).

Analysing repeated sampling every 5 to 7 months, about 13.8 % of the shedders which underwent at least three faecal examinations would have been detected by one sampling, additional 16.6 % by two samplings and further 49.0 % by three samplings. 20.7 % of the shedders would not have been detected by three subsequent samplings (Table 1).

Table 1. Effect of repeated sampling on the detection rate of faecal shedders.

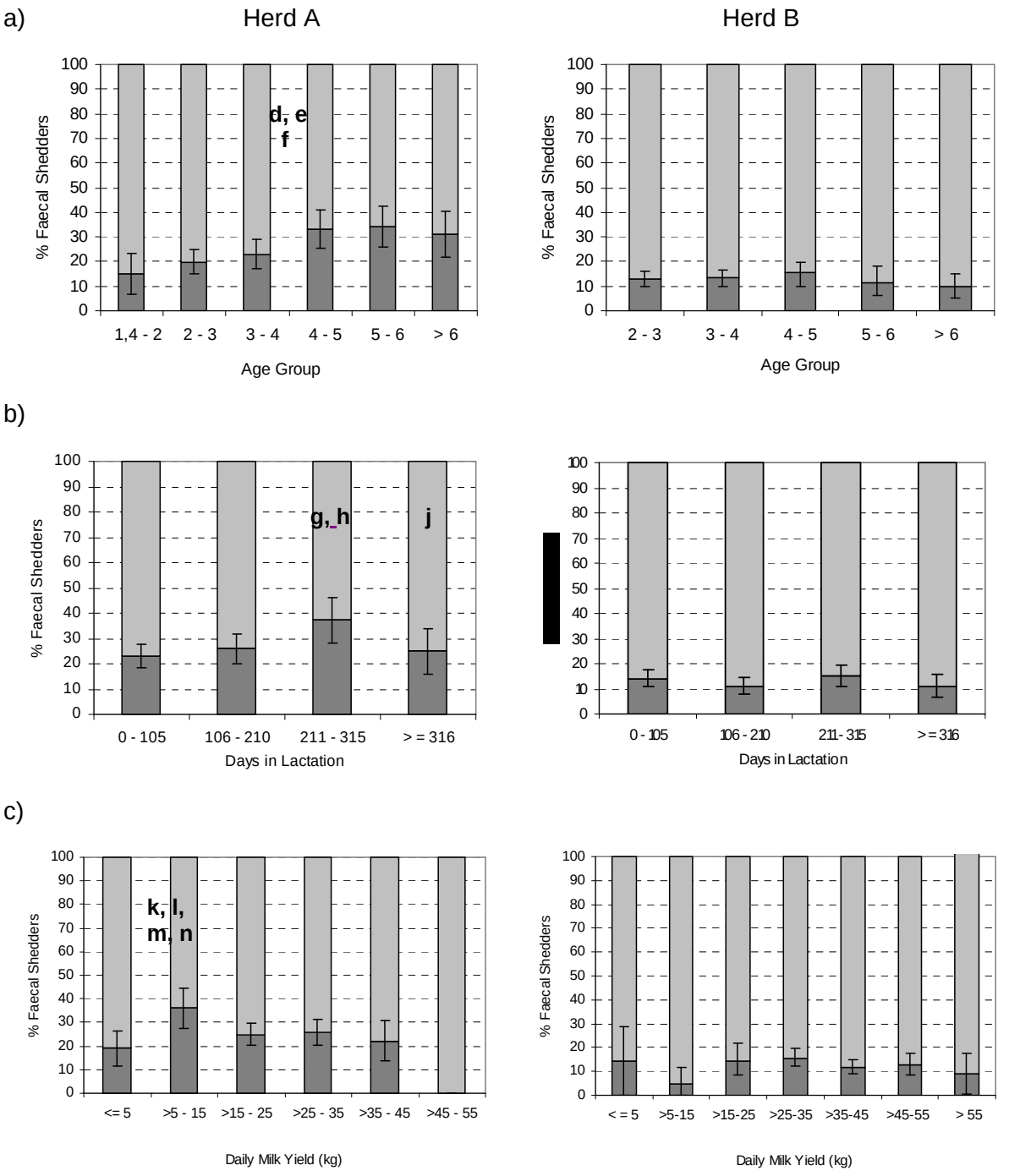
Results of examination			Detected faecal shedders	
I	**II**	**III**	**Number**	**%**
1[1]	1	1	20	13.8
1	1	0[2]	3	
1	0	1	3	
0	1	1	18	
		Σ	24	16.6
1	0	0	7	
0	1	0	13	
0	0	1	51	
		Σ	71	49.0
0	0	0	30*	20.7
		Total	145	100

*[1]faecal culture positive, [2]faecal culture negative,*positive result on 4th sampling*

DISCUSSION

In the current field study faecal shedding of Map was examined for diverse age cohorts, for varying lactation periods and for different classifications of daily milk yields at the sampling day. Marked differences between the two herds were found in the distribution of faecal shedders within these classes. In herd A the percentage of positive faecal culture results was significantly higher in 4 to 5 year old animals (33 %) compared to younger cattle. In herd B no significant differences between the five age groups were found. On average, 12.6 % of the examined animals in every age group were shedding Map. Differences in herd management, especially regarding the removal of known shedders seem to be one possible reason for this. An increase of faecal shedding of Map with higher age and progress of the disease was reported in natural dairy herds by Nielsen and Toft (2006) and Berghaus et al.

(2007). Accumulation of shedders and therefore in addition higher infection rates may occur in herds without systematic culling of already detected shedders.



Significant results of Fisher´s Exact Test are:

d	*Age 1.4-2 years vs. 4-5 years*	*p = 0.002*	***k***	*DMY ≤ 5 kg vs. >5-15 kg*	*p = 0.003*
e	*Age 2-3 years vs. 4-5 years*	*p = 0.003*	***l***	*DMY >5-15 kg vs. >15-25 kg*	*p = 0.011*
f	*Age 3-4 years vs. 4-5 years*	*p = 0.024*	***m***	*DMY >5-15 kg vs. >25-35 kg*	*p = 0.026*
g	*LS 0-105 d vs. 211-315 d*	*p = 0.003*	***n***	*DMY >5-15 kg vs. >35-45 kg*	*p = 0.017*
h	*LS 106-210 d vs. 211-315 d*	*p = 0.026*			
j	*LS 211-315 d vs. ≥ 316 d*	*p = 0.047*			

Fig. 2. Percentage of faecal shedders in herd A and B depending on age (a), lactation stage (b), and daily milk yield (c)

Dairy cows pass through different performing stages during lactation. Stabel et al. (2003) postulated, that the energy deficiency around parturition can lead to immunosuppression followed by increased faecal shedding. However, they could not find a significant rise in faecal shedding before parturition and during the first weeks of lactation. In our study faecal shedding at four different lactation periods was compared. We found a significant increase in the detection of Map in bovine faeces in herd A during the third lactation stage while in herd B there were no obvious differences. A clear explanation for this is still lacking.

A decrease in the average milk yield of paratuberculosis infected animals has been reported recently (Beaudeau et al., 2007), however, the influence of high productivity on faecal shedding of Map by dairy cows has not been studied so far. In herd A, the highest proportion of faecal shedders could be found in the group with a DMY of >5-15 kg. This seems to reflect the negative effect of paratuberculosis infection on milk production rather than an increase of shedding due to higher milk yield. This is supported by the finding in herd B that animals with very high milk yield are not more likely to shed Map than animals with lower milk yields.

Because only two herds were included, only limited data are available in this study. All of the findings may be biased by various factors. However, the data indicate that factors that influence the percentage of faecal shedders/ faecal shedding seem to be herd specific. In addition, we could demonstrate increased detection rates of faecal shedders if sampling was repeated. Three analysed samples seem to be optimum choice.

CONCLUSIONS

In conclusion, individual factors that influence faecal shedding of MAP seem to be herd specific. In the present study, no general, preferential time point for sample collection could be identified. Although older animals may have a higher risk of MAP excretion, faecal culture is also successful in young dairy cattle and these animals should be examined too. One way to increase the detection rate of faecal shedders is repeated sampling.

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A cultivation ring trail for *Mycobacterium avium* subsp. *paratuberculosis* in three certified laboratories

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ABSTRACT

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

INTRODUCTION

Paratuberculosis is a chronic granulomatous enteritis in ruminants. It is caused by infection with MAP in the intestinal mucosa and lymph nodes. The presence of MAP is usually shown by faecal culture on different solid media or by polymerase chain reaction (PCR) based on the MAP specific insertion sequence IS*900*.

MATERIALS AND METHODS

Collection of samples

Faeces samples from cows with and without clinical symptoms of paratuberculosis (chronic diarrhoea and weight loss) were sent to the laboratories for cultivation and/or PCR. The samples were from a faecal collection at one of the laboratories (lab A) stored in a freezer at -20^0 C for 3 months and then at -80^0 C for 2 months.

Cultivation of samples

Lab A: Faeces samples (2 g) were decontaminated ad modum Beerwerth (1967) [4% sodium hydroxyde for 10-15 min., spin for 15 min. (1000 g), discard supernatant, dilute pellet with 8 ml of 5% oxalic acid and 0.1% malachite green for 15 min., spin for 15 min. (1000 g), discard supernatant, dilute pellet with 4 ml of amphotericin B (50µg/ml) and neomycin (50µg/ml), settle for 10-15 min.] and cultivated on modified Löwenstein-Jensen (L-J) medium described by Jørgensen (1982). The L-J's were read once a week for 10 weeks. The identification of MAP was based on growth rate, colonial morphology, acid-fast staining, dependence on mycobactin and presence of IS*900* by PCR.

PCR was performed as described earlier by Ahrens et al. (1995): In short tubes were washed down with 1 ml PBS and then spun down (20,000 g for 5 min.). Supernatant was discarded, and the pellet was resuspended in 100-200 µl PBS and disrupted by boiling.

Lab B: Same procedure as Lab A, but the media was Herrolds Egg Yolk Medium (HEYM) produced by Difco/Becton Dickinson, Sparks, Maryland, USA as described by Nielsen et al. (2004).

Lab. C: Same procedure as Lab A without the final decontamination step (Neomycin/Amphotericin B solution) and with HEYM (Becton Dickinson). PCR was performed directly on faecal samples and after culture. In short DNA was extracted by beadbeating with zirconia/silica beads followed by purification of the lysate with a modified QIAamp protocol (QIAamp DNA Stool Mini Kit, Qiagen) as described by Herthnek (2006).

RESULTS

	Lab. A Original culture	Lab. A Culture	Lab. A Culture + PCR	Lab. B Culture	Lab. C Culture	Lab. C Culture + PCR	Lab. C Direct PCR
Medium	LJ	LJ	LJ	HEYM	HEYM	HEYM	HEYM
Positive	14 (1)*	0	10 (2)*	6	12	14 (1)*	13
Negative	16	30	20	24	18	16	17

* In brackets are number of positive samples not found by the other methods

DISCUSSION

In this study the methods to demonstrate the presence of MAP in faeces gave quite different results. This is also shown by others (Nielsen et al., 2004; Whitlock et al., 2002). The decontamination procedures showed that the use of neomycin/amphotericin B in combination with freezing might damage MAP. In this study HEYM seemed superior to L-J on samples after freezing, while L-J performed as good on fresh samples. PCR enhanced the sensitivity. This shows that combination of methods gives the highest sensitivity.

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

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ABSTRACT

A cross-sectional study was conducted from March through August 2006 in dairy herds in Fars province, southern Iran to determine the herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection and associated risk factors. Bulk-tank milk samples and management information were collected from 110 dairy herds in the province. Among study populations, 12 herds (11%, 95% CI: 5-17%) were positive for MAP infection based on IS*900* nested PCR. Statistical analysis using multivariable logistic regression showed that contamination of udders of periparturient cows with manure (OR=6.4, P=0.02) and history of suspected cases of Johne's disease in the herd (OR=6.7, P=0.04) were significantly associated with herd infection status. No relationship between breed, herd size and other management practices was found in this study. It is recommended to conduct further epidemiologic studies to determine cow-level prevalence and risk factors, and to evaluate the economic consequences of the MAP infection in the region.

INTRODUCTION

Johne's disease is a chronic disease of ruminants worldwide caused by the bacterium *Mycobacterium avium.* subsp*. paratuberculosis* (MAP). The Johne's disease in the dairy community has long been considered as an economically-important disease by reports of the negative impact on milk production and the overall economic health of dairies (Johnson et al., 2001; Beaudeau et al., 2007).

Diagnostic tests such as ELISA, agar gel immunodiffusion (AGID) test and fecal culture are being used commonly. Also PCR methods, targeting MAP specific insertion sequence (IS*900*) or other species-specific genes have been developed to increase sensitivity and specificity of diagnosis. On the other hand, presence of the pathogen in milk has been confirmed by several researchers (Sweeney et al., 1992; Grant, 2003). It was shown that MAP could be detected directly from quarter milk and bulk-tank milk by IS*900* PCR (Pillai and Jayarao, 2002; Buergelt and Williams, 2004). Therefore, application of PCR for milk samples from individual cows or at herd level as bulk-tank milk have proven useful for prevalence estimation of infection with MAP by some studies (Corti and Stephan, 2002; Stabel et al., 2002).

The aim of the present study was to estimate the current herd-level prevalence of MAP infection in dairies located in the Fars province, southern Iran using IS*900* nested PCR on bulk-tank milk samples, and to identify possible associated risk factors.

MATERIALS AND METHODS

Study population

A cross-sectional study was conducted in the Fars province, southern Iran to determine the prevalence of MAP infected-herd in the region. Overall 110 herds were included in the study.

A structured questionnaire was used to collect data about general herd information such as herd size and breed as well as information about possible risk factors for MAP infection. Most factors were compiled from the literature.

Extraction of DNA and PCR process

Milk samples were collected from well mixed bulk-tank of study herds and brought cooled to the laboratory. For detection of MAP, 50 ml was stored at -20ºC up to time of investigation.

Extraction of DNA was undertaken as described by Stabel et al., (2002) with minor modifications.

IS*900* nested PCR was conducted as described by Corti and Stephan with minor modification (Corti and Stephan, 2002). For samples with 413 bp band and suspected samples, 3 µl from the primary amplification were used for nested PCR. The primers are shown in Table 1.

Table 1. Primers used in this study

Primer name	Sequence
P90	5′-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3′
P91	5′-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3′
AV1	5′-ATG TGG TTG CTG TGT TGG ATG G-3′
AV2	5′-CCG CCG CAA TCA ACT CCA G-3′

Statistical analysis

Confidence limits (95%) for prevalence were calculated using standard normal distribution. The unit of statistical analysis was herd. To evaluate associations between suggested risk factors and herd level status for MAP infection (outcome variable), univariable and multivariable logistic regression analysis was done. Factors with P-value <0.2 in the univariable analysis were included in the multivariable logistic regression analysis.

Multicollinearity amongst putative risk factor variables was assessed using Spearman Rank correlation coefficients. All computed coefficients were less than 0.40. Also correlation between each independent variable and linear combination of other variables was investigated using variance inflation factor (VIF). All VIF were less than 1.2, revealing lack of significant collinearity in the dataset. The multivariable logistic regression model was constructed using a step-wise backward elimination approach based on significant change in -2 log-likelihood. Those variables with Wald statistic values with P<0.05 in the final model were considered as statistically significant. To account for clustering of herds in each district, location of herds (area) was forced to remain in the model and presence of two-way interaction between location and the other included variables was investigated. All statistical analysis was conducted using SPSS software (Version.11.5).

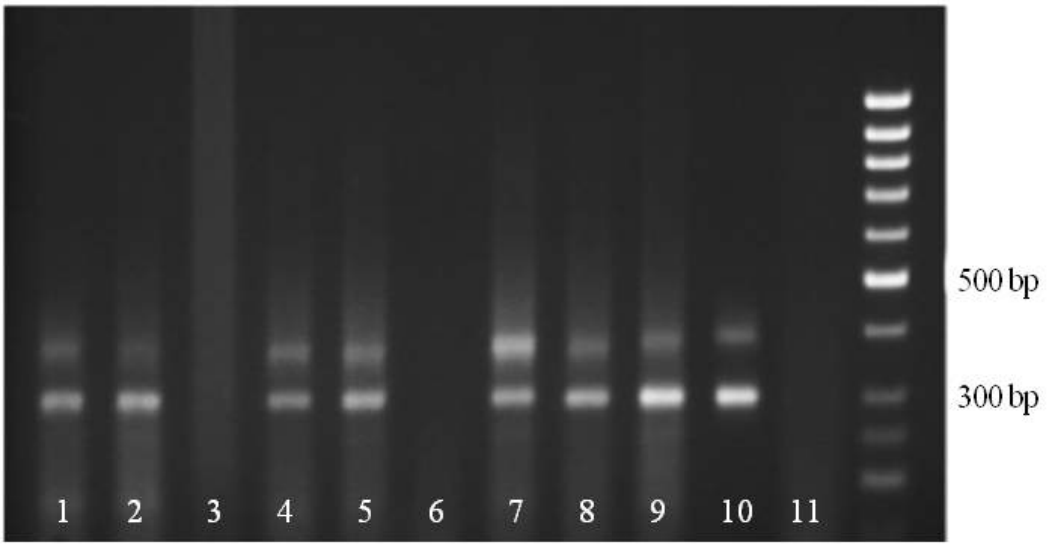

Fig 1. Nested-PCR products for IS*900* in bulk-tank milk from Fars province (southern Iran) dairy herds. L1, L2, L4, L5, L7, L8 and L9 positive samples, L3 and L6 negative samples, L10 positive control, L11 negative control and L12 100 bp marker.

RESULTS

A single band of 298 bp for each of the positive milk sample was detected by PCR amplification of the MAP specific insertion sequence IS*900* and subsequent agarose gel

analysis of the amplified products (Fig 1). Among 110 bulk-tank milk samples, 12 (11%, 95% CI: 5-17%) were positive in IS*900* nested PCR.

The prevalence of positive milk samples in the three district of Fars province were different ranging from 8.6 to 23% which was not significant (χ^2=3.3, df=2, P=0.19).

With univariable analysis six factors were selected based on P value <0.2 (Table 2) and included in the multivariable step-wise logistic regression analysis. In the final logistic model, two out of six factors were significant at the 0.05 level. Location of herds was retained in the model although it was not significant. No significant interaction was detected in the regression model. The Wald statistic, standard error, odds ratios and the 95% confidence level for the factors are provided in Table 2. Herds with low sanitation of periparturient cows as measured by high contamination of udders with manure had 6.4 times the odds of being positive for MAP infection. Also herds with history of suspected cases of Johne's disease had 6.7 times more likely to be positive by PCR than herds without such history.

Table 2. Multivariable logistic regression analysis of risk factors for *Mycobacterium paretuberculosis* infection in 110 dairy herds in the Fars province, southern Iran based on IS900-PCR in balk-tank milk samples

Risk factor	Wald statistic	SE	P	OR	95% CI
Contamination of udders with manure					
Yes	5.18	0.81	0.02	6.38	1.29-31.49
No	-	-	-	1.00	-
History of Johne's disease in the herd					
Yes	3.91	0.96	0.04	6.70	1.02-44.16
No	-	-	-	1.00	-

DISCUSSION

The overall herd-level prevalence of MAP in dairy herds investigated was approximately 11% based on IS*900* nested PCR on bulk-tank milk samples. History of suspected cases of Johne's disease and low sanitation of periparturient cows as measured by contamination of udders with manure, both indicate a higher likelihood that a herd is infected with MAP infection. It is critical to conduct in-depth epidemiologic studies to identify cow-level prevalence and economic consequences of MAP infection throughout the country.

Though several potential risk factors were not significant in the present study, as suggested by Berghaus et al. (2005), this does not necessarily imply that they are not important. Considering the various environmental, nutritional and management conditions over the world, some factors may work as important causal complement for MAP infection in one region and might not be so important in another region.

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

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INTRODUCTION

In many countries both tuberculosis (TB) and paratuberculosis (PTB) occur concurrently and there is a need to understand the real interference of bovine TB in PTB-ELISAs before recommending those assays for large scale screening. This interference was recently demonstrated in a study using *M. bovis*-infected cows, with no signs of PTB originating from PTB-free herds and demonstrated that following ELISA and immunoblot analysis some proteins are shared between *M. bovis* and Map (Marassi et al*.,* 2005).

In order to reduce such serological interference and increase the specificity of ELISA tests, recent studies have focused on the development of tests using new purified immunogenic and species-specific antigens. MPB70 and MPB83 are *M. bovis*-specific proteins that have been evaluated with promising results. Regardless of this, novel antigens could be helpful in diminishing cross-reactions between Map and *M. bovis* (Olsen et al., 2001; El-Zaatari et al*.,* 2002; Waters et al*.,* 2004).

The aim of this study was to evaluate the performance of two purified recombinant *M. bovis*-specific proteins, MPB70 and MPB83, using an ELISA assay in order to differentiate bovine tuberculosis from paratuberculosis infection.

MATERIALS AND METHODS

Cattle – Two TB-free herds, comprising 340 and 150 adult crossbred autochthon dairy cattle, respectively, were studied. A history of chronic and intermittent diarrhoea, wasting and occasional deaths in the previous six years led us to investigate paratuberculosis in these herds. In addition, six herds where a TB control program is being conducted were studied in order to obtain *M. bovis* – infected animals.

Bacterial culture for M. bovis and histopathology **-** Lymph nodes and lung samples were collected and handled for this purpose. For histopathology fixed tissue samples were stained by both hematoxylin-eosin (HE) and Ziehl-Neelsen (ZN). For bacteriology examination, samples were refrigerated for 48 hours and inoculated in Löwenstein -Jensen with pyruvate slopes, incubated at 37ºC and observed weekly for 10 weeks.

Bacterial culture of faecal and tissue samples for Map **-** From the 490 animals of both herds of Group A, 130 faecal samples were randomly taken from adult cows (age >3 years) over a period of 24 months and cultured for Map. Faecal samples were processed by the centrifugation protocol (Ristow et al*.,* 2006).

Post mortem examination of PTB clinically affected animals **-** Three adult cattle presenting clinical signs of PTB were necropsied and examined for the presence of gross lesions typical of Map infection. Tissue samples were taken from both lesioned and non-lesioned tissue and processed for both bacteriology and histopathology

Confirmation of isolates by PCR **-** Map isolates were confirmed by using PCR based on IS*900*. DNA extraction was described elsewhere (Ristow et al*.,* 2006). Amplification was performed as described (Ristow et al*.,* 2007), using primers specific for the insertion sequence IS*900* (Bio Synthesis, USA) and the Taq Platinum ® polymerase system (Invitrogen, USA).

ELISAs **-** The recombinant proteins MPB70 and MPB83 were purified and used separately as capture antigens in ELISAs as previously described (Lightbody et al*.,* 2000; McNair et al*.,* 2001). Cut-off points based on OD readings were calculated using ROC analysis and concordance of tests conducted using *kappa* index (κ).

RESULTS

Cattle - In order to evaluate the potential of ELISAs to distinguish between Map and *M. bovis* infections in cattle, two distinct populations of animals were selected. In Group A (n = 23), animals coming from TB-free herds with history of PTB were faecal culture positive for Map. In this group, six animals were in the clinical stages of Map infection while 17 were sub-clinically affected. In Group B (n = 48), animals were skin test positive to bovine tuberculin and when necropsied there was evidence of *M. bovis* infection seen either as the presence of lesions typical of bovine tuberculosis or by the recovery of *M. bovis* from tissue samples.

Evaluation of MPB70 and MPB83 by ELISA - Capture ELISAs based on either MPB70 or MPB83 were used to measure antibody responses. In Group A (Map positive), ten animals reacted to MPB70 (43%) and nine to MPB83 (39%). The mean ELISA OD value for the animals in the clinical stages of infection was 0.162 compared to 0.05 for those animals sub-clinically affected. In Group B (*M. bovis* positive), 37 animals reacted to both MPB70 and MPB83 (77.08%). Results of MPB70 and MPB83 ELISA are depicted in Tables 1 and 2. Our results indicate that both antigens presented very similar results, with a concordance (*kappa* index) of 0.91. The difference between the mean OD value for Group A and Group B measured against the same antigen was highly significant ($p<0.01$). Control sera reacted as expected, i.e. very low ODs (<0.06) for both ELISAs.

Table 1. Results of MPB70 ELISA used to measure antibody responses in Map infected cattle (Group A, n = 23) and *M. bovis* infected cattle (Group B, n = 48).

	***M. bovis* culture positive**	***Map* positive (faeces)**	**Total**
ELISA positive	37	10	47
ELISA negative	11	13	24
Total	48	23	71

Table 2: Results of MPB83 ELISA used to measure antibody responses in Map infected cattle (Group A, n = 23) and *M. bovis* infected cattle (Group B, n = 48). The difference between serum samples taken from *M. bovis* or Map culture positive and which were positive to MPB83 was statistically significant ($p <0.01$).

	***M. bovis* culture positive**	***Map* positive (faeces)**	**Total**
ELISA positive	37	9	46
ELISA negative	11	14	25
Total	48	23	71

ELISA cut-off values - Cut-off points were determined from OD readings and analysed by ROC analysis using isolation and recovery of *M. bovis* as an indicator of infection in tested cattle. At a cut-off point of 0.06, for both antigens (MPB70, MPB83) each ELISA test had a sensitivity of 52.2% and a specificity of 77.1%.

DISCUSSION

Map shares several antigens with other *mycobacteria*, including *M. bovis*. Reports show that PTB can compromise the specificity of bovine tuberculosis diagnostic tests, and natural infection with Map was demonstrated to lead to false-positive reactions in TB skin tests (Buddle et al*.,* 2003). Bovine TB also interferes on the efficacy of diagnostic tests, but this phenomenon has not yet been widely evaluated (Olsen et al*.,* 2001). More recently, a cross reactivity between *M. bovis* and Map antigens in PTB-ELISAs was demonstrated using sera of *M. bovis*- infected and Map-free cows (Marassi et al*.,* 2005)*.*

Several recombinant proteins have been tested as antigens in order to improve ELISA specificity. AhpC, AhpD and 14kDa proteins were used in an ELISA in order to discriminate PTB from TB infected-cattle, with promising results (Olsen et al*.,* 2001). A fusion protein comprising ESAT-6 and CFP-10 was used in an ELISA system with the purpose of improving the detection of specific antibodies to bovine TB, detecting even early stages of the infection (Waters et al*.,* 2004).

Immune responses to MPB70 and MPB83, proteins derived from pathogenic strains of *M. bovis* culture filtrates, are both representative of a strong antigen-induced CMI response in the early stages of the tuberculosis infection. MPB70 is the major secreted antigen of *M. bovis*, while MPB83 is a cell wall lipoprotein (Juarez et al*.,* 2001). MPB70 is specific to *M. bovis* since cattle infected with Map exposed to *M. avium* presented no detectable serum antibodies to it (Lightbody et al*.,* 2000).

In the present study, ELISAs based on either MPB70 or MPB83 were used to identify the antibody status of animals from either *M. bovis* or Map infected herds. Using these ELISAs, more *M. bovis* infected cattle were positive to both MPB70 and MPB83 compared to Map infected cattle, and the mean OD values for each group were statistically different. Using a cut-off value of 0.06, sensitivity was defined at 52.2% and specificity at 77.1%.

A relatively high proportion of Map infected cattle were also ELISA positive to MPB70 and MPB83 (43% and 39% respectively). This contrasted strongly with those animals which were infected with *M. bovis*, where 77% were antibody positive to both MPB70 and MPB83. In addition to a greater number of antibody positives, the mean OD values in the *M. bovis* infected group was greater than that for the Map infected group, with the difference between the mean values statistically significant. While these higher recognition rates within the *M. bovis* infected group indicate the usefulness of these proteins as diagnostic reagents, they lack total specificity.

There was a much higher antigen recognition frequency in the *M. bovis* compared to the Map infected group. The mean OD values for each antigen were significantly higher than that for the Map infected group ($P \leq 0.01$) and in addition, there was no significant difference between the mean values for MPB70 or MPB83 ($\kappa = 0.91$). These data indicate that either antigen could be used in an ELISA to detect antibodies to *M. bovis*.

In group A, six animals were in the late stage of paratuberculosis infection, presenting clinical symptoms, while 17 were sub-clinically infected. Mean OD of the six ill animals was 0.162, while for the sub-clinically infected cows was 0.050. This difference is highly significant ($P \leq 0.01$) and demonstrates that high ODs are more common in clinically infected animals. Consequently, cross reactions are more probable to occur in those animals since at this stage humoral responses to Map are more detectable, a feature of mycobacterial infections.

In conclusion, our results indicates that MPB70 and MPB83 clearly detect *M. bovis* specific antibodies more often in tuberculous cows than in paratuberculous cattle, and therefore can be considered as valuable tools to differentiate between these two infections using serological assays. The confusion caused by antigenic cross-reactivity between bovine paratuberculosis and tuberculosis may be resolved using purified recombinant antigens derived from each micro-organism, in order to increase the specificity of serological assays. The future combination of assays, each based on different antigens, has therefore potential as a diagnostic tool, taking into account the evolution of these infections, the individual variation of immune responses and herd history.

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

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INTRODUCTION

Since cross reactions with environmental mycobacteria were commonly reported in the first ELISAs, an absorption step of bovine sera with a suspension of killed environmental mycobacterium *Mycobacterium phlei* showed to be efficient for reducing such false positive reactions and therefore improving the test's specificity without reducing the sensitivity.

Some studies suggest that several atypical mycobacteria widely recovered from pastures could be ingested by cattle and possibly cause cross reactions in antibody tests for paratuberculosis (Norby et al., 2005). Those atypical mycobacteria have been demonstrated to induce humoral immune response in cattle that contribute to false-positive serologic reactions even in commercially available preadsorbed serum ELISAs (Osterstock et al., 2005). In Brazil, the most frequently isolated environmental mycobacteria are *M. fortuitum*, which is ubiquitous in the soil in south and southeast regions of the country, where dairy cattle breeding is more common (Leite et al., 2003).

Due to the difference in prevalence of environmental mycobacteria and their role in the specificity of diagnosis tests, we designed a pilot study to consider the hypothesis that the use of a local strain of mycobacteria could be useful as an alternative instead of, or combined with *M. phlei*, and therefore increase the specificity of ELISAs tests.

MATERIAL AND METHODS

Study design

A panel of 10 negative and four positive sera selected from our collection was used. One positive and one negative control serum, kindly offered by Dr. Michael Collins (Wisconsin, USA) were also included. All the animals that provided the positive and negative sera had their status confirmed by bacteriological culture. All the sera were tested by an ELISA protocol developed in our laboratory that uses protoplasmic paratuberculosis antigen (PPA) (Marassi et al., 2005). This assay presented 100% sensitivity and 83.5% specificity, being comparable (κ >0.5) to commercial tests (Marassi et al., 2007). Absorption of bovine sera was performed in three distinct ways: using *M. phlei* only, *M. fortuitum* only or a combination of *M. phlei* + *M. fortuitum.* With the exception of the preadsorption step, the exact same protocol, and a cut-off value of 0.35, was considered in all assays.

ELISAs

M. phlei-ELISA was performed as previously described (Marassi et al., 2005). A lyophilized commercial *M. phlei* was reconstituted in saline solution in order to obtain a 5mg/mL final concentration, according to manufacturer's instructions (Allied Monitor, USA). Ten micro liters of the suspension were mixed with an equal volume of suspect sera and incubated for 60 minutes at 37℃ with constant agitation. After t hat, the suspension was diluted in 1mL of TBST (Tris (Sigma) 10 mM, 0.9% NaCl, 0.2% Tween 20), and incubated overnight at 8℃. Sera and the *M. phlei* solution were used in a final dilution of 1:100 each in the ELISA for paratuberculosis.

M. fortuitum

ELISA was performed using a standard strain (ATCC strain 6841) and cultivated as described in routine protocols. *M. fortuitum* was diluted as above in order to correspond to the commercial *M. phlei* solution (5mg/mL) and used in the exact same conditions as described above. In the *M. phlei* + *M. fortuitum*-ELISA, sera were mixed with a solution

containing the same quantities of *M. phlei* and *M. fortuitum* that together presented a final concentration of 5mg/mL. The protein concentration founded in 5mg/mL of *M. fortuitum* was analyzed by the bicinchoninic acid (BCA) analysis (Pierce BCA Protein Assays Kit, USA), which demonstrated that *M. fortuitum* solution presented a protein concentration equivalent to the *M. phlei* commercial solution.

SDS-PAGE

In order to compare the protein pattern of *M. phlei* and *M. fortuitum* protein extracts (5mg/mL) of each were separated by sodium dodeylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel. Ten microliters of each sample were mixed with 10µL of sample buffer (Tris-HCl 62.5 mM/ pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol; 0.002% bromophenol blue). After boiling for 10 minutes, different volumes of the samples were loaded into slots: 2µL, 4µL and 8µL. Gels were stained by Comassie-blue 250.

Statistics

Chi-square test (x^2) was used to compare the different protocols. Concordance between protocols was calculated using Kappa test (k).

RESULTS AND DISCUSSION

Negative and positive control sera (one each), four sera of culture positive animals and 10 sera of culture negative animals were tested in three different conditions: as usual with *M. phlei*, with *M. fortuitum* and with a combination of *M. phlei* and *M. fortuitum* in the same concentration and volume, in the preadsorption step. When the *M. fortuitum* preadsorption was used, four sera (two positive and two negative) plus the positive control serum presented lower ODs when compared to the standard assay (*M. phlei*-ELISA), possibly indicating a higher efficiency in eliminating unspecific antibodies, which could lead to cross-reactions with environmental mycobacteria and consequent false-positive results.

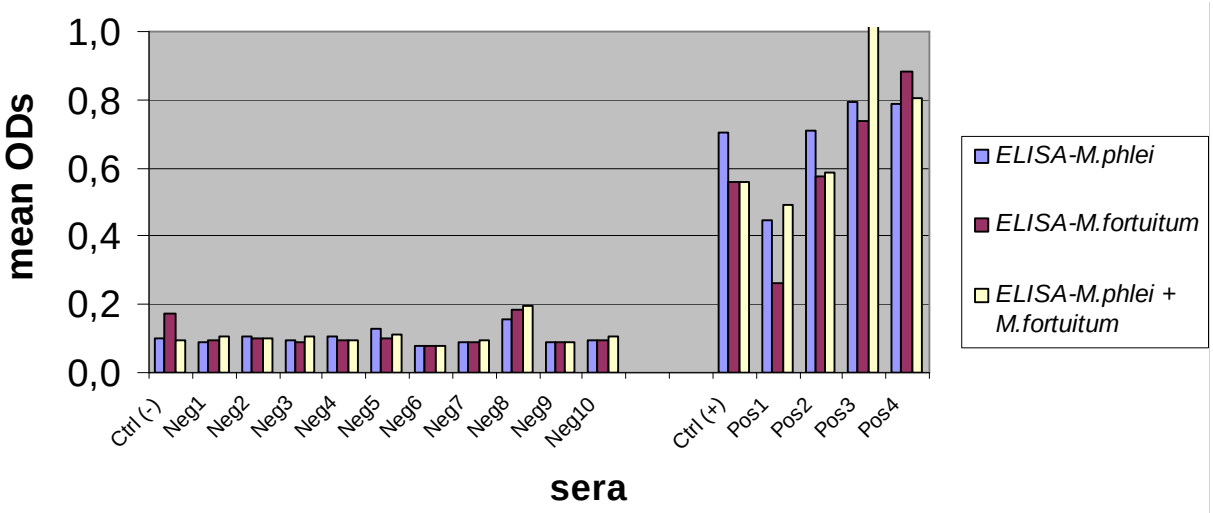

Fig. 1. ELISA results of culture positive and negative bovine sera for paratuberculosis using *M. phlei* and *M. fortuitum* alone or associated at the preadsorption step.
Ctrl (+): control positive serum / Ctrl (-): control negative serum; Neg 1-10: Sera from culture negative animals; Pos 1: Sera from borderline positive animal; Pos 2-4: Sera from culture positive animals

In spite of the overall reduction on ODs values observed at the *M. fortuitum*-ELISA, three positive sera remained presenting much higher values (mean = 0.650) than negative sera (mean= 0.150), as expected. Only one positive serum became negative with an OD value of 0.261 (cut-off = 0.35). This serum, when tested by *M. phlei*-ELISA, presented an OD value of 0.440, which is lower than the mean ODs of the positive sera used in this study. This same serum presented an OD of 0.489 when preadsorbed with the *M. phlei* + *M. fortuitum* suspension. This serum was obtained from an old cow that, in spite of being culture-positive

and, consequently, presenting a PTB-positive status, might be immunologically sub-responding. Therefore, we classified the serum as being borderline. Statistical analysis revealed that, in spite of one serum having its final result altered, the assays using different preadsorptions were demonstrated to be comparable ($P<0.01$) and no difference on efficacy could be detected between them ($\kappa > 0.8$).

On the other hand, two of the positive sera and four of the negative sera preadsorbed with the *M. fortuitum* + *M. phlei* solution presented higher ODs than with the standard assay. Nevertheless, with this preadsorption step, no serum changed its final status and correlation between both tests was also high for those samples ($\kappa > 0.8$). In spite of these differences, variation on ODs values observed among the three preadsorption assays was not significant ($P< 0.01$).

The inclusion of a preadsorption step employing *M. phlei* was mandatory for increasing specificity of paratuberculosis ELISAs without interfering in the sensitivity. Nevertheless, since atypical mycobacteria interfere with the results even in preadsorbed commercially available serum ELISAs (Osterstock et al., 2005), other ways of reducing such interference must be achieved.

Protein patterns of both *M. phlei* and *M. fortuitum* were compared after separation by SDS-PAGE. This analysis demonstrated a very similar pattern of proteic bands. This finding suggests that some antigens may be shared between both microorganisms, leading to cross reactions. This is not unexpected, since it has been widely demonstrated that several mycobacteria species share proteins and other antigens (Bannantine et al., 2002). Therefore, it reinforces in a biochemical point of view the possibility of using *M. fortuitum* preparations as an alternative for the preadsorption step in paratuberculosis ELISAs.

M. fortuitum is a fast-growing mycobacterium with few requirements for its culture. It is also very frequent as soil inhabitant in many countries and can be easily obtained and maintained by mycobacteria laboratories worldwide. Although only few sera were used in this study, these preliminary results suggest that *M. fortuitum*, alone or combined with *M. phlei*, may be considered as an alternative for the preadsorption step of ELISAs for paratuberculosis, with comparable results from those obtained with the standard assay that uses *M. phlei* at the preadsorption step. All the tested assays were capable to reduce cross-reactions with environmental mycobacteria and no significant difference was observed in the sensitivity or specificity of the assays in this study.

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

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ABSTRACT
The objective of the present study was to evaluate the association between interferon-gamma (IFN-γ) test results in young stock and ELISA status as adult cow among animals with different status in faecal culture (FC). The study was conducted in a three-year period, during which blood for IFN-γ testing was sampled from heifers in 18 Danish dairy cattle herds. In addition, milk samples collected after calving were analysed for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) antibodies (Ab) and faecal samples were cultured. Among 1180 FC negative cows, 2.8% of IFN-γ positive heifers became ELISA positive cows, whereas among 77 FC-positive cows a significantly higher proportion of 29.5% were ELISA positive ($p<0.001$). Eleven of the 77 FC-positive animals were ELISA positive without previously being IFN- γ positive. Generally, the results concur with the generally accepted pathogenesis, but some results suggest that either some infected animals are not infected as young or that the IFN-γ is not very sensitive.

INTRODUCTION
Paratuberculosis is a chronic, granulomatous enteric infection caused by MAP in ruminants. Eradication of MAP in cattle herds is complicated by lack of accurate diagnostic tests for early diagnosis of infected animals. Available diagnostic methods include detection of MAP by cultivation, cell-mediated immune responses by IFN-γ assays on blood samples or Ab in milk and blood by ELISA.

MATERIALS AND METHODS
During a three year study period, blood was repeatedly sampled from 15-24 months old heifers in 18 Danish dairy cattle herds infected with MAP, and analysed the following day by a whole blood IFN-γ test supplemented with IL-12 (Jungersen et al., 2005). After calving, milk samples were analysed for MAP Ab by ELISA (Nielsen, 2002) three times per year per animal. For the present analysis, the result of the latest available ELISA test was used. Faecal samples were cultured on Herrold's egg yolk medium (Nielsen et al., 2004) once per year from adult cattle to describe the MAP shedding status of the cows. Animals were retrospectively grouped by their FC status.

INTERPRETATION
Animals were considered FC-negative if all samples were negative at culture. The IFN-γ test was considered positive if IFN-γ ≥ 1000 pg/ml in PPDj stimulated and IL-12 potentiated blood samples. The ELISA test result was considered positive if $OD_{Corrected} > 0.3$.

RESULTS
The results are presented in Table 1. Of the 77 FC positive animals, 13 animals were tested both IFN-γ and ELISA positive. However, 31 heifers that had been tested IFN-γ positive were not ELISA positive later on. Conversely, 11 cows that were ELISA positive had been tested negative as heifers by the IFN-γ test. A large part of the tested animals were FC negative. Of 1180 FC negative animals, 17 were both IFN-γ and ELISA positive. Nearly half of the FC negative animals, or 593 heifers, were tested positive only by IFN-γ. Fifty of the FC negative animals were only ELISA positive. Among the FC positive, 57% were previously positive by IFN-γ, whereas 52% of the FC negative animals had been tested positive by the early IFN-γ test.

Table 1. The distributions of tests-results from ELISA and IFN-γ stratified by FC.

FC positive cows (N=77)			FC negative cows (FC=1180)		
	ELISA^{+}	ELISA$^{\div}$		ELISA^{+}	ELISA$^{\div}$
IFN-γ^{+}	13	31	IFN-γ^{+}	17	593
IFN-γ$^{\div}$	11	22	IFN-γ$^{\div}$	50	520

Figure 1 illustrates that among FC negative cows, 2.8% of the heifers, that were previously IFN-γ positive, became positive in ELISA, whereas a significantly ($p< 0.001$) higher proportion of 29.5% became ELISA positive among FC-positive cows.

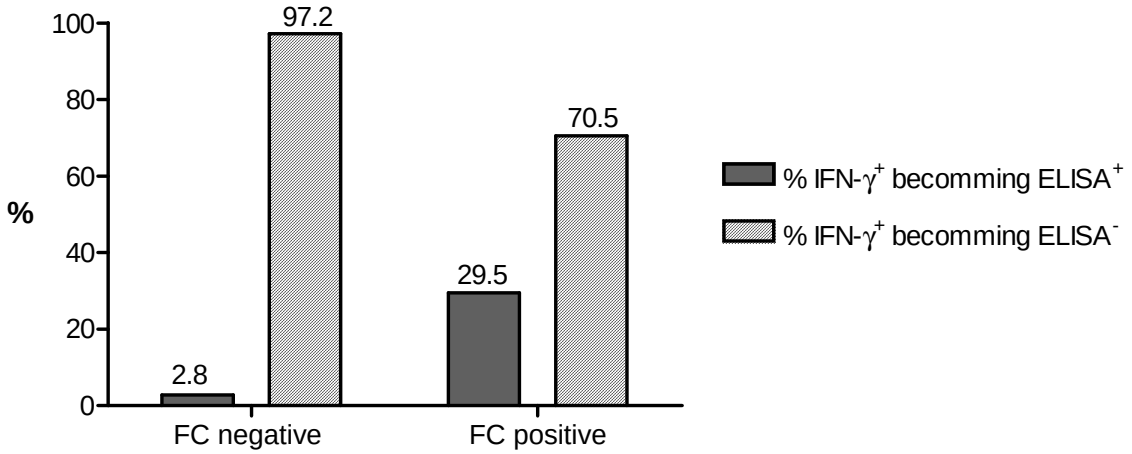

Fig. 1 The distribution of IFN-γ positive heifers becoming ELISA positive, and IFN-γ positive heifers becoming ELISA negative, both stratified by FC status.

DISCUSSION AND CONCLUSION

The significantly higher proportion of IFN-γ positive animals that tested positive by ELISA among FC positive, correspond to the generally accepted pathogenesis, where animals shedding MAP are more likely to have antibodies. However, among FC-positive, 11 animals (33% of all IFN-γ negative) with a positive ELISA were previously IFN-γ negative, suggesting that either: a) not all shedding animals are infected as young animals or b) that the IFN-γ test is not very sensitive.

This study only included MAP infected herds and hence the specificities of the tests were not evaluated. In addition, the true infection status of cows can at present only be obtained by post mortem histopathology. Moreover, all tests have limitations at certain points during progression of MAP infection. Further evaluation and optimisation of the IFN-γ test using new and more specific antigens is necessary for diagnosis in young animals. An association between milk antibody and IFN-γ may not be expected until a specific and sensitive IFN-γ test has been developed.

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

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ABSTRACT

United States Department of Agriculture (USDA) regulations have stated that an organism-based test (culture/PCR) is the official test for determining the infective status of an animal for Johne's Disease. Recent method evaluation tests performed for laboratory approval for the Voluntary Bovine Johne's Disease Control Program (VBJDCP) indicate multiple culture methods 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 USDA licensed diagnostic kits used for Johne's disease detection.

Based on the results of the last 11 years of proficiency tests for detecting *Map,* several methods have been chosen for further evaluation. These methods included different decontamination methods involving sedimentation or centrifugation, and different media including solid and liquid which have been used by multiple laboratories. Preliminary evaluation based on proficiency test results indicate that centrifugation methods are more sensitive than sedimentation decontamination methods, and liquid media methods are faster than methods using Herrold's Egg Yolk (HEY) media with mycobactin J. More PCR methods are being introduced and evaluated by different laboratories each year. The Tetracore VetAlert™ Johne's Real-Time PCR is the only USDA licensed PCR Kit available in the United States.

Varied growth performances in the solid media used with different culture methods were also noted during the last 11 check tests. There are now 2 commercial sources of HEY available in the United States which were evaluated along with in-house media for growth performance. Tissue culture flasks containing the same amount of HEY media and inoculums were evaluated and shown to have isolated more MAP colonies in an 8 week time period than tubes containing equal volume of HEY media in the same time.

Keywords: cattle, culture methods, feces, proficiency tests

INTRODUCTION

United States Department of Agriculture (USDA) regulations have stated that an organism-based test (culture/PCR) is the official test for determining the infective status of an animal for Johne's disease. Recent method evaluation tests performed for laboratory approval for the Voluntary Bovine Johne's Disease Control Program (VBJDCP) indicate multiple culture methods 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 USDA licensed diagnostic kits used for Johne's disease detection.

The goals of the NVSL Laboratory approval program for Johne's Disease include the following: (1) Standardize the methods for antibody and organism based tests; (2) Provide information to the lab on their own testing performance in relation to other labs; (3) Maintain a list of approved labs for state programs and producers who participate in the VBJDCP; and (4) Establish minimum standards for diagnostic testing and interpretation of Johne's Disease test results in different species of animals. The Johne's Disease Laboratory Approval Program is voluntary. Failure on the check tests does not prevent testing by the participating labs. The VBJDCP requires states to use NVSL approved laboratories for testing samples. Annual participation is required to maintain approved status.

MATERIALS AND METHODS

Laboratory participation has increased every year since the program started in 1996. Laboratories from the United States, Canada, The Netherlands, Denmark and Sweden have participated during the last 11 years (Table 1). The fecal test panel consisted of approximately a fourth negative (autoclaved) samples, a fourth TNTC (too numerous to count) and the remaining half with different levels of organisms which were less than 100 CFU per sample (Table 2).

Table 1. Check Test Participants

	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
No. labs	33	41	48	48	52	60	64	72	76	68	71
U.S.	32	40	44	42	48	55	56	64	68	63	65
Canada	1	1	3	5	4	4	6	5	6	4	5
Netherlands	0	0	1	0	0	1	0	0	0	0	0
Denmark	0	0	0	1	0	0	2	2	1	0	0
Sweden	0	0	0	0	0	0	0	1	1	1	1

Table 2. Fecal Panel Contents

	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Negative	10	4	7	6	6	6	7	7	7	7	7
Positive	20	16	18	19	19	19	18	18	18	18	18
>50	7	7	5	6	10	6	8	8	3	4	7
11-49	7	3	8	4	4	3	5	6	7	8	6
1-10	6	6	5	9	5	10	5	4	8	6	5
+ control	0	0	0	0	0	0	0	0	1	1	1

The criteria for laboratory approval included the following: (1) a lab must identify 100% of the negative samples – no false positives; (2) a lab must identify 100% of the TNTC (too numerous to count) positive samples – no false negatives; (3) a lab must identify 70% of the remaining positive samples which have 1-50 organisms; (4) the lab should have a minimum score of 85%; (5) A valid sample is one in which 70% of the labs had a consensus of agreement.

Each laboratory submitted 12 tubes of Herrold's Egg Yolk Medium (HEYM) with mycobactin J for evaluation. Each tube of media was evaluated for pH, sterility and growth performance. The recommended pH was 7.0 to 7.5. Sterility was checked at 24 and 48 hours and weekly for 6 weeks. Growth performance was checked weekly for 6 weeks. HEYM with mycobactin J was inoculated with 0.5 McFarland standard cultures of *Map* ATCC 19698, *M. avium* ATCC 25291 and *M.* i*ntracellulare* ATCC 13950.

RESULTS

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 HEYM with mycobactin J (Tables 3 and 4). Each year more labs are submitting results based on direct PCR on feces and the

number of approvals has increased each year (Table 5). Currently the Tetracore VetAlert™ Johne's Real-Time PCR is the only USDA licensed PCR Kit available in the United States.

Table 3. Lab approval for organism based tests – fecal culture

	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
No. labs approved	26	16	33	35	45	50	47	56	62	55	83
No. labs not approved	2	12	11	6	2	12	5	8	14	22	15

Table 4. Fecal culture by methods

	1999	2000	2001	2002	2003	2004	2005	2006	2007
No. methods approved	33	35	45	60	57	69	62	56	83
Centrifugation	21	29	36	40	40	42	35	30	44
Sedimentation	11	4	6	4	5	4	3	3	2
TREK ESP	0	0	1	5	7	15	15	12	23
BACTEC 460	1	2	2	1	4	3	3	2	3
BACTEC MGIT 960	0	0	0	0	1	5	6	9	11
No. methods not approved	11	6	2	12	5	8	14	22	15
Centrifugation	5	5	2	10	4	7	7	9	6
Sedimentation	4	1	0	1	0	0	0	0	0
TREK ESP	0	0	0	0	0	0	3	10	7
BACTEC 460	2	0	0	1	1	1	1	2	0
BACTEC MGIT 960	0	0	0	0	0	0	3	1	2

Table 5. Lab approval for organism based tests – PCR direct on feces

	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
No. labs approved	1	0	2	4	8	8	7	15	16	22	36
No. labs not approved	5	4	1	2	2	8	6	5	5	7	6

Varied growth performances in the solid media used with different culture methods were also noted during the last 8 check tests. There are 2 commercial sources (Becton Dickinson & Remel) of HEY with mycobactin J available in the United States which had been evaluated along with in-house media for growth performance. Labs that used media produced by Becton Dickinson only had to submit the lot numbers that were used. This is the main reason that the number of labs who participated has decreased over the last 3 years (Table 6). Media evaluation was discontinued in 2007 since only BD media is being used by the majority of labs in the U.S.

Table 6. Media evaluation

	1999	2000	2001	2002	2003	2004	2005	2006
No. labs	12	20	21	8	8	5	2	2
Sterility	12 OK	20 OK	21 OK	8 OK	8 OK	5 OK	2 OK	2 OK
pH test	7-7.5	6.9-7.6	6.6-7.2	6.8-7.3	6.9-7.2	7.0	7.0	7.0
Growth performance	P+2 to P+4	P+2 to P+4	P+2 to P+4	P+4	P+1 to P+4	P+1 to P+4	P+1 to P+4	P+1 to P+4

The list of NVSL approved laboratories is published each year in the United States Animal Health Association Proceedings – Johne's Committee report and posted on the following web sites: NVSL web site: http://www.aphis.usda.gov/animal_health/lab_info_services/downloads/ApprovedLabs_Johnes_organism.pdf; NIAA web site: http://www.johnesdisease.org/Labs/certifiedlabs.htm; and the JDIP Web site: http://www.jdip.org/2007_Johnes_approved_labs_organism.pdf. The list of NVSL approved laboratories and those labs which failed are also sent to the National Johne's Epidemiologist and the Designated Johne's Epidemiologist from each state.

CONCLUSIONS

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 (BACTEC 460, TREK ESP, and BACTEC MGIT 960) which have been used by multiple laboratories. Based on the media evaluation of the last 8 years, HEYM with and without mycobactin J which are produced by 2 commercial companies [Remel and Becton Dickinson (BD)] are used by over half of the participating laboratories. BD media appears to isolate more *Map* organisms (>100 cfu / tube) than Remel media in comparison testing by several labs and on the media performance test. HEYM produced in-house by the participating lab had more varied results and fewer organisms were isolated (10-100 cfu). The Tetracore VetAlert™ Johne's Real-Time PCR kit is performing well compared to other PCR methods developed in-house. Coupled with the Tetracore MAP Extraction System, this kit can be used for direct fecal analysis and culture confirmation of *Map* organisms from bovine samples.

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

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INTRODUCTION

Johne's disease (JD) is a serious infection of animals caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Johne's is not a priority in India. Therefore, information on prevalence in 465.50 million domestic ruminants have not been estimated nor realized, mainly due to lack of indigenous diagnostic kits. Diagnosis is difficult due to long incubation, absence of characteristic symptoms, problems in cultivation of MAP and non-specific results in Johnin test. Until 1989, when IS*900* was discovered in the MAP genome, characterization was difficult. IS*900* is specifically present in 14-18 copies in MAP (Green et al., 1989) facilitating specific identification in PCR. However, PCR is still not a convenient test to standardize mainly due to difficulties in isolation of genomic DNA. MAP has rigid cell wall which is very difficult to break (Garrido et al., 2000; Hammer et al., 2000). Therefore, MAP cells must be primarily prepared before DNA isolation and selection of method of isolation of mycobacterial DNA essentially determines success of PCR. The best method for isolation of mycobacterial DNA is a combination of physical, chemical and enzymatic methods (Fus et al., 2003; Hosek et al., 2006), successfully employed by van Soolingen et al. (1993). However, a loopful of culture as starting material is required. But minute colonies of MAP were usually lost on prolonged incubation / contamination / drying of media and desired loopful growth has rarely been available (Kumar et al., 2007). Attempts to isolate DNA from a few colonies were consistently negative. The current study aimed to compare routinely used a standard DNA isolation method with a new non-chemical (physical) method and further characterization by IS*900* PCR.

MATERIALS AND METHODS

A total of 139 colonies (characterized on the basis of slow growth, mycobactin J dependency and acid-fastness) from tissues of animals, tissues and stool samples of human beings, un-pasteurized milk, commercial pasteurized milk and fecal samples of dairy cattle were used in the study. Colonies were extremely minute and fewer in number (1-4) and rarely luxuriant. There was homogeneity (colonies size and numbers) in samples processed by two methods (Table 1).

Table 1. Number of colonies per culture processed by method 1 and 2

Colonies number	Method 1	Method 2
1	10	10
2	15	23
3	29	28
4	15	9
Total	69	70

Preparation of M. paratuberculosis template DNA from culture

Method 1

DNA was isolated using the method described by van Soolingen et al. (1991) for 69 cultures.

Method 2

Another 70 cultures were subjected to DNA recovery using new freeze-thaw method. MAP growth (single to pauci-bacillary) was harvested in 100µl of triple distilled water or normal

saline. The suspension was heated at 90^{o}C in water bath for 10 min, followed by snap cooling in ice for 10 min. The steps were repeated 3 times. Eppendorf was centrifuged at 2432 x *g* for 5 min, and the supernatant was used directly as template DNA in IS*900* PCR.

Estimation of DNA quantity
Pico-green analysis was used to determine the DNA concentration in culture samples processed by Methods 1 and 2.

DNA amplification
Template DNA prepared by Methods 1 and 2 were subjected to IS*900* PCR using Vary primers (Vary et al., 1990). PCR reaction mix (50µl) contained 0.2 m*M* each of the 4 dNTPs, 1µ*M* primers, 1.5 m*M* $MgCl_2$, 1U of Taq polymerase (Qiagen), and template DNA (1.0 µl) isolated by *Method 1* and *2*. The Q-solution was used in PCR after optimization in our laboratory as recommended by manufacturer. PCR conditions were as described by Vary et al. (1990). PCR products were separated in agarose gel (2%), stained with ethidium bromide and visualized (Gel Doc, Alpha Innotech).

RESULTS

Method 1 yielded DNA (concentration 13.86-18.54 ng/µl) from 23 (33.3%) cultures (Table 2). DNA was isolated from cultures containing 4 and more visible colonies and from 10 samples containing at least 3 visible colonies. Using Method 2, DNA was recovered from all 70 cultures and the concentration of DNA was 3.0 to 10.0 ng/µl).

IS*900* was amplified from the 10 of the 23 DNA samples isolated by Method 1, and from 63 of the 70 DNA samples isolated by Method 2, and the IS*900* positive samples were characterized as MAP. The samples from which DNA was isolated originated from 16 human samples, 13 pasteurized milk samples, 7 un-pasteurized milk samples and 34 fecal cultures, PCR confirmed 14 (87.5%), 13 (100%), 7 (100%), and 29 (85%) of the samples as MAP, respectively (Table 3).

Table 2. Estimation of the quantity of DNA obtained by method 1 and 2

Colonies Number	Concentration of total DNA recovered (ng/µl)	
	Method 1	Method 2
1	-	3.0 - 4.0
2	-	3.5 - 5.4
3	-	5.0 - 8.0
4	13.86-18.54	8.0 -10.0

Table 3. Comparison of IS*900* PCR of Method 1 and 2 for DNA isolation

Samples	**Source and place**	**Cultures**	**DNA Recovery**	**PCR Positives**
Method 1				
Milk (Un-pasteurized)	Cattle , Ludhiana	45	15 (33.3)	3 (6.6)
Feces	Blue bulls, Farah	9	2 (22.2)	1 (11.1)
Tissues	Goats, Farah	15	6 (40.0)	6 (40.0)
Total		69	23 (33.3)	10 (14.5)
Method 2 (New method)				
Milk (Un pasteurized)	Cattle & buffalo, Farah & Mathura	13	13 (100.0)	13 (100)
Milk (Pasteurized)	Market, Mathura, Agra, New Delhi	7	7 (100.0)	7 (100)
Feces	Cattle, Mathura	34	30 (88.2)	29 (85.3)
Tissues / Stools				
Human	Human CD patient (New Delhi) & in contact persons (Farah)	16	14 (87.5)	14 (87.5)
Total		70	64 (91.4)	63 (90.0)

Figures in parenthesis are per cent

DISCUSSION

MAP can be differentiated phenotypically from *M. avium* and *M. sylvaticum* by dependence on mycobactin (Thorel et al., 1990) and geno-typically by presence of multiple copies of the insertion element IS*900* (Green et al., 1989). Diagnosis of JD depended on detection of MAP in clinical samples. Optimization of isolation of genomic DNA from MAP is difficult therefore lot of variability has been observed in results. Culture of MAP though being a 'Gold standard' is hampered by primary MAP colonies growing extremely slowly. MAP from different animal species has diverse requirements of medium components (Merkal and Curran, 1974).

During large-scale culture of MAP from different species including human beings, difficulties were encountered in our lab. It led to non-availability of a loopful of MAP cultures for DNA isolation. Therefore, use of Method 1 for isolation of DNA (van Soolingen et al., 1991) from cultures with low quantities of MAP colonies was counter productive, and only multi-bacillary cultures could be characterized.

In sheep the paucibacillary form of MAP predominates and prolonged incubation up to years is required to grow the organism (Whittington et al., 1998). In large number of cultures MAP colonies were few and never grew in size despite prolonged incubation and they were lost due to drying of medium or contamination. Isolation of DNA from few minute colonies by Method 1 was less successful due to loss of DNA in the multi-step procedure, which resulted in variability in sensitivity of PCR employed by different workers (vander Giessen et al., 1992).

Low sensitivity of the PCR reaction for detection of MAP in tissues, faeces, milk and culture samples is attributed to false negative results due to difficulties involved in preparatory phase (bacterial concentration). DNA extraction from few bacilli often leads to false negative results. A rapid and sensitive method to detect viable MAP in milk or other dairy products is important for risk to human beings. PCR targeting IS*900* is promising alternative for specific detection of MAP in different clinical samples within short time. The cell wall of MAP is difficult to break down causing additional problems in DNA isolation. There is no single efficient protocol for DNA extraction (van Soolingen et al., 1991; Boom et al., 1990; Bose et al., 1993). In the present study, a new method was adopted wherein DNA was successfully recovered from a single tiny colony of MAP, without using chemicals, enzymes and hectic multi-step process of DNA isolation. The new method increased the sensitivity of PCR manifolds. Therefore, it helped to overcome low sensitivity of PCR assay in Method 1, due to low yield of DNA from tiny colonies. It was presumed that repeated freeze and thaw method lysed sufficient proportions of MAP cells, thereby allowing the DNA release and detection by PCR. Method 1 yielded DNA only from samples containing more than 3-4 colonies. Method 2 recovered DNA from every sample and concentration of DNA ranged from 3 to 10 ng/µl. The concentration of DNA was sufficient to detect MAP in PCR. In routine testing 2-5.0 ng/µl DNA concentration has been standardized in PCR in our laboratory. Method 2 was detected MAP in pasteurized milk and saved MAP isolates for other purposes that had few colonies even after prolonged incubation. Comparative results indicated better sensitivity of Method 2 over Method 1 (multi-step DNA isolation process). Success of PCR amplification depended on quality and quantity of template DNA. Method 1 was applied unsuccessfully in extraction of sufficient DNA from pauci-bacillary cultures. Though Method 2 was used for DNA recovery from MAP colonies in this study, it could be employed directly on clinical samples.

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

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INTRODUCTION

Control of Johne's disease (JD) is hampered in India due to lack of suitable diagnostic test. Culture usually takes up to 4 months and ovine strains of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are more difficult to cultivate. As compared to feces, culture of target tissues is more confirmatory. However, culture is inadequate to distinguish MAP from *M. avium* and other closely related mycobacterial species. IS*900* PCR is rapid test for identification of MAP, however sensitivity is highly variable. False-negative reactions result in low sensitivity, which may be due to unsuccessful DNA isolation and presence of inhibitors. Cell wall of MAP is difficult to break causing problems in DNA isolation step. Thus, selection of suitable method for isolation of DNA is crucial for detection of MAP. This study compared 4 methods for isolation of good quality DNA of MAP from intestinal tissues and compared their sensitivity with tissue culture.

MATERIALS AND METHODS

Animals and tissues

Twenty-five intestinal tissues from farm goats with clinical JD collected. Tissues were swollen and corrugated and positive in culture for MAP bacilli.

Culture and DNA isolation

Tissue was homogenized in 15 ml sterilized distilled water and allowed to settle for 3-4 hours at room temperature, and the supernatant was distributed into 5 tubes (4 for DNA isolation and 1 for culture). Five ml of supernatant was decontaminated overnight with 0.9% HPC (Hexadecylpyridium chloride). Sediment was inoculated on Herrold's Egg Yolk Medium (HEYM) supplemented with mycobactin J as per Singh et al (1996). *G*enomic DNA isolated from each tissue using 4 methods.

Method I

Supernatant was pelleted, washed twice with PBS and DNA was isolated as per method of van Soolingen et al. (1991) (employed for MAP cultures).

Method II

Supernatant was decontaminated (0.9% HPC) over night and sediment (1 ml) was pelleted and washed twice with PBS. Pellet was subjected to DNA isolation by the method of van Soolingen et al. (1991).

Method III

Supernatant pelleted and washed twice with PBS. Pellet was digested with 400 µl of tissue lysis solution (2% Triton-X, 1% SDS, 100mM NaCl, 0mM Tris HCl) [pH 8.0] and 5 µl of proteinase K (20 mg/ml), and incubated for 30 min at 50^0C. Saturated phenol (400 µl) was added, mixed thoroughly and centrifuged for 5 min at 10,000 rpm. Aqueous layer was transferred to fresh tube, and equal volume of chloroform-iso-amyl alcohol (24:1) was added and mixed thoroughly and centrifuged for 5 min at 10,000 rpm. This step was repeated and aqueous layer was transferred to a fresh tube. Double volume of chilled absolute ethanol was mixed and stored overnight at -20^0C. DNA was recovered by centrifuging samples for 10 min at 12,000 rpm. Pellet washed with 70% ethanol, air-dried and suspended in 30 µl TE buffer [pH 8.0].

Method IV

The supernatant was first decontaminated as per method II and DNA was isolated using Method III. Genomic DNA was purified by Qiagen kit and checked for quality and quantity on agarose gel and by spectrophotometry, respectively.

IS900 PCR

DNA obtained from 4 methods was screened by IS*900* PCR using Vary Primers. PCR reaction mix (50 μl) contained; 0.2 mM each of the 4 dNTPs, 1μM each of the 2 primers, 1.5 mM $MgCl_2$, 1 U of Taq polymerase, and template DNA (50-100 ng). PCR reactions were conducted (M J Research, PTC- 200) under following conditions: 94°C for 4 min; 30 cycles at 94°C for 10 sec, 61°C for 10 sec, 72°C for 10 sec and finally 72°C for 10 min. PCR products were separated in 2% agarose gel, stained with ethidium bromide, and the expected 229 bp PCR products were visualized by gel document system. DNA from known local MAP culture of Indian 'Bison type' strain (S-5) was positive control and sterilized distilled water as negative control. Expected size of amplicons (229 bp) obtained in all 4 methods were further confirmed by sequencing of PCR products and by subjecting assembled sequence data to BLAST (NCBI).

Statistical analysis

Relative sensitivity, specificity, and kappa values were calculated comparing each PCR test (using different DNA isolation methods) with tissue culture.

RESULTS

Tissues culture

Multibacillary colonies were recovered in all 25 intestinal goat tissues.

IS900 PCR

The 10, 7, 12 and 12 DNA samples extracted by method I, II, III, and IV, respectively, were not intact. However, all 25 samples were subjected to IS*900* PCR and specific 229 bp amplicons (Fig. 1) were obtained from 15, 18, 13, and 13 DNA extracted by methods I, II, III, and IV, respectively (Fig. 2). As compared to other 3 methods, method II was most efficient for isolation of DNA of MAP from tissues. Relative sensitivity of PCR using DNA from 4 different methods with 'tissues culture' was 60, 72, 52 and 52% for methods I, II, III and IV, respectively. Results of PCR using DNA from methods I, II, III and IV on comparison with culture gave Kappa scores of 0.60, 0.72, 0.52 and 0.52, respectively.

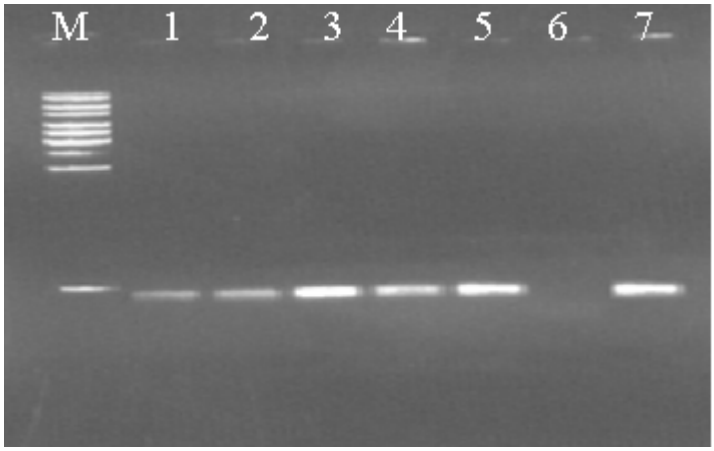

Fig. 1. MAP specific amplicons using IS*900* PCR for MAP in tissues. Lane M: 100bp DNA marker, Lane 1-5: test samples, Lane 6: negative control, Lane 7: positive control.

DNA Sequencing of IS900 PCR products

PCR products (229 bp) purified by glass filter plate and sequenced using Big Dye Terminator v3.1 kit (Applied Biosystems, CA). Sequenced products were analyzed in 3730 DNA Analyzer (Applied Biosystems, CA). Assembled sequence data was subjected to BLAST (NCBI) and results confirmed presence of MAP (Fig. 3).

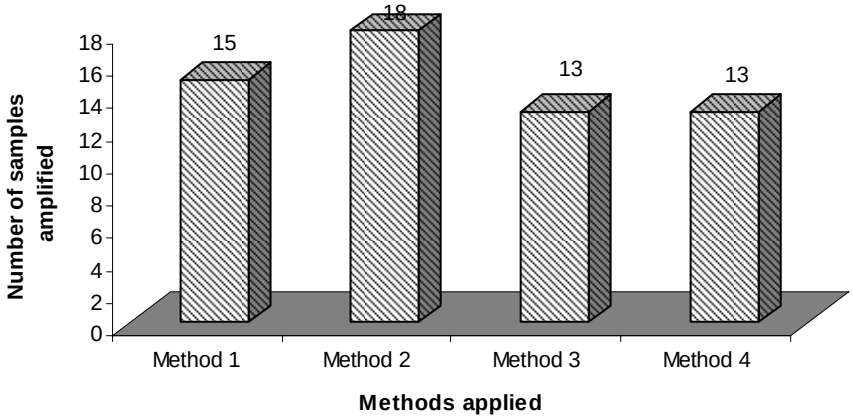

Fig. 2. Results of IS*900* PCR to detect MAP infection in tissues samples using four different methods of MAP DNA isolation methods

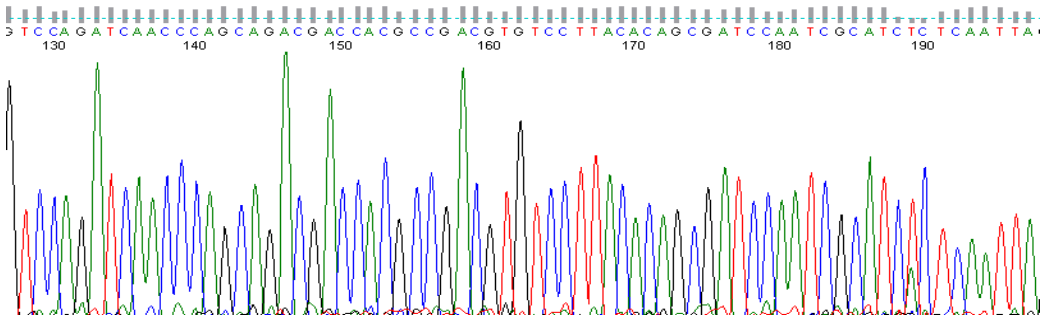

Fig. 3. Sequencing of PCR products (229 bp) amplified by IS*900* PCR

DISCUSSION

Rapid and early detection of MAP infection is important for control of JD. It reduces the risk of transmission of infection to healthy animals and human beings. MAP is difficult to control if detected late in herds. Isolation of MAP by culture is most definitive, but it takes up to 16 weeks (Singh et al., 1996). Direct amplification of MAP DNA from clinical samples has potential to remove time required for culture but it suffers from false negative results due to poor recovery of DNA, thick lysis resistant cell wall of MAP and presence of inhibitory substances in clinical specimens. To address some of the limitations, different methods of cell wall lysis and removal of inhibitors have been developed. Culture is not sensitive, especially in sheep and goats (Choy et al., 1998). Dimareli-Malli et al. (1997) reported sensitivity of culture in intestinal samples of goats and sheep as 74.0 and 46.0%, respectively. Mesenteric lymph nodes of goats and sheep had sensitivity of 60.0 and 36.0%, respectively. On comparing the confirmed histo-pathological changes with culture, 54.0% sensitivity was reported by them. Poor sensitivity of culture may be due to presence of small number of bacilli in sub-clinical stage of disease or presence of spheroplastic form in tissues (Chiodini et al., 1986; Miller et al., 2002). In the present study, culture of tissues of goats having clinical signs of JD and prior positive status in fecal culture showed 100% sensitivity to detect MAP in necropsy tissues. All tissues were positive in culture, therefore was reference test to check efficiency of 4 methods of DNA isolation.

In this study efficacy in terms of extraction of MAP DNA by 2 classical methods, method I and method III (using tissues lysis buffer) with modification (treatment with HPC in method II and IV) were compared. Electrophoresis of DNA isolated by methods I and II showed that

DNA were of good quality than isolated by other 2 methods. Accordingly, results of PCR from methods I and II were better than other 2 methods. Kumar et al. (2007) also used method II for detection of MAP in kids and of 9 intestinal tissues 3 (33.3%) were positive by IS900 PCR that was in agreement with results of culture and path morphology. Sivakumar et al., 2005 compared efficacy of culture and IS*900*-PCR for the detection of MAP. Of 20 (4.9%) animals showing histological lesions suggestive of paratuberculosis, 14 (70%) and 6 (30%) were positive in PCR and culture, respectively. PCR was more sensitive than culture in detection of sub-clinical paratuberculosis in water buffaloes.

Treatment of samples with HPC prior to DNA isolation proved to be more efficient as earlier described by Pislak et al (2003) than both the classical DNA isolation methods (except method IV by which same number of samples were amplified as by method III). Treatment of tissues with HPC has been used for decontamination. HPC an anionic detergent has influence on cell wall that probably enabled better DNA isolation. Decontamination also helped in concentration of MAP. Though culture is as sensitive as PCR, differences may still occur because live undamaged bacilli are necessary for culture, but only qualitatively preserved DNA is required for PCR (Altwegg, 1995). Sensitivity of method II with tissues culture was 72%, which is in close agreement with 79% sensitivity reported by Dimareli-Malli et al. (1997) against histopathological study of tissues. In this study, assessing the agreement of PCR tests to tissues culture, only method II showed substantial agreement, whereas K values for method I, III and IV suggested good agreement with culture results. Although, method II lie in the same categories of ‘good agreement’ with method III and IV, the proportional agreement is nearly on the borderline (0.60) of substantial agreement. This further indicated the crucial role of HPC in isolation of DNA form tissues for detection of MAP. Method II was most efficient. This is first report of HPC assisted DNA isolation from MAP.

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Evaluation of indigenous ELISA kit and status of *Mycobacterium avium* subsp. *paratuberculosis* infection in farm and farmer's goats in India with respect to screening of target tissues by culture and PCR

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INTRODUCTION

India has 16.2% (120 million) of the world's goat population (3.2% growth rate), despite 60.0% annual removal (43.0% slaughter and 17.0% mortality). Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a primary cause of wasting in goats. In India per goat productivity is much below world and Asian averages. Many countries have National JD eradication programs to safeguard animal productivity, international market and human population. The prevalence of JD in India has not been estimated due to lack of priority and diagnostic kits. Johne's is endemic in farm and farmer's goatherds in India (Singh et al., 1996, Singh et al., 2007, Kumar et al., 2007 a and b, Singh and Vihan, 2004). Diagnosis is difficult due to absence of characteristic symptoms and non-specific results in Johnin test, therefore, fecal culture is usually employed. In endemic herds, many times detection of MAP in fecal samples may not be associated with disease since animals receive daily dose of bacilli through contaminated feed, fodder, water and soil and may passively excrete MAP in feces. Therefore, mesenteric lymph nodes (MLN) and intestine near ileo-caecal junction (target tissues) were screened to know true estimates of MAP in farm and farmer's goats sacrificed in Mathura city of North India. An 'Indigenous ELISA kit' developed for goats was evaluated with respect to culture and PCR.

MATERIALS AND METHODS

Animals and tissue samples

Fifty eight goats; (35 from a goat farm in Mathura and 23 farmer's goats from Ajmer, Rajasthan sacrificed in Mathura for meat) were sampled. Forty six tissues and 23 serum were collected from farmer's goats in six visits to slaughterhouse, Mathura during 2005. Farm samples belonged to goats located at Central Institute for Research on Goats, Mathura. Seventy tissues from MLN and intestines and 35 serum samples were collected from goats culled from farm herds in 2005.

Culture of tissues

Culture standardized at Microbiology lab (Singh et al., 1996) was used for screening of 116 tissues. MAP colonies were primarily identified on the basis of mycobactin J dependency, acid fastness and slow growth.

DNA isolation and IS900 PCR (decontaminated pellets/ cultures)

Modified method of DNA isolation used, wherein decontaminated sediment (<1.0 ml) left after inoculation of solid medium was pelleted after centrifugation at 10,000 rpm for 10 minutes. Pellet was washed in PBS and was starting material for isolation of DNA. Pellets and cultures were processed for DNA isolation as per van Soolingen et al., (1991). PCR was standardized using specific IS*900* primers **(**Vary et al., 1990**)** to characterize MAP colonies. Presence of specific 229 bp PCR amplicon in 1.8% agarose ethidium bromide gel electrophoresis confirmed presence of MAP.

Screening of serum by ELISA kit

The indigenous ELISA kit developed for goats (Singh et al., 2007) using a protoplasmic antigen (PA) from native MAP 'Bison type' genotype (Sevilla at al., 2005) of goat origin was used for screening of serum samples. Positive and negative controls were selected from goats, which were positive and negative in culture in the earlier study. OD values were

transformed to S/P ratios for JD status (Collins, 2002). Goats in the strong positive category were considered positive for JD. Both culture and PCR were used to screen tissues and goat was considered positive for MAP infection or JD, if any of tissues was positive in culture and / or PCR (True Positives). Kappa statistics was applied to estimate agreement between different tests. Kit was evaluated with respect to tissues culture and tissues PCR.

RESULTS

The prevalence of *Mycobacterium avium* subspecies *paratuberculosis* was 52, 38, and 47% in the goat population in North India using culture, PCR and ELISA kit, respectively. The prevalence was 65, 39 and 65% in farmer's and 43, 37 and 34% in farm goats using culture, PCR and ELISA kit, respectively. Based on tissue-culture, the prevalence of MAP was 40 and 33% in intestine and MLN, respectively. Using PCR, 26 and 24% of intestine and MLN, respectively were positive. Culture and PCR together detected 62% goats infected. Using 3 tests (tissues culture, PCR and ELISA), 69% goats were detected as infected. Using 2 tests combinations, 62, 64 and 59% goats were positive in culture and PCR, culture and ELISA and PCR and ELISA, respectively. In terms of S/P ratios, 47% goats were positive in ELISA, of which 74 and 56% were detected in culture and PCR, respectively. Collectively in low positives, positives and strong positives categories, 81% goats were detected by ELISA, of which 53 and 45% were detected positives in culture and PCR tests, respectively. In farm goats, true prevalence of MAP (culture and PCR) was 54%. However, 51% of the goats were detected in culture and ELISA and PCR and ELISA combinations. The prevalence in farmer's herds was 74%. Based on culture and ELISA, and PCR and ELISA, 83 and 70%, goats were positive. Using kappa, there was substantial agreement between direct PCR (kappa= 0.32, proportion agreement: 0.65) and ELISA (kappa=0.42, proportion agreement: 0.70) with respect to tissue culture. ELISA test also showed 'substantial agreement' with direct PCR (kappa=0.33 and proportion agreement: 0.67). Sensitivity and specificity of ELISA with respect to tissues culture and tissue PCR was 67 and 75% and 68 and 67%, respectively. PCR with respect to culture had 57% sensitivity and 73% specificity. The 39 sheep sacrificed for mutton production were also sampled from slaughter house, Mathura. Sensitivities and specificities calculated using data on 97 animals (58 goats and 39 sheep), the sensitivity and specificity of ELISA with respect to culture and PCR were 65 and 79% and 63 and 66%, respectively. PCR with culture had 60 and 81% of sensitivity and specificity respectively.

DISCUSSION

Tissues have been used less frequently to estimate prevalence of JD in domestic livestock. Screening of tissues depends on many factors, which are host dependent (livestock species, age of infection, status of disease, concurrent infections, resistance to disease etc). A popular method of screening is by fecal culture, which some times may over or under estimate due to irregular shedding. Therefore, true estimates of JD are based on detection of MAP in tissues (Perez et al., 1996). Culture and PCR of tissues together detected 62% goats, which can be taken as 'True prevalence' of MAP in goats and was significantly higher than three tests used individually. Limited information on prevalence of MAP in farm and farmer's animals showed wide variations (Singh et al., 1996). Using culture of tissues the prevalence of MAP was higher in farmer's herds as compared to farm herds. This may be due to screening of farmer's goats, which were procured from Rajasthan and sacrificed in Mathura for production of meat for government supplies. Pre-slaughter examination of goats showed that animals in extreme debilitated condition (advance clinical cases of JD). Farm goats screened were also discarded from herds on health grounds (suspected cases of JD). These goatherds have been reported to be endemic for JD (Vihan et al., 1989, Singh, 1998, Singh et al., 1996, 1998 and 2000, Kumar et al., 2007a and b). Goats screened were suspected for JD (suffering from chronic weakness), therefore, high presence of MAP in target tissues showed that JD is the predominant cause of weakness, emaciation and low production in Indian goats. High prevalence of MAP in farmer's herds counters the age old myth that farmer's herds clean as compared to farm herds with respect to JD. Most of the goats positive in culture were confirmed as MAP by IS*900* PCR. A high prevalence may be

due independent screening of two tissues (MLN and intestine). Recovery of MAP was higher in intestinal tissues as compared to MLN. Comparatively, the 'Indigenous ELISA kit' showed substantial agreement with the other two tests, and was not more sensitive than culture and PCR. Of 47% goats detected by ELISA, more than 75% were positive in culture and PCR. Therefore this ELISA can be used for screening of goats against JD both in farm and field. However, the presence of culture and PCR positive goats in all the categories of S/P ratio exhibited the spectral nature and need for use of multiple diagnostic tests. The study showed an alarming high presence of MAP in goatherds and underlines the need for urgent surveillance and control of JD in India.

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Real-time PCR testing of pooled fecal samples: comparison to HEYM culture

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One important aspect of controlling paratuberculosis in cattle is the identification of infected animals for removal from the herd. Organism detection methods such as culture or PCR have the advantage of yielding quantitative results, so that the animals shedding the largest number of organisms in feces can be identified. Removing those animals that are heavy shedders or "super-shedders" can significantly reduce the level of environmental contamination with *Mycobacterium* subsp *avium paratuberculosis* (MAP), and thus reduce transmission to susceptible animals. However, particularly in large herds, fecal testing of individual animals can be costly, and may also challenge laboratory capacity for handling large numbers of samples. Pooling of fecal samples offers a method to enhance diagnostic efficiency, both with respect to cost to the producer as well as laboratory capacity. However, since pooling of samples involves possible dilution of feces from infected cows with feces from uninfected cows, the question is raised whether this dilution will cause a reduction in sensitivity to detect infected cows. Fecal culture methods also have the disadvantage of prolonged incubation time required to cultivate the MAP organisms, which may require 30 days or more in both currently available liquid culture systems or 16 weeks on solid media. In contrast, PCR methods can provide results within hours. Quantitative real-time PCR (RT-PCR) methods are preferred for this purpose, as they facilitate distinguishing between heavy- and light-shedding animals, in a fashion similar to culture. The objective of this study was to compare the results of RT-PCR testing with results of culture on solid media for MAP detection in pooled bovine fecal samples. The second objective of this study was to compare the results of RT-PCR and culture for pooled samples to results obtained from the individual samples that comprised those pools. The third objective was to identify a "cut-off" value to select which RT-PCR-positive pools would likely "harbor" a heavy-shedding animal, and thus be selected for further individual sample testing of the pool "members."

Individual fecal samples from 736 cows in four dairy herds were processed for MAP detection by culture and RT-PCR. A 2g aliquot of each fecal sample from an individual cow was suspended in 35 ml sterile water. To create 1:5 sample pools, 5 ml of the fecal water from 5 different individual samples were combined and mixed thoroughly. Culture of individual samples was performed on a 5 ml aliquot of the original fecal-water tube, while pooled cultures were performed on a 5 ml aliquot obtained from the pooled fecal water tube. For the purposes of this investigation all individual and pooled fecal samples were cultured using the standard three day culture preparation protocol with four tubes of HEYM. The 5 ml fecal water suspension (pooled or individual) was transferred to 25 ml of BHI and incubated overnight; centrifuged at 900 X G for 30 minutes and the pellet was re-suspended in 1 ml of antibiotic brew on day 2 and incubated overnight. On the third day the sample was vortexed and approximately 200 ul inoculated on the surface of each of four tubes of HEYM with mycobactin J. The RT-PCR testing of individual and pooled fecal samples was performed using the Tetracore Vet Alert™ Johne's Real-Time PCR assay according to manufacturer's instructions for individual samples. From the pooled fecal water tubes, The remaining 20 ml of the pooled fecal water tube was centrifuged at 2,300 X G for 30 minutes, decanted and the pellet re-suspended in water and processed according to manufacturer's recommendations for RT-PCR, which includes bead-beating and spin column chaotropic separation for DNA isolation. Similarly, 20 ml of the original fecal water suspension from individual samples was processed for RT-PCR detection of MAP. The DNA amplification was performed using a Cepheid Smart Cycler® in duplicate wells. The number of amplification cycles to reach

detection threshold (Ct) was recorded for each sample. The Ct value is inversely proportional to the amount of MAP DNA in the original sample, with values >42 cycles considered negative.

Of the 148 fecal pool samples representing 736 individual cows, 34 pools were RT-PCR positive on both wells. An additional 24 pools had one of two wells positive on RT-PCR. When only one of two wells is positive, this usually represents samples with low MAP concentration that is near the limit of detection. The samples positive in only one well in this study represented lower concentrations of MAP with Ct values between 37 and 42, the cut-off value for a negative sample. A comparison of HEYM culture results and RT-PCR results for the 148 fecal pools are shown in Table 1.

Table 1: Comparison of culture and RT-PCR results on pools of five fecal samples.

	PCR Pos (2 well)	**PCR pos (1 well)**	**PCR NEG**	**Total**
Culture POS	14	4	0	18
Culture NEG	20	20	90	130
Total	34	24	90	148

As can be seen in the table above, RT-PCR was more sensitive than culture to detect pools with MAP. While it is possible that the RT-PCR test may have detected MAP DNA or non-viable organisms that would not grow in culture, the performance of the RT-PCR on fecal pools was similar to our prior experience with individual samples. Samples that had higher RT-PCR Ct values, or that were only positive in one well (ie near the lower limit of PCR detection) were often negative on culture. Those samples with faster (lower) Ct values were more likely to be positive also on culture, reflecting the greater number of MAP in those samples.

Of the 34 RT-PCR pools positive in 2 wells, a subset of 19 had individual samples within the pools tested. Of those 19 pools, 17 had at least one individual sample also positive by RT-PCR, while 2 pools had all 5 of the individual samples negative despite the pool being positive. This phenomenon has also been observed with culture testing of pools, and is presumed due to non-homogeneous mixing of MAP especially in low-concentration samples. Of the 18 culture-positive pools in this data set, 4 pools had none of the individual samples culture-positive. Similar results were obtained in the 9 pools that had one well positive on RT-PCR. Of those 9 pools, 7 had at least one individual sample RT-PCR positive, and 2 had all 5 individual samples negative despite the pool being positive. There were 16 pools that were negative on RT-PCR that had individual samples tested. Of these, 5 pools did have one animal RT-PCR positive, and these tended to be high Ct value (low shedder) individuals. In all, there were 27 individual samples that were positive for MAP on HEYM culture (out of 736 samples), and 35 individual samples positive on RT-PCR (out of 220 tested).

When optimizing use of a pooled fecal sample strategy, the decision regarding how many of the positive pools to be retested for individual samples will have a major impact on the cost savings and efficiency of the pooling strategy. For example, with the current sample of 148 pools, 58 pools were positive on at least one well in the RT-PCR system. Thus, in a 5-sample pooling strategy, with individual retesting on all samples positive in at least one well, an additional 240 individual tests would have to be performed. Using this strategy, 26 of the 27 culture-positive individual cows would have been identified, and the only cow not identified was a very low shedder (one colony on 1 HEYM tube). If the strategy was to trade sensitivity for increased efficiency/decreased cost, the strategy might be to retest only those pools that were positive in both wells. Thus, 170 individual samples from 34 pools would be retested, at a cost savings of 30%. This strategy would still identify 22 of the 27 culture positive individual cows, and the 5 cows not identified were, as before, all low shedders. Finally, if an even more stringent criterion for retesting were used, additional cost savings could be realized with minimal sacrifice in sensitivity, especially for detecting heavy shedding cows. In this sample set, if the strategy were to only retest pools that yielded a moderately strong positive result, Ct<34, then only 8 pools would be retested, requiring the testing of

only 40 samples at a cost savings of 80% compared with the first strategy. Using this strategy, 13 of the 27 culture positive cows would still be identified, but most significantly, all cows shedding more than 7 CFU/tube (140 CFU/g) would have been identified. Thus the only cows “missed” by this strategy would be those lower shedders having less impact on the environment.

In conclusion, the use of a commercially available RT-PCR with pooled fecal samples offers a very economical, flexible, rapid and sensitive method to identify those MAP infected cattle at the greatest risk to spread MAP infection to herd-mates. Only individual samples in pools with the highest concentration of MAP (lowest Ct values) need to be tested for MAP, thus significantly reducing the testing cost for the herd. Furthermore, the “decision maker” can adjust the “cut-off” point for determining which pools are retested individually, depending on the trade-off between stringency and cost that is appropriate for the specific herd in question.

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In vitro effect of antibiotics on *Mycobacterium avium* subspecies *paratuberculosis* in Herrold's medium

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ABSTRACT

The isolation of *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is considered the "Gold Standard" diagnostic test for paratuberculosis. Due to high contamination rates with an observed average of 30% after conventional fecal culture from beef cattle herds, we address this problem by identification of *Pseudomonas aeruginosa* as the most common contaminant. After antibiotic susceptibility testing, the following antibiotics were efficient to control the growth of *Pseudomonas aeruginosa*: enrofloxacin, florfenicol and gentamicin. To check the *in vitro* breakpoint inhibition effect of these antimicrobial agents to *Map*, clinical isolates of paratuberculosis were tested to examine their growth responses in Herrold's medium supplemented with these antibiotics at serial dilutions. Inhibition effect on *Map* was observed for enrofloxacin and gentamicin, florfenicol showed the minimal inhibitory effect to clinical isolates of *Map* at the proposed dilution ranges.

INTRODUCTION

Paratuberculosis is an insidious, chronic, granulomatous enteritis of ruminants and other animals caused by *Mycobacterium avium* subsp. p*aratuberculosis* (*Map*). Clinically affected cattle develop chronic diarrhea and lose weight. The infection spreads mainly by the oral-fecal route, animals are more susceptible to infection shortly after birth and typically do not show outward signs of disease before they are 3-5 years old. In cattle, microbiological culture of the organism from feces, serves as identification of infected animals. This method is considered the reference assay (gold standard), against which other tests are compared. The disadvantages of using fecal culture are prolonged incubation times and contamination is often a problem when culturing fecal specimens. The estimated 33% sensitivity of the fecal culture method is adversely affected by the growth of contaminant bacteria. In a beef herd with paratuberculosis problem a whole herd fecal culture was conducted with overgrowth problem primarily identified as *Pseudomonas aeruginosa*, the average number of tubes that became contaminated ranged 30%.

The purpose of this communication was to identify antimicrobial agents that minimally growth of *Map* yet reduce the growth of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Study Herd and sample process

Fecal samples from a beef herd identified as having paratuberculosis were processed as previously described. Briefly, fecal samples (4 g) were decontaminated into 50-ml tubes containing 30 ml sterile, 0,9% hexadecylpyridinium chloride in brain hearth infusion broth (HPC-BHI), vortexed for 30 min and left in an upright position at room temperature for 30 min, after settling the entire supernatant fraction was removed and placed in a 50 ml tube, after overnight decontamination at 37℃ samples wer e centrifuged 1,700 g for 30 min, supernatant were removed and the pellet resuspended in 1 ml antibiotic solution (100 µg/ml nalidixic acid, 100 µg/ml vancomycin, 100 µg/ml nystatin) and incubated overnight at 37℃. Sample suspension (0.2 ml) were inoculated onto 2 tubes of Herrold´s Egg Yolk Medium (HEYM) containing 100 µg/ml nalidixic acid, 100 µg/ml vancomycin, 100 µg/ml nystatin.

Pseudomonas aeruginosa strain and susceptibility testing

A strain of *Pseudomonas aeruginosa* was isolated in cetrimide agar from contaminated tubes with HEYM cultured with feces from a beef herd with known paratuberculosis. For *in vitro* qualitative antibiotic sensitivity of *Pseudomonas* aeruginosa isolate, the disk diffusion method was used with these antibiotics: cefalexin, augmentin, cloxacillin, ceftiofur, ampicillin,

gentamicin, vancomycin, nalidixic ac, enrofloxacin and florfenicol. The zone of bacterial growth inhibition was recorded. For quantitative activity, the minimum inhibitory concentration (MIC) in a tube dilution procedure was used in a series of tubes containing cetrimide agar mixed with serially diluted antibiotic solutions. Solutions of gentamicin and enrofloxacin were performed in sterile distilled water, while florfenicol was dissolved in dimethyl sulfoxide (DMSO). Twofold dilutions of the antibiotics were performed in order to obtain concentration from 1.95 µg/ml to 1000 µg/ml (1.95 µg/ml, 3.9 µg/ml, 7.8 µg/ml, 15.6 µg/ml, 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500µg/ml, 1000µg/ml), 10 tubes with cetrimide free of antibiotics and with DMSO alone were used as control. The *Pseudomonas aeruginosa* colonies were suspended in sterile distilled water with an equivalent turbidity to the density of McFarland 0.5 barium sulphate. This standard inoculum was applied with a sterile cotton swab. After incubation at 36℃ for 2 days, to deter mine which concentration completely inhibited the growth of *Pseudomonas aeruginosa*, tubes were observed and the MIC was recorded as the first tube in which growth of the organism has been inhibited.

Map strains and susceptibility testing

The study involved 2 clinical strains of *Map* that were isolated from fecal specimens submitted to the laboratory for cultural examination. The strains were identified on the basis of phenotypic characteristics. The quantitative drug susceptibility tests were performed on those antibiotics with sensitivity effect against *Pseudomonas aeruginosa*. Each of two strains were suspended in distilled water, this suspension was inoculated in two sets of 10 tubes with HEYM containing mycobactin J (2mg/L) and serial dilution of one of these three antibiotics: enrofloxacin, gentamicin and florfenicol from 1.95 µg/ml to 1000 µg/ml were diluted serially as described in *Pseudomonas aeruginosa* with a set of 10 tubes free of antibiotics and with DMSO as control. Each of 10 tubes was inoculated with 200 µl of standard inoculum similar to *Pseudomonas aeruginosa*. The inoculated slants were incubated at 37℃ and for bacterial growth the surf ace were observed visually for 1.5 month. The MICs of inoculated tubes containing several sequential dilutions of each drug was the lowest concentration of the antimicrobial agent that inhibited the colonies growing on the drug-free control media.

Statistical methods

Fisher´s exact test was used to analyze the interaction between antibiotics and the *in vitro* growing effect to *Map* and *Pseudomonas aeruginosa*.

RESULTS

In the qualitative antibiotic sensitivity against *Pseudomonas aeruginosa* the next antibiotics demonstrated a zone of inhibition around the discs: enrofloxacin, gentamicin and florfenicol. To these antibiotics, the growth end points MIC for *Map* were read when organisms reached good macroscopic growth in tubes with antibiotics and control tubes after 1.5 months of incubation.

The presence of *Pseudomonas aeruginosa* was confirmed by the appearance of colonies with pigmentation in cetrimide agar (a selective medium for *Pseudomonas aeruginosa*), and the observation of Gram stained smears. The identification of *Map* colonies were based on the observation of acid-fast organisms from smears stained with Ziehl-Neelsen method and phenotypic characteristics as time required to develop a visible colony with typically morphology resembling *Map*.

MICs were as follow: gentamicin 15.62 (µg/ml), enrofloxacin 7.8 (µg/ml) and florfenicol 1000 (µg/m). The breakpoints for *Pseudomonas aeuginosa* were read after 2 days of incubation with the next MIC results: gentamicin 31.25 (µg/ml), enrofloxacin 7.8 (µg/ml) and florfenicol 250 (µg/ml). These results are summarized in Table 1. No statistical significant interactions was found between *Pseudomonas aeruginosa* and *Map* with gentamicin, enrofloxacin and florfenicol. Enrofloxacin present the breakpoint for *Pseudomonas aeruginosa* and *Map* at the same concentration (7.8 µg/ml), gentamicin have a MIC for *Map* (15.62 µg/ml) below to the end point of *Pseudomonas aeruginosa* (31.25 µg/ml), florfenicol

shows activity against *Pseudomonas aeruginosa* at 250 µg/ml, for *Map* MIC limits could not be established because it was beyond the proposed range.

Table 1. Susceptibilities of *Map* and *Pseudomonas aeruginosa* (*Ps. aeruginosa*) to florfenicol, gentamicin and enrofloxacin.

Tube	Florfenicol		Gentamicin		Enrofloxacin	
	(*Ps. aeruginosa*)	(*Map*)	(*Ps. aeruginosa*)	(*Map*)	(*Ps. aeruginosa*)	(*Map*)
1	1000	1000 [g]	1000	1000	1000	1000
2	500	500 [g]	500	500	500	500
3	250 *	250 [g]	250	250	250	250
4	125 [g]	125 [g]	125	125	125	125
5	62.5 [g]	62.5 [g]	62.5	62.5	62.5	62.5
6	31.25 [g]	31.25 [g]	31.25 *	31.25	31.25	31.25
7	15.62 [g]	15.62 [g]	15.62 [g]	15.62 *	15.62	15.62
8	7.8 [g]	7.8 [g]	7.8 [g]	7.8 [g]	7.8 *	7.8 *
9	3.9 [g]	3.9 [g]	3.9 [g]	3.9 [g]	3.9 [g]	3.9 [g]
10	1.9 [g]	1.9 [g]	1.9 [g]	1.9 [g]	1.9 [g]	1.9 [g]

(*) MIC breakpoints to the corresponding tube with antibiotic concentration expressed in (µg/ml)
([g]) Growing in the presence of antibiotics

DISCUSSION

The bacteriologic culture of feces in bovine paratuberculosis remains the reference assay, but some problems in culturing *Map* are the slow growth of the organism, the degree and the nature of contamination. In this case we have identified *Pseudomonas aeruginosa* as the most common contaminant. The isolation of *Pseudomonas* aeruginosa as contaminant seems not to be casual, other reports describes an unusual level of contamination caused also by *Pseudomonas aeruginosa* in HEYM tubes inoculated with fecal samples. We used BHI in the decontamination process for these studies, previous work suggests that BHI may not be a required component in the decontamination procedures, the exclusion of BHI from the isolation protocol, may result in increased *Map* detection rate.

After having performed qualitative antibiotic sensitivity, enrofloxacin, gentamicin and florfenicol were found to have activity against *Pseudomonas aeruginosa*. In the MIC results, gentamicin and enrofloxacin demonstrated discernible growth end points for *Pseudomonas aeruginosa*, 31.25 µg/ml and 7.8 µg/ml respectively but similar MIC breakpoints were observed for *Map*. This *in vitro* antimicrobial susceptibility of *Map*, excludes the use of these antibiotics in processing methods as initial decontaminant or be incorporated in HEYM as selective agents. Florfenicol, an analogue of chloramphenicol, possess an antimicrobial spectrum similar to that of chloramphenicol, and has been reported to have superior *in vitro* bactericidal activity compared with chloramphenicol. In addition this last antibiotic serves as a frame of reference for *Map*, because it has already been incorporated as a component in selective media for *Map* isolation. Florfenicol was effective controlling the growth of *Pseudomonas aeruginosa* at 250 µg/ml, the MIC for *Map* could not be determined as it was near or above their tested dilution schedule (up to 1000 µg/ml). In previous reports a slight depression of the *Map* growth at 200 µg/ml using chloramphenicol as selective agent was reported during the first 8 weeks of incubations in the number and size of colonies with recuperation after 12 weeks. To evaluate if florfenicol is harmful to *Map*, upper full range relevant MIC limits should be performed *in vitro* with these and other isolates of *Map*. This experience was designed to be done with more statistical relevant number of clinical isolates but in a previous pilot work not all isolates grew well in subcultures. Discrepancy in capacity of growth between field strains, mainly the speed of growth and the erratic behavior prevented the inclusions of a higher number of field isolates. The harmful effect of any agent for a selective isolation of mycobacteria can be evaluated as a frequency of isolation from clinical isolates or the time required for appearance and number of clearly visible colonies. In this case tubes with antibiotics were read when organisms reached macroscopic growth equal to that of control tubes, this happened after 6 weeks of incubation apparently and no inhibitory effect of florfenicol was observed to *Map*. To establish any harmful effect of

florfenicol for *Map* further studies should be performed at higher MIC concentration with clinical samples and to evaluate the speed of growth and number of colonies in comparison with control medium.

CONCLUSION

Due to the *in vitro* inhibitory growing effect of gentamicin and enrofloxacin observed for *Map*, these antibiotics can not be used to prevent and control the overgrown problem caused by *Pseudomonas aeruginosa*. Florfenicol should further be evaluated to assure that it is harmless at the recommended concentration to stop the growing of *Pseudomonas aeruginosa*.

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Note from the editor: This paper was presented only as an abstract at the 9ICP. It was not presented as a poster or an oral presentation. Therefore, the 9ICP delegates had no possibility of discussing the study.

Interference of intradermal tuberculin tests on the serodiagnosis of paratuberculosis in cattle

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INTRODUCTION

ELISA-based methods are widely used worldwide (Marassi et al., 2005). Map shares several antigens with *M. bovis*, but few studies have focused on the interference of *M. bovis* infection in PTB tests (Paolicchi et al., 2003). This interference was recently demonstrated in a study using *M. bovis*-infected cows, originating from PTB-free herds (Marassi et al., 2005). In countries where TB remains to be a serious problem, periodic intradermal tests are mandatory, but the possibility that regular testing of non-infected cattle may determine false-positive reactions on PTB-ELISAs have not yet been evaluated.

MATERIALS AND METHODS

Herd – The herd selected for this experiment was confirmed as TB and PTB free using standard tests (intradermal tests for TB and ELISA and feces culturing for PTB, performed on all animals).

Cattle – Sixty-three animals were studied. Animals were divided into three groups of 17 animals, plus a control group with 12 animals. Group A was tested with single intradermal tuberculin test (ITT), using only bovine PPD; group B, tested with comparative ITT using both bovine and avian PPDs; group C, inoculated only with avian PPD, and group D, as control, inoculated with PPD diluents.

Bacterial culture of faecal samples for Map – All the animals were cultured for Map. Faecal samples were processed by the centrifugation protocol, as previously described (Ristow et al*.,* 2006). Samples were inoculated onto slopes of Herrold's egg yolk agar with and without mycobactin J (2mg/L- Allied Monitors - USA) and also with antibiotics (nalidixic acid 50 µg/mL, amphotericin B 50 µg/mL, vancomicin 50 µg/mL). Slopes were incubated at 37ºC and observed in a two weeks-interval for 24 weeks.

PTB ELISAs - The in-house ELISA protocol (ELISA-PPA) was performed as previously described (Marassi et al., 2007). ELISA-Pourquier is a commercial ELISA for paratuberculosis diagnosis and was performed according to the manufacturer's instructions.

RESULTS AND DISCUSSION

All the 63 animals were negative at both ITT and Map culture. In the present study, sera of 63 animals were submitted to ELISA-PPA, and three (4.76%) were reactive. One of those animals belonged to Group A, suggesting that animals submitted to single ITT (inoculated with bovine PPD) may suffer immune interference and show false-positivity to PTB-ELISA. Single ITT is currently performed as screening test and is recommended as the main tool for the detection of infected animals in Brazil and in many other countries (Lilenbaum, 2000). The two other false-positive animals were from Group B, tested with comparative intradermal test. No animal from Group C, inoculated only with avian PPD, was reactive. It suggests that the cross reactivity presented by the animals from Group B may probably be credited to the sensitization with bovine PPD.

It is well-established that tuberculin testing of animals alters the immune status of the animal and can affect both cellular and humoral responses. It causes a period of desensitization, usually considered as 60 days long, during which the cellular response to further tuberculin tests is depressed (Monaghan et al., 1994). The interference of *M. bovis* antigens in the serodiagnostic of PTB in tuberculous cattle, leading to false positive reactions, has already been described (Olsen et al., 2001; Marassi et al., 2005), as well as the influence of other mycobacteria, e.g. environmental species (Osterstock et al., 2005).

Table 1. Reactivity and Increase of S/P at two ELISAs according to the tested groups

	ELISA-PPA		ELISA-commercial	
Groups	Positive	Increase	Positive	Increase
A	1	7	2	6
B	2	10	3	6
C	-	12	-	10
D	-	-	-	-
TOTAL	3	29	5	22

Beside those animals that showed cross-reactivity to PTB-ELISA, some cows had an evident increase in S/P values. From the 63 tested animals, 29 (46%) demonstrated this phenomena, which could be observed in animals from all tested groups, except for the control group. When those animals that presented an important increase in ELISA titres are considered together with those that reached the cut-off point and consequently presented reactive results, we can suggest that cross reactions in PTB-ELISA due to anti-*M. bovis* antibodies which are induced by bovine PPD inoculation may be a very frequent event in countries where both infections occur, which is being undervalued.

Cross reactivity was not observed until 15 days post-inoculation (p.i.). In spite of the detection of antibody enhanced titres until 90 days p.i. in two animals, the majority of cross reactions were observed between 30 and 60 days p.i. This finding indicates that animals routinely submitted to ITT for TB diagnosis can be under effect of PPD stimulation of antibodies and present false-positive reactions to PTB-ELISA up to 90 days after PPD inoculation.

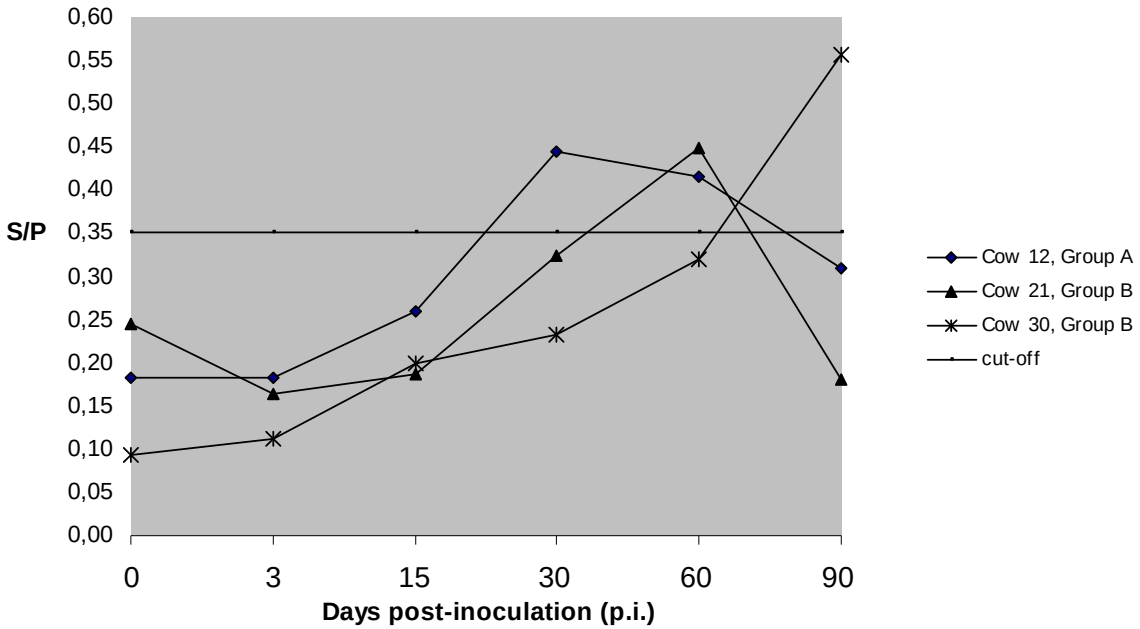

Fig. 1. Evolution of S/P values of three positive cows at PTB-ELISA according to the days post-inoculation of PPD.

Thirty-six samples that presented sero-conversion or an important increase in antibodies titres detected by ELISA-PPA were selected to be confirmed with an accredited ELISA kit. A strong correlation (k = 0.78) between the results was observed. Not only the occurrence of false positive reaction was confirmed, but also the moment when those reactions were more frequent, i.e. between 30 and 60 days p.i. It confirms that the interference of the PPD inoculation as well as the occurrence of cross-reactions derived from it may also be observed in commercial tests.

Concluding, we demonstrate that intradermal tuberculin tests, both single and comparative test, widely used for the *in vivo* diagnosis of bovine TB, may temporarily

interfere in the immune status of the animal and determine cross reactions with other mycobacteria. That interference may lead to false-positive reactions in either ELISAs widely used for the serodiagnostic of paratuberculosis. Therefore, in order to avoid such occurrence, cattle should not be bled for PTB serodiagnostic for a period of at least 90 days after tuberculin testing.

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

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ABSTRACT

The aim of this study was to evaluate the diagnostic-test characteristics of microscopic examination of Ziehl-Neelsen-stained faecal smears for acid-fast Mycobacteria (ZN-test) and serum-ELISA in cattle suspected of clinical paratuberculosis.

Test results of all cattle tested simultaneously by ZN-test and serum-ELISA between April 2003 and April 2006 were analysed with latent-class models for evaluation of diagnostic tests in two populations without a gold standard. Sampled cattle were divided into two populations in two different ways using known risk factors for clinical paratuberculosis: region and age. Priors for sensitivity and specificity of tests were based on the literature; uninformative priors were used for prevalence's in the various populations.

Posterior estimates of sensitivity, specificity, and positive and negative predictive values of the ELISA were always higher than those of the ZN-test, irrespective of the population and choice of model.

It is concluded that the ELISA is preferred to the ZN-test to confirm the presumptive diagnosis of clinical paratuberculosis. Little diagnostic information can be gained by performing the ZN-test in addition to the ELISA.

INTRODUCTION

Testing cattle with clinical signs of paratuberculosis is an important element of surveillance for paratuberculosis. In many infected herds, control of paratuberculosis infection is only initiated after detecting clinical paratuberculosis cases. Diagnostic characteristics of tests used to confirm a clinical presumptive diagnosis are therefore important, not only in managing the individual patient, but also for paratuberculosis control on the herd-level and on the national level.

To support culling decisions in cases suspected of paratuberculosis, a fast confirmation of the clinical presumptive diagnosis is preferred. Therefore, cattle suspected of paratuberculosis are often tested by methods such as Ziehl-Neelsen-stained faecal smears for acid-fast Mycobacteria (ZN-test) and serum-ELISA. The sensitivity in clinically affected cattle and the specificity of the ZN-test have been estimated at 49.3% and 83%, respectively (Ris et al., 1988; Zimmer et al., 1999). However, only small numbers of cattle were included in these studies. The sensitivity of the serum-ELISA used at the Animal Health Service laboratory (ELISA Paratuberculosis Antibody Screening, Institut Pourquier, Montpellier, France) has been estimated at 28.0% to 40.8% in faecal culture-positive cattle (van Maanen et al., 2002; Collins et al., 2005). To our knowledge, this ELISA has not been evaluated in clinical paratuberculosis cases. Therefore, and because of the rather small sample sizes in the studies on the ZN-test, it is difficult to give a clear advice on the preferred choice of test and interpretation of test-results in cases suspected of clinical paratuberculosis.

The aim of the present study was to evaluate the diagnostic-test characteristics of the ZN-test and the serum-ELISA in cattle suspected of clinical paratuberculosis.

MATERIALS AND METHODS

Samples and laboratory tests

Test results of all cattle tested simultaneously by ZN-test and serum-ELISA for paratuberculosis between April 2003 and April 2006 were retrieved from the laboratory information system of the Animal Health Service.

For the ZN-test, five grams of faeces were suspended in tap water and filtrated through a tea-strainer. The filtrate was mixed with a sodiumhypochlorite solution, kept overnight, and centrifuged. A smear was made of the top layer of the sediment, fixated in hot air, stained in

a carbolfuchsine solution, decolorized, and then stained in a methylene-blue solution. Samples were considered positive if at least 2 groups of at least 3 acid-fast Mycobacteria were detected at microscopical examination for 10 minutes.

Serum samples were tested with the ELISA Paratuberculosis Antibody Screening [Institut Pourquier, Montpellier, France] according to the manufacturers' instruction. Results of samples with a sample to positive control (S/P) ratio ≤0.90 were considered negative, results of samples with an S/P≥1.10 were considered positive and results of samples with 0.90<S/P<1.10 were considered inconclusive.

Herd-level and animal-level data

For each sample, the unique herd identification number and the animal identification were retrieved, as provided at the submission of the sample. In our analyses, inconclusive test results were assumed to be negative. For each herd, the region of the country was retrieved. If a sample was uniquely identified, the date of birth of the animal was retrieved the national cattle identification & registration (I&R) database (Nielen et al., 1996).

Analyses

To estimate the diagnostic-test characteristics of both ZN-test and ELISA, Bayesian latent-class models for evaluation of two tests in two populations described by Branscum et al. (2005) were adapted. Three models were used, with conditional independence of the ZN-test and ELISA (model 1), conditional dependence of the ZN-test and ELISA (model 2) or conditional dependence of the tests in infected cattle, but conditional independence in non-infected cattle (model 3). The models were run with the freeware program WinBUGS version 1.4.1 (Lunn et al., 2000).

For each of the three models, the population of cattle tested with both tests was divided in two ways into two subpopulations with a presumed different prevalence of infection using known risk factors for paratuberculosis: age (<4 years versus ≥ 4 years) and region (North of the Netherlands versus other parts). Uninformative prior distributions (Uniform between 0 and 1) were used for the proportion of paratuberculosis cases within each subpopulation. Prior β(s+1, n-s+1) distributions for sensitivity (specificity) of tests were created based on the number s of test-positive (test-negative) and total number n of infected (non-infected) cattle tested in various studies (Table 1).

Table 1. Prior distributions for the sensitivity (Se) and specificity (Sp) of microscopic examination of Ziehl-Neelsen stained faecal smears (ZN) and ELISA

Test	Parameter	Prior distribution	Mode	Percentiles			Reference
				2.5%	50%	97.5%	
ZN	Se	β(38, 39)	0.493	0.383	0.493	0.604	Zimmer et al. (1999)
	Sp	β(16, 4)	0.833	0.604	0.810	0.939	Ris et al. (1988)
ELISA	Se	β(44, 14)	0.768	0.642	0.762	0.859	Egan et al. (1999)
	Sp	β(2133, 4)	0.999	0.996	0.998	0.999	van Maanen et al. (2002).

RESULTS

For 892 cattle suspected of clinical paratuberculosis, results of both ZN-test and ELISA were available: 250 ZN-positive and ELISA-positive, 12 ZN-positive and ELISA-negative, 260 ZN-negative and ELISA-positive, and 370 ZN-negative and ELISA-negative cattle. Age at sampling could be retrieved for 665 cattle and region for all 892 cattle.

In the analyses with subpopulations by age group, point estimates of the overall proportion of true paratuberculosis cases ranged from 0.605 to 0.673 (Table 2). Models 2 and 3 resulted in slightly higher estimates of the proportion of true paratuberculosis cases than model 1. The posterior estimates of the diagnostic sensitivity, specificity and positive and negative predictive values of the ELISA were always higher than those of the ZN-test, irrespective of the choice of model (Table 3). Similar results were obtained in analyses with subpopulations based on region (data not shown).

Table 2. Estimated median proportions (95% CI) of 665 cattle with clinical signs resembling paratuberculosis divided in subpopulations by age.

Model	Proportion (95% CI) of cattle with paratuberculosis		
	Overall	Within subpopulation	
	(n=665)	< 4 yrs (n=230)	≥ 4 yrs (n=435)
Model 1	0.605 (0.565, 0.647)	0.473 (0.406, 0.542)	0.676 (0.626, 0.725)
Model 2	0.673 (0.611, 0.757)	0.527 (0.445, 0.620)	0.751 (0.679, 0.847)
Model 3	0.650 (0.597, 0.713)	0.508 (0.432, 0.590)	0.725 (0.663, 0.798)

Table 3. Estimated sensitivity (Se), specificity (Sp), negative predictive value (PVN) and positive predictive value (PVP) of microscopic examination of ZN-stained faecal smears (ZN) and ELISA in analyses using subpopulations based on age (<4 yrs versus ≥4 yrs of age).

Test	Test characteristic	Model		
		Model 1	Model 2	Model 3
ZN	Se	0.477 (0.431, 0.524)	0.436 (0.380, 0.489)	0.476 (0.430, 0.521)
	Sp	0.980 (0.956, 0.994)	0.976 (0.946, 0.993)	0.978 (0.951, 0.993)
	PVN	0.550 (0.498, 0.598)	0.457 (0.341, 0.538)	0.502 (0.416, 0.568)
	PVP	0.974 (0.941, 0.992)	0.975 (0.944, 0.992)	0.976 (0.947, 0.993)
ELISA	Se	0.920 (0.880, 0.951)	0.829 (0.750, 0.888)	0.856 (0.796, 0.903)
	Sp	0.998 (0.996, 1.000)	0.998 (0.996, 1.000)	0.998 (0.996, 1.000)
	PVN	0.891 (0.832, 0.935)	0.740 (0.570, 0.843)	0.789 (0.673, 0.866)
	PVP	0.999 (0.997, 1.000)	0.999 (0.998, 1.000)	0.999 (0.997, 1.000)

DISCUSSION

In this study, posterior estimates of the sensitivity, specificity and positive and negative predictive values of the ELISA were higher than those of the ZN-test. This indicates that the ELISA is superior to the ZN-test to confirm the presumptive diagnosis of clinical paratuberculosis. The positive predictive value of the ELISA was estimated at 0.999 (0.997, 1.000) meaning that very little diagnostic information can be gained with the ZN-test if the ELISA has a positive result. Also, if the ELISA has a negative result, the likelihood of gaining diagnostic information with the ZN-test is very small, because the ZN-test was positive in only 3% of ELISA-negative cases. Furthermore, the ZN-test is very laborious, can not be automated and is expensive in comparison to the ELISA. The results of this study indicate that the ELISA alone is a cost-effective test to confirm the presumptive diagnosis of clinical paratuberculosis.

The posterior estimates of the sensitivity of the ZN-test were comparable to previously published estimates (Zimmer et al., 1999; Ris et al., 1988). However, the posterior estimates of specificity (>95%) in this study were higher than the estimate of 0.83 (95% CI: 0.59, 0.96) of Ris et al. (1988), which may be related to the strict criterion used to declare a sample positive in the present study in the present study (at least 2 groups of at least 3 acid-fast Mycobacteria). The posterior estimates of the sensitivity of the ELISA in this study were ≥80%, broadly in line with previous estimates in clinical cases of 77% to 87% (Sweeney 1995; Egan et al. 1999), but higher than the estimate of 50% by Bech-Nielsen et al. (1992). The posterior estimate of the specificity of the ELISA was in line with previous estimates (van Maanen et al., 2002; Collins et al., 2005).

The assumption of conditional dependence of tests had no practical consequences for the preferred choice of test, because with each of the models, the test-characteristics of the ELISA were more attractive than those of the ZN-test. Constant test accuracy across the subpopulations within an analysis was assumed in the present study. This assumption was considered to be justified, because only the final stage of the infection-and-disease process, i.e. clinical disease, was studied. However, even if test accuracy would differ between

subpopulations, our analysis still applies. Then the resulting estimates can be interpreted as average values across both populations rather than population-specific values, which would still be of interest (Branscum et al., 2005).

CONCLUSION

It is concluded that the sensitivity, specificity, and positive and negative predictive value of the ELISA in cattle suspected of clinical paratuberculosis are higher than those of the ZN-test. Therefore, to confirm the presumptive diagnosis of paratuberculosis, the ELISA is preferred above the ZN-test. If the ELISA is used to confirm this presumptive diagnosis, little diagnostic information can be gained by performing the ZN-test as well.

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

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INTRODUCTION

The pathology of ovine paratuberculosis has been described by several authors and histological examination is considered one of the best diagnostic techniques in sheep (Pérez et al., 1996). However, only a few studies have compared histopathological results with other techniques such as culture, polymerase chain reaction (PCR) and serology. Diagnosis based on the detection of IS*900*-specific sequences of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by PCR from faeces, tissues and blood are alternative methods considered to be very quick, and highly specific (Garrido et al., 2000; Juste et al., 2003). In order to better understand the association of histopathological, microbiological and serological techniques in the paratuberculosis diagnosis, a pathological study in 30 sheep with clinical compatible signs was performed. Results of this study were compared with the other techniques such as culture, PCR and serology.

MATERIAL AND METHODS

A total of 30 adult sheep of both sexes with suspected paratuberculosis were obtained from 30 different flocks in the Trás-os-Montes e Alto Douro region in the North East of Portugal. Sheep were subsequently diagnosed on the basis of a combination of clinical, pathological, microbiological and serological findings. Prior to euthanasia, blood samples to be used in serological procedures and DNA extraction were collected from each sheep. Faecal samples weighing approximately 5 g were collected directly from the rectum using disposable gloves, transferred to individual sterile plastic containers, frozen, and then stored at -20ºC until processed. Sheep were subjected to a full necropsy. The intestinal wall was carefully examined and segments of the ileum, jejunum, ileocaecal valve and mesenteric lymph nodes were removed aseptically for bacteriological examination and DNA extraction, frozen, and stored at -20ºC until used. Tissue samples were fixed on a 10% buffered formol-saline-solution. Fixed tissue samples for histopathology were processed using routine techniques. Tissue extracts were embedded in paraffin, sectioned at 4 µm, stained with haematoxylin and eosin (HE) and the Ziehl–Neelsen (ZN) technique. Histopathological lesions in the ileum, ileocaecal valve, jejunum and mesenteric lymph nodes which relate to the type of cell infiltrated and the presence of acid-fast organisms, were observed and recorded. Processing of faeces and tissues for culture was based on the method of Juste et al. (1991) and Aduriz *et al*. (1995). Positive colonies were confirmed by IS*900* PCR and PCR-REA IS*1311* confirmed that they were S strains.

Specific MAP DNA was detected following the extraction of genomic DNA from frozen samples of faeces, tissues and blood. The PCR analysis procedure for colonies, faeces, tissues and blood has been previously described in detail by Garrido et al. (2000) and Juste et al. (2003). All 30 sera samples were subjected to a commercial ELISA Kit according to the manufacturers instructions (ELISA paratuberculosis screening test®, Institut Pourquier).

RESULTS

Twenty-six of the 30 (87%) sheep showed gross findings of intestinal mucosa thickening, and 20 out of 30 (67%) animals exhibited enlarged mesenteric lymph nodes. Twenty-one out of 30 (70%) animals showed lesions suggestive of paratuberculosis. Lesions associated with infection were classified as proposed by Pérez et al. (1996). Of the 21 sheep with histological lesions, 2 (9.5%) had focal lesions characterized by small granulomas. Multifocal lesions appeared in 3 (9.5%) animals with granulomas in the lymphoid tissue and in the intestinal *lp*.

Sixteen (76%) sheep showed diffuse lesions. Animals were divided into different subtypes according to the main cell type present in the infiltrate and the amount of acid-fast bacilli: 9 sheep (53%) presented diffuse non-lymphocytic granulomatous enteritis, and 8 (47%) showed diffuse lymphocytic granulomatous enteritis (Figs. 1, 2, 3 and 4). MAP colonies were recovered from faeces and tissue samples from only 2 and 6 of the 30 sheep, respectively. MAP was detected in 4 sheep by faeces PCR (13%), and in 19 (63%) sheep, by tissue PCR. PCR in blood revealed 7 (23%) infected animals. Three (10%) animals showed antibodies against Map. Histological examination and PCR in tissue both revealed that 16 animals out of 26 were infected with MAP. The 2 sheep which showed positive on faecal culture had also histological lesions typical of PTBC. Mycobacteria were isolated by tissue culture in 6 sheep. These animals had also histopathological lesions consistent with paratuberculosis. Histological examination of the same tissue samples examined by both culture and PCR identified 6 animals as positive. Five animals with no histological evidence were also culture negative in a variety of samples but were detected by PCR in faeces, blood or tissues. The 3 animals that gave positive results using serology were also detected by histological examination. Two animals were detected solely by means of histological examination.

DISCUSSION

The most sensitive tests were histological examination (80%) and PCR in tissues (73%). The least sensitive test was faecal culture (8.3%). All tests were assumed to be 100% specific.

Histological examination and PCR in tissues allowed a greater number of positive animals to be detected when compared with the results obtained by culture or serology. The results of this study suggested that histological examination and PCR in tissues were more sensitive than PCR in faeces, bacterial culture, or ELISA in the clinical detection of MAP.

CONCLUSION

A limitation of this study was the inclusion of only 30 suspected animals, therefore limiting the generalizations that can be made from the study results. However, the results suggest that a combination of the testing methods, especially histopathology, PCR in tissue and PCR in blood, improve diagnostic sensitivity, are relatively inexpensive, and are able to detect the majority of clinically affected sheep.

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

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BACKGROUND

Mycobacterium avium subspecies *paratuberculosis* (*Map*) is an important pathogen causing ruminant paratuberculosis and human infection. paratuberculosis is characterized by chronic granulomatous enteritis, persistent diarrhea, progressive wasting, and finally death, and has resulted in significant economic losses to the dairy and cattle industries worldwide. Furthermore, the bacteria are speculated to be the cause of human Crohn's disease. One of the difficult issues in diagnosis and research of the infection is their significant slow growth. Even by using specially enriched Herrold's egg yolk medium (HEYM) with Mycobactin J, it takes 3 to 4 months to see minimal colonies. However, in a susceptibility test for anti-bacterial substance, evaluation of disinfectant or bactericidal activity of macrophages, for example, we need to know their viability for efficacy as soon as possible. Carboxyfluorescein diacetate succinimidyl (CFDA) and carboxyfluorescein diacetate succinimidyl ester (CFDA/SE) labelling has been used previously to study the adhesion of labelled bacteria to host cells and the uptake of labelled substrates by various cells using flowcytometry analysis and viability test of several bacteria, however no study was reported on *Map*. Therefore, we investigated the application of a viability test of *Map* by CFDA or CFDA/SE.

RESULTS

The results revealed that CFDA is a useful reagent as a fluorescent probe to determine the quantitative viability of *Map* and as a tracer. CFDA/SE labelling heat-killed *Map* as well as live bacilli. Incubation of *Map* with CFDA at 100µM for 30min was practically the optimal condition for the viability test.

CONCLUSIONS

CFDA staining with fluorescent measurement is a useful tool in various required tests of viability, such as the evaluation of antibiotics, disinfectant, other sterilization conditions, and the bactericidal effect of activated phagocytes for *Map*.

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

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

Culture of *M. paratuberculosis* from blood

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Between exposure to *Mycobacterium avium* subsp *paratuberculosis (M. ptb)* and expression of clinical disease, there is a long subclinical phase in which shedding of the organism in faeces occurs intermittently. Current diagnostic tests are unable to provide sensitive and specific diagnosis during the early stages of the disease.

Detection of *M. ptb* DNA in blood, and culture of *M.ptb* from milk, liver, mammary tissue, spleen, foetal tissues, reproductive tissue, and extra intestinal lymph nodes, indicate that at some stage in the disease, bacteraemia occurs. Demonstration of the organism in blood from infected sheep and cattle by PCR has sparked recent interest in developing a diagnostic test based on culture or molecular detection of the organism from blood.

Various methods of processing blood prior to culturing in Bactec 12B culture media were compared using whole blood spiked with a known quantity of a sheep strain of *M.ptb*. The culture results and factors influencing the ease of processing were used to choose a method to be applied to samples from naturally and experimentally infected sheep. The chosen method utilises the intracellular location of the organism, concentrating the *M.ptb* by collecting the white blood cells. This allows efficient removal of the red blood cells which can be inhibitory to the growth of *M.ptb* in liquid media.

Preliminary findings indicate that *M.ptb* can be isolated from blood of a low proportion of animals following exposure, and before development of clinical signs. Results will be presented from two trials with 152 sheep.

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

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BACKGROUND

Diagnosis of infection with *Mycobacterium avium* subsp. *paratuberculosis* (Map), the causative agent of Johne's disease in ruminants, is currently performed by culture or serology. These tests are only reliable in advanced stages of the disease and, moreover, detection of Map by culture requires long incubation times. A commercial kit using mycobacteriophage D29 to detect *M. tuberculosis* in human sputum samples (FASTplaqueTB™) has recently been adapted for rapid identification of viable Map in pure culture as well as in spiked milk samples. AIM: To evaluate the usefulness of this kit as a simple, rapid, and quantitative means to detect viable Map bacteria in fecal samples from veterinary sources.

METHODS

We first compared the susceptibility of different Map isolates to the bacteriophage provided in the kit, using only the FASTPlaqueTB™ assay reagents. In total, 8 Map isolates were tested. Ten-fold dilutions were studied in triplicate with the phage lysis kit and in parallel by conventional culture on 7H11 agar plates allowing quantification of both plaque forming units (pfu) and colony forming units (cfu), respectively.

Using various processing and decontamination procedures, we then applied the mycobacteriophage method to detect Map in bovine fecal samples that were spiked with a phage-susceptible Map strain as well as in culture-positive (paraJEM Map culturing system and PCR confirmation) samples from bovine Johne's disease.

RESULTS

While some of the isolates (5/8) gave almost equal numbers of cfu/ml and pfu/ml, other isolates (3/8) were poorly quantified with the bacteriophage assay as demonstrated by low pfu/cfu ratios. In fecal samples from cattle infected with Map, the modified FASTPlaqueTB™ system was not successful in detecting viable Map bacteria, even in non-decontaminated specimens. This was also the case for Map-spiked fecal samples.

CONCLUSIONS

Inhibitory components in bovine fecal matter and/or unsuitable decontamination procedures interfere with the detection of Map in feces by mycobacteriophage D29. Moreover, some isolates of Map have variable susceptibility to infection and lysis, and thus detection, by the D29 mycobacteriophage. Overall, these studies suggest that the routine use of this kit to detect viable Map bacteria might not be suitable for all types of samples without modification, especially where low bacterial counts are expected.

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

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In a considerable proportion of cattle infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the bacterium can be isolated from tissues other than intestine and associated lymph nodes, including certain tissues with potential for human consumption. Should evidence legitimize MAP as a food safety risk, methods will be needed to differentiate cattle with MAP infection confined to intestinal tissues from those with disseminated infection (DI). The objective of the present study was to determine if the severity of histologic lesions of small intestine and mesenteric lymph nodes could be used to classify DI status of cattle. Forty culled dairy cows from four MAP-infected herds were enrolled in the study. Conventional bacteriologic culture was performed on samples of feces collected immediately prior to euthanasia, and 15 tissue specimens collected during postmortem examination. Ileum, jejunum, mesenteric lymph node, and ileocolic lymph node were collected postmortem for histological evaluation. A grading system was developed to categorize the level of severity of granulomatous inflammation in these tissues; grades of 0 to 3 were assigned based on the quantity and micro-anatomical distribution of leukocytes. Cows were classified as “infected” if MAP was only isolated from feces, intestine, or gut-associated lymph nodes. Cows were classified as “DI” if MAP was isolated from other tissues. Twenty-eight (70%) of enrolled cows were infected. Twenty-one cows (75% of infected, 52.5% of all enrolled cows) had DI. The proportion of cows with DI increased with inflammation grade of each tissue. The sensitivity/specificity of granulomatous inflammation lesions that exceeded grade 1 for classifying DI status were 76/95%, 71/100%, 67/94%, and 62/82% for ileum, jejunum, mesenteric lymph node, and ileocolic lymph node respectively. The presence of DI in cows with low-grade or nonexistent intestinal lesions suggests that DI may occur in an early stage of disease. Since most cows with intestinal lesions of moderate or greater severity have DI, histologic evaluation of intestinal tissues may be a useful means to selectively identify and exclude a large proportion of cows with DI from the human food chain.

Development of a novel enzyme-linked immunosorbent assay for the diagnosis of Johne's disease

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Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), has a significant economic impact on the US dairy cattle industry. Use of enzyme-linked immunosorbent assays (ELISA) to identify cattle for further fecal culture testing or for culling is listed as a recommended method for JD control in dairy and beef herds. However, several recent reports estimated diagnostic sensitivities of currently available ELISAs to be only 13.5 to 27.8%. For example, by using a Bayesian non-gold standard analysis, the diagnostic sensitivities of two current ELISAs were estimated to be 26-27%. Recently, it was predicted that if the diagnostic sensitivity of currently available ELISAs could be improved to 80%, then their use could result in an effective reduction of JD prevalence, higher level of milk production, and higher annual net revenue per cow.

We developed a novel ELISA, called EVELISA, for the detection of MAP infections in cattle and is highly sensitive identifying 97.4% of fecal-culture positive cattle compared to a currently marketed ELISA that identified 50%. However, when 37 serum samples from a herd with a high rate of false-positives were tested by the EVELISA as well as a currently available ELISA, both ELISAs found more than 70% of the samples to be positive for JD. The false-positive rate of the EVELISA was reduced significantly to 26.1% when the serum samples were pre-absorbed with *M. phlei*. By using the fecal culture method as the gold standard, empirical diagnostic sensitivity of the EVELISA using *M. phlei* absorption (absorbed EVELISA) was 97.1%, whereas that of a current ELISA was 48.5%. Moreover, a Bayesian non-gold standard analysis revealed that the absorbed EVELISA had a significantly higher level of diagnostic sensitivity (82%) than that of a current ELISA (22%). These data indicate that this novel ELISA is highly sensitive and may improve the effectiveness of JD control measures.

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

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

Radiometric culture of farm slurry – inexpensive tool for the detection of paratuberculosis in dairy cattle herds

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OBJECTIVES

To evaluate the usefulness of the radiometric culture of faecal slurry for rapid and inexpensive screening of dairy herds for paratuberculosis.

EXPERIMENTAL DESIGN

Samples of faecal slurry were collected from yards immediately after milking from 70 herds in which paratuberculosis had previously been diagnosed. Results of the last blood ELISA test that was performed as part of the Victorian Bovine Johne's Disease Test and Control Program were also obtained from each herd.

Faecal material was pushed together across the full length and breadth of the yard and mixed using a shovel or a shed scraper and triplicate samples were collected from each farm. After initial processing, two sub-samples were derived from each replicate. Subsequently, 6 sub-samples from each herd were submitted for culture and decontaminated using the double incubation method. Three of the 6 sub-samples were decontaminated at 37^{o}C and the other 3 were decontaminated at 42^{o}C. After decontamination, each sub-sample was inoculated into a BACTEC 12B bottle supplemented with egg yolk, mycobactin J and PANTA, and incubated for at least 12 weeks at 37^{o}C. Cultures showing growth were subcultured to demonstrate mycobactin dependency and tested by the polymerase chain reaction for the presence of IS*900*.

RESULTS

M. paratuberculosis, or its DNA, was identified in faecal slurry from 50 of the 70 (71.4%) herds with paratuberculosis. The radiometric culture of slurry detected 53% of herds with low (0 to 1.5%) seroprevalence, 75% of herds with moderate (1.6 to 3%) seroprevalence and 100% of herds with high (>3%) seroprevalence. The growth of *M. paratuberculosis* in cultures of slurry from herds with high seroprevalence was detected significantly earlier than that observed in cultures from herds with low and moderate seroprevalences. These results show that this test is a good indicator of the prevalence of infection.

Of the 210 paired sub-samples of slurry that were decontaminated at 37^{o}C and 42^{o}C, 175 produced concordant results. Among the 35 pairs of sub-samples with discordant results, *M. paratuberculosis* was detected in 23 (65.7%) sub-samples decontaminated at 42^{o}C and in 12 (34.3%) sub-samples decontaminated at 37^{o}C. This indicates that the decontamination at 42^{o}C offers a sensitivity advantage.

From these preliminary findings using replicate samples it has been estimated that about 50% of infected herds would have been identified with the culture of a single sample.

CONCLUSIONS

The radiometric culture of farm slurry has the potential to be a useful and inexpensive tool to screen dairy herds for paratuberculosis.

Application of culture and PCR to bulk milk for detection of paratuberculosis in dairy cattle herds

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OBJECTIVES

To evaluate the usefulness of the polymerase chain reaction (IS*900* PCR) and radiometric culture of bulk milk for screening of dairy herds for paratuberculosis.

EXPERIMENTAL DESIGN

Samples of bulk milk from 70 herds with paratuberculosis and 15 herds considered as free of infection were tested for *Mycobacterium paratuberculosis* using the IS*900* PCR and radiometric culture.

RESULTS

From each herd triplicate samples of milk were tested by the PCR. Of the 210 samples from the 70 herds with paratuberculosis, one gave a positive PCR reaction. The survey of milking practices revealed that a filter of a milking system on the farm that had the positive PCR result was always dirty. The PCR gave negative results in all 45 samples from the 15 herds considered as free of infection. No *M. paratuberculosis* was detected in any cultures of bulk milk from the 70 herds with paratuberculosis or the 15 herds considered as free of the disease.

The analytical sensitivities of the PCR and culture are 1 organism/mL of milk and 10 organisms/mL of milk, respectively.

The positive PCR result was obtained from only one of three samples of the bulk milk so the estimated concentration of *M. paratuberculosis* in bulk milk from the herd tested positive by the PCR was less than one organism/mL.

CONCLUSIONS

M. paratuberculosis could not be readily detected in the bulk milk from herds with infected cattle by either PCR or culture. This indicates that the hygiene measures in milk collection are effective and that in Australian conditions testing of bulk milk may not be a useful method to identify herds with paratuberculosis.

Reproducibility of results in batches of three ELISA kits for the diagnosis of paratuberculosis in cattle: recommendations for kit evaluation criteria

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INTRODUCTION

In Australia, the ELISA technology is used in the National Johne's Disease Market Assurance Program for Cattle to assess herds for paratuberculosis. Currently, there are three commercially available ELISA kits that are approved for testing cattle for this disease in Australia. New batches of the ELISA kits are subjected to independent evaluation to assess reproducibility of the assay performance prior to release of a kit for diagnostic purposes.

OBJECTIVE

To validate criteria for the evaluation of new batches of three ELISA kits.

EXPERIMENTAL DESIGN

Three batches of each of the three ELISA kits were evaluated over a period of three years. Specificity sera from 180 cattle from a region considered as free of paratuberculosis and sensitivity sera from 40 cattle with paratuberculosis were tested following the kit manufacturer’s recommendations.

RESULTS

The average CVs of OD values within a plate (among wells), between plates and between batches of the three kits were 6.8% (range 3.73 to 9.12%), 9.3% (5.35 to 14.9%) and 13% (8.8 to 16.98%), respectively. The overall average agreement of diagnostic classification for all kits and batches was 99% (98 to 100%). The overall average specificity and sensitivity for all kits and batches were 99.75% (98.53 to 100%) and 78.6% (70.6 to 90.9%), respectively.

CONCLUSIONS

Data derived from this study was used to formulate acceptance criteria for evaluation of new batches of the ELISA kits. The high reproducibility of results warrants the use of these tests in market assurance programs to consistently assess level of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle herds.

Application of three ELISA kits to bulk milk for detection of paratuberculosis in dairy cattle herds

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OBJECTIVE
To evaluate the usefulness of testing bulk milk samples using 3 commercially available ELISA kits for rapid detection of paratuberculosis in dairy cattle herds.

EXPERIMENTAL DESIGN
Duplicate samples of bulk milk from 70 herds with paratuberculosis and 15 herds considered as free of infection were tested using 3 commercially available ELISA kits supplied by Prionics, Institut Pourquier and Svanova .

RESULTS
The 3 ELISA kits showed limited ability to discriminate between herds with paratuberculosis and herds considered as free of the disease. Of the 70 herds with paratuberculosis, 9 (13%) tested positive by the Porquier ELISA and 12 (17%) tested positive by the Prionics ELISA when results were interpreted using a cut-off value that theoretically offers 95% specificity (2 SD + mean). However, at this cut-off value the Porquier ELISA gave a positive reaction in one of the 15 herds considered as free of paratuberculosis. Consequently, the results were also interpreted using a cut-off value of 3 SD + mean, which offers a test of approximately 99% specificity. Both the Porquier ELISA and the Prionics ELISA at the 3 SD cut-off detected 6 of the 70 (8.6%) herds with paratuberculosis. The Svanova ELISA consistently produced a high non-specific reaction in one sample of milk from the 15 herds considered to be free of paratuberculosis and was subsequently excluded from further analysis.

CONCLUSIONS
ELISA technology applied to bulk milk samples is not a sensitive method for the identification of dairy herds affected by paratuberculosis. With further evaluation and refinement the technology may be suitable for the identification of herds with a high prevalence of infected cows.

Determination of optimal conditions to test bovine milk for antibodies against *Mycobacterium paratuberculosis* using an ELISA test

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OBJECTIVES

To determine optimal volume and type of milk samples for the Paracheck ™ ELISA, a test that was developed to assay serum or plasma for antibodies against *Mycobacterium paratuberculosis*.

EXPERIMENTAL DESIGN

Samples of milk and blood were collected from 12 cows that previously tested positive by the ELISA. Of the 12 cows, 11 tested positive by faecal culture and/or histopathology and one had no bacteriological or histopathological evidence of infection. In the first experiment, duplicate samples of serum, whole milk and skim milk the 12 cows were assayed for antibodies using a commercial bovine ELISA. Each replicate was diluted 1:20 in absorbing buffer and tested as recommended by the manufacturer. In the second experiment, 3 sets of duplicate samples of whole milk from 5 cows with paratuberculosis and one cow with no evidence of infection were assayed for antibodies. One set was diluted 1:20 in absorbing buffer (25 µL sample/475 µL absorbing buffer), the second set was diluted 1:10 (50 µL sample/450 µL absorbing buffer) and the third set was diluted 1:5 (100 µL sample/400 µL absorbing buffer).

RESULTS

In the first experiment, there was no significant difference ($P<0.05$) between the mean OD values in whole and skim milk. The average coefficient of variation (CV) between OD values in whole and skim milk was 4.8%. Although there was strong positive correlation between OD values in samples of serum and that measured in corresponding samples of whole milk and skim milk, only 7 of the 11 cows with paratuberculosis gave positive reactions in both the whole and skim milk. The OD values in samples of serum were significantly higher than that measured in corresponding samples of whole milk and skim milk.

In the second experiment, the mean OD values in whole milk samples diluted 1:20, 1:10 and 1:5 were 0.217, 0.340 and 0.549, respectively. The latter was similar to the mean OD in corresponding samples of serum. Of the 5 cows with paratuberculosis, all tested positive when the test was applied to serum and samples of milk diluted 1:5. In comparison, only one of the 5 cows gave a positive reaction when samples of milk diluted 1:20 were tested. The negative control showed a slight, negligible increase in OD values when larger volumes were tested. The one cow with no evidence of infection gave consistent negative results throughout the testing.

CONCLUSIONS

The likelihood of detecting infected animals increases when the ELISA is applied to large volume samples of milk. The whole milk is a suitable sample as the differences between the OD values in samples of whole and skim milk are negligible and similar to normally expected well-to-well variation.

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

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OBJECTIVES
To evaluate three methods of DNA extraction from milk for the IS*900* polymerase chain reaction (PCR) and compare analytical sensitivities of the PCR and modified double incubation radiometric mycobacterial culture (RMC) method.

EXPERIMENTAL DESIGN
The comparative evaluation of the three DNA extraction methods (Beadbeater, InstaGene and Qiagene) and determination of the detection limits of the RMC and PCR were carried out on triplicate samples of milk inoculated with serial ten-fold dilutions of *Mycobacterium paratuberculosis*.

RESULTS
Among the three protocols of DNA extraction from milk, the Beadbeater method was the most efficient procedure for the preparation of *M. paratuberculosis* DNA template for the IS*900* PCR. The average detection limit of the Beadbeater PCR system was about 70 viable *M. paratuberculosis* cells/50 ml sample. The InstaGene and Qiagene (QIAamp DNA Stool Kit) methods produced average detection limits by PCR of 600 and 700 cells/50 ml sample, respectively. The analytical sensitivity of the RMC was about 700 viable cells/50 ml sample.

CONCLUSIONS
The analytical sensitivity of the Beadbeater PCR system is sufficient for this test to be used for the detection of low levels of *M. paratuberculosis* contamination in milk. Further evaluation of this test on diagnostic samples is warranted.

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

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A possible mode of transmission for the ruminant pathogen *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from cattle to humans is via milk and dairy products. Although controversially, MAP has been suggested as the causative agent of Crohn's disease and its presence in consumers milk might be of concern. Isolation of MAP has been reported from milk of infected cows, bulk tank milk and from pasteurized milk. For screening of farm bulk tank milk, a method to detect MAP in milk with real-time PCR was developed.

With this method, both pellet and cream fraction of the milk were harvested for analysis. The bacteria were lysed enzymatically and by mechanical disruption and the DNA was extracted by robotized magnetic bead separation. The analytical sensitivity was determined to 100 organisms per ml milk (corresponding to less than 10 CFU per ml, as CFU measurement usually underestimates the actual numbers) for samples of 10 ml, although as few as 10 organisms per ml milk was detected in three of four replicates in spiked milk samples.

The method was applied in a study of 55 dairy herds to compare PCR of farm bulk tank milk to culture of environmental samples for detection of MAP in the herds. In this study, 17 herds (31%) were negative with both methods, 21 herds (38%) were positive with environmental culture but negative with milk PCR, one herd (2%) was positive with milk PCR but negative with environmental culture and 16 herds (29%) were positive with both methods. Hence the sensitivity for detection of MAP in a herd was considerably higher for the environmental culture method than PCR testing of farm bulk tank milk.

From the 37 herds that were proven positive by culture of environmental samples, 89 tank milk samples were tested. Eighteen of these milk samples (20%) tested PCR positive and altogether 16 of these 37 herds (43%) had at least one positive tank milk sample. By comparison with spiked milk samples, it was concluded that the positive milk samples contained low numbers of MAP, usually less than 100 organisms per ml and never more than a few hundred organisms per ml.

The results indicate that although MAP may be shed into milk or transferred to milk by faecal contamination, it will only occur in low numbers in the farm bulk tank milk due to the dilution and it can be assumed to often fall below the detection limit. Thus, PCR detection of MAP in milk would be less suitable for herd prevalence testing, but useful for control of MAP presence in milk, in order to avoid transfer to humans. The results also suggest that the level of MAP in the bulk tank milk of Danish dairy herds with paratuberculosis is low.

Detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* in ovine and bovine faeces by direct quantitative PCR

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In this study, a new test based on a novel faecal nucleic acid extraction method and an IS*900*-based real-time quantitative PCR (QPCR) method was developed and evaluated for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) DNA in ovine and bovine faecal samples. Both the Cattle (C) and Sheep (S) strains of MAP were detected by the QPCR assay, and no cross reactions were detected with 51 other species of mycobacteria including 10 which contained IS*900*-like sequences. One copy of IS*900* fragment cloned into plasmid pCR2.1 and 1 fg of MAP genomic DNA were consistently detected, while in spiked faecal samples the detection limit was 10 viable MAP (S strain) per one gram of negative ovine faeces. A total of 506 individual ovine faecal samples with known culture (BACTEC) results and histological status were tested in Australia, and a total of 666 bovine faecal samples cultured by Herrold's egg yolk medium (HEYM) were tested in Japan. In ovine samples, the QPCR assay detected 68 of 69 (98.6%) BACTEC culture positive faeces and there was a strong relation between time to detection in culture and DNA quantity measured by QPCR (r=-0.70). Furthermore, when DNA quantities detected by the QPCR were analysed on the basis of histological classification, faecal samples representing sheep with multibacillary lesions showed significantly higher levels of MAP DNA than samples from sheep with paucibacillary lesions or no lesions, suggesting that the QPCR test could be used for estimation of the risk of transmission. In bovine samples, 54 of 60 (90.0%) HEYM culture positive faeces were detected by the QPCR. MAP DNA was also detected from some culture negative faecal samples from sheep and cattle exposed to MAP, suggesting that the QPCR has very high analytical sensitivity for MAP in faecal samples and detects non-viable MAP in faeces. None of the faecal samples from 176 sheep and 508 cattle that were not exposed to MAP were positive in QPCR.

Evaluation of four commercial bovine-ELISA kits for the diagnosis of paratuberculosis in dairy goats

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Goat paratuberculosis is a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), characterized by enteritis, progressive loss of body weight, and decrease in production, especially in dairy goats. The disease is worldwide distributed and in Chile the prevalence is suspected to be high in dairy herds with intensive management systems and specialized breeds for milk production. There is no single test able to detect all infected animals and the faecal culture and the ELISA test are the most widely used tests to diagnose the disease. However, faecal culture is laborious, expensive and takes a long time to give a result and, therefore, the ELISA test, despite its low sensitivity, is the best alternative to be used as a diagnostic tool in Chilean dairy goat herds. The main objective of this study was to evaluate the sensitivity and specificity of four commercial bovine-ELISA kits for the diagnosis of paratuberculosis in dairy goats. A total of 379 serum samples from dairy goats >2 years old with known infection status belonging to the bank of sera of the Paratuberculosis Laboratory, Microbiology Department, Universidad Austral de Chile, were analyzed. All sera were previously collected from animals in four infected and four non-infected dairy goat herds from different regions of the country. Serum samples were simultaneously assayed for anti-*Map* antibody using four different commercial bovine-ELISA kits (A, B, C, and D), and performed after manufacturers' recommendations. Sensitivity and specificity of each test were calculated by means of 2x2 tables and for comparison between tests the Z test was used. Association and agreement between two kits were determined by means of the McNemar Chi square and the Kappa tests, respectively. Positive results were obtained in 49 (12,9%) samples assayed with kits A, 33 (8,7 %) for kit B, 46 (12,1 %) for kit C, and 42 (11,1 %) for kit D. Test sensitivity varied between 69,4% (B) and 77,7% (A, C, and D). Test specificity was 100% for all four kits. The McNemar P value showed statistic differences between kits A and B, B and C, and B and D but no difference between kits A, C, and D. The highest *kappa* value was 0,806 for kits A and D, a high agreement between these two kits. These results suggest that ELISA test developed for diagnosis of paratuberculosis in cattle can be equally used for diagnosis in goats though differences in sensitivity and specificity exist between kits, in particular when applied to low shedder animals, being kit D, the only licensed ELISA kit for goats, the most accurate test for detecting low shedder animals. Consequently, the ELISA test can be recommended for the diagnosis of paratuberculosis in dairy goat herds as a more inexpensive and confident alternative diagnostic test.

Evaluation of four commercial ELISA kits for the diagnosis of bovine paratuberculosis in dairy herds of southern Chile

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Four commercial ELISA kits for bovine paratuberculosis were evaluated using individual serum samples from 53 dairy cattle in 10 infected dairy herds and 368 dairy cattle in 11 paratuberculosis-free herds of southern Chile. Blood samples were collected in 10 ml Vacutainer tubes and the sera frozen at -20°C until tested in duplicate following manufacturer's recommendations. The infection status of the infected herds was determined by fecal culture on a single sampling and for the paratuberculosis-free herds by two consecutive 100% negative fecal cultures one year apart. Fecal samples were collected via rectum using individual polyethylene sleeves, transported to the lab, and cultured within 24h on home-made Herrold's Egg Yolk Medium (HEYM) with mycobactin J (3 tubes) and HEYM without mycobactin J (1 tube), using the centrifugation method. Prior to culture, 2g of each fecal sample was decontaminated with HPC and an antibiotic solution containing nalidixic acid, vancomycin, and amphotericin B. A 0.15 ml aliquot of each suspension was used to inoculate all HEYM tubes which were incubated at 37°C for 16 weeks. Colonies resembling *M. paratuberculosis* and showing mycobactin-dependence were tested and confirmed by IS*900* PCR technology. The following cutoff values recommended by the manufacturers were used for sensitivity and specificity analysis of each kit: S/P ≥ 0.25 (kit A), S/P% ≥ 70 (kit B), (OD-neg) ≥ 0.100 (kit C), and S/P% ≥ 70 (kit D). The specificity of the four ELISAs were 99.73% (kit A), 99.18% (kit B), 99.46% (kit C), and 98.76% (kit D). The sensitivity of the four kits for detecting fecal culture-positive cows were: 32.08% (kit A), 37.74% (kit B), 41.51% (kit C), and 37.74% (kit D). Receiver Operating Characteristic (ROC) analysis showed that kit C performed much better than the other three kits as the AUC values for the four kits assayed were 0.899 (kit A), 0.818 (kit B), 0.945 (kit C), and 0.854 (kit D). Assay agreement between kits was high (*kappa* 0.842 to 0.908) for categorical interpretations (positive or negative); the following *kappa* values were calculated for all four kits: A vs B = 0.843; A vs C = 0.843; A vs D = 0.842; B vs C = 0.908; B vs D = 0.907; and C vs D = 0.952. According to these results all four paratuberculosis ELISA kits evaluated were similar in sensitivity and specificity but kit C was most accurate.

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

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Paratuberculosis was diagnosed in a herd with 160 goats that had performed sanitation for paratuberculosis. Sanitation was carried out by establishing a new herd from goat kids snatched from their dams, culling of all the adult goats, exclusion of the goat kids from potentially contaminated pastures and cleaning and disinfection of pens and outdoor areas. The goats were tested when approximately 10 and 23 months old, using an interferon gamma (IFN-γ) test from Biosource, but no IFN-γ positive goats were identified. Three years following sanitation some two- and three-year-old goats began loosing weight and had a reduced milk production just after kidding. One goat died, and paratuberculosis was diagnosed based on histopathology, presence of large amounts of acid fast rods, detection of *Mycobacterium avium* subsp. *paratuberculosis* by culture and IS*900* by PCR. The herd was then tested with two months intervals using the IFN-γ test and the Pourquier ELISA. Post mortem examination of selected goats revealed significantly enlarged intestinal lymph nodes with necrotic cortical areas and severe intestinal lesions compatible with paratuberculosis, located particularly in the proximal jejunum. Culturing of feces from two-and three-year-old goats identified *M. a. paratuberculosis* in more than one-third of the animals. The ELISA and the IFN-γ test had the same ability to identify culture positive animals (about 80 %), while more culture negative goats were positive on IFN-γ testing than on ELISA. Further more, some culture positive animals were negative on both ELISA and IFN-γ testing, indicating that shedding can occur without any detectable immune response. IFN-γ and ELISA testing of goats less than 18 months old rarely gave positive results. Some goats that were negative on the IFN-γ test at 23 months of age, tested positive on the ELISA one year later. As expected the IFN-γ test was the most sensitive in detecting sub-clinically infected animals. However, the ELISA also performed quite well, and the time from positive IFN-γ results are seen, until goats become ELISA positive, appears to be short. In addition some culture positive goats had only antibody responses and no detectable IFN-γ response. Results from testing in this herd suggest that the Pourquier ELISA can be well suited for screening goat herds for paratuberculosis. However, the disease progress might have been unusually fast in this herd, compared to herds with a well-established infection, and this could explain why antibody responses were seen earlier than expected.

Novel diagnostic criteria of the Interferon-gamma test for bovine paratuberculosis

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Interferon-gamma (IFN-ɣ test based on cell-mediated immunity against *Mycobacterium avium* subspecies *paratuberculosis* (Map) infection has been reported to be one of the useful diagnostic methods for bovine paratuberculosis. Several criteria for interpretation of IFN-γ test using purified protein derivative (PPD) antigens and mitogens have been applied and discussed, however, it seems that the generally accepted interpretation criteria for the IFN-γ test are not established yet. In this study, the IFN-γ test of whole-blood stimulation with mycobacterial PPDs and Concanavalin A (Con A) was evaluated with samples from experimentally infected calves and different age groups of cattle in Japan.

Johnin and tuberculin PPDs were prepared from Map strain Kag-1, which had been isolated in Japan and adapted to the protein free medium, and *M. bovis* BCG strain Tokyo respectively. After 24 hours incubation of heparinized blood with PPDs or ConA, the culture supernatant samples were tested for IFN-ɣ concentration using ELISA with monoclonal and polyclonal antibodies against bovine IFN-ɣ̃ The concentrations of IFN-γ were calculated according to the formula of the dose-response curve obtained from the same ELISA using a recombinant bovine IFN-ɣ̃ After 1 to 2 months from inoculation of Map, IFN-γ was detected by stimulation with johnin PPD (jPPD) in experimentally infected calves, thereafter those production of IFN-γ have lasted for more than two years. The blood samples from healthy cattle showed higher concentration of IFN-ɣ against the stimulation of ConA than those of PPDs. On the contrary, the stimulation with jPPD induced the highest IFN-ɣ production in experimentally and spontaneously infected cattle compared to those of *M. bovis* PPD (bPPD) or ConA. On the basis of the results obtained from experimentally infected calves and more than 1,000 cattle from herds infected with or without paratuberculosis in Japan, the following criteria of the IFN-γ test seems to be useful for the early diagnosis of bovine paratuberculosis. 1) IFN-ɣ is detected by ConA stimulation (positive control), 2) concentration of IFN-γ induced by jPPD is higher than that of ConA, 3) jPPD/bPPD ratio of IFN-ɣconcentration is higher than 1.5, and 4) no-antigen control/jPPD ratio is less than 0.1. The samples that satisfied these all conditions are interpreted as IFN-γ test positive.

Recombinant enoyl-CoA hydratase (echA) antigen of *Mycobacterium avium* subspecies *paratuberculosis* expressed in *Escherichia coli* can be used for serological diagnosis of Johne's disease

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The phage library of *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) strain ATCC19698, which had been constructed using the Zap Express cloning vector (Infect. Immun. 73:3778-82, 2005), was screened with sera from calves infected with *Map* in order to detect the *Map* antigens with eliciting humoral immunity. The serum used for screening of the phage library was absorbed with plaques from non-recombinant phages, *Escherichia coli* and *Mycobacterium phlei* organisms to remove cross-reacting antibodies. After the screening of about 4 x 10^5 plaques, we have finally cloned one recombinant phage expressing *Map* antigen which strongly react with serum antibodies from infected calves. The *Map* DNA insert cloned into the phage vector was excised out of the phage into the form of the phagemid vector with *E*. *coli* strain XLOLR, and a part of 5' end of the insert DNA was sequenced using T3 universal primer. The sequence results indicated that the insert *Map* DNA contained *Map* gene encoding "echA12_2", an enoyl-CoA hydratase protein (echA), which plays a role for effective ATP synthesis in stationary phase survival. Therefore, the coding sequence of *Map* echA gene was amplified from DNA of *Map* strain ATCC19698 by PCR, and cloned into *p*QE plasmid vectors to obtain the recombinant *Map*-echA protein (rMap-echA). Sera from experimentally infected calves strongly reacted with the rMap-echA with immunoblotting, whereas sera from uninfected cattle did not. Although the homologous gene of *M. avium* subspecies *avium* (*Maa*) showed 99% nucleotide sequence homology with echA12_2 gene of *Map*, ELISA using the rMap-echA indicated high specificity without any cross-reaction to bovine hyperimmune sera against *Maa* and other related *Mycobacterium* species. These results suggest that the ELISA using the rMap-echA antigen may be useful for the diagnosis of Johne's disease.

A review of diagnostic accuracies of ELISA and faecal culture in cattle

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Infections with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) can be latent for years without affecting the animal, but the infection may result in the animal becoming infectious and developing clinical disease. Diagnosis can be a challenge primarily in latent stages of the infection, and because different decision makers have different target conditions for a diagnosis. The objective of this study was to provide a critical review of reported accuracies of ELISA and faecal culture (FC) tests used for diagnosis of three defined target conditions in cattle: MAP infected, MAP infectious and MAP affected animals.

For each target condition and test, sensitivities (Se) and specificities (Sp) were summarised. The diagnostic test information varied substantially for tests of the same type and make, particularly ELISA. For affected and infectious animals, the Sp of FC was set to 1.0 by definition. Se reported for FC in infectious and affected cattle were 0.74 and 0.70, respectively, whereas Se for infected cattle were 0.23 to 0.29. Se for ELISA were in the ranges 0.50 to 0.87 for affected, 0.24 to 0.94 for infectious and 0.07 to 0.39 for infected cattle, but Se of ELISA should always be interpreted with Sp, which also varied considerably.

The variation in reported Se and Sp may primarily be a reflection of the choices of the test-evaluators regarding weighing of either Se or Sp, study design and population. Comparison of the various tests accuracies was generally not possible, but stratification of test-evaluations by target condition improved the interpretation of the test accuracies. Infectious and affected animals can often be detected, but Se for infected cattle is generally low. A main conclusion of the review was that the quality of design, implementation and reporting of evaluations of tests for paratuberculosis was generally poor. Particularly, there is a need for better correspondence between the study population and target population, i.e. the subjects chosen for test evaluation should reflect the distribution of animals in the population, where the test is intended to be used.

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

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Johne's disease (JD) in ruminants is caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). JD has caused significant economic loss in affected farms. The worldwide distribution of MAP and its potential link to Crohn's disease in humans raises a public health concern. To facilitate more widespread acceptance of JD control programs, inexpensive diagnostic method that requires less time, inexpensive equipment and that are user friendly must continue to be developed. Diagnosis of JD is done by either detecting MAP in feces or infected tissues or detecting an immune response. Common assays used to detect MAP antibodies include: compliment fixation (CF), agar gel immunodiffusion (AGID), and enzyme linked immunoabsorbent (ELISA) assays. In this study, a polyaniline-based conductometric biosensor was developed for the rapid detection of antibodies to MAP in cattle serum. The conductometric biosensor consists of three components; the biological sensing element, the transducer and the detector element. MAP antigen served as the biological sensing element, polyaniline (Pani) was the transducer and an ohmmeter was used as the detector element. The biological element and the transducer are immobilized in a membrane with four regions; sample application, conjugate, capture and absorption regions. The detection technique is based on the antigen-antibody coupling reaction with Pani as the conductive transducer. Pani is conjugated to the heavy chains of the monoclonal anti-bovine IgG (AB/IgG) forming Pani-AB/IgG, which is positioned in the conjugate region. MAP purified proteins, serving as capture antigens; are immobilized on the nitrocellulose capture region. Subsequently, silver electrodes are fabricated on both sides of the capture region. 0.1 ml of the sample is added to the application region and allowed to flow through the other regions through capillary movement. Theoretically, Pani-AB/IgG in the conjugate region binds to the bovine IgG present in the sample. The conjugated bovine IgG-Pani-AB/IgG flow through the capture zone where MAP specific antibodies are captured by immobilized MAP purified proteins. As MAP specific antibodies are captured, the attached Pani structures form a bridge across the electrodes flanking the capture region. This bridge closes an electrical circuit that can be measured as a decrease in electrical resistance as more antibodies are captured. In this proof of concept study, JD positive cattle sera, as determined by standardized ELISA technique, were compared to serum samples that were negative for JD. The average resistance for JD negative samples was 92.875 kΩ (Kilo ohms) (range 101.3-72.1 kΩ) while the average resistance for JD positive samples was 53.338 kΩ (range 66.4-37.0 kΩ). These initial studies demonstrate that a conductometric biosensor can be fabricated to detect antibodies to MAP. Further testing to optimize the biosensor performance and test with large numbers of samples is underway.

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

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Deer farming in Chile began in the 1960s. Currently there are about 3,000 fallow deer and about 7,000 red deer distributed mainly in southern regions of Chile. Most animals were introduced from Germany mainly for hunting purposes and meat production. The aim of this study is to report the first isolation of *Mycobacterium avium* subsp *paratuberculosis (Map)* in a Chilean deer farm established in 1996. Most animals were imported from a German deer herd with breeding stock but some came from Argentinean herds. The herd comprised 180 red deer (*Cervus elaphus*) and 200 fallow deer *(Dama dama*) which lived commingled but were separated by gender into adjacent but different pens. Diagnosis of paratuberculosis was based on clinical signs, gross pathology, histopathology, and isolation of *Map* from feces and tissues. An adult hind red deer died suddenly in poor body condition without any other clinical signs. At necropsy, samples taken for parasite examination were negative, however, thickening of the small intestine and hyperactive mesenteric lymph nodes resembling paratuberculosis were observed. Fecal and tissue samples were collected for bacteriology and histopathology. Two grams of feces and tissue were cultured for *Map* using HEY medium with and without mycobactin J, and incubated at 37 ºC for up to 5 months. Two months later, four fallow deer of the same farm were euthanized because poor body condition, macroscopically examined, and fecal and tissue samples collected and processed as before for *Map* isolation. All Mycobactin-dependant colonies resembling *Map* were tested for IS*900* using PCR technology. One out of 5 fecal samples and all tissue samples examined were positive for *Map*, and confirmed by PCR. Tissue samples of liver, ileum and lymph nodes were fixed in formalin 10% and stained with hematoxylin and eosin and Ziehl-Neelsen stains for histopathological examination. Gross lesions and histopathology were characteristic of paratuberculosis. Thickening of the small intestine and calcification of the mesenteric lymphatic vessels were evident. Intense macrophage infiltration with many acid-fast bacteria in the lamina propria of the small intestine and mesenteric lymph nodes were observed. Necrotic focuses with acid fast bacilli were also present in the liver. These cases of paratuberculosis are the first reported in farmed deer in Chile.

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

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OBJECTIVE

The aim of this study was to follow within-herd Map infection using an absorbed ELISA and other diagnostic methods.

METHODS

An ovine herd (n=1000), endemically infected by Map, was bleeded twice a year for 3 years. Animals were killed at 5 years of age, and different tissue samples and a final serum sample were taken. Serum samples were tested by ELISA (ID Screen® Paratuberculoisis Indirect, ID VET, France), and tissue samples were examined with other diagnostic techniques.

RESULTS

The poster will present and analyse the results in detail, allowing a better understanding of Map serology in ovine herds.

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

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

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

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Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of paratuberculosis. The primarily affected hosts are domestic ruminants, but paratuberculosis has also been reported in wild animal hosts. In Chile, the infection has been confirmed in cattle, sheep and goats, but there is no information about paratuberculosis in wildlife animals. The broad range of hosts affected by *Map* implies a possible intraspecies transmission, as well as wildlife reservoir. Guanaco is the only wild ungulate species widely distributed across the Patagonian steppe, sharing grazing land with domestic sheep. The aim of this study was to detect *Map* infection in a free ranging wildlife animal species in Chile, using conventional diagnostic tools, as well as new molecular confirmation technology. Faecal samples were obtained from 501 guanacos populating the Rusffin area, Timmaukel County in Tierra del Fuego Island in August 2006. The sampling was synchronized with a controlled hunting activity carried out by a private company under the Ministry of Agricultural (SAG) supervision. Faecal samples were collected post mortem right after hunting and cultured on a homemade HEY medium with and without mycobactin J following the procedure recommended by the Cornell University. Colonies resembling *Map* and showing mycobactin-dependence were confirmed by Real -Time PCR based on IS*900* and F57. Twenty one out of 501 (4.2%) animals sampled were positive for *Map* all of which were confirmed by Real -Time PCR IS*900* and F57. This represents the first isolation of *Map* from a free-ranging wildlife animal in Chile. These findings support an increasing body of evidence that indicates that a wide diversity of wildlife species as well as domestic ruminants can become infected with *Map*. In a control or eradication program of this disease, it is of special importance to know how to control the transmission. The presence of a wildlife reservoir of the disease has to be considered for the potential transmission to livestock. However, for free-ranging wildlife, the most likely initial source of infection is the shared range with domestic species, given the higher prevalence of Johne's disease in the latter species, an issue to be determined in the Chilean situation.

Comparative proteomic analysis of mycobacterial tuberculins and identification of *Mycobacterium avium* subspecies *paratuberculosis* antigens with diagnostic potential

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Accurate immunodiagnosis of bovine paratuberculosis is among others hampered by the lack of specific antigens. One of the most frequently used antigen preparations is purified protein derivative, also known as tuberculin, produced from heat processed culture filtrates. This crude extract has limitations when used in diagnostic assays due to the presence of cross reactive antigens. The aim of the current study was to analyse the qualitative composition of tuberculins of the major mycobacterial pathogens and subsequently identify novel paratuberculosis specific antigens.

Using one dimensional gel electrophoresis followed by tandem mass spectrometry analysis of purified protein derivatives from *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Mycobacterium avium* subspecies *avium* (MAA) and *Mycobacterium bovis* we identified 156, 95 and 132 proteins respectively. Subsequently, comparative sequence analysis led to the selection of a MAP specific protein (MAP1718c). This protein as well as MAP3515c (4 AA difference with the homologous protein in MAA) and MAP1138c (LprG, the homologue of an interesting *M. tuberculosis* antigen) were expressed as recombinant proteins in *E. coli* for use in lymphocyte proliferation assays and serum antibody ELISA. While lymphocyte proliferation responses did not indicate substantial diagnostic potential of the antigens tested, the antibody titers measured by ELISA specific for MAP1138c, but not MAP1718c and MAP3515c, in serum from paratuberculosis infected cows (N=20) were significantly higher ($p<0.05$) than those in serum from control animals (N=20), despite the conserved nature of this protein.

In conclusion this study showed that a combination of proteomics and genomics starting from complex protein mixtures can reveal novel antigens supporting the development of more accurate diagnostics in mycobacterial diseases.

Sensitivity and specificity of unique 'Multi-species indigenous ELISA kit' with respect to fecal, milk and tissues culture for the diagnosis of Johne's and Crohn's disease in India

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

Variability of repeated Johne's Disease milk ELISA test results in Canadian dairy herds

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Commercially available milk ELISA tests for Johne's disease (JD) are vary in sensitivity and specificity depending on the disease status of the cow. The objective of this study was to explore and describe subsequent test results from cows previously positive on the AntelBio Johne's Milk ELISA test. The cut point for a positive milk ELISA test was set at an optical density (OD) of 0.1.

The study was conducted retrospectively utilizing the CanWest DHI milk ELISA records from 2,398 cows in 128 herds in the Canadian provinces of Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia. The study included all cows in these herds that had been tested at least twice between March 2005 and April 2007. The cows were in lactations one to eleven.

Of all cows tested, 87 cows had a positive test result and were tested on at least one subsequent occasion. Of these, 36 (41%) tested negative and 51 (58.6%) tested positive for JD at a second milk ELISA test (27 to 632 days after the first test). Nine cows were tested three times, with five of these testing positive at all three tests. Of the four remaining cows, two tested negative on test two and three, while one cow changed from positive to negative and one from suspicious to positive between test two and three. One cow had four and another cow five subsequent positive tests within the period of one year. The OD of the test score and subsequent changes in test status were independent ($p=0.1852$). Neither the lactation number nor the breed of the cows (Guernsey, Jersey, Holstein) were associated with the occurrence of changes in the test status ($p > 0.1$), nor were the differences in OD between tests of the 87 cows associated with interval between the tests ($p > 0.6$).

The variability in the milk ELISA test scores and their test interpretations, even in cows with initially high OD test scores, underscore the limitations of this test as a diagnostic tool for individual cows.

Comparison of liquid culture to solid media for quantification of *Mycobacterium* in tissues following experimental infection

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When experimental models of MAP infection are employed to investigate the efficacy of vaccination or other therapeutic strategies, quantification of tissue concentrations of MAP is desirable. This has most commonly been achieved through processing tissue samples for culture, and plate counts of MAP colonies grown on solid media such as Herrold's Egg Yolk Media (HEYM). This procedure requires up to 16 weeks of incubation, whereas growth in liquid media can be achieved in a much shorter time period. The time required for the mycobacterial growth to trigger detection in an automated liquid culture system (Time To Positive, TTP) is correlated to the amount of MAP in the original inoculum. The purpose of this study was to compare the use of automated liquid culture of MAP, using the MGIT system (Becton-Dickinson) to conventional quantification of MAP using HEYM, using tissues obtained from calves experimentally infected with MAP.

Twelve male Holstein calves were given an oral challenge of 10^9 CFU of MAP on days 21 and 22 of life. Calves were euthanized at 100 days of age, and 33 tissue samples (intestine, mesenteric lymph nodes) were processed for MAP culture. Tissue samples were disrupted with a stomacher, decontaminated with HPC, and plated on 4 tubes of HEYM (200 ul inoculum per tube) or in one tube of liquid MGIT media (100 ul/tube). The number of colonies per tube of HEYM were counted after 16 weeks of incubation. The MGIT tubes were incubated until signalled positive by the Bactec unit. Time to positive was recorded, and converted to a CFU value by use of a standard curve constructed by plotting time to positive vs. CFU/ml for inocula created from known concentration MAP suspension.

Of 396 samples, 3 were lost to contamination and excluded from analysis. Of 393 remaining samples, 269 (68%) were positive on both culture systems, 83 were negative on both systems (21%), for a total of 89% agreement between methods. Of the discordant samples, 36 were positive on HEYM only, and 5 were positive on MGIT only. The CFU as determined by HEYM was significantly (but inversely) correlated with the TTP in MGIT ($r = -0.96$).

On the basis of these results, automated liquid culture detection of MAP in tissue samples provides a more rapid method to quantify tissue concentration of MAP, compared with HEYM. Comparable results are obtained with both methods. In this experiment, in which half of the calves received MAP vaccination prior to challenge, differences in MAP tissue colonization of experimental vs. control groups was detected at a similar level of statistical significance for the two culture methods.

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

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

Ability of simple and nesting Polymerase Chain Reaction to detect *Mycobacterium avium* subspecies *paratuberculosis* DNA using blotted tissue impressions of bovine tissue: A new technique for rapid identification

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Simple and nesting polymerase chain reaction, both readily identify *Mycobacterium avium* subspecies *paratuberculosis* (Map) DNA in blotted tissue impressions done on bovine tissue of cows with documented Johne's disease.

Fourteen cows, primarily derived from a Holstein herd located in Alachua County (Florida, USA) were subjected to necropsy. Each animal had been documented to have clinical Johne's disease confirmed by ELISA titer and nesting PCR done on one or more of its biological fluids. Tissue samples from ileum and mesenteric and ileocecal lymph nodes were tested by P90P91 and J1J2 primers (simple and nested PCR respectively).

The objective of this note is to report the results when blotted tissue impressions of bovine tissue obtained from Map diseased dairy cows are probed with primers P90P91 for IS*900* versus primers P90P91 which are followed by a second set of primers J1J2.

Samples were first probed with primers P90P91 which recognized a 413 bp sequence of *Map* insertion sequence IS*900*. A second set of primers, J1J2 which overlapped and spanned a 333 bp region within the insertion sequence was then used as nested PCR. Sections were stained with hematoxylin-eosin stain. At least one representative section was stained with acid-fast stain to determine the density of bacilli present.

The J1J2 primers tested positive in 11 instances in which P90P91 primers had tested negative, and P90P91 primers tested positive in one instance not identified by the J1J2 primers. The addition of the J1J2 set of primers appears to extend the sensitivity of PCR analysis for Map DNA in bovine tissue. Simple PCR using primers P90P91 identified Map in 64% of the ileocecal lymph nodes, 69% of the mesenteric lymph nodes and 57% of the ileal tissue samples from cows with documented Map enteritis. The addition of the J1J2 set of primers identified 100% of ileocecal lymph nodes, 100% of the mesenteric lymph nodes and 86% the ileal tissue. The blotted tissue impression method offers an additional means of rapid identification of Map within diseased tissue.

Diagnostic performance of PARACHEK® for the detection of antibodies against *Mycobacterium avium* subsp*. paratuberculosis* in milk and serum of Dutch dairy cattle

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Efficient control of Johne's disease is dependent on early detection and removal of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infected cows to prevent spreading of the disease and associated economic losses to the cattle industry. Testing schemes optimized for cost and ease of implementation are often based on screening of milk samples and ELISA methodology rather than the use of blood serum samples with ELISA. The goal of this study was to determine the diagnostic performance of the PARACHEK® *Mycobacterium paratuberculosis* antibody test kit (Prionics AG, Schlieren, Switzerland) using milk and blood serum samples compared to the culture results of feces for MAP bacteria. For this purpose milk, blood serum and fecal samples were collected from 531 dairy cows in the Netherlands. These cows derived from 10 dairy herds. Eight herds had a recent history of paratuberculosis and two farms had a confirmed status of freedom of paratuberculosis infection based on their participation in a Dutch herd-certification program (1). Serum samples were collected from all 531 cows and stored at -20°C until analyzed. Milk samples were obtained from lactating cows (n=483) and stored at -20°C until further use. Fecal samples (n=531) were obtained directly from the rectum of the cows. The feces samples were decontaminated (3) and subsequently cultured in the TREK ESP para-JEM Culture System II (TREK Diagnostic Systems, Cleveland, Ohio, USA). All samples detected by the system and all samples not yet detected by the system at the conclusion of the experiment (49 days) were further investigated via Ziehl Neelsen staining and PCR methodologies (2). Milk and serum samples were analyzed using the indirect enzyme immunoassay PARACHEK® to detect antibodies against MAP. The manufacturer has two different protocols for milk and blood serum samples using the same kit. The samples were analyzed according to the manufacturer's instructions.

The results showed that in total 4.0% of the cows (21 out of 531) were culture positive. The sensitivity of the PARACHEK® with regard to culture positive cows was 52.4% (11/21) in serum and 57.9% (11/19) in milk samples. The specificity was 98.6% (503/510) and 98.9% (445/450), respectively. None of the cows from certified MAP-negative herds were determined as positive with the PARACHEK® either with serum or with milk samples. This suggests that false positive determinations from herds with a recent history of paratuberculosis may in fact indicate truly infected cows. These cows are possibly only negative in culture analysis due to intermittent or low mycobacterial shedding in feces, i.e. levels which are below the detection limit of the culture method.

The agreement between the results from milk and serum samples was excellent with a proportion of agreement of 0.98 and a weighted kappa value of 0.843.

In conclusion, the results of this study indicate that surveillance of cattle herds with the PARACHEK® using either serum or milk is an efficient, cost effective and reliable method to detect paratuberculosis in infected herds. In combination with good farm management, this test can contribute substantially to the reduction in prevalence of Johne's disease in cattle herds.

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Correlation between the occurrence of sero-doubtful and sero-positive reactions and the presence of DNA-MAP in milk

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Poland is one of the countries without the clinical form of paratuberculosis. Studies conducted in the north-eastern region of Poland revealed the presence of the subclinical form of the disease in cattle stock. This triggered further analyses of cattle stock infection and consumer exposure to Mycobacterium paratuberculosis (MAP). To this end, serological tests and DNA-MAP isolation from raw milk have been performed.

The study conducted in a cattle stock of over 200 cows revealed a relationship between the presence of antibodies in blood serum and the presence of DNA-MAP in milk samples.

Samples of blood serum and udder milk were taken from 103 cows over 3 years old. The ELISA (SVANOVIR^{tm} Para-TB-Ab, Svanova) test produced 10 sero-positive results and 19 sero-doubtful ones, indicative of paratuberculosis. DNA isolation with a Qiamp DNA mini kit (Qiagen) produced a positive result in 21 samples.

Nine samples were taken from animals with a sero-doubtful reaction observed in the ELISA test, while 5 – from the animals with a sero-positive reaction. The remaining 7 samples were from the animals with MAP-negative serological reaction. This confirms the periodical presence of MAP tubercles in milk, as in the case of antibodies in blood, whose level fluctuates. Analysing this relationship will contribute to better diagnostics of *M.paratuberculosis* infections in milk cattle stock.

Development of ELISA method using purified protein derived from *Mycobacterium avium* subspecies *paratuberculosis* isolate from Japan

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The immunological diagnosis of Johne's disease is performed by an ELISA test and Johnin test. For the production of the diagnostic antigen for the tests, P-18 strain, classified as *Mycobacterium avium* subsp. *avium* (*Maa*) on the basis of genetic properties, has been used. Hence, in the present study, we used Kag-1 strain of *Mycobacterium avium* subsp. *Paratuberculosis* (*Map*), an isolate from Japan, to produce an ELISA antigen and examined its sensitivity and specificity in antibody detection.

Sera from 141 fecal culture-positive and 103 fecal culture-negative cattle were used to examine the sensitivity and precision of the partially purified ELISA antigen from Kag-1 strain of *Map*. The sensitivity was 80.1% in ELISA and an agreement of 88.1% was found between the ELISA method and fecal culture method (Kappa value: 0.765, $P<0.01$). In addition, in the fecal culture-positive cattle, the ELISA-positive ratio was 71.8% for light shedders, 80.9% for intermediate shedders, and 92.9% for heavy shedders. The average ratio was 79.4%. In examination of cross reactions with bovine *Mycobacterium* antibodies, the purified ELISA antigen revealed week cross reactions with antisera immunized by BCG Tokyo strain of *M. bovis* and Kumamoto-8 strain of *Maa*, and a strong cross reaction with an antiserum immunized by S-7 strain of *M. intracellulare*. A western blotting that uses infection serum of ATCC 19698 strain and Kag-1 strain of *Map* showed colored bands mainly at 56 kDa and 29 kDa.

The above results indicate that the purified protein from Kag-1 strain of *Map* can be used as an antigen for ELISA.

Proteomic analysis for biomarkers for Johne's Disease in sheep serum by SELDI-TOP mass spectrometry

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Surface enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry has facilitated the discovery of disease specific protein profiles from different biological samples, such as serum and tissues, in a variety of diseases of man. These results have raised the possibility that protein profiles may become a powerful diagnostic tool and may be applicable to Ovine Johne's Disease (OJD).

In the first phase of this study, SELDI-TOF MS experiments have been rigorously optimized by using sheep serum applied on four different ProteinChip® Array surfaces, in combination with nine different binding/washing buffers and five different sample dilutions. The reproducibility study showed the range for mean within-chip coefficient of variation for peak intensity determined from up to 18 peaks using sera from 8 sheep was 13% - 18% and for mass accuracy was 0.01% - 0.02%. Corresponding values between-chip were 13% - 23% and 0.02% - 0.03% respectively.

Based on the results of the optimization experiment, a large scale biomarker discovery experiment has been conducted. We used SELDI-TOF MS to identify potential proteomic biomarkers from sheep serum that can differentiate between sheep infected with *Mycobacterium avium* subsp. *paratuberculosis*, uninfected sheep and those previously vaccinated with Gudair^{TM}. Univariate and two independent multivariate data analysis procedures: Linear Discriminant Analysis (LDA) and Classification and Regression Decision Tree (CART), have been used to develop classification models between contrasting populations. A panel of key polypeptides has been selected using both models for identification and further analysis.

To identify the serum proteins found using SELDI-TOF MS, a number of chromatographic procedures, include gel filtration, affinity and ion exchange chromatography are being used in a protein purification scheme. A 13.6 kDa protein, transthyretin, which was down-regulated in both infected and vaccinated serum samples, has been identified by MS/MS.

Bulk milk contamination by *Mycobacterium avium* subsp. *paratuberculosis* and related risk factors

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INTRODUCTION

The aim of the present paper is to evaluate, in dairy cattle infected herd, the resultant risk of contamination of bulk milk by *Mycobacterium avium* subsp. *paratuberculosis* (Map).

MATERIALS AND METHODS

This study was carried out in 52 dairy herds, previously classified as positive for Map infection by ELISA test (Institut Pourquier), performed on individual blood samples of cows over one year of age. When the number of sero-positive animals was below a statistical threshold, the positivity was confirmed by faecal culture (Arrigoni et al., 2006).

On the basis of the prevalence of sero-positive animals, the herds were classified as:

- "low prevalence" herds (<5% sero-prevalence);
- "moderate prevalence" herds (5-15%);
- "high prevalence" herds (>15%).

The bulk milk of the infected herds was repeatedly sampled, each farm being submitted to an average of 3.5 samples, at a frequency interval of at least 30 days, with the aim of sampling all the lactating cows, resulting in a total of 183 bulk milk samples being collected.

The culture was performed following the method suggested by Dundee et al. (2001). PCR tests were performed, after magnetic separation (Adiapure kit, Adiagene), by a nested "home made" protocol (Taddei et al., 2006). An anamnestic questionnaire was offered to each farmer, to evaluate the risk factors for bulk milk contamination by Map.

RESULTS

From the total of 183 samples, 176 were submitted to analysis, while 7 were tested because the milk curdled. Twenty (11.4%) samples were positive and distributed as follows:

- 3 positive (4.4%) were obtained from 68 samples from "low prevalence" herds;
- 10 positive (10.8%) were obtained from 93 samples from "moderate prevalence" herds;
- 7 positive (46.7%) were obtained from 15 samples from "high prevalence" herds.

Overall, 11 herds of 52 assessed (21.2%) had at least one positive sample, and were distributed as follows:

- 3/21 (14.3%) "low prevalence" herds;
- 5/27 (18.6%) "moderate prevalence" herds;
- 3/4 (75.0%) "high prevalence" herds.

FARM DATA IN RELATION TO MILK CONTAMINATION

A total of 49 anamnestic questionnaires were collected from infected farms, of which 11 were producers of contaminated milk; in 3 cases it was not possible to obtain the data.

Herd size

On the basis of the number of cattle over 12 months of age, the herds examined were classified as: small (<100 heads), mid (101-200 heads) and large sized (>200 heads) herds. Map was detected in bulk milk of 5 out of 11 mid sized herds (45.5%), in 4 out of 20 large herds (20.0%), and 2 out of 18 small herds (11.1%).

Incidence of clinical cases
In 42.1% of infected farms producing uncontaminated milk and in 72.7% of infected farms producing contaminated milk, clinical cases of paratuberculosis were recorded. Moreover, while the disease had a low incidence in the first case (lower than 2% in 97.4% of cases), in farms producing contaminated milk the incidence was over 2% in 54.6% of cases. All the farms in which the incidence of clinical cases was over 5%, in addition to those in which clinical cases in heifers were registered, produced contaminated milk.

Bedding conditions
In general, the hygienic conditions of bedding were good or quite good. Relevant differences between farms producing contaminated and uncontaminated milk were not registered.

Udder hygiene
In 87% of infected farms producing uncontaminated milk, udders were free of dirt or only slightly dirty, while in 13% were moderately dirty, but never very dirty. Among farms producing contaminated milk, the percentage with dirty udders (from moderately to very dirty) was 36%.

Milking system employed
Milking in tie stall barns, being carried out in the same environment where the cows live, is generally considered a risk factor for faecal contamination of milk. From our study, 18% of infected farms adopted this kind of milking. In spite of this, no farms producing contaminated milk belonged to this category. On the contrary, all the farms producing contaminated milk had milking parlours, 54% of which were fishbone parlours, which in theory should give the best safeguard against faecal contamination of milk.

Milking machine and bulk cooling tank hygiene
In 91% of farms producing contaminated milk, the bulk bacterial count (BBC) was <50,000 cfu/ml and in 100% BBC was <100,000 cfu/ml. Therefore the BBC cannot be considered a significant indicator for milk contamination by Map, as presumably, given that the BBC is generally a consequence of milking machine and bulk cooling tank hygiene, as well as rapid and proper refrigeration. The BBC can only be minimally influenced by milking routine hygiene and by udder hygiene, which are directly related to faecal contamination of milk.

Milking hygiene
Among farms producing contaminated milk, 27.3% did not clean the udder properly, while 72.7% did, 45.5% of which did so in an excellent way (cleaning with disinfecting towels and wiping with individual paper towels); the hygienic measures adopted in these herds were equal or superior in comparison to farms producing uncontaminated milk.

Milk filtration
In more than 90% of farms, milk was filtered, with the filters being changed at least daily.

Risk factor analysis
To assess the risk of milk contamination by Map, odds ratios (ORs) were estimated using multiple logistic regression. After exploratory analysis, only occurrence indicators (sero-prevalence, incidence of clinical cases), herd size and udder hygiene could be included in the logistic model. Because of the low number of observations, only the high incidence of clinical cases (>2%) and the number of adult cattle between 101 and 200 resulted significantly associated with Map contamination of milk.

DISCUSSION AND CONCLUSIONS

Culture of bulk milk samples, although producing promising sensitivity results under experimental conditions, proved problematical due to frequent contamination by environmental microorganisms, *M. porcinum* in particular (Taddei et al., 2005).

If performed on bulk milk, PCR appears more rapid and sensitive than culture, because it revealed milk contamination by Map more efficiently than culture (PCR 10.8% vs. culture 1.1%) in samples from proven infected herds.

In total, 21.2% of infected farms tested positive by culture and/or PCR.

The presence of Map in milk is related to sero-prevalence: a higher percentage of milk samples from high sero-prevalence herds tested positive (46.7%), compared to those exhibiting moderate or low sero-prevalence (10.7% and 4.4% respectively). It should be noted that repeated sampling greatly increased the percentage of positivity.

From the anamnestic data collected, the risk of milk contamination appears directly related both to the infection occurrence in the herd (sero-prevalence and high clinical case incidence, particularly in young animals), and to udder hygiene. The mid size of the herd (101-200 cattle >12 months) seems to be another risk factor. Anyway, neither the ideal hygienic measures in routine milking, nor proper milk filtration were effective in preventing the presence of Map in milk.

Therefore, in order to reduce the milk contamination risk by Map, suitable actions should be taken into consideration: on the one hand the infected cows should be culled, in particular the “heavy shedders”, and on the other the hygienic, sanitary and managerial measures should be implemented to reduce the faecal contamination of udders.

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

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INTRODUCTION

Paratuberculosis, an infectious and contagious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), is reported all over the world. In Europe, the herd prevalence varies between 7 and 55% (IDF, 2001). Given that the herd prevalence estimation is fundamental for planning a control programme, we have carried out a survey on an Italian northern region, Lombardia, in which 42% of the overall Italian milk production is concentrated.

MATERIAL AND METHODS

In the period between October 2003 and March 2005, we carried out a serological survey for the detection of Map antibodies in blood samples of 38,478 cows over one year of age, belonging to 391 dairy herds of the Lombardia Region; the tests for paratuberculosis were carried out on the samples collected by the Veterinary National Health Service for Brucellosis and Leukosis eradication programmes.

The 391 sampled herds, corresponding to nearly 3% of the 15,111 dairy herds of the Lombardia Region, were stratified for geographical distribution and size. In the selected herds, the number of dairy cows over 12 months varied between 10 and 584.

The blood samples were submitted to a screening analysis using a commercial ELISA kit (Institut Pourquier, Montpellier, France); all the reactive (positive and inconclusive) samples were analysed using a verification ELISA test (Institut Pourquier, Montpellier, France).

It was established that the herds with a minimum number of sero-positive animals (over a statistical threshold) were considered as infected, taking into consideration the following parameters:

- Sensitivity 45%
- Specificity 99%
- Lower prevalence in infected herds 5%
- Confidence limits 95%
- Minimum herd-level specificity 95%

Two different values of herd prevalence were calculated:

- *Apparent prevalence*: calculated by classifying a herd as positive if it contained at least one positive animal;
- *Corrected prevalence*: calculated by classifying a herd as positive if it contained a number of sero-positive animals over the threshold.

The sero-positive cows belonging to the "inconclusive herds" (number of ELISA-positive animal below the threshold), were submitted to confirmation by faecal culture.

The confirmation by faecal culture, performed by a sedimentation method (Taddei et al., 2004), was introduced as a further control to avoid the possible underestimation in herds with a very low prevalence.

RESULTS

The *apparent prevalence* of infected herds was 43.7%, while the corrected prevalence was 16.1%. On the whole, the sero-positive animals were 982 over 38,487 submitted to analysis (2.6%).

A total of 108 herds (27.6%) were classified as “inconclusive”; the sero-positive animals belonging to 73 of these herds were submitted to faecal culture, while in the other 35 herds it was impossible, due to the immediate slaughtering of the reactors.

This further research, directed to minimize the underestimation of the corrected prevalence, can only partially reduce it, mainly because it was impossible to sample all the inconclusive herds, and also because the faecal culture has a low sensitivity; for this reason it is possible that a percentage of infected sero-positive animals, negative to faecal culture, were infected but shedding an undetectable number of Map in their faeces.

On the whole, 185 faecal samples were collected and submitted to culture, of which 43 tested positive. Out of 73 inconclusive herds submitted to faecal culture, 12 turned out positive and 61 negative.

Taking into consideration these results, the number of infected herds went up to 75 and the corrected prevalence from 16.1% to 19.2% (95% C.I.: 15.4%-23.4%). In smaller herds (10-50 cows > 12 months) the corrected prevalence was 7.8%, while in medium sized herds (51-100 cows > 12 months) it went up to 20%, and to 37% in bigger herds (>100 cows > 12 months).

Analysing the data of the “in-herd” prevalence, only 9.3% of the infected herds show a high prevalence (>15%), while respectively the 50.7% and the 40.0% of the infected herds are medium (5-15%) and low (<5%) prevalence herds.

The results of this survey, if compared with the data reported by previous surveys carried out in Veneto (Robbi et al., 2002)(apparent prevalence 64.9%, corrected prevalence 26.7%, percentage of seropositive animals 3.5%) showed lower results. Otherwise, the data observed in Lombardia were similar to that of the Lazio Region (Lillini et al., 2005), where the apparent prevalence was 42% (corrected prevalence undetermined) and the percentage of sero-positive animals 2.4%.

Furthermore, the risk of a herd being infected is directly related to the herd size, confirming the results of the survey carried out in Veneto; this could be due, apart from a higher probability of detecting a positive animal in a bigger herd, to a higher frequency of purchasing animals in big herds, or to the modern breeding technologies (free stalls) that enhance the risk of infection.

In general, the “in-herd” prevalence appears low (90.7% of herds show a sero-prevalence lower than 15%), and this should be regarded as favourable for the farmers that intend carrying out a control plan in their own herds.

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Results from paratuberculosis surveillance under government regulation in Austria

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INTRODUCTION

Control and prevention of important livestock diseases has been a top priority in Austria throughout the last decades. Eradication programs of bovine tuberculosis, brucellosis, enzootic bovine leukosis and infectious bovine rhinotracheitis under government regulation have been completed successfully. Austria is a member of the European Union that has been declared free of these diseases. A compulsory control program for bovine virus diarrhea started in 2004, and in April 2006 paratuberculosis (Johne's disease) in cattle, sheep, goat and farmed deer became a notifiable disease.

Contrary to the diseases mentioned above, the eradication of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in ruminants is hampered by enormous difficulties with the diagnosis of subclinically infected animals. Therefore the main target of the Austrian paratuberculosis surveillance program is the detection and elimination of clinically affected animals and the implementation of hygienic and management measures to reduce economic losses at the farms affected.

MATERIALS AND METHODS

Clinical signs typical of paratuberculosis mostly develop after several years of incubation. They include weight loss, emaciation and chronic diarrhea. For laboratory verification of clinically suspicious animals, blood and feces samples of live animals, or tissue samples from the intestine, the intestinal lymph nodes and the hepatic lymph nodes of slaughtered or perished animals, have to be sent to the national laboratory for paratuberculosis. Blood samples are tested by ELISA (Idexx, USA) for paratuberculosis-specific antibodies, and a commercially available real-time PCR (Adiagene, France) is used for the detection of MAP in feces and tissue samples.

RESULTS

The number of animals examined in accordance with this regulation and the results are listed in Table 1. Forty-three cattle turned out positive by both methods and 10 cattle tested positive in just one laboratory procedure. Either blood or feces/tissue samples were available for an additional 10 cattle, which tested positive. The only paratuberculosis infection in a small ruminant was confirmed by real-time PCR from tissue. Table 2 lists the positive results by laboratory method.

Table 1. Examinations and results between April 06 and July 07

	Cattle		Sheep		Goat		Farmed deer	
	Examined	**Positive**	**Examined**	**Positive**	**Examined**	**Positive**	**Examined**	**Positive**
Animals	257	63	20	1	2	0	-	-
Farms	100	35	1	1	2	0	-	-

Table 2. Results in cattle by laboratory method

Method	Number of samples	Positive (%)
ELISA	253	48 (19)
Real-time PCR feces	173	52 (30)
Real-time PCR tissue	38	11 (29)

The age of cattle which tested positive ranged between 14 months and 213 months, the average being 67 months. More than 50% of the animals which tested positive were cattle of the Limousin breed.

DISCUSSION

Despite two serological cross-section studies for MAP antibodies in cattle carried out in Austrian cattle herds between 1995-97 and 2002/03, the true prevalence of MAP and the economic impact on Austrian livestock are still far from clear (Baumgartner et al., 2005). As a consequence, paratuberculosis became a notifiable disease, with diagnosis and destruction of clinically diseased animals financially supported by government. From the current point of view, it seems that paratuberculosis is a major problem in cattle herds of Limousin breed. Because of intermittent shedding and pre-mortal antibody decrease, a combination of ELISA and real-time PCR is applied. Eight PCR-positive cows had a negative ELISA result, and 2 ELISA-positive animals were PCR negative. In contrast to culture, which takes at least four weeks, the result from real-time PCR is available within one day. The duration of laboratory procedures is important especially in clinically diseased animals which are subject to this regulation.

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Investigation of paratuberculosis status based on comparative analysis of serology and faecal culture in a dairy herd in Thuringia (Germany)

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ABSTRACT

In Thuringia, a federal state of Germany, paratuberculosis is being monitored by a voluntary control programme. Within the framework of this programme, a dairy herd of about 400 cattle with clinical problems of untreatable chronic diarrhoea was tested for the first time to detect paratuberculosis.

The objective of the present investigation was to determine the status of paratuberculosis in this herd, to analyse the diagnostic significance of an absorbed ELISA and the faecal culture, and compare it with age and lactation.

Cattle older than 24 months (n=279) were included in the study. In faecal culture, 93 cows (33 %) were detected as shedders and in ELISA, 39 cows (14 %) were positive or doubtful. Of the 93 shedders, only 31 cows (33 %) were classified as positive or doubtful by the ELISA. Almost half of the shedders were in the first or second lactation period. No significant differences in age of cows between serological negative and positive shedders were observed. The average milk production of shedders was significantly reduced ($p<0.001$) by approximately 1000 kg per lactation period, which corresponds to 10 % of the milk production. There was a high prevalence of paratuberculosis in the herd. Faecal culture is still the most suitable method to determine the individual status of paratuberculosis in a herd as most of the shedders were not detected by ELISA.

In view of the economic losses through reduced milk production, a herd management based on testing of the individual animals by faecal culture was found necessary to institute an effective control programme for paratuberculosis in infected herds.

Key words: age, cattle, ELISA, faecal culture, lactation monitoring, paratuberculosis, prevalence, serology.

INTRODUCTION

Paratuberculosis (PTB) is an economically important infectious disease of dairy cattle, causing untreatable diarrhoea, reduced milk production and progressive weight loss (Chiodini et al., 1984). The determination of infection status at herd level is necessary to implement control measures to reduce the transmission of the infection to susceptible cattle. Although PTB is widely prevalent, there is a lack of adequate studies on prevalence at herd or national level due to the difficulty of diagnosis. Despite the fact that the bacterial culture of MAP is time consuming (12-16 weeks), it is generally considered to be the most accurate method with 100 % specificity and 38–55 % sensitivity (Sockett et al., 1992; Whitlock et al., 2000). The absorbed serum ELISA is rapid and substantially economic, but its sensitivity and specificity are in the ranges of 28–29 % and 95–100 %, respectively (Collins et al., 2005).

In Thuringia, a federal state of Germany, a voluntary control programme exists for the monitoring of paratuberculosis using ELISA und faecal culture as diagnostic tools. In the framework of this programme, a dairy herd of 400 cattle with clinical problems of untreatable diarrhoea was tested for the first time to determine the paratuberculosis status of the herd, to analyze the diagnostic significance of an absorbed ELISA and faecal culture, and to compare the results with the age, lactation and the milk yield.

MATERIALS AND METHODS

Samples: Blood and faecal samples were collected from cattle (n=279) older than 24 months.

Faecal culture: Culture was carried out with 3 g faeces according to the AVID method (www.dvg.net). Briefly, following 24 h decontamination with 0.75 % (w/v) hexadecyl-pyridinium chloride, the samples were incubated on Herrold's egg yolk medium supplemented with mycobactin J for 12 weeks. The growth of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) colonies was confirmed by amplification of the MAP specific insertion sequence IS*900* by PCR (Englund et al. 1999). A sample was regarded as positive if one or more MAP colonies were detected.

Serology: The serological analyses were performed with EDTA plasma. The ELISA (IDEXX) included a preabsorption step with *Mycobacteria phlei* to detect specific antibodies of MAP in the serum. Classification of results was according to manufacturer instruction in negative, doubtful and positive samples. Samples classified as positive or doubtful were summarised.

Milk yield: Total milk yield was calculated based on monthly measurement.

Statistic: For statistical analysis an unpaired, two-tailed t-test was used.

RESULTS AND DISCUSSION

Faecal culture and serology

In faecal culture, 93 of 279 tested cattle were detected as shedders equivalent to 33 % of the tested cattle. In comparison to the cultural confirmation of MAP, specific MAP antibodies were detected by ELISA in samples of 39 cattle equivalent to 14 % of tested cattle. The results of faecal culture and serology in relation to average age and milk yield are shown in Table 1. Of the 93 shedders, only 31 cows (33 %) were classified as positive or doubtful by the ELISA. Sixty two shedders were not detected by ELISA and 8 serologically positive or doubtful tested cattle (2.9 %) were cultural negative. The percentage of serological positive results in faecal culture with more than 100 CFU/tube was much higher for serological positive cattle (48.5 %) in comparison to serological negative cattle (14.5 %) (Fig.1a).

Table 1. Diagnostic results of faecal culture and serology related to average age and milk production (Mean ± Standard deviation)

	Culture negative		Culture positive	
Serology	**Negative**	**Positive/ Doubtful**	**Negative**	**Positive/ Doubtful**
Numbers of cattle tested	178	8	62	31
Age (months)	50.5 ±18.8	55.4 ± 14.2	46.0 ± 17.6	50.6 ± 11.7
Milk yield (kg/ lactation period)	9741 ±1542	9983 ± 652	8784 ± 2010	8575 ± 1602

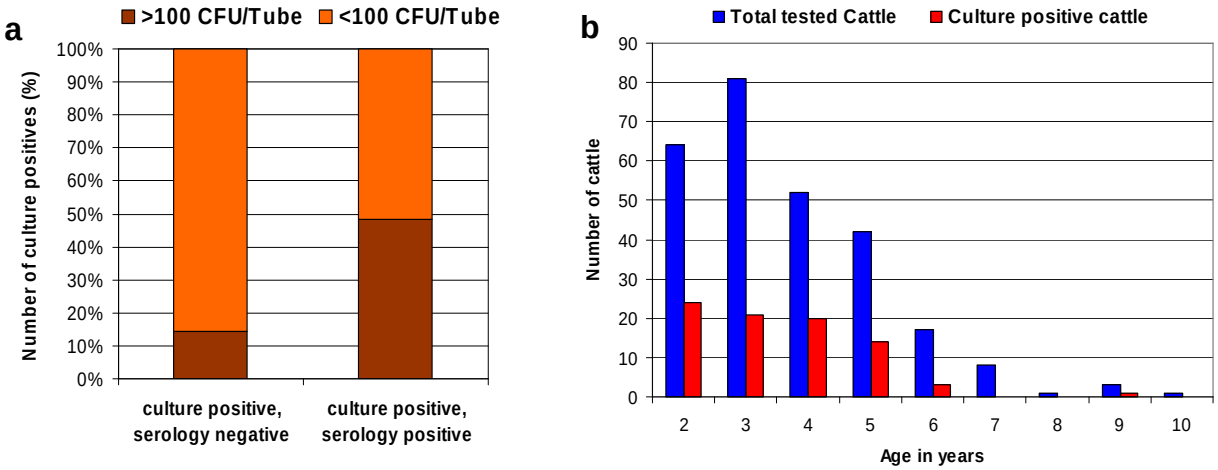

Fig. 1. a) Percentage of faecal samples with high CFU-amount concerning serological results, **b)** Fractions of shedders of every age group in relation to total tested cattle

The cultural method detected more cattle positive in comparison to ELISA, which might be due to the facts that animals either did not seroconvert or might have transplacental infections leading to a lack of immunological recognition of paratuberculosis infection (Seitz et al., 1989). It is also possible that the culture method detected animals in the early stage of the infection with low antibody response. A decrease in the "cut-off" value of ELISA might have increased the proportion detected. However this was not assessed in the present study.

The false positive results of ELISA might be due to shedding of bacteria below the detection limit at the time of sampling. It is widely known that the sensitivity of faecal culture is limited due to intermitting shedding, inhomogeneous distribution of MAP in faeces and losses during decontamination process.

Age

The evaluation of data concerning age of tested cattle showed no significant differences ($p > 0.1$) between serological negative and positive shedders (46.0 and 50.6 months, respectively, see Table 1). Almost half of the shedders were between two and three years old and hence were in their first or second lactation period (Fig. 1b).

The presence of more shedders during first or second lactation period indicated the onset of the clinical disease at a young age disproving the old concept of occurrence of clinical disease at later stages of life.

Milk yield

The mean milk production of shedders was significantly reduced ($p<0.001$) by approximately 1000 kg per lactation period (8719 kg compared to 9749 kg of non-shedders), which corresponded to 10 % of the milk yield (Fig. 2). A reduced lactation was also observed when comparing milk yield of serologically positive or doubtful cattle (8802 kg) with serologically negative cattle (9481 kg).

Significant impact of paratuberculosis infection on milk yield has been widely reported (McKenna et al., 2006). Non-significant influence of serological status on milk yield might be due to lower number of serological positive subjects in the present study.

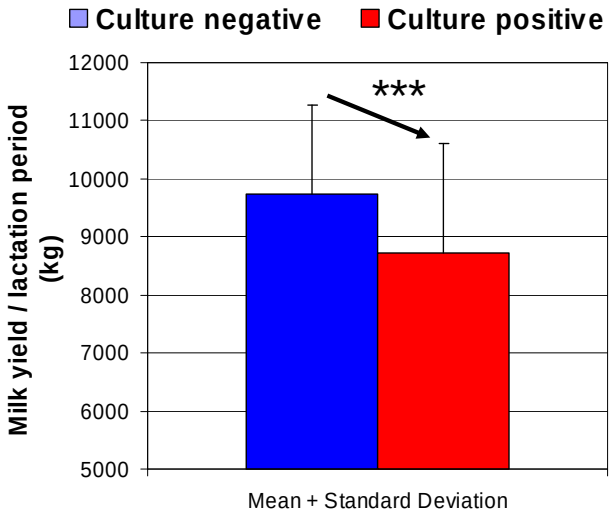

Fig. 2. Milk yield per lactation of shedders compared with non-shedders (*** $p<0.001$, two-tailed, unpaired t-test)

CONCLUSIONS

There was a high prevalence of paratuberculosis in the herd. Faecal culture is still the most suitable method to determine the individual status of paratuberculosis in a herd as most of the shedders were not detected by ELISA. In view of the economic losses through reduced milk production, a herd management based on testing of the individual animals by faecal culture was found necessary to institute an effective control programme for paratuberculosis in infected herds with high prevalence.

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Preliminary evaluation of a field trial on the use of vaccination in dairy cattle farms with paratuberculosis

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INTRODUCTION

In Spain, even though paratuberculosis vaccination in sheep and goats has been allowed and even recommended, its use in cattle has been opposed by the animal health authorities, because of its potential interference with the diagnosis of tuberculosis. The increased incidence of this disease in dairy herds in a moment in which they are greatly improving their productive efficiency in other aspects, as well as the nearly complete eradication of TB has led the local Animal Health authorities in the Basque Country to endorse a field trial designed by NEIKER and supported by a national research grant and the manufacturer of the Spanish paratuberculosis vaccine, CZV. This study required a strict follow-up agreement with the farmers and began in 2003 in one single farm.

MATERIALS AND METHODS

Herds and conditions

Six dairy herds (five Holstein Friesian and one Jersey herd) were vaccinated. Two other Holstein Friesian herds were left as unvaccinated controls and the program was based on detection and culling of positive animals. At the beginning comparative intradermal test of whole herd, blood and faecal sampling for ELISA, culture and PCR to all older than two years old was realized.

One ml of Silirum® (CZV) was applied in the dewlap to all animals present in the farm at the moment of joining the trial, and then to all heifer calves intended for replacement during their first month of life. A clinical follow-up was performed at one month post-vaccination at the beginning, and then blood and faecal samples were taken on a yearly schedule at the same time that comparative intradermal test was realized.

All culling were reported, and samples from the ones slaughtered within the Basque Country were taken for pathology and bacteriology. Records from the breeders association regarding milking monthly controls, total lactation and days of lactation were obtained and analyzed comparing pre-and post-vaccination data. At least 15% of culling animals tested at slaughter.

To be included in the vaccination program, the herds should have been free of tuberculosis for at least 5 years, the annual clinical incidence had to be over 5% and the infection had to be confirmed by culture and/or pathology. Also the local administration signed two agreements, one of them with the farmer not to sell vaccinated breeders and provide information, and the second one with the practitioner to collaborate in clinical recording, sampling and vaccine application.

Vaccination nodule size

The nodule sizes were classified in three categories: small (less than 2.5 cm), medium (between 2.5-5 cm) and large (more than 5 cm). The nodule size was reviewed 15 days post-vaccination and one year post-vaccination

Study of the immune response

The development of the humoral immune response was studied by a commercial ELISA (Pourquier).

Microbiological studies

Faecal samples were processed by cross pooled culture as described previously and cultured in Herrold's Egg Yolk medium and in Lowënstein-Jensen medium both supplemented with mycobactin J for 20 weeks.

Initially conventional IS*900* PCR (Adiagene) was used but in the last sampling was replaced for the IS*900* Real Time PCR produced by Adiagene.

RESULTS

No relevant clinical effects were observed apart from the formation of a small nodule at the point of inoculation that tended to disappear or at least decrease after a few months (Fig. 1). Comparative tuberculin testing one year and more after vaccination yielded one positive result for bovine TB, pending of confirmation (Fig. 2).

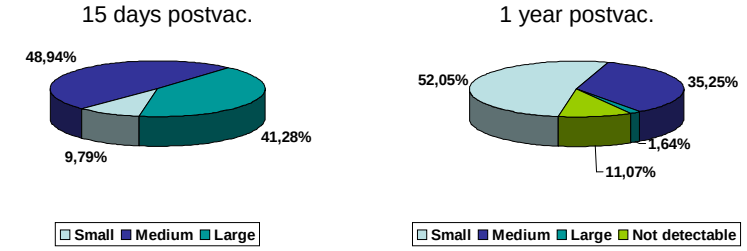


Fig 1. Nodule size 15 days post-vaccination and 1 year post-vaccination

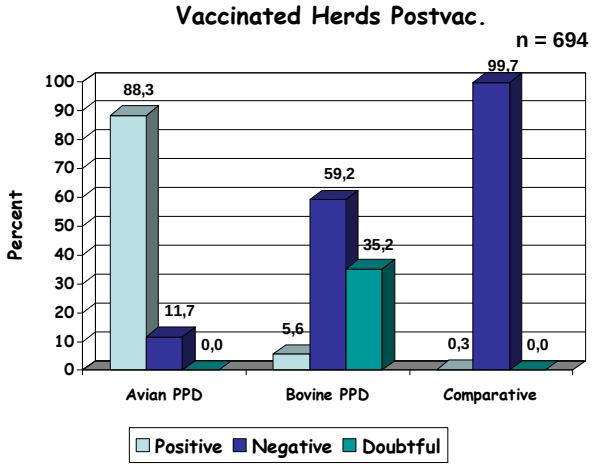


Fig 2. IDT reactions with the two antigens and with the comparative test

Initially, we saw an increase in antibodies due to the vaccine effect, with a progressive reduction 12 months later. Two exceptions have to be noted. The first one in the Pokop herd in which new animals were introduced in the farm 5 months before the 24 months Post-vaccination sampling and changed the expected results. The second one appeared in Endai herd and have yet to be analyzed yet because of no management change was reported (Fig. 3).

In the control herds the dynamic of antibodies was influenced by the culling program adopted in each one, so when only shedders were slaughtered no reduction was observed in the antibody levels.

The proportion of animals excreting bacteria were

The proportion of animals excreting bacteria were 11% as detected by PCR within 12 months post-vaccination (Fig 4). No general reduction was observed by faecal culture, but the 19% of the whole positive animals detected in the first sampling were heavy shedders, which disappeared 12 months after vaccination (Fig 5).

In the control herds, the reduction of shedders was lower than in the vaccinated herds. Also in these herds, a large number of animals were culled based on ELISA and IS*900* PCR

results, so we can say that the reduction of shedders was faster and cheaper with vaccination than with test and culling.

Up to now, there has been a reduction in the number of clinical cases in all the vaccinated farms. Of all the animals older than 24 months that have been culled or died after the first sampling, we have examined 34 (22.4%). Advanced lesions of paratuberculosis have been found in 5 cows (14.7%) of which 4 were Jersey (40%) and 1 Holstein (4.2%). We have to take into account that the Jersey breed is more susceptible and the hygiene practices of this farm were deficient. The clearest effect has been a reduction in the culling rate of first parity cows, which was 46% according to the milking records.

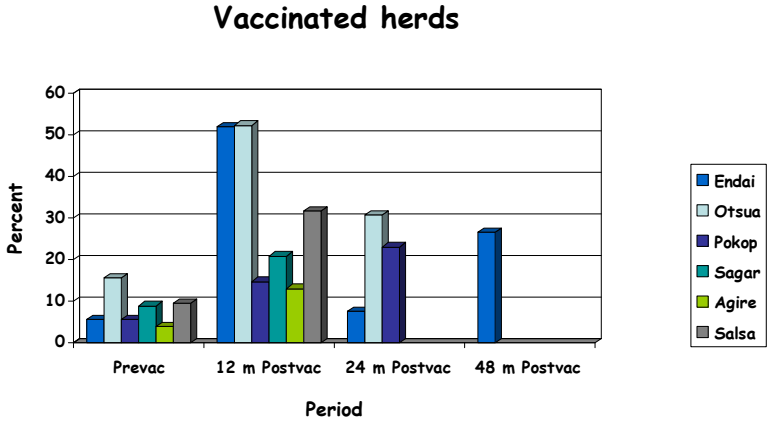


Fig 3. Dynamic of antibodies in the vaccinated herds

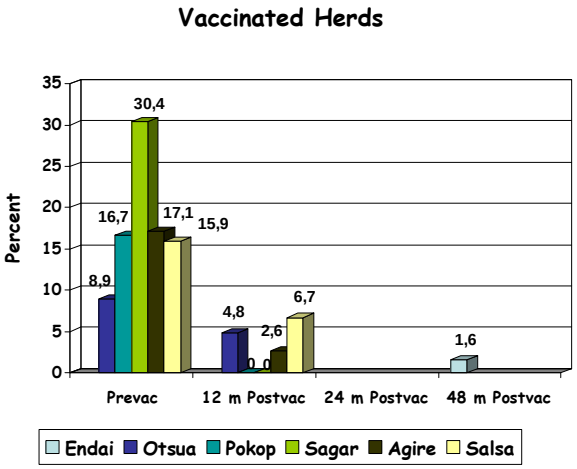


Fig. 4. Percentage reduction of shedders detected by PCR

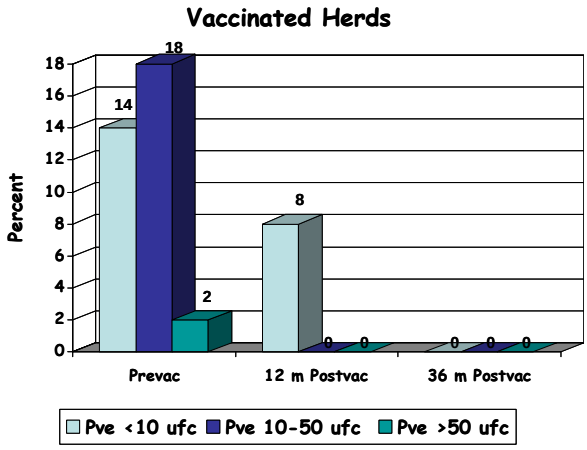
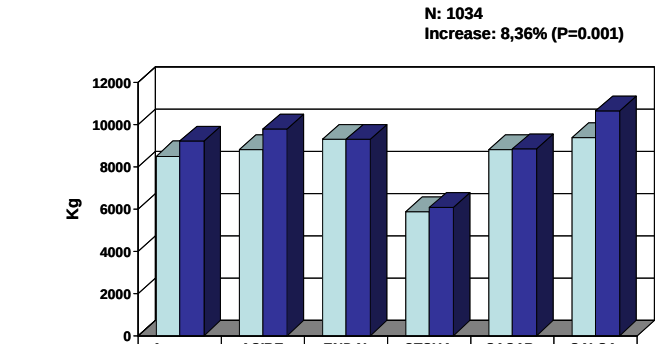


Fig. 5. Reduction in the level of excretion

Milk production was increased by an average 709 kg per cow after vaccination, but varied greatly according to number of lactation and farm. Given the trial design it cannot solely attributed to paratuberculosis control achieved with the vaccine (Fig 6).



	Average	AGIRE	ENDAI	OTSUA	SAGAR	SALSA
Pre-vaccination	8489	8791	9279	5845	8791	9369
Post-vaccination	9199	9784	9285	6050	8854	10649
Difference	710	993	6	205	63	1280

Fig 6. Milk production in vaccinated herds

DISCUSSION

The results observed in this preliminary study in tuberculosis free herds indicate that vaccination in cattle is a useful tool for the control of paratuberculosis. The possible interference with the tuberculosis eradication programs is solved using the comparative intradermal test. Vaccination in commercial farms has brought back to normal the replacement rate and has probably increased the overall milk production per cow without relevant adverse effects in clinical terms. In the period that we have been working with these herds the reduction of shedders has been faster and cheaper in vaccinated herds than in controls. These results are clearly visible to the farmers that feel very satisfied with the program and enthusiastically collaborate in the research aspects of this program.

CONCLUSIONS

There was no relevant clinical effect caused by vaccination. Vaccinations nodules were visible in 1.6% of the vaccinated animals one year after vaccination.

Comparative TB IDT interference was observed only in 1 animal, where there is pending of confirmation. Avian reactivity reached about 50% and slowly decreased. The results of ELISA seemed to decrease by one year after vaccination.

The milk production apparently increased by on average of 710 kg per cow and lactation. The culling rate of young cows apparently decreased.

There is still a need to complete the five year follow-up, but new farmers are currently asking to join the program.

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Paratuberculosis vaccination in cattle: Culling follow-up

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ABSTRACT

Eight dairy herds under study (six vaccinated and two unvaccinated control herds) were followed up and samples of 15% of the fallen stock and slaughtered in abattoir animals were collected. Immunological, microbiological and histopathological studies were carried out.

Two positive blood cultures (5.4%) and one positive muscle culture (2.5%) were obtained from three animals with clinical symptoms of paratuberculosis. These results suggest a significant role of bacteraemia in the spread of Map in infected animals. These findings should be considered in future studies on the pathogenesis of paratuberculosis and on human exposure to Map.

INTRODUCTION

The relationship between paratuberculosis and Crohn's disease has been discussed along the time and there are different studies which link both diseases (Mishina et al., 1996; Naser et al., 2000a; Naser et al., 2000b; Greenstein, 2003; Bull et al., 2003). Although the main way of introduction of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in the human food chain seems to be milk (Grant et al., 2002; Ayele et al., 2005; Ellingson et al., 2005), dairy products (Ikonomopoulos et al., 2005) and water (Mishina et al., 1996; Pickup et al., 2005), meat from infected livestock can also play a significant role as a possible source of infection. In Crohn's disease Map bacteraemia has been described (Naser et al., 2004), but this fact has not received enough attention in the study of paratuberculosis. In this way, we will present our results proving the bacteraemia in Johne's disease and the presence of viable Map in muscle.

MATERIALS AND METHODS

Animals in study

Six dairy herds (five Holstein Friesian and one Jersey herd) were vaccinated. One ml of Silirum® (C.Z.V.) was administered to the whole herd and later to the replacers. Two other Holstein Friesian herds were left as unvaccinated controls with detection and culling of positive animals.

A 15% of the culled animals of every herd were followed up. These included fallen stock and slaughtered in abattoir animals from which samples of faeces, gut tissues (ileocaecal valve, proximal ileum, medium ileum, distal ileum, proximal jejunum, medium jejunum and distal jejunum), mesenteric lymph nodes (ileocaecal L.N. and mesenteric caudal jejunal L.N.), blood, milk and muscle were collected. Blood, milk and muscle samples were not obtained from every animal. Up to now 32 cows from vaccinated herds and 16 from control herds have been analyzed.

Study of the immune response

The evolution of the humoral immune response was studied by a commercial ELISA (Pourquier).

Microbiological study

Samples of faeces, gut, lymph nodes, blood and muscle were processed as described previously (Juste et al., 1991) and cultured in Herrold's Egg Yolk medium with mycobactin J and in Lowënstein-Jensen medium with micobactin J for 20 weeks. Positive isolates were confirmed by Ziehl-Neelsen staining and by IS*900* PCR.

Histopathological study

Samples of gut and lymph nodes were processed by standard techniques and stained with haematoxylin-eosin and Ziehl-Neelsen methods. The classification of histopathological lesions was performed according to the previous classification of González et al. (2005).

RESULTS

The evolution of the humoral immune response is presented in Table 1. An increase in the proportion of positive results to ELISA was observed in vaccinated animals as well as in controls, probably due to the effect of the vaccine in the first ones and due to the progression of the disease toward advanced cases in the last ones. The mean time from vaccination to culling was 265 days (49-587days) and in

this period the control group suffered an increase in the frequency of ELISA positive results of 24%. In contrast, the vaccinated group had an increase of 76%.

Table 1. Evolution of the humoral immune response

	Controls		Vaccinated	
Group	**Initial sampling (N) %**	**Slaughter (N) %**	**Initial sampling (N) %**	**Slaughter (N) %**
ELISA positive	(9) 64%	(12) 80%	(9) 32%	(13) 57%
ELISA negative	(5) 36%	(3) 20%	(19) 68%	(10) 43%
	(14)	(15)	(28)	(23)[1]

[1] Blood samples could not be obtained from fallen stock

The proportion of animals culled /died due to paratuberculosis was 50% lower in the vaccinated compared to the control group (Table 2). Furthermore, 5 of the 6 vaccinated animals culled with clinical symptoms of paratuberculosis were Jersey cows from a severely affected herd that probably were in an advanced phase of the disease in the moment of vaccination. If this comparison is restricted to the Holstein breed, the proportion of vaccinated animals culled / dead with clinical symptoms of paratuberculosis was reduced an 85% in vaccinated animals.

Table 2. Reasons for culling / death

Reason	**Vaccinated (N) %**	**Vaccinated Holstein (N) %**	**Controls (N) %**
PTB	(6) 19%	(1) 6%	(6) 38%
Low production	(4) 13%	(4) 22%	(1) 6%
Unknown	(2) 6%	(0) 0%	(7) 44%
Others	(20) 63%	(13) 72%	(2) 13%
Total	(32)	(18)	(16)

Histopathological findings of the lesions in culled / dead animals are summarised in Table 3. The more severe and with heavier bacterial load forms, that is diffuse intermediate and diffuse multibacillary forms, were reduced 81% and 44%, respectively, in the vaccinated group. If this comparison was restricted to the Holstein breed, the proportion of diffuse intermediate and diffuse multibacillary forms were reduced 82% and 100% in vaccinated animals, respectively

Table 3. Histopathological lesions

Lesion	**Vaccinated (N) %**	**Controls (N) %**
No PTB	(18) 56%	(1) 11%
Focal	(2) 6%	(2) 22%
Multifocal	(3) 9%	(1) 11%
Diffuse paucibacillary	(2) 6%	(0) 0%
Diffuse intermediate	(2) 6%	(3) 33%
Diffuse multibacillary	(4) 13%	(2) 22%
Autolytic	(1) 3%	(0) 0%
Total	(32)	(9)

Table 4. Evolution of the bacterial load by culture

	Vaccinated			Controls		
Sample	**Pos**	**N**	**% Pos**	**Pos**	**N**	**% Pos**
Faeces	7	31	22.58	6	9	66.67
ICV	7	12	58.33	0	0	0.00
Lymph nodes	16	31	51.61	8	9	88.89
Ileum	14	31	45.16	8	9	88.89
Jejunum	10	31	32.26	7	9	77.78
Intestinal tissues	47	105	44.76	23	27	85.19

Table 4 shows the frequency of infection as determined by culture of faeces and tissue samples. A reduction of 66% (92% in Holstein breed) in the frequency of positive faecal cultures was observed when comparing controls and vaccinated animals. The reduction of the frequency of infection detected in intestinal tissues was slightly lower: 47% (68% in the Holstein breed).

Culture of muscle and blood samples (Table 5) yielded a total of 3 Map isolates (one isolate in muscle of a Jersey cow and two isolates in blood of Holstein cows). That is 2.5% of the cultured muscle samples and 5.6% of the cultured blood samples showed viable Map.

Table 5. Bacterial load in muscle and blood

	Vaccinated			Controls			All		
Sample	**Pos**	**N**	**% Pos**	**Pos**	**N**	**% Pos**	**Pos**	**N**	**% Pos**
Muscle	1	25	4.0%	0	15	0.0%	1	40	2.5%
Blood	1	21	4.8%	1	15	6.7%	2	36	5.6%

DISCUSSION

The reduction of the clinical symptoms in the vaccinated herds points out a reduction of the culling / death rate due to paratuberculosis after vaccination.

On the other hand, the predominance of focal / multifocal type lesions with low bacterial load in vaccinated animals points out a reduction of the faecal shedding of Map as other studies have shown before (Körmendy, 1994; Windsor et al., 2002; Reddacliff et al., 2006). Therefore, vaccination of a herd can achieve a reduction of the bacterial load in the environment that could lead to the control of the disease in the herd.

The detection of viable Map in blood and muscle could have important consequences in public health, since today all the attention has been put on milk and dairy products as source of infection for man.

CONCLUSIONS

The different techniques yielded consistent results and provided values that allowed the quantification of the protective effect of the vaccine.

Vaccination of the whole herd showed a clear favourable effect on the vaccinated animals.

The finding of viable Map in blood and muscle would point out an underestimation of the role of bacteraemia in the pathogenesis of paratuberculosis. In human Crohn's disease presence of viable Map in blood has been demonstrated (Naser et al., 2004). These findings should be considered in future studies on the pathogenesis of paratuberculosis and on human exposure to Map.

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New cases of Johne's disease and Map infection in two dairy herds from the States of Pernambuco and Rio de Janeiro, Brazil

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INTRODUCTION

Bovine paratuberculosis or Johne's disease is a chronic enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), leading to malabsorption, cachexy and death. It is not a new disease in Brazil. Otávio Dupont, a Belgian but Brazilian naturalized veterinarian, was the first one to report this disease among Flemish cattle imported into Rio de Janeiro in 1915. After that, there were other reports in the states of Rio de Janeiro, Santa Catarina, and Rio Grande do Sul between the 1950s and 1980s, by pathologists who regarded it as an exotic or sporadic disease associated with the import of infected animals (Gomes, 2002).

In the late 1990s, new cases arose and Map was isolated from herds in the Brazilian states of Minas Gerais, Paraíba, Rio Grande do Sul (Gomes, 2002), Pernambuco and Rio de Janeiro, being regarded as a mycobacteriosis that could quickly deteriorate the health of cows and compromise the production of milk and derivatives. Collins (1997) stated that Map infection can be transmitted by the milk and that it may be hazardous to the health of consumers.

Epidemiological studies were carried out to estimate the prevalence of Map infection in herds from the states of Mato Grosso do Sul and São Paulo, São Paulo, Rio Grande do Sul (Gomes, 2002; Gomes et al., 2005; Gomes et al., 2007), Rio de Janeiro, Pará and Minas Gerais (personal communication). Most serological surveys revealed the presence of infection in virtually all of the tested herds. However, most of these estimates have been overestimated, since in countries affected by bovine tuberculosis, *M. bovis* or environmental mycobacteria can interfere with the serological tests used for the diagnosis of bovine paratuberculosis, mainly with ELISA.

The aim of this case report was to provide a clinical and pathoanatomical description of Johne's disease, based on Map isolation and on the estimated prevalence of infection in two infected dairy herds from the Brazilian states of Pernambuco and Rio de Janeiro.

MATERIAL AND METHODS

Cases in Rio de Janeiro and Pernambuco

Four cows were autopsied in Rio de Janeiro (RJ) and one in Pernambuco (PE). Fragments of lymph nodes, small intestine, large intestine, and of several other organs, were collected; the samples were fixed in 10% formaldehyde buffer and processed using conventional methods for histopathological analysis. Sections from the intestines and from mesenteric lymph nodes were stained with Ziehl-Neelsen. Stool, tissue and serum samples from the RJ and PE herds were sent to the Laboratory of Bacteriology of the School of Veterinary Medicine of Universidade Federal do Rio Grande do Sul for detection of antibodies against Map.

Sample treatment and culture

Eight stool samples and terminal ileum samples (1 to 2 g) from the PE herd and 85 stool samples and one tissue sample from the RJ herd were treated and processed according to the modified classic Cornell technique, as described in Stabel (1997).

The samples were inoculated into four tubes containing Herrold's egg yolk medium (HEYM), two with mycobactin and the other two without it. The cultured samples were kept in a stove at 37ºC and were checked fortnightly for 5 months.

The following criteria were used for Map identification: time of microbial growth, morphology, and mycobactin dependence (Manning & Collins, 2001). The isolated colonies were submitted to polymerase chain reaction (PCR) for amplification of insertion sequence IS*900* specific to the colony with suspected Map and for confirmation of the species, as described in Ayele et al. (2005).

Herd ELISA

A total of 170 serum samples were collected from the RJ herd and 130 from the PE herd. Commercially available indirect ELISA (Allied Monitor, Fayette, MI, USA) with protoplasmic antigen (PPA-3) was used for the identification of antibodies against Map. The immunoassay was carried out according to the protocol supplied by the antigen manufacturer (Allied Monitor, Fayette, MI, USA).

RESULTS AND DISCUSSION

The RJ herd included 240 Girolanda cows, 131 of which were older than 3 years. The owner reported the disease among adult cows only, in both lactating and dry cows, which was characterized by intermittent chronic watery diarrhea, initially olive green and then blackish in color, often passed profusely, causing progressive emaciation, partial or total reduction in milk production and enlargement of superficial lymph nodes. The disease progressed within weeks or months, always leading to death. Between 2005 and 2007, nine cows showed clinical signs of the disease and eventually died. On the farm, there had been frequent cases of chronic and recurrent mastitis, reduction in milk production, and reproductive disorders. The herd had a previous history of bovine tuberculosis.

The first cases of the disease were observed in September 2005. The herd belonged to the Zona da Mata region and comprised 250 lactating or dry cows, five of which were aged 4 to 5 years and presented with symptoms that suggested the disease. The owner stated that the animals had chronic and profuse diarrhea characterized by watery consistency and a blackish color and that was refractory to antimicrobial therapy. Besides diarrhea, other symptoms (signs) included emaciation with muscular atrophy (despite adequate nutrition) and reduction in milk production. The herd also had a previous history of tuberculosis.

The five autopsied animals (4 from the RJ herd and 1 from the PE herd) showed fair to poor nutritional status; the serosa of small intestine had a cerebroid appearance, with thick and corrugated membranes and irregular reddish areas, with some foci of whitish dots on the corrugated surface. The mesenteric and ileocecal lymph nodes were enlarged.

In both herds (RJ and PE), there was pronounced diffuse, granulomatous inflammatory infiltrate in the small intestine, from the duodenum to the large intestine, and in the mesenteric lymph nodes. The infiltrate was constituted chiefly of macrophages, lymphocytes, plasma cells, eosinophils, epithelioid cells and several Langhans giant cells. The villi, which were collapsed especially at the top, showed remarkably dilated lymphatic vessels, some of which were filled with exfoliated inflammatory cells and mucus. Ziehl-Neelsen staining revealed a large amount of acid-fast bacilli within macrophages and within Langhans giant cells, which were lying free in the interstitium of the mucosa and submucosa of the small intestine and in the mesenteric lymph nodes.

The clinical findings of chronic diarrhea in adult cows associated with weakness and reduction in milk and beef production have been frequently described in bovine paratuberculosis.

The HEYM allowed isolating two positive samples for *Mycobacterium avium* subsp. *paratuberculosis* and three suspected ones among 86 stool and tissue samples from the RJ herd and four positive samples and three suspected ones from the PE herd.

Of the five samples isolated from the RJ herd and the four samples isolated from the PE herd, only 2 and 4 amplified the IS*900* genetic sequence of Map, respectively.

Out of 170 samples from the PE herd submitted to indirect ELISA, 55 (32.3%) were positive and 20 were suspected of Map (11.7%). Of 130 samples from the RJ herd, 57 (43.8%) were positive and 19 (18.4%) suspected. Map was isolated in several Brazilian dairy herds, mainly in the states of Rio de Janeiro, Paraíba, Minas Gerais, Santa Catarina and Rio

Grande do Sul; however, this is the first time that classic Johne's disease is identified in a dairy herd in the state of Pernambuco and that Map is isolated from animals with clinical signs and from subclinically infected animals in the same herd with prevalence of infection estimated by indirect ELISA. The estimated infection rate is high; however, one must consider the possible interference of antibodies against other mycobacteria, especially against *M. bovis*, since both herds have a past history of tuberculosis.

CONCLUSIONS

Map was isolated and identified by culture and PCR reaction in dairy cows with clinical signs of paratuberculosis in Pernambuco and Rio de Janeiro, and antibodies against the causative agent were found in a large proportion of subclinically infected cattle.

The prevalence rate obtained by ELISA was high compared with international data. This suggests that further studies are necessary so that control programs can be implemented for dairy herds on a nationwide basis. The estimated infection rate is high and worrying since we have a previous history of tuberculosis and there is no program for protecting our cattle herd from paratuberculosis and from milk-borne diseases.

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Note from the editor: This paper was presented only as an abstract at the 9ICP. It was not presented as a poster or an oral presentation. Therefore, the 9ICP delegates had no possibility of discussing the study.

Effects of infection by *Mycobacterium avium paratuberculosis* (*Map*) on fertility of dairy cows

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ABSTRACT

This study aimed at quantifying the variation in fertility of dairy cows according to their *Map*-infection status. The hypothesis of an indirect effect of the infection on fertility was set. Fertility was measured by the non-return rate at first and second services. A non-return was defined as the absence of another artificial insemination (AI) after the first one while the cow was still present. Three different statuses were defined based on both individual and herd test results: positive cow, negative cow in a negative herd and negative cow in a positive herd. 237,612 AI from 72,135 cows in 1,470 herds were studied by logistic regression after adjusting on known factors influencing reproduction. Non-return rate was higher for infected cows compared to negative cows from negative herds (OR of 1.14, or +3.2 point of % of non-return rate). This increase was higher for parity 1 cows (OR of 1.20, or +4.4 point of % of non-return rate) than for other parities. The effects were lower when comparing positive cows to negative cows in the positive herds. Looking at these observations, the hypothesis of *Map*-effect based on the relation between *Map*-infection, production and reproduction is formulated. Due to the lack of protein absorption in the intestine, the milk production is reduced. In the early stages of the infection, this could lead to a lower negative energy balance that could be associated with improved fertility.

INTRODUCTION

In affected herds, Johne's disease can lead to reduced milk yield, lower slaughter value of clinically infected cows and mortality or premature culling of sick animals. A precise appraisal of the production losses attributable to the infection is needed to evaluate the economic losses in dairy herds. Estimates of reduced milk yield associated with *Map*-infection are abundant (Beaudeau et al., 2007; Gonda et al., 2007; Johnson et al., 2001; Kudahl et al., 2004) with a decrease of mean milk yield estimated between 500 and 1400 kg per cow in the lactation when the infection is detected. A decrease is sometimes noticed as soon as the first lactation (Nielsen et al., 2006). Conversely, there are only few studies analysing the effect of *Map*-infection on fertility and their results are contradictory (Haddad et al., 2003; Johnson-Ifearulundu et al., 2000; Kostoulas et al., 2006). It is all the more difficult to estimate the effect of the infection that the diagnostic of the disease is not easy: first of all because of the low sensitivity of the tests but also because even if the infection occurs early, the diagnostic is always postponed in time.

The objective of this study was to assess if there is an effect of *Map* infection on fertility and more precisely to quantify the difference of fertility between infected and non-infected dairy cows. Since the infection develops with age, we studied the effect by parity in order to see if there is an effect at early stage of infection.

MATERIAL AND METHODS

A retrospective study was performed to compare individual reproductive performance of *Map*-infected or non-infected dairy cows. The data were obtained between January 1, 1999 and February 28, 2007 from herds located in western France that were monitored for *Map*-infection, enrolled in the official Milk Recording scheme and used artificial insemination (AI).

Assessment of fertility

Fertility was assessed by the outcome variable « non-return-to-service » opposite to « return-to-service ». A non-return was defined as the absence of another artificial insemination after a given service while the cow was still present in the herd. Only returns after first and second

services were taken into account. A new insemination after a third service or more is indeed dependent upon the farmer decision in France because in the region of the study, farmer generally has to pay for a fourth service whereas he does not for the second or third.

Map infection status

Map-status can be defined at individual and herd levels based on individual tests. At the herd level, a herd was considered positive as soon as one test was positive. A herd was considered negative when all the tests were negative. At the individual level, a positive cow was a cow with at least one positive test and a negative cow had all its tests negative independently of the type and number of tests performed (ELISA on serum, PCR, Faecal culture, Ziehl-Neelsen staining). Finally, cows were classified into 3 groups: positive cows, negative cows in positive herds and negative cows in negative herds.

Selection of data

Heifers were excluded because their fertility differs from that of cows. In order to limit classification bias for infection, cows vaccinated against paratuberculosis were excluded. Several exclusions were done in order to get rid of cows or lactations with missing information or to get rid of special cases. We also chose to exclude cows culled early after an insemination (less than 200 days after an AI) in order to limit the risk of misclassifying a cow as non-return when she is culled early without knowing if she is pregnant or not (assuming that when kept more than 200 days and in absence of AI, cows were likely to be pregnant). Another reason to exclude cows that were culled less than 200 days following AI is because farmers in France must cull positive cattle quickly following a positive test result if they wish to receive some compensation for her. Our final data set was composed of 237,612 AI from 72,825 cows in 1,472 herds.

Modelling

The effect of the *Map*-infection cow status on fertility was studied by logistic regression after adjusting on several independent variables described as risk factors for fertility traits in the literature. The confounding factors taken into account were calving-to-service interval, rank of service within lactation, month and year of service, lactation number, herd, inseminator and bull. Diseases, feeding and reproduction management were not recorded and were assumed to be taken into account in the herd effect.

Two models were run. Positive cows were first compared to negative cows in negative herds and then to negative cows in positive herds.

For each variable, and each class, the logistic regression model provides odds-ratio (OR) adjusted for other explanatory variables. ORs were converted into relative risks (RR) using Beaudeau and Fourichon's method (Beaudeau and Fourichon, 1998) and the effects in % of non-return rate were calculated from RRs estimates.

RESULTS

The results reported here are the ones for the model excluding early culling and adjusting for milk yield.

Whichever the parity-group, *Map* positive status was significantly ($p<0.05$) associated with non-return-to first and second services (Fig. 1). Non-return rate was significantly increased in positive-testing cows (OR = 1.14 or +3.2 point of % of non-return rate) compared to negative cows from negative herds. A similar trend of lower magnitude was found when comparing positive cows to negative cows from positive herds (OR = 1.11).

For positive cows in first lactation compared to negative cows from negative herds, the effect was greater in first lactation and tended to decrease with each successive lactation (OR of 1.20; 1.09 and 1.05 or +4.4; +2.1 and +1.2 point of % for lactation 1, 2 and 3 and more respectively). When comparing cows in positive herds, the ORs were always lower (even if above 1) than when comparing positive cows to negative cows of negative herds. The association between *Map* status and non-return-to-service was not significant for positive cows in 3rd lactation or more compared to negative cows of the same herds.

All the adjustment factors had an effect on non-return-to-service as described in literature. Results were comparable for all parities.

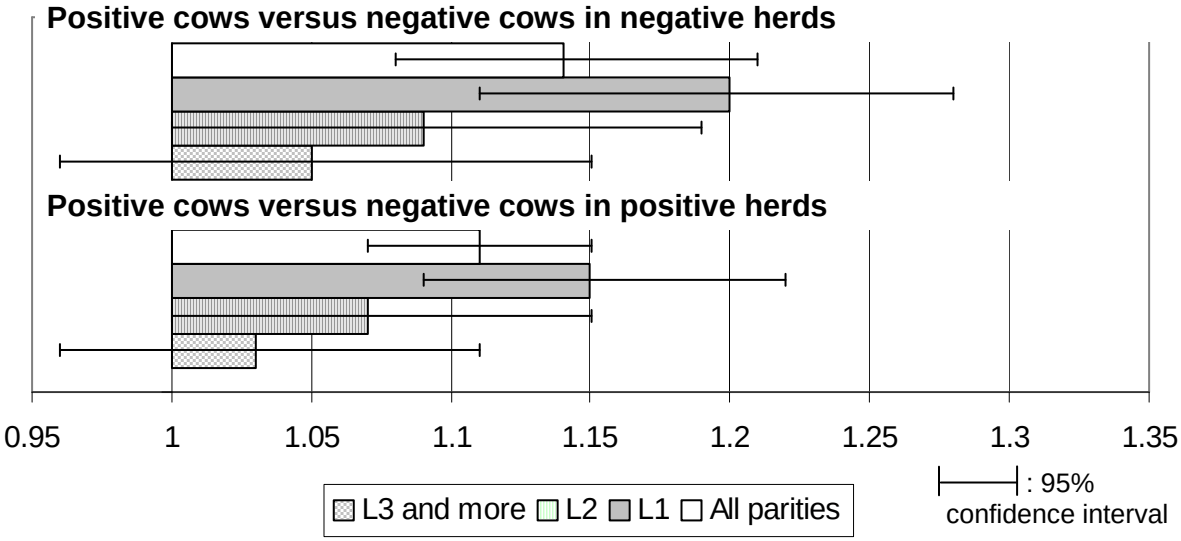

Fig. 1. Odds ratio of non-return to service according to the *Map*-infection status of cows (by parity) for the model excluding early culling and adjusting for milk yield

DISCUSSION AND CONCLUSION

Map positive status as here defined was associated with a higher non-return rate after first and second services. The effect of *Map* positive status on fertility varied with age. The non-return rate was higher for young cows and decreased for older cows, suggesting that the effect of infection which results in improved fertility starts early. These results were not expected, as fertility barely improves when concurrent disease happens for diseases frequently investigated.

The effect was lower, but still positive, when comparing positive cows to negative cows of the same herds. This is consistent with the assumption that the bias of misclassifying a cow as negative is higher in a positive herd. In order to prevent the selection bias from the farmers, all the AI were taken into account as soon as the first lactation, before the *Map*-infection status was known.

Milk production is known to influence the fertility of cows. A relation between production and reproduction functions could participate in the biological mechanism relating paratuberculosis to fertility, as both functions depend on the nutrition of cows. *Map* infection influences milk production and milk production influences reproduction. Including simultaneously the two variables could drive to over-adjustment. The model was therefore studied with and without the milk production variable as a confounding factor. The effect was higher when not adjusting on milk production (results not shown).

Hypotheses on possible biological mechanisms underlying the positive effect of *Map* infection on fertility are made. Present knowledge on *Map* does not support a direct effect of the infection on the reproductive tract or on embryo or foetus viability. Tropism for the reproductive tract, or lesions in calves born from infected dams are indeed not reported. A likely possible biological mechanism connecting infection to reproduction could be indirect and bring into play the effect of *Map*-infection, production and reproduction. At early stage of the disease, *Map*-infection causes lesions on the intestine where proteins absorption takes place. The infection could then provoke a decrease of protein absorption (Patterson and Barett, 1968) that could lead to a decrease of milk yield particularly in first lactation. This decrease in yield is associated with a fall of energy use bringing to a reduction of the negative energy balance (normally increasing in early lactation). This lower negative energy balance could then possibly be associated with a better fertility, particularly in first lactation.

The link between *Map*-infection, milk yield and fertility could evolve with animal age since paratuberculosis develops with time. In young animals, intestinal lesions are small. Energy absorption is likely not influenced at first by *Map* infection since energy absorption takes place in the rumen while *Map* lesions are on the intestine. Furthermore, feed intake seems not to be affected by *Map* infection at least in early stages of the disease contrary to other health disorders. As infection get in a more advanced stage, general status could be affected because of the greater decrease of nutrient absorption. Finally, acute granulomatous enteritis and thickening of intestinal mucous lead to a significant digestive dysfunction. In the long term, significant decrease of nutrient absorption and concurrent diseases could then impair fertility.

The study was mainly performed on herds involved in control programs. The majority of the cows were then subclinically infected and generally culled before symptoms appear. This could explain why fertility was always improved in positive cows of the present study.
This study demonstrates that *Map* infection may have an effect on fertility in dairy herds varying with age, which could occur in the absence of any clinical sign attributable to paratuberculosis. It seems now important to study the presence of a potential link between infection and negative energy balance in order to validate the proposed hypothesis.

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

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INTRODUCTION

India has highest population of cattle (185 millions) in the world. Cows contribute 44.0% to total milk production in the country. However, per animal productivity is much below Asian and world averages. Due to ban on cow slaughter number of low and un-productive cows is increasing day by day, which has negative impact on their popularity as dairy animals. Their number has reduced by 0.5 million in last 2 years (185.5 million in 2004). Cows in India are valued as 'Holy' due to medicinal attributes in their milk, urine and dung. *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the cause of Johne's disease (JD) is primarily responsible for low productivity. It is estimated that nearly 40.0% of US dairy herds are infected with MAP and losses to dairy industry exceed 1.5 billion/year (Wells and Wagner, 2000). Information on production losses and prevalence of JD in cows at National level has not been estimated. This is mainly due to low priority accorded to JD and lack of indigenous diagnostic kits. However, limited information is available on prevalence of MAP in dairy cows in India (Singh et al., 2007a; Bapat and Bangi, 1985; Nain et al., 1985; Kulshreshtha et al., 1980) using traditional tests (johnin, postmortem, rectal pinch).

This study aimed to estimate the prevalence of Johne's disease (MAP infection) in dairy cows and evaluate 'Indigenous goat based ELISA kit' for the diagnosis of bovine Johne's disease (BJD) in cows belonging to diary herds (government and private). The ELISA kit, originally developed for the screening of goats was validated in cows with respect to fecal culture. Specific IS*900* PCR was used to characterize MAP colonies.

MATERIALS AND METHODS

Dairy herds

Lactating cows from private dairy farms (Krishna Balram Goshala, Malwiya Goshala, Panchayati Goshala), government district dairy demonstration farm (DDD Farm, veterinary college, Mathura) and cows reported for treatment in the clinics of veterinary college, Mathura were screened. Condition of cows was poor and showed clinical symptoms of JD (weakness, low milk yield, body weights decreasing, skin rough and poor in body condition, feces loose and semi-solid, frequent diarrheal episodes etc.).

Samples

Fecal and serum samples were collected from 120 cows from dairy farms. Further 104 serum samples were collected from these dairies.

Processing of fecal samples

Fecal smears were stained by Ziehl-Neelsen (ZN). For isolation of MAP, the method of Singh et al. (1996) was followed. Colonies were identified on the basis of cultural characteristics, slow growth, mycobactin J dependency, acid fastness and bacterial morphology.

Processing of serum samples

'Indigenous ELISA kit', initially developed for goats (Singh et al., 2007) was standardized in bovines (Yadav et al., 2007) and used. OD values were transformed to S/P ratio. Cows in strong positive category were considered positive for JD.

DNA isolation and IS900 PCR

DNA from colonies was isolated by method of Singh et al. (2007b). DNA was amplified by PCR using specific IS*900* primers. Presence and yield of specific PCR product (229 bp) was analyzed by 1.7% agarose ethidium bromide gel electrophoresis.

RESULTS

Of the 537 cows available, 120 (33.9%) were sampled from 3 dairy farms. Prevalences of MAP were 28.3 and 20.8% by fecal culture and serum ELISA kit, respectively. Herd-wise, 20, 30 and 29% cows were positive in fecal culture, and 30, 50 and 17% in ELISA kit from Krishna Balram Gosala, Malwiya Gosala and DDD Farm Mathura, respectively. Fecal examination of 120 cows by direct microscopy (DM) showed that 30.8% cows were positive. The 14.1% cows were positive in both tests, however, 14.1 and 6.6% cows were detected positive exclusively in culture and kit, respectively (Table 1). Two tests showed 79.1% agreement (positives+negatives). Kappa statistics when applied to estimate agreement between culture and kit there was nearly perfect agreement (proportion agreement = 0.79, kappa value = 0.442). 'Indigenous goat based ELISA kit' had 50.0% sensitivity and 90.6% specificity with respect to fecal culture. MAP colonies were characterized using specific IS*900* PCR. Of the, 34 (28.3%) cultures screened, 29 (85.2%) were positive for MAP by IS900 PCR. Of the 224 serum samples of cows from 5 dairy herds, 23.6% were positive for MAP antibodies by ELISA kit. In S/P ratio, 6.6, 3.5, 5.3, 60.7 and 23.6% cows were in negative, suspected, low positive, positive and strong positive categories, respectively with regard to their JD status (Table 2). Herd-wise, 36.3, 35.0, 16.0, 29.0 and 33.3% cows were positive from Krishna Balram Goshala, Malwiya Goshala, DDD Farm Mathura, Panchayati Goshala and Kothari Veterinary Hospital, respectively (Table 3).

Table1. Comparative detection of MAP in cows, by fecal culture and ELISA kit

Tests	**Combinations**			
	1	**2**	**3**	**4**
Fecal Culture	+	-	+	-
Serum ELISA	+	-	-	+
Total – 120 (Percent)	17 (14)	78 (65)	17 (14)	8 (6.6)

Table 2. Johne's disease status of cows by indigenous goat based ELISA kit (S/P ratio)

Disease status	**S/P Ratio**	**Number of cows (%)**
Negative	0.00 - 0.09	15 (06.6)
Suspect	0.10 - 0.24	8 (03.5)
Low Positive	0.25 -0.39	12 (05.3)
Positive	0.40 - 0.99	136 (60.7)
Strong Positive	1.0 - 10.0	53 (23.6)
Total		224

Table 3. Screening of dairy cow herds for MAP infection using goat based ELISA kit

Herds	**Cows (number)**	**ELISA kit (% positives)**
Krishna Balram Goshala	22	8 (36.3)
Malwiya Goshala	20	7 (35.0)
Panchayati Goshala	27	8 (29.6)
DDD Farm	125	20 (16.0)
Kotharai Veterinary Hospital	30	10 (33.3)
Total	224	53 (23.6)

Sensitivity – 50.0% and Specificity – 90.6%

DISCUSSION

Of the 537 cows available (3 private dairy farms, 1 government and rest individual cows) in the Mathura city of South UP (India), revealed moderately high prevalence of JD using fecal culture and indigenous ELISA kit. Fecal culture was more sensitive than indigenous goat based ELISA kit adapted to screen cattle. However, other workers (Bapat and Bangi, 1985, Nain et al., 1985, Kulshreshtha et al., 1980) reported low prevalence of JD in cattle using

traditional tests (Johnin, necropsy and microscopic examination). Kumar et al. (2007) reported very high sensitivity of fecal culture in goats over this ELISA kit. This may be due to poor condition of the animals and severe damage to the lymphoid organs (lymph nodes and Peyer's patches) by the MAP. Disease has been reported to be endemic in ruminant population of this region (Singh et al., 1996; Yadav et al., 2007; Sharma et al., 2007; Kumar et al., 2007; Singh et al., 2007b and c) using these sensitive tests (ELISA kit, fecal culture and PCR). Using fecal culture only moderate prevalence of JD was recorded, though cows were poor in condition and showed advance clinical signs of the JD. However in other studies using milk culture in the same herd (DDDF, Mathura) Sharma et al., (2007) reported very high prevalence of JD. This again indicated the high damage to intestinal tissues of these cows, therefore at the time of sampling cows were probably in very advance stages of the disease. Culture of milk yielded high prevalence of MAP in dairy cattle from Agra region (Sharma et al., 2007). They further reported that using milk culture and milk ELISA prevalence was higher in government diary as compared to private dairies. In this study comparatively in ELISA kit the sero-prevalence was higher in private dairies as compared to government dairy, which again indicated severe damage to lymphoid organs by the bacilli. In different dairy herds the prevalence of JD was moderate using fecal culture and showed less variation between herds. But using ELISA test (serum), these variations in prevalence were more. This may be due to less number of samples used for comparison between fecal culture and serum ELISA test. Fecal culture of feces was superior test and has been considered 'Gold standard' in JD. However, indigenous ELISA kit (using antigen of goat origin) could be employed as 'herd screening test to screen dairy cows against Johne's disease in India. This may be probably due to sharing of unique MAP 'Bison type' genotype by domestic livestock in India (Sevilla et al., 2005). The ELISA kit was never over sensitive as compared to culture (feel in this case and milk Sharma et al., 2007). Using Kappa statistics there was nearly perfect agreement (proportion agreement = 0.79, kappa value = 0.442) between culture and kit. 'Indigenous goat based ELISA kit' had 50.0% sensitivity and 90.6% specificity with respect to fecal culture. The sensitivity and specificity were comparable to the commercial ELISA kits available globally. The indigenous kit was cost effective as compared to imported kits. Of the 224 serum samples of cows from 5 dairy herds, 23.6% were positive for MAP antibodies by ELISA kit. Similar moderate prevalence was reported in cattle population of Uttar Pradesh and Punjab (India) in randomly selected villages, by Singh et al. (2007a). In S/P ratio, 6.6, 3.5, 5.3, 60.7 and 23.6% cows were in negative, suspected, low positive, positive and strong positive categories, respectively with regard to their JD status (Table 2). Depending on the purpose the cows in positive categories could also be considered positive for JD. In larger number of serum samples ELIS it also gave uniform prevalence of JD between different dairies in Agra region. Herd-wise, 36.3, 35.0, 16.0, 29.0 and 33.3% cows were positive from Krishna Balram Goshala, Malwiya Goshala, DDD Farm Mathura, Panchayati Goshala and Kothari Veterinary Hospital, respectively (Table 3). MAP colonies from fecal culture were characterized using specific IS*900* PCR. Of 34 (28.3%) cultures screened, 29 (85.2%) were positive for MAP by PCR. Using a new method of DNA isolation > 90.0% colonies (single to few, minute and stunted colonies) could be specifically characterized as MAP by IS*900* PCR (Singh et al., 2007d).

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Development of "Colostrum Pasteurizer"

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BACKGROUND AND OBJECTIVE

Paratuberculosis, caused by *Mycobacterium paratuberculosis*, is an important chronic enteric disease of domestic cattle. The major routes of infection are the oral ingestion of contaminated manure, milk, and colostrum. Infection from contaminated colostrum is the most significant route. This route has three times higher rate of infection than from ordinary milk (Robert et al., 1995).

Prevention of infection via contaminated colostrum will play an important role towards controlling paratuberculosis. Development of colostrum pasteurization protocols which inactivates pathogens without affecting important the beneficial fractions of colostrums is the objective of the studies.

ESTABLISHMENT OF COLOSTRUM PASTEURIZATION

1. Incubating temperature

a) Physical property

Colostrum was placed in micro tubes and incubated at 56, 60 or 63°C, respectively for 30 minutes. After 30 minutes incubation in each temperature, the physical appearance of colostrum was examined visually. As shown in Fig.1, colostrum incubated at 63°C coagulated completely and was unable to be fed to newborn calves. Although incubation at 60°C slightly increased its viscosity, incubated colostrum can be fed to calves.

56°C 60°C 63°C

Fig.1. Physical property of colostrum after incubation at 56, 60 and 63°C for 30min

b) Inactivation of Pathogens

Two strains of *M. paratuberculosis*, ATCC19698 and field isolate were used to inoculate colostrum. Each inoculated each strain was incubated at 56°C and 60°C respectively, for 30 minutes. Samples were taken and inoculated onto Herrold's Egg Yolk Agar with Mycobactin to test the inactivation of *M. paratuberculosis*.

Incubation at 56°C could not inactivate *M. paratuberculosis* (Fig.2).

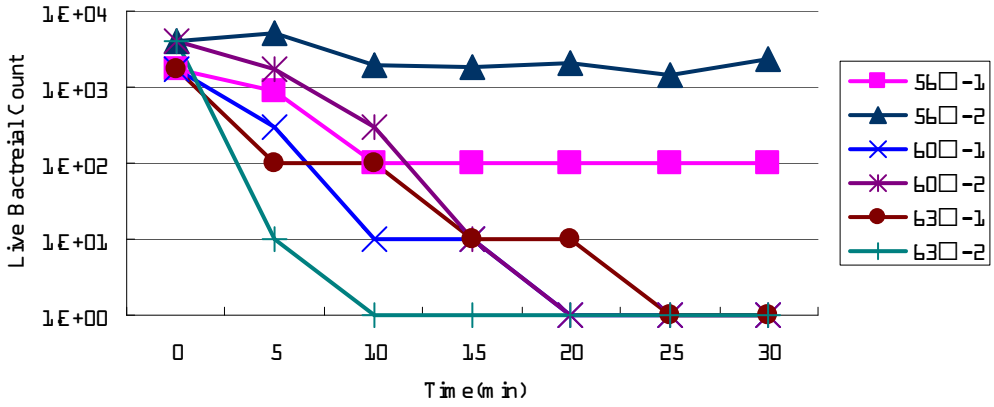

Fig. 2. Viable count of *M. paratuberculosis* in experimentally inoculated colostrum

Since *M. paratuberculosis* could not be inactivated at 56°C, inactivation test of other pathogens were conducted only at 60°C. Two of each field isolate of *Mycoplasma bovis* (MB), *Listeria monocytogenes* (LM) and *Salmonella* Typhimurium (ST) were used to be examined at 60°C for 30 min. incubation could inactivate them. As shown in Table 1, all of the pathogens tested, except one strain of *Listeria monocytogenes*, were inactivated with incubation at 60°C for 30 minutes. Two field isolates of Bovine Leukemia virus were inactivated by incubating at 56°C for five minutes (data not shown).

Table 1. Titer of inoculated pathogens during the incubation at 60°C

	Incubation time(min) at 60°C						
	0	**5**	**10**	**15**	**20**	**25**	**30**
MB #1	1x107	ND	ND	ND	ND	ND	ND
MB #2	1x107	ND	ND	ND	ND	ND	ND
LM #1	1x106	1x105	1x105	1x104	1x103	1x102	10
LM #2	1x106	1x105	1x102	1x102	1x102	ND	ND
ST #1	1x106	1x101	ND	ND	ND	ND	ND
ST #2	1x106	1x104	ND	ND	ND	ND	ND

ND: not detected

c) Affection for important beneficial fractions of colostrum. In order to test that the incubation at 60°C for 30 minutes will not affect any beneficial fractions of colostrum, several important fractions were tested. As shown in Table 2, all of the fractions were not changed before or after incubation.

Table 2. Concentration of beneficial fractions before and after incubation at 60°C for 30 min

	IgG(mg/ml)	**IgM(mg/ml)**	**IgA(mg/ml)**	**Lactoferrin(ug/ml)**	**Vit. A(ug/ml)**
Before incubation	66.0	6.9	7.4	650	7.86
After incubation	66.0	5.0	7.4	650	7.79

Other fractions such as total solids content, percentage of milk fat, total protein and lactose were not affected before or after treatment. Antibody titer against BCV, IBR, PI3, RS, BVD and Ad7 were unchanged before or after treatment (data not shown).

DEVELOPMENT OF "COLOSTRUM PASTEURIZER" DEVISE

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

Two colostrums, collected from different cows were pasteurized respectively using this prototype "Colostrum Pasteurizer" at one dairy farm. Pasteurized colostrums were tested to confirm inactivation of mastitis causing bacteria, such as coliform, *Streptococcus* spp. and *Staphylococcus* spp. and also they were tested to confirm that the process did not to affect important beneficial fractions of colostrum such as immunogloblin, lactoferrin, gamma GTP, vitamin A and Vitamin E.

As shown in Table 3, coliform, *Streptococcus* spp. and *Staphylococcus* spp. could not be cultured after pasteurization. Although the concentration of IgM decreased significantly very much, other fractions tested were decreased only slightly (Table 4).

These pasteurized colostrums were actually fed to newborn calves. Serum samples were taken from these calves before being fed the colostrum, and again at 7 and 24 hours after feeding. There was no gamma globulin fraction detected before feeding colostrum. At seven hours after feeding, it increased to 0.4 g/dl, and at 24 hours it increased to 2.4 g/dl,

which showed that the calves could absorb enough of the gamma globulin from colostrum (Table 5).

Table 3. Viable Bacterial Count before and after treatment

		Viable Bacterial Count	
		Before treatment	**After treatment**
Colostrum 1	Coliform	ND	ND
	Staphylococcus	1x103	ND
Colostrum 2	Coliform	1x101	ND
	Staphylococcus	1x102	ND
	Streptococcus	1x103	ND

ND: not detected

Table 4. Concentration of beneficial fractions before and after treatment (average of two colostrums)

	IgG (mg/ml)	**IgM(mg/ml)**	**IgA(mg/ml)**	**γ-GTP(IU/L)**	**Vit. A(ug/ml)**
Before incubation	46.8	2.5	5.9	20846	2.4
After incubation	45.5	0.45	5.4	20145	2.0

Table 5. Concentration of serum protein before and after feeding colostrum

	Alb(g/dl)	**α-glb(g/dl)**	**β-glb(g/dl)**	**γ-glb(g/dl)**
Before feeding	2.9	1.1	0.6	0.0
7 hours after feeding	3.0	1.3	0.7	0.4
24 hours after feeding	2.4	1.0	1.0	2.4

IgG, IgM, IgA concentration in the serum were also tested by single radial immunodiffusion. There were no detectable IgG, IgM and IgA in the calves' serum before feeding the colostrum. The concentration level of each fraction was raised by absorbing each fraction (Table 6). As shown in Table 7, concentration of total cholesterol, Vitamin A and gamma GTP were raised 24 hours after feeding the colostrum. Antibody titer against Bovine Adenovirus type 7, Bovine Parainfluenza Virus Type3 and Bovine coronavirus were raised considerably after 24 hours (Fig. 3).

Table 6. Concentration of serum protein before and after feeding colostrum

	IgG(mg/ml)	IgM(mg/ml)	IgA(mg/ml)
Before feeding	ND	ND	ND
7 hour after feeding	11.2	0.5	2.7
24 hour after feeding	28.8	1.8	3.7

ND: not detected

Table 7. Concentration of total cholesterol, vitamin A and γ-GTP before and after feeding colostrum

	T-cho(mg/dl)	**Vitamin A(mg/ml)**	**γ-GTP(IU/ml)**
Before feeding	18.3	6.8	9.7
7 hour after feeding	21.4	6.7	253.1
24 hour after feeding	34.1	15.4	781.8

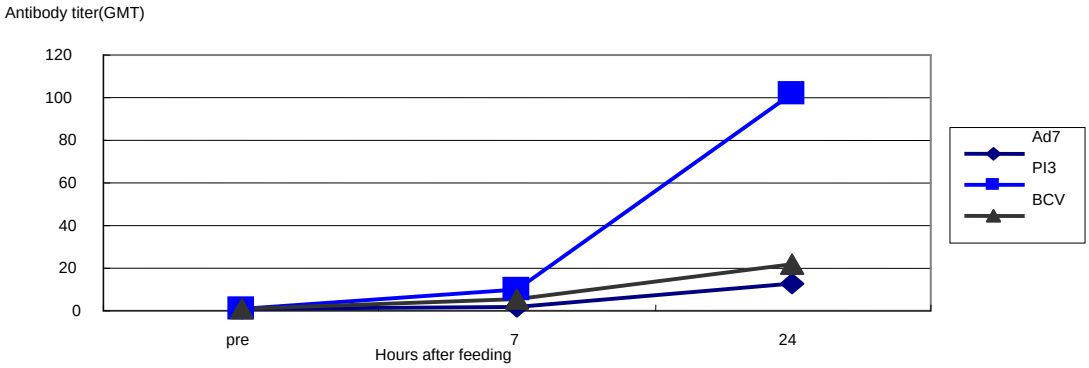

Fig. 3. Serum antibody titer before and after feeding colostrum(average of three calves)

USE OF "COLOSTRUM PASTEURIZER" ON DAIRY FARMS

Modifications to prototype were made and a commercial model has been marketed since November, 2005. At present, there has been no report of transmission of Paratuberculosis from colostrum treated by "Colostrum Pasteurizer". As shown in Table 8, field reports indicate a decrease in the frequency of diarrhea in calves fed pasteurized colostrum. These reports suggest the use of "Colostrum Pasteurizer" reduces incidences of diarrhea by deactivating diarrhea-causing pathogens.

Table 8. Frequency of diarrhea before and after feeding "Colostrum Pasteurizer" treated colostrum

Farm 1	**# of calved**	**# of diarrhea**	**% diarrhea**
Before treatment	90	20	22%
After treatment	92	2	2%

Farm 2	**# of calved**	**# of diarrhea**	**% diarrhea**
Before treatment	142	35	25%
After treatment	125	5	4%

CONCLUSION

Prevention of infections through contaminated colostrum will contribute to paratuberculosis eradication programs in Japan. Additionally, pasteurized colostrum will prevent other infectious diseases caused by contaminated colostrum such as leukemia, and decrease calf diarrhea cases by decreasing total bacterial count in colostrum.

Evaluation of indigenous ELISA kit with tissue culture and PCR for the estimation of Ovine Johne's disease (OJD) in farmers' flocks in India

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INTRODUCTION

Despite of low priority, a substantial population of sheep (62.5 million) exists in India. Johne's (JD) is a most serious disease of animals caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Eppleston et al. (2005) reported high mortality rates (1.8 to 17.5%) in sheep farms in Australia and of these, 74.0% deaths were due to JD.

Several countries have national policies for control and eradication of JD, in order to safeguard productivity of their flocks and international market. In India, sheep are reared in extensive management in large flocks as family tradition. Despite low productivity, information on prevalence of OJD in farmers' flocks is not known in the country. Few reports exist based on Johnin, microscopic examination and histo-pathology in farm flocks (Reddy et al., 1984). Though Ayub et al. (1988) reported highest morbidity in farmers' flocks due to enteric infections and debility, but animals have rarely been investigated for JD.

The current study was conducted to estimate the real prevalence of MAP using sensitive culture and PCR (in intestine and mesenteric lymph nodes tissues) and ELISA in sheep belonging to farmer's flocks. The three tests were also compared for the diagnosis of Johne's disease in sheep.

MATERIALS AND METHODS

Animals and samples

Sheep (39) from farmers' flocks of Ajmer, Rajasthan were sampled in 2005 from slaughterhouse in Mathura city. Intestine near ileo-caecal junction (ICJ) and associated mesenteric lymph nodes (MLN) were screened for the presence of MAP.

Diagnostic tests

The culture method of Singh et al. (1996) was followed to screen tissues. A modified method of isolation of DNA was used. Decontaminated sediment (<1.0 ml) left after inoculation of solid medium was pelleted by centrifugation at 10,000 rpm for 10 minutes. Pellets were washed with PBS and used for isolation of DNA as per van Soolingen et al. (1991). DNA was amplified using specific IS*900* primers. Presence and yield of specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis.

Serum samples were screened using indigenous ELISA kit, originally developed for goats (Singh et al., 2007). Protoplasmic antigen from native MAP 'Bison type' genotype (Sevilla et al., 2005) of goat origin was used in kit. OD values were transformed to S/P ratios and sheep in strong positive category were considered positive for JD.

In culture and PCR both target tissues (intestine and MLN) were screened and sheep was considered positive if any of the tissues were positive in culture and/or PCR. Based on the number of colonies (cfu) per sample, tissues were classed pauci- or multi-bacillary.

RESULTS

Of 78 tissues, live cultivable MAP was recovered from 64% of the sheep (41% intestine and 39% MLN). Only 15% sheep were positive in both tissues, whereas 26 and 23% of the sheep were independently positive in intestine and MLN, respectively. In PCR, 49% of the sheep were positive. The 13% sheep were positive in both tissues and 18% each independently positive in intestine and MLN. Culture and PCR together detected 69% sheep positive for MAP (64% in culture and 49% in PCR). The 44% sheep were positive in both tests (21% in culture and 51% in PCR).

Using the ELISA kit, 46% of the sheep were positive. Combined with culture, 69% sheep were detected positive (46% in ELISA and 64% in culture). Independently, 5 and 23% of the sheep were positive in ELISA and culture, respectively, and 44% were detected by both the tests. The ELISA and PCR detected 67% sheep positive (46% in ELISA and 49% in PCR), and 28% sheep together. Independently, 18 and 21% sheep were detected in ELISA and PCR, respectively. The ELISA detected 46, 31, 13, 8 and 3% sheep as strong positives, positives, low positives, suspected and negatives, respectively. Of positive sheep in ELISA, 89 and 61% correlation was with culture and PCR, respectively. Correlation was 75% in negative and suspected sheep though culture and PCR positives were seen in all categories (Spectral disease).

Based on kappa scores, tissue culture had 'substantial agreement' both with direct PCR (kappa: 0.49, proportion agreement: 0.74) and ELISA (kappa value: 0.45, proportion agreement: 0.71). ELISA also showed 'substantial agreement' with direct PCR (kappa value: 0.29 and proportion agreement: 0.65). Using 3 tests (culture, PCR and ELISA), 74% sheep were detected positive. In combinations of 2 tests, 69, 69 and 67% sheep were positive in culture and ELISA, culture and PCR and ELISA and PCR, respectively. Independently, 64, 49 and 46 sheep were positive in culture, PCR and ELISA, respectively.

DISCUSSION

The present study reports a very high presence of MAP in farmers' sheep flocks by culture of tissues. Reported low prevalence was mainly due to use of less sensitive tests like, Johnin, rectal pinch, necropsy. Singh et al. (1996) reported high (37.7%) prevalence of MAP in fecal culture in farm sheep. Recovery of MAP was higher from intestinal tissues as compared to MLN. Similar high prevalence of MAP in intestinal tissues than in MLN by tissue PCR was reported by Gwozdz et al. (1997). Culture of intestine and MLN together detected 93% of the total 69% sheep positive for MAP by PCR. Addition of PCR only marginally increased the prevalence of MAP. Processing of intestine and MLN tissues independently was recommended. Of the 69% positive sheep, 2 tests together detected 44%, whereas, 21 and 5% were missed in PCR and culture, respectively. Chaitaweesub (1999) also reported that culture from individual sheep was more sensitive than serological testing. Sensitivity of tissue culture by Kurade et al. (2004) was poor.

Direct PCR also revealed a high presence of MAP in tissues of farmers' flocks. MAP primarily infects the intestine near the ileo-caecal junction and associated lymph nodes with gross lesions of thickening and corrugations of intestinal mucosa, lymphatic cording and lymph-adenopathy (Reddy et al., 1984). High recovery of MAP from tissues was due to different MAP genotype or high optimization of culture tests (Singh et al., 1996) or endemicity of MAP in Indian sheep population.

The 'ovine' genotype is known for difficulties in recovery in artificial medium (Whittington et al., 2001). Kumar (2002) also reported high recovery of MAP from farm sheep. This was due to presence of 'Bison type' geno-type in sheep and other ruminants (Sevilla et al., 2005). Robbe-Austerman et al. (2005) reported similar high recovery of MAP in tissues culture from INF-γ positive sheep. Thus screening of target tissues (intestine and MLN) by culture was the most sensitive and accurate method to estimate true prevalence of MAP and also associated with active infection. Comparison of PCR with culture and ELISA showed that PCR had a better correlation with culture (26% mis-match) as compared to ELISA (38% mis-match). PCR and CFT had similar results in the study of Ikonomopoulos et al. (2005).

A freeze-boiling PCR protocol was evaluated with conventional tests in fecal samples of sheep and showed sensitivity of 94% and specificity of 92%). Sensitivities of culture and PCR, with respect to the 3 tests were 93 and 70%, respectively. Specificities of culture and PCR were considered 100.0%, (Gwozdz et al., 1997).

Comparative evaluation of ELISA kit with direct tissue PCR and tissues culture revealed 67% sensitivity and 83% specificity. Compared to tissues culture, ELISA sensitivity and specificity were 64 and 86%, respectively, and compared to PCR, ELISA sensitivity and specificity was 58 and 65%, respectively. Sensitivity of this kit was comparable to commercial

ELISA kits (Collins et al., 1991). Kurade et al. (2004) used an antigen from MAP (TEPES) strain, imported from UK 40 years back from UK. This may be a reason for poor sensitivity of the ELISA, despite using antigen in high concentration. The ELISA kit detected only 2 sheep as false positives, therefore this kit using protoplasmic antigen from MAP 'Bison type', can used as screening test.

Positive sheep detected by ELISA had high correlation with culture (89% positive) and PCR (61% positive). A similarly high correlation was seen in negatives and suspected categories, as compared to low positives and positive (S/P ratio). This may be due to clinical and sub-clinical stage of JD in sheep screened. High correlation between positivity in ELISA and tissues culture and PCR was in clinical (strong positive) and anergy stage (negatives and suspected) as compared to sub-clinical stage (low positives and positives). Variable sensitivity of commercial ELISA kits has been reported in clinical and sub-clinically infected cattle by Collins et al. (1991). The ELISA like the PCR had a lower sensitivity compared to tissues culture and was not over sensitive (Kumar, 2002). Variable sensitivity of ELISA has been reported in multi-bacillary and pauci-bacillary conditions (Clarke et al., 1996). Owing to high specificity of serological tests (Perez et al., 1996) in sheep, they could be used for the screening and control of JD sheep flocks. JD is spectral disease and difficult to diagnose. Therefore, large number of studies evaluated multiple diagnostic tests in different livestock species.

In this study, comparison of 3 tests showed that single tissues culture was comparable to multiple diagnostic tests (2 or 3), in detecting MAP infection in sheep. Using 2 tests, tissues culture and PCR was superior to culture and ELISA and PCR and ELISA combinations, in that order. Using 3 tests, only 2 sheep extra were detected as compared to 2 tests combinations. Kurade et al. (2004), also reported high detection rate for JD by using 2 tests.

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

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INTRODUCTION

In India commercial goat farming created employment opportunities for rural and urban youth. Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis*, is one of the most serious infections of farm ruminants worldwide. Morbidity rates are high and distributed over time. Therefore, they may go un-noticed and never be estimated despite low per animal productivity. Due to lack of information on prevalence and losses, JD has low priority. However, disease has been reported from public farm herds (Mathur et al., 1981, Kumar et al., 1988, Koul et al., 1989, Sharma et al., 1987, Srivastava and More, 1987, Singh et al., 1996, Tripathi and Parihar, 1999, Goswami et al., 2000) but rarely from private farms. Information on outbreaks of JD in goats is not available in India. This study reports a rare outbreak of severe weakness and diarrhoea due to Johne's disease in a newly established commercial goat farm using indigenous ELISA kit and culture.

MATERIALS AND METHODS

History

A rural entrepreneur established a commercial goat farm in his village (Akosh, Mathura) by taking loan (Sept, 2004). He purchased 40 pregnant goats from local markets. Goats gave birth to 40 kids at his farm. In January 2006, he had 150 goats, when an incidence of weakness and diarrhoea occurred. Diarrhoea continued despite usual treatment and the condition of the goats got worse. Milk production was reduced and 13.3% goats aborted. On a visit (Feb. 3, 2006) the problems of weakness, weight loss, loss of appetite, off-feed and off water in goats was reported. Fifty goats (40 young and 10 adult) died in 1 month (Jan. to Feb., 2006) from clinical JD, due to weakness, diarrhoea and tympanitis. Morbidity was 100% and mortality 33%. The entire herd (100 goats; 35 purchased, 25 new adults and 40 kids) was affected by the severe outbreak of clinical Johne's disease. Body condition was extremely poor and hide bound (> 60%), continuous diarrhoea not responding to drugs (18%; 10 kids and 8 adult goats). Animals were dull, depressed, anaemic, unable to stand (5.3%), exhibiting symptoms of advanced stage of clinical JD (Photo 1 and 2). The farm was poorly managed, unhygienic and goats mixed freely (sick or healthy, young or adult). Milk production was reduced by >75.0%. There was 40–50% reduction in body weights. At the second visit (April 4, 2006), another 14% goats had died due to weakness and diarrhoea.

Photo1. Adult goats with clinical signs of JD (Weakness, debility, emaciation, diarrhoea)

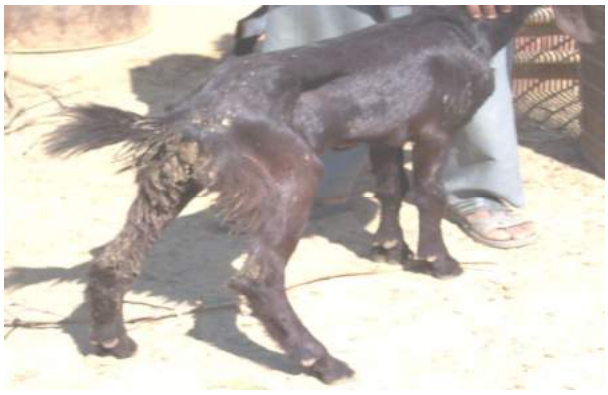

Photo 2. Kid suffering with chronic diarrhoea

Samples, culture and ELISA

Thirty-five faecal and serum samples from 15 kids and 20 goats were collected. Faecal samples were cultured as per Singh (1998). MAP colonies were identified on the basis of culture characteristics, mycobactin J dependency, acid fastness and slow growth. Based on the number of colonies (cfu) per sample, goats were categorized as pauci- and multi-bacillary. Serum samples were screened by a new ELISA (Kumar et al., 2007) developed using semi-purified, soluble, indigenous PPA antigen from native 'Bison type' genotype of MAP of goat origin (Sevilla et al., 2005). S/P ratios were calculated from the OD values. Animals in positive and strong positive category of S/P ratio were considered positive reactors in ELISA.

RESULTS

Of 35 faecal samples, 77% were positive in culture. Individually, 75% adult goats and 80% kids were positive. None of the samples were contaminated during incubation and no fast growing mycobacterial colonies observed. The majority of the colonies were pauci-bacillary (66%) and 11% were multi-bacillary. In kids and adult goats, the majority (23/27) of sero-reactors were pauci-bacillary, and only a few cases (4/27) were multi-bacillary or super-shedders. Among the pauci-bacillary conditions, the majority (31%) were in the 1–2 colonies category. In ELISA, 40% goats were positive. The 20% each were low positives, suspected and negatives. However, of the 14 positive in ELISA, 10 were detected in culture. Similarly, in low positive and suspected groups, 6 and 5 were detected by culture. Six cultures were also detected in negative goats (Table 1). In kids, 40, 30, 20 and 10% were positives, low positives, suspected and negatives, respectively and of these 5, 5, 3 and 2 were detected in fecal culture. In adult goats, 40, 6.6, 20, and 33% were positive, low positive, suspected and negatives, respectively and of these except 3 goats all were detected in culture. Comparative evaluation of fecal culture with ELISA kit showed that fecal culture detected, 27 (77%), goats as compared to 14 (40.0%) positive by ELISA. Independently, 49 and 11% cases were detected by fecal culture and ELISA, respectively (Table 2). None of the goats were in the strong positive category.

Table 1. S/P ratios and faecal culture in clinical cases of JD in goats and kids

Disease status	S/P Ratio	Animals	Culture Positive	Type of Culture
Negative	0.00-0.09	7 (20%)	6 (17%)	6PB*
Suspect	0.10-0.24	7 (20%)	5 (14%)	1MB#/4PB
Low Positive	0.25-0.39	7 (20%)	6 (17%)	2MB/4PB
Positive	0.40-0.99	14 (40%)	10 (29%)	1MB/9PB
Strong Positive	1.0-10.0	0 (0%)	0 (0%)	
Total animals		35	27 (77%)	4MB/23PB

*PB- Paucibacillary, #MB-Multibacillary

Table 2. Comparative evaluation of indigenous ELISA kit and faecal culture

Test	Combinations			
	1	2	3	4
Faecal culture	+	-	+	-
ELISA*	+	-	-	+
Total - 35	10 (28.5%)	4 (11.4%)	17 (49%)	4 (11%)

*Strong positive and positive categories in S/P ratio taken as positive

DISCUSSION

JD continues to increase in absence of screening and control strategy and endemic in farm herds (Singh, 1998). Reduced body weight gain, lowered milk production, abortions, increased kidding intervals, higher culling rates, high morbidity and mortality rates are common in farm herds but outbreaks of JD are rarely reported. Singh (1998) reported high morbidity and mortality in a new commercial goat farm located at Jhansi, UP. Barbari goats were transported from Mathura region (endemic for JD) and were eventually closed due to

this outbreak of JD. The 13% abortions were due to weakness and goats were unable to carry foetus (negative for brucellosis by SAT).

Using method of Singh (1998), culture had higher sensitivity than ELISA. Culture detected 77% animals' positive from the 100% clinical cases of JD and only 23% cases of clinical JD were missed. It may be due to advance cases of JD where mucosa gets denuded. Concentration and decontamination improved sensitivity of culture. Detection of large number of pauci-bacillary cases also revealed high sensitive of culture in detecting infected goats. Prevalence has been reported from farm herds in North India (Singh, 1998). Low prevalence reported (Tripathi and Parihar, 1999, Goswami et al., 2000) may be due to use of less sensitive tests (Johnin, fecal examination). Lowered sensitivity of faecal culture (Tripathi et al., 1999) may be due to variation in standardization. 'Bison type' genotype has been reported first time in India from domestic ruminants including goats using IS*1311* PCR-REA (Sevilla et al., 2005) may have a preference for HEY medium. Presence of super-shedder goats rapidly contaminates environment and premises for other healthy animals.

The ELISA used in this study has been developed using indigenous PPA from native and unique 'Bison type' genotype of MAP infecting goats in this region. In the ELISA, PPA was used at very low concentration (0.01µg/well). Commercial kits had lowered sensitivity than indigenous ELISA kit (Kumar et al., 2007). Low positivity reported by Goswami et al., (2000) in ELISA may be due to use of antigen from MAP 'TEPS' strain procured from UK 40 years back for preparation of Johnin. Low titers in a large number of goats (48% in low positive and suspected range and 18% as negative), of which most of them (81%) were detected in fecal culture may be due to damage to immune system, hypo-proteinaemia (protein loosing entropy) and anergy, Thus, goats in low positive sero-reactivity can also be considered as positive for JD. There were no goats in the strong sero-positive category. Therefore, goats in the positive category were taken as positive in ELISA. Correlation between positives in ELISA and faecal culture was (71%). Lowered sensitivity of ELISA kit is partly due to very high sensitivity of culture test, which detected large number of pauci-bacillary cultures (65.6%). Kit has been used for the screening of kids (Kumar et al., 2007). These findings confirmed the outbreak as that of Johne's disease caused by MAP. Outbreaks of Johne's disease are rare and comparable information not available. Indian 'Bison type' strain of MAP was highly pathogenic in goats and takes the form of outbreak, if infected goats are transported or stressed (e.g. to establish new commercial farms).

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

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INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) causing Johne's disease (JD) in goats in India has been characterized as 'Bison type' (Sevilla et al., 2005). JD is endemic in goatherds in India (Singh et al., 1996). Control based on test and removal for past 28 years in herds located at Central Institute for Research on Goats did not reduce prevalence. Instead prevalence continued to increase despite best of feeding and health care. Efficacy of 'indigenous vaccine' compared with 'commercial vaccine' for the control of disease in natural cases of clinical to advance clinical JD in farm goats.

MATERIALS AND METHODS

Goats

Seventy-one ready to cull (weak/diarrheic) naturally infected (positive in ELISA and fecal culture) farm goats had clinical to advanced clinical signs of JD. The goats were divided randomly into 3 groups; 'Bison' (Indigenous vaccine), Gudair (Commercial vaccine) and Sham Immunized consisting of 29, 24 and 18 goats, respectively. The goats were 2 to 4 years old and kept under semi-intensive management. They were extremely poor in condition (body weights below average weights of normal goats, Photo 1e). The skin was rough, hard, non-pliable and dry. JD was major cause of culling in these herds.

Vaccines

A new 'indigenous vaccine' (using highly virulent native field 'Bison type' strain of MAP from a terminally sick farm goat) and a 'commercial vaccine' ('Gudair^{TM}', Spain) were used. The 'Indigenous vaccine' contained 2.5 mg ($12x10^8$ bacilli) per ml of heat inactivated (72^oC for 2 hrs) MAP 'Bison type' suspended in aluminum hydro-oxide gel. The commercial vaccine contained MAP (strain 316F) with Montanide adjuvant. Goats with clinical to advanced clinical form of JD were vaccinated. The dose of each vaccine was 1 ml (sub-cutaneous) for goats. 'Sham immunized goats were given 1 ml sterilized PBS.

Samples

Serum was collected at zero and 30 days post vaccination (dpv). Blood samples of 9 goats (3 from each groups) were collected at zero and 30 dpv. The samples screened by ELISA and Lymphocyte Transformation Test (LTT). Nitric oxide (NO) was estimated in serum of all goats. Improvements in physical traits (body condition and body weights), mortality and morbidity were recorded. Detailed necropsy of goats died conducted. Sick goats were treated symptomatically.

Body condition

Body weights analyzed statistically using one-way analysis (ANOVA) at 5% level of significance. Body scores (heart girth, post girth, loin width, leg circumference, body length, height, slaughter weight, condition of skin and hair and skin thickness and pliability were measured before sacrifice of goats.

Mortality

Mortality rates were statistically analyzed by χ^2 test and Preventable fraction (PF value) calculated for each vaccine. Morbidity rates were also recorded.

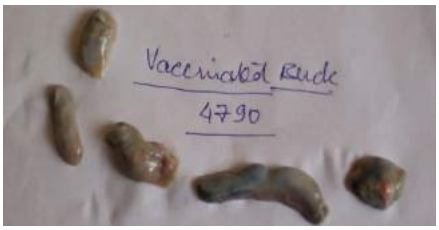

Photo 1a. Regression of lesions in MLN and normal inestine of vaccinated animal

Photo 1b. Enlarged and swollen MLN and thickened and corrugated inestine of sham-immunized animal

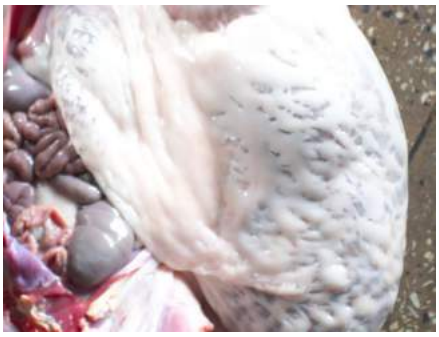

Photo 1c. Excess omental fat in vaccinated (Bison) animals

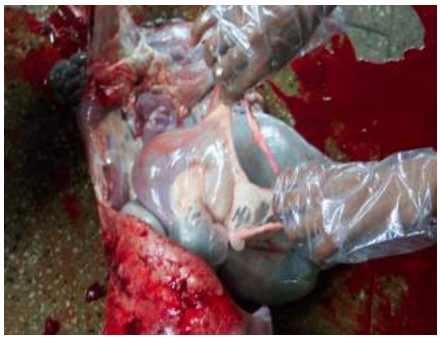

Photo 1d. Very little omental fat in sham-immunized animals

Photo 1e. Animals prior to vaccination

Photo 1f. Animals 7 months post vaccination

Cellular immune response

PBMCs isolated and LTT was performed as described by Uma et al. (1999). Stimulative indexes (SI) of MAP antigen stimulated lymphocytes of goats' were determined and average SI values for each group compared.

Nitric oxide (NO) estimation

NO was estimated in serum as described by Sastry et al. (2002). OD values transformed into OD index values. Means of OD index were compared in the 3 groups through a student *t* test at each sampling interval post vaccination.

Humoral immune response
ELISA titers (OD values) were converted to S/P ratios. Goats in positive and strong positive categories were considered positive for MAP. OD values were transformed to an OD index (Corpa et al., 2000). Means of OD indexes were compared among the 3 groups by ANOVA at each sampling interval post vaccination.

Shedding of bacilli
Feces collected at zero and 180 dpv were screened by culture for excretion of MAP. Differences in fecal culture positive goats were expressed in statistical terms by chi-square test.

Gross lesions
Internally goats examined for presence of fat layer on visceral organs, visceral fluid, enlargement and thickness of mesenteric lymph nodes (MLN) and condition of small intestinal mucosa from ileo-caecal junction upwards.

RESULTS

Body condition
There was marked overall improvement in body conditions (body coat regained luster, shining, pliability, regeneration of hairs, eyes were shining bright) of vaccinated goats as compared to 'Sham immunized' (Photo 1f). Average body weights gained at the end of 7 months post vaccination (mpv) were 5.00, 4.95 and 3.73 kilogram (kg) in 'Bison type', 'Gudair' and 'Sham-immunized' groups, respectively. More goats lost weight in 'Gudair' (8) and 'sham-immunized' (7) groups, as compared to 'Bison type' (5). The T-test revealed that the difference in body weights between 'Bison type' and 'Sham-immunized' groups was almost most significant ($p=0.05$). The difference in body weights between 'Bison type' and 'Gudair' groups was less significant ($p=0.1$). The difference in body weights between the 'Gudair' and 'Sham-immunized' groups was not significant ($p=0.1$).

Mortality rates
Mortality rates were lowest in 'Bison type' followed by 'Gudair' and 'sham immunized' goats. JD was the major cause of death in 'Sham immunized' followed by 'Gudair' and 'Bison type' groups. The proportions of goats dying due to JD were 71, 17 and 14% in the 'sham-immunized', Gudair' and 'Bison type' groups, respectively. Outbreak of contagious ecthyma occurred in vaccinated goats. In 'sham-immunized' group besides diarrhea, pneumonia was frequent cause of sickness as compared to vaccinated goats.

Cellular immune response
Vaccinated goats showed good response to stimulation with protoplasmic MAP antigen than 'Sham-immunized' group at 2 mpv. Stimulative Index (SI) was higher in goats vaccinated with 'Bison type' vaccine.

Nitric oxide
Vaccinated goats had higher NO concentration than 'Sham immunized' (Fig. 2). In 'Bison type' group, peak was seen at 45 dpv and then it declined. In 'Gudair', NO concentration showed similar trend At 15 dpv 'Bison type' goats had significantly higher ($P<0.05$) NO than 'Sham immunized'. 'Gudair' goats also had higher NO at 15 DPV compared to 'sham-immunized' but increase was not significantly different. After 15 dpv vaccinated groups had significantly higher ($P<0.05$) concentration compared to 'sham immunized' and at 45 dpv 'Bison type' goats had NO concentration significantly higher ($P<0.05$) than 'Gudair' goats. In 'sham Immunized' goats there was no regular pattern or goats showing decreasing trends in the NO concentration.

Humoral immune response

At all sampling intervals vaccinated goats had significantly higher ($P<0.05$) antibodies compared to 'sham-immunized'. At 75 dpv and 90 dpv the antibody titers of 'Bison type' goats was significantly higher ($P<0.05$) than 'Gudair'. 'Sham Immunized' goats did not show any pattern in pre- and post-vaccination antibody titers.

Shedding of bacilli

At 0 dpv, 89, 79 and 79% of the goats were positive for MAP in 'sham immunized', 'Bison type' and 'Gudair' groups, respectively. Vaccines reduced fecal shedding and at 7 mpv 17 and 29% goats were excreting MAP in feces of 'Bison type' and 'Gudair' goats, respectively. In the 'sham-immunized' group, all 18 goats were positive for shedding MAP in feces at 7 mpv.

Gross lesions

Five of 7 goats dying in the 'Sham Immunized' group had gross lesions of JD (Photos 1b and 1d). Two goats died 2 mpv in 'Gudair' group showed regression of lesions (reduction in size of MLN and regeneration of fat layer, Photo 1a). There was marked improvement in a goat of 'Bison type' group, which died 5.15 mpv (carcass healthy and extensive fat seen on mesentery and over visceral organs Photo 1c).

DISCUSSION

This study showed that vaccination was effective in clinical to advanced clinical cases of JD in any age group. There was complete turn around in the condition of ready to cull goats after vaccination. Regeneration of fat and regression of lesions in intestine and MLN was predominantly observed in vaccinated goats. Traditionally, JD vaccination is practiced during the first weeks of an animal's life, on the basis that protection would be conferred for first contact with mycobacteria (Saxegaard and Fodstad, 1985). Corpa et al., (2000) observed incomplete response in 15-day-old kids but immunity was higher in adults. Perez et al. (1995) also reported very good results in controlling JD by vaccination of adult animals. Corpa et al. (2000) reported vaccination modified inflammatory response causing limitation of progression of granulomatous lesions in infected goats and in reduction of bacterial shedding. Juste et al. (1994) also reported in vaccinated animals lesions formed were regressive type granulomas (tuberculoid forms) located exclusively in intestinal organized lymphoid tissues, whereas in those non-vaccinated, lesions can spread to other areas of intestine causing severe enteritis.

Lympho-proliferative responses to antigen stimulation have been widely used as *in vitro* correlates of cellular immunity (Pitchappan et al., 1991). Proliferative response was higher in PBMCs from vaccinated goats than of 'sham-immunized'. SI was higher for PBMCs of 'Bison type' goats stimulated with protoplasmic MAP antigens. Significantly higher NO concentration was seen in vaccinated goats and was maximum in 'Bison type' and least for 'sham-immunized'. Increased production of NO induced by antigen may cause effective immune response towards MAP and can lead to inhibition of MAP in macrophages. Significant increases in serum antibodies was seen in vaccinated goats. Peak titers' were attained at 60 dpv that gradually declined. This pattern was absent in 'sham-immunized' group. Comparative antibody titers were higher in the 'Bison type' group than 'Gudair'. Antibody responses may be indicator of degree of activation of immune system against mycobacteria (Corpa et al., 2000).

Vaccines significantly reduced mortality. Vaccination reduced fecal shedding in significant number of vaccinated goats. The numbers of goats that had fecal culture negative status were higher in the 'Bison type' group. Uzonna et al. (2003), using vaccine prepared from field isolates, reduced fecal shedders more than that of commercial vaccine. In this study also an indigenous vaccine provided better response in controlling JD in naturally infected clinical cases of JD in goats. Vaccines had therapeutic effect in clinical JD. 'Bison type' vaccine contained aluminum hydro-oxide gel as adjuvant, as compared to 'Montanide' in 'Gudair', still it was superior in controlling MAP infection in advanced to very advanced cases of JD in goats.

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The effects of control measures for paratuberculosis on sero-prevalence and clinical disease in Northern Italian dairy herds

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ABSTRACT

The effects of a control program for paratuberculosis were assessed during a six years period (2001-2006). The survey involved 8 dairy herds located in Northern Italy, with a number of animals aged >24 months ranging from 224 to 784, mean 437 (SD = 154), median 411. Globally, a mean of 3,497 (SD = 328) animals aged >24 months per year were monitored during the six years period. Cows showing diarrhoea were readily detected, isolated and culled after laboratory confirmation. Cows were tested by an indirect blood serum ELISA during the dry period. Calves were fed only with colostrum from sero-negative cows and with milk submitted to thermic treatment (70℃ for 40 min).

Based on the total number of animals > 24months of age, historical means (from 1995 to 2000) of the sero-prevalence and prevalence of clinical cases were 12.6% and 5.5%, respectively. Statistical analysis of sero-positivity and clinical cases trends after the beginning of the control program showed a significant ($p<0.01$) reduction of both parameters during the six years period. In particular, the sero-prevalence decreased from 12.7% to 7.3% and clinical cases decreased from 5.3% to 3.2%.

Following the use of the pasteurizers, a strong reduction of neonatal diarrhoea was observed in all the herds. In addition, the calf's mean weight gain/day moved from 500 g to 900 g. Conversely, an increase of myodystrophy cases was detected after two months of age. The problem was solved by adding selenium and vitamin E to the pasteurized milk.

INTRODUCTION

Paratuberculosis is a chronic enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), characterized by a long incubation period and with a mostly fatal outcome. Bovine paratuberculosis is one of the most important diseases affecting cattle, due to the relevant direct and indirect costs disease-associated (Kennedy and Benedictus, 2001). An increasing interest on this disease is coming from farmers who became aware of the economic impact of the disease on their business. Consequently, there is an increasing request of paratuberculosis control programs to be applied to the cattle-breeding activity.

The transmission of the infection to susceptible animals is considered to occur mainly via the faecal-oral route. Colostrum or milk, either contaminated by faeces or infected because originating from infectious cows, are important means of infection (Sweeney, 1996). These considerations and the fact that young animals are more susceptible than adults to the infection (Taylor, 1953), entail that a correct control program should not leave out of consideration the good management of calves.

In paratuberculosis control, herd management changes aimed at minimizing the transmission risks are probably more effective than test-and-cull approaches. A simulation model indicates that, under certain situations and especially in the face of low milk prices or for large herds, not testing could be an economically viable strategy (Dorshorst et al., 2006).

The aim of the present study was to evaluate the effects of economically viable control measures, applied to Northern Italian dairy herds, on sero-prevalence and prevalence of clinical disease for a term of years.

MATERIALS AND METHODS

Animals and samples

The survey involved 8 dairy herds located in Northern Italy, with a number of animals aged >24 months ranging from 224 to 784, mean 437 (SD = 154), median 411. Globally, a mean of 3,497 (SD = 328) animals aged >24 months per year were monitored during a six years period (2001-2006). Blood serum samples were collected in silicone coated tubes. After

clotting at room temperature, serum was separated by centrifugation at 1250 x g for 5 min and stored at –20℃ until tested.

Testing protocol

Sero-positivity rate and prevalence of clinical paratuberculosis per year were recorded in each herd involved in the study. Blood serum samples were collected during the dry period from all animals aged >24 months. Sero-positivity was evaluated by a commercial indirect blood serum ELISA (Paratub.Serum-S, Institut Pourquier, Montpellier, France) according to manufacturer instructions.

Control program protocol

The control program started in year 2000. In the herds involved, no particular control measures were already present. However, calves were separated from the dams after birth and kept in individual calf pens until 60 days of age. Older calves were kept in groups of 5-20 animals. Few modifications of the indicated original management practices were made. In particular, cows showing diarrhoea were readily detected, isolated and culled after laboratory confirmation. Cows were tested by the indirect blood serum ELISA during the dry period. Calves were fed only with colostrum from sero-negative cows and with milk submitted to thermic treatment.

Milk treatment

The pasteurization unit was a batch pasteurizer, able to handle up to 80 litres of milk for each heating cycle, comprised of a stainless steel container with a heating coil and a stirring system to allow a vigorous agitation and an even heating of the milk. Milk temperature was constantly monitored by a thermocouple and a display during treatment, gradually rose to 70℃ in 75 min and then kept further at that level for 40 min. After heating, milk temperature was lowered in the pasteurizer to 4℃ in 30 min.

Statistical analysis

Temporal variations of sero-positivity and clinical cases were analyzed by Friedman's test.

RESULTS

Based on the total number of animals aged >24 months from all the herds, historical means (from 1995 to 2000) of the sero-prevalence and prevalence of clinical cases were 12.6% and 5.5%, respectively. Statistical analysis of sero-positivity and clinical cases trends after the beginning of the control program showed a significant ($p<0.01$) reduction of both parameters during the six years period. In particular, the sero-prevalence decreased from 12.7% to 7.3% and the prevalence of clinical cases decreased from 5.3% to 3.2% (Fig. 1). The sero-prevalences and the prevalences of clinical cases for each herd during the six years period are shown in Figs. 2 and 3.

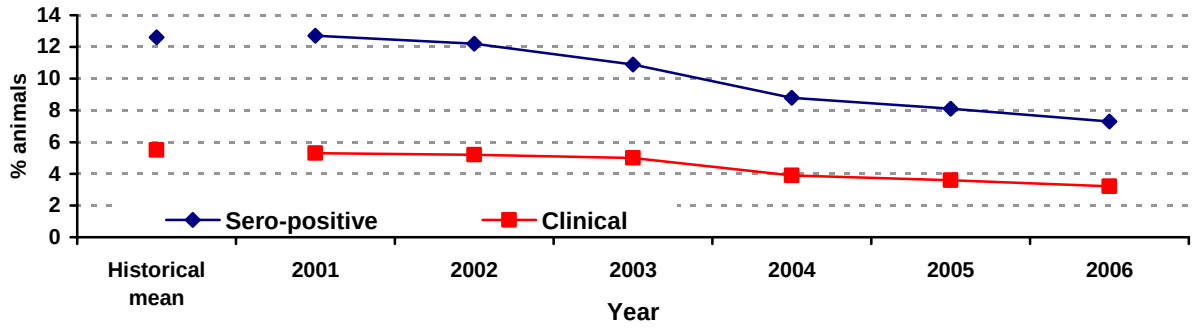

Fig. 1. Sero-prevalence and prevalence of clinical cases among animals >24 months of age from 8 dairy herds

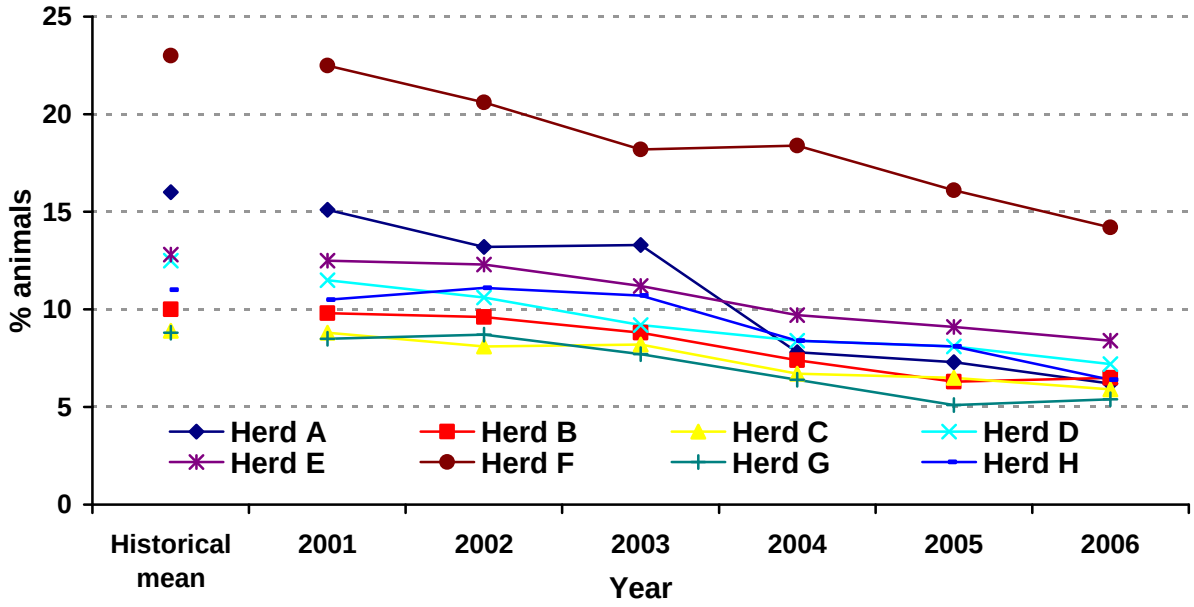

Fig. 2. Sero-prevalence among animals >24 months of age for each herd

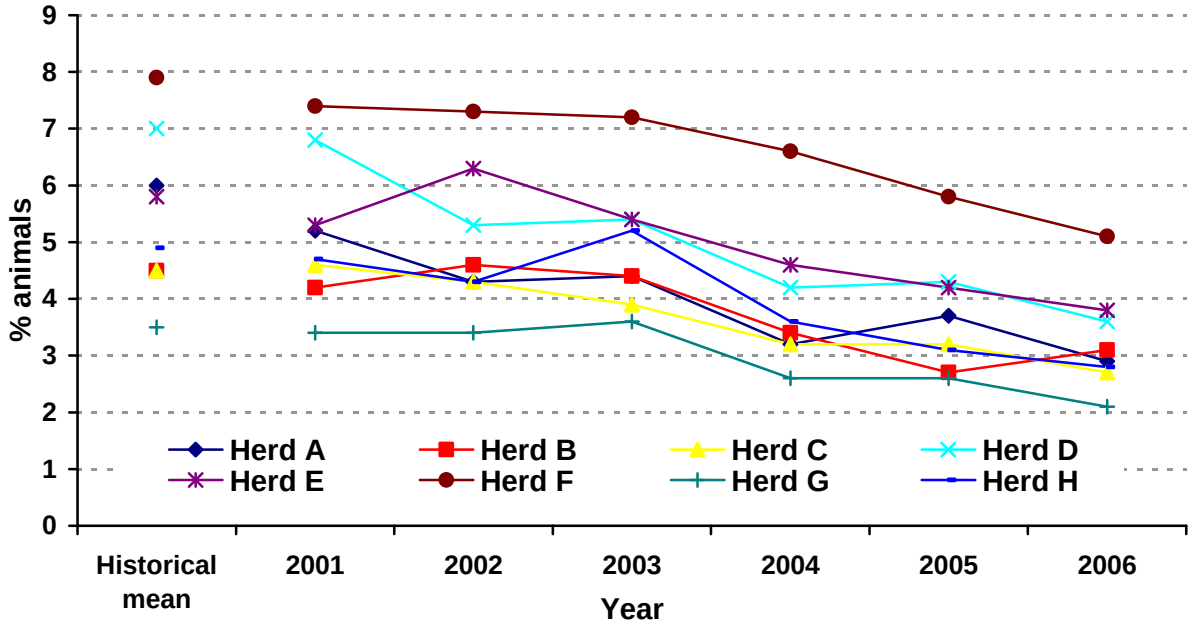

Fig. 3. Prevalence of clinical cases occurring in each herd

DISCUSSION

In the control program protocol we used heat-treated transition milk to fed calves, avoiding mastitis milk to reduce antibiotic or endotoxin contamination. When the transition milk volume available was not adequate, saleable bulk tank milk was added and heat-treated as well. The use of pasteurized whole milk, compared to a standard milk replacer or to non-pasteurized milk, offers several benefits, among which a better performance in terms of calf weight gain and improved calf immune function and health (Godden et al., 2005; Jamaluddin et al., 1996).

We decided to use a pasteurization unit for each herd involved in the control program. Thus, milk was pasteurized and fed within a couple hours of harvest, making not necessary the use of a complex chilling and transport system. Moreover, a simple batch pasteurizer was used, being the least expensive option. Sanitization procedures for the pasteurization equipment were similar to those used for the milking system present in each herd. The

pasteurization of milk at 72℃ for 15 sec could be adequate to kill MAP if properly done by a continuous-flow pasteurizer (Stabel et al., 1997). However, some considerations led us to adopt a more aggressive thermic treatment. The precision of our batch pasteurizer was probably lower than those of a continuous flow pasteurizer and an extended treatment can also obviate to pasteurization failures due to human errors, such as farm staff not trained to follow carefully the protocols. Moreover, the heat-treating of milk was not so problematic as in case of colostrum, where a lower temperature and a longer time should be used in order to preserve the activity of colostral antibodies (McMartin et al., 2006; Godden et al., 2006). Our milk treatment protocol was adequate to kill MAP, because the bacterium was not recovered from milk inoculated with MAP at a concentration of 10^4 CFU/ml.

As side effect, following the application of the pasteurizers, a strong reduction of neonatal diarrhoea was observed in all the herds. In addition, the calf's mean weight gain/day moved from 500 g to 900 g. This agreed with literature, where is reported that calves fed pasteurized milk had fewer days with diarrhoea and greater average weight gain than calves fed non-pasteurized milk (Jamaluddin et al., 1996). Conversely, an increase of myodystrophy cases was detected after two months of age. The problem was solved by adding selenium and vitamin E to the pasteurized milk.

The introduction of testing was necessary to single out sero-negative cows for MAP-free colostrum collection. Considering sero-prevalences of the herds involved (Fig. 2), testing with a low cost test can be regarded as an economically viable option as indicated by a reported prediction model (Dorshorst et al., 2006). In consideration of the improved control measures, aimed at reducing MAP transmission, sero-positive animals were retained in the herds, thus maintaining profits from milk sales, while those showing clinical signs, less productive and being a major source of infection, were culled.

The application of the protocol permitted to obtain a significant progressive reduction of both sero-prevalence and clinical cases in all the herds involved in the study (Figs. 2 and 3). Apparently in contrast to reported data, indicating that the effect of control measures cannot be measured with an ELISA until after more than 3 years (van Weering et al., 2005), globally we recorded a weak reduction of sero-prevalence as early as 2 years after control program started (Fig. 1). This could be partially explained by the fact that we tested animals aged >24 months instead of animals aged >36 months, so that after 2 years some of the tested animals had grown up under conditions of preventive management.

CONCLUSION

In conclusion, this field study showed that simple control measures, compatible with farmers needs and with the economics and logistic condition of the herds involved, were successful in reducing sero-prevalence and prevalence of clinical paratuberculosis and can be considered an economically viable control strategy.

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Seroprevalence of paratuberculosis in selected population of ruminants in India

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ABSTRACT

The objective of this study was to estimate the sero-prevalence of paratuberculosis in selected population of ruminants from different parts of India using a highly specific commercial ELISA kit (Institut Pourquier, France). Out of 1822 sera screened, 22.5%, 20%, 11.6% and 4% of 414 indigenous cattle, 264 cross-bred cattle, 465 sheep and 359 goats, respectively, were found to be positive. A total of 320 buffalo sera from central west (n= 80) and northern (n= 240) parts of India did not show antibody prevalence. It was concluded that Pourquier ELISA was a sensitive and highly specific test and paratuberculosis was widely prevalent in ruminant population of several parts of India.

INTRODUCTION

Paratuberculosis (Johne's disease), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been recognised as a major disease of ruminants worldwide for over a century. In India, Johne's disease has been occurring since early 1930s and became prevalent in many parts of the country by 1950s (Tripathi et al., 2002). Assessment of the disease at several government farms in most state has been based on single intradermal johnin test, and faecal and tissue smear examination. Of late, prevalence studies were also based on faecal/ tissue culture (Tripathi and Parihar, 1999). Sero-prevalence of disease has also been reported based on an in-house developed ELISA (Rajukumar et al., 2001; Sivakumar et al., 2005; 2007). A number of commercial ELISA have been developed for specific detection of MAP antibody in cattle, sheep and goats with differing but acceptable level of sensitivities and specificities (Collins et al., 2005).

In the present study, sensitivity and specificity of Pourquier ELISA kit (Institut Pourquier, France) was accessed on serial experimental and natural sera and then applied to sera from cattle, sheep, goats and buffalo from different parts of India.

MATERIALS AND METHODS

Collection of experimental and natural sera from sheep and goats

Experimental paratuberculosis has been produced in sheep and goats in our laboratory (Kurade et al., 2004; Munjal et al., 2005). Serial sera available in laboratory from 12 sheep (n=36) and 4 goats (n=14) were used in this study to assess sensitivity of the ELISA. Additionally, 30 sera available from naturally infected goats from Institute's Goat and Sheep farm were also used. All the naturally and experimentally infected animals were either positive on faecal/tissue culture and or histopathology.

Sample collection for assessing the specificity of the test

For assessment of diagnostic specificity of Pourquier ELISA, sera and mesenteric lymph nodes were collected from 98 animals (50 sheep and 48 goats) from the Bareilly slaughterhouse whose lymph node culture for MAP was negative.

Collection of natural sera from domestic ruminants

A total of 1822 sera were received on ice from small and large ruminant populations of different parts of the country. Details of sera received from different species of animals are presented in Table 1. Cattle sera originated from central-west, northern and southern parts of India, and sheep and goat sera were from organised farms of Rajasthan and Uttar Pradesh

states, respectively, and buffalo sera from central west and northern part of India. Cattle belonged to indigenous breeds (Ongole, Deoni) and Jersey and HF cross. Buffalo sera were from Murrah and Surti breeds.

Table 1. Results of ELISA on different ruminant species

Species/ Breed	Indigenous cattle	Cross-bred cattle	Sheep	Goat	Buffalo	Total
No. of sera tested	414	264	465	359	320	1822
No. of positive sera	93	53	54	15	0	215
Percentage (%)	22.5	20	11.6	4	0	11.8

Infection status of the animals

Infection status of the animals was confirmed by exhaustive histopathology of small intestines and the associated lymph nodes and/or culture of terminal ileum and lymph nodes as gold standard. All bacterial cultures were carried out on HEYM medium supplemented with mycobactin J (Tripathi et al., 1999). Mycobactin J dependent growths were tentatively considered positive and were subsequently confirmed by IS*900* PCR (Tripathi et al., 2006). The procedure for samples collection for histopathology has already been described (Kurade et al., 2004; Munjal et al., 2005). H & E sections were prepared and examined for the presence of characteristic granulomatous lesions. Adjacent sections were stained by ZN and immunoperoxidase method for mycobacterial demonstration.

ELISA Procedure

The test procedure and interpretation of the result was done according to the manufacture's instructions. Results were expressed as sample to positive (S/P) ratio after correction with the negative control. Any sample with S/P equal to or greater than 70% was considered to be positive.

RESULTS

Sensitivity of ELISA

The sensitivity on experimental sera was 56% (28/50), whereas on natural sera, it was 73.3% (22/30). The naturally infected animals were mostly in the clinical stages, whereas experimentally infected animals had moderate and severe infection of paratuberculosis. The overall sensitivity was found to be 62.5%.

Specificity of ELISA

Two sheep showed positive ELISA results (S/P ratio between 70 and 80 %.). Thus specificity of the assay was about 98% based on 98 sheep and goat sera from slaughterhouse, whose MLN tissue samples were negative on bacterial culture.

Test sera

The results of the assay on the field sera from different animals are presented in the Table 1. The sero-prevalence of disease in various animals varied between 4 and 22.4%. There was no substantial difference between indigenous cattle and cross-bred cattle, albeit, it was higher in the indigenous cattle. The assay could not detect any positive case in buffaloes.

DISCUSSION

There appears to be no reports of assessment of any commercial ELISA for detection of antibodies to MAP infection in ruminants in Indian conditions. In the present study, firstly, the sensitivity and specificity of Pourquier ELISA was determined on serial experimental and natural sera from sheep and goats of known infection status. The sensitivity of the test was found to be higher (62.5%) than those reported earlier (about 55%). Our results compared well with a most recent study from Australia (Gumber et al., 2007), wherein sensitivity of Pourquier ELISA was 77.8% on known positive samples against only 47.8% on infected flock samples with overall sensitivity of 56.4%. The higher observed sensitivity was perhaps due to

inclusion of histopathology also as a parameter of infection (gold standard) as reported previously (Gumber et al., 2007). In India, there are no certified paratuberculosis negative farms, whose samples can be used for determination of specificity of ELISA. Therefore, 98 sera with negative MLN culture results were used for specificity estimation in this study. Slaughterhouses receive sheep and goats mainly from local rural population, which may not be free from paratuberculosis infection, although the incidence of the disease is very low. Therefore, it is possible that 2 sheep reacting positively in ELISA but negative in MLN culture were either mildly infected or might have sensitised with other mycobacteria.

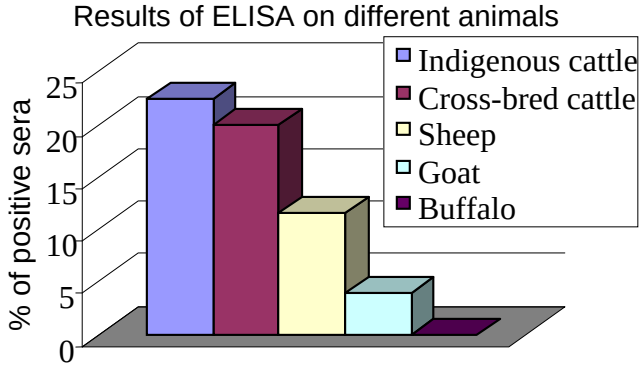

Fig.1. Percentage sero-prevalence of paratuberculosis in different ruminant species

Screening of sera from cattle revealed that the disease was equally prevalent in indigenous and cross-bed cattle in India. The result suggested that disease was prevalent in north, east and central west part of India, and a central programme should be initiated to contain the disease. Data also suggested that disease was widely prevalent in sheep and goats also. None of the buffalo sera was positive, while in a previous study (Sivakumar et al., 2007), a sero-prevalence of ~14% has been reported. This might be due to the fact that conjugate used in the kit reacts specifically to cattle, sheep and goat IgG, but not to buffalo IgG. Further studies are needed to evaluate this ELISA kit on buffalo sera.

CONCLUSION

The ELISA kit was found to be highly sensitive and specific. The results suggested that paratuberculosis was prevalent in different parts of the country, which needs institution of control and eradication programme.

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Paratuberculosis in sheep from Serra da Estrela Region, Portugal

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INTRODUCTION

This work is part of a National Project (AGRO 786), started in September 2004 and with the duration of three years, aiming the study of Paratuberculosis incidence in sheep herds in one of the best Portuguese cheese production region, Serra da Estrela.

Several diagnostic parameters were used and correlated with each other: clinical signs, serological results, pathological lesions at necropsies, histopathological tests, Ziehl-Neelsen staining, immunohistochemical technique, direct PCR and bacteriological culture.

MATERIALS AND METHODS

Thirteen sheep herds were selected and submitted to an epidemiological inquiry focused on the herd size, animal movement, clinical history and sanitation measures, type of bedding, manure management and human involvement. ELISA and AGID tests were performed in 2 562 animals. Live and and *post mortem* examination were done and several tissue samples of 33 ewes and 1 ram, 30 of them previously submitted to screening ELISA and AGID tests, were collected for histopathological and immunohistochemical analysis. Bowel tissues with lesions were subjected to direct detection of *Mycobacterium avium* subsp. *paratuberculosis* by PCR, according to the ADIAVET kit, and also cultured in selective media for bacteriological isolation. From 144 sero-reacting animals, faeces were sampled, stained by Ziehl-Neelsen method and cultured in selective media for bacteriological isolation.

RESULTS

The serological survey revealed that 234 samples (9.1%) were positive in ELISA and 30 (1.2%) in AGID. From the 13 herds, only two of them did not show positive serological results.

From 34 animals submitted to necropsies, 27 presented clinical signs compatible with the disease. Typical lesions of paratuberculosis were observed in 28 animals (Figs. 1 and 2): mucosa folds, lymphangiectasia lesions, lymphangitis and lymph nodes enlargement. However, macroscopic valvular lesions were noticed only in a very low percentage of animals.

Histophatological lesions were present in 21 animals and in 18 of them acid-alcohol resistant bacilli were observed (Fig. 3). The predominant microscopic lesion was granulomatous lymphadenitis, followed by the granulomatous inflammation in the intestine and the ileocecal valve mucosa and submucosa. Only two animals were tested by immunohistochemistry and showed positive results (Fig. 4).

From 28 PCR positive samples collected at necropsies, *Mycobacterium avium* subsp. *paratuberculosis* was isolated in 20 of them. The results are expressed in Fig. 5.

Acid-fast bacilli were found in 26 faecal samples but, until now, *Mycobacterium avium* subsp. *Paratuberculosis* was only isolated in 1 of them.

Mycobacterium avium subsp. *paratuberculosis* isolates are being molecular typed by single nucleotide polymorphisms of *gyrA* and *gyrB* genes.

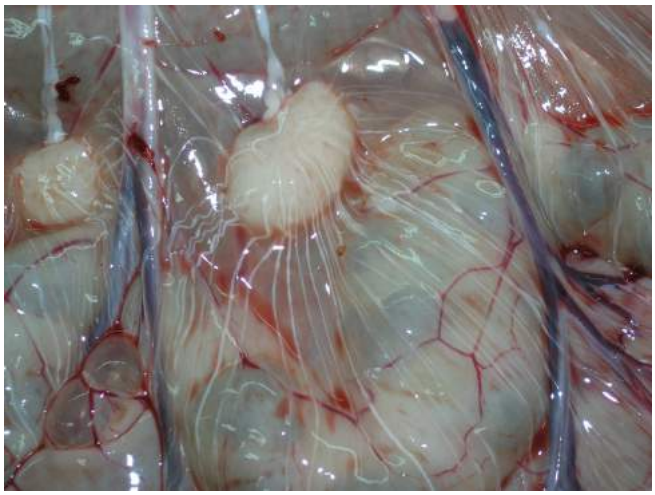

Fig. 1. Macroscopic exam. Mesenteric lymph nodes enlargement, thickened of lymphatic vessels and atrophy of mesenteric fat.

Fig. 2. Macroscopic exam. Diffuse thickening of the mucosa, which is folded into transverse rugae.

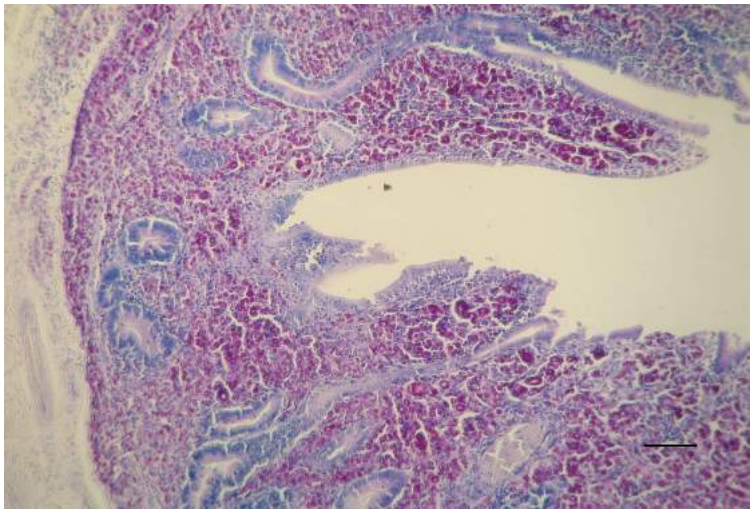

Fig. 3. Ziehl-Neelsen staining. Diffuse giant cells infiltrate with multiple alcohol-acid-resistant bacteria, in the lamina propria of the intestinal villi (Bar = 50µm).

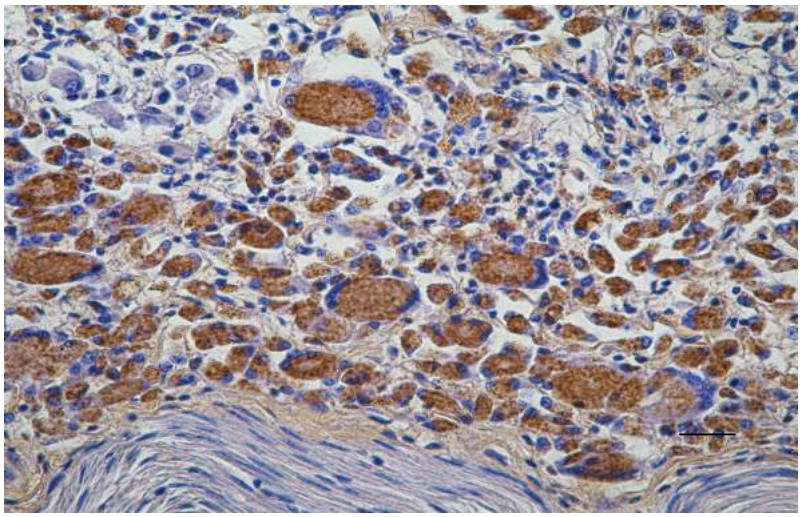

Fig. 4. Imunohistochemistry. Diffuse giant cells and epithelioid macrophages infiltrate, positive for MAP antibody, in the intestinal submucosa. (Bar = 200µm).

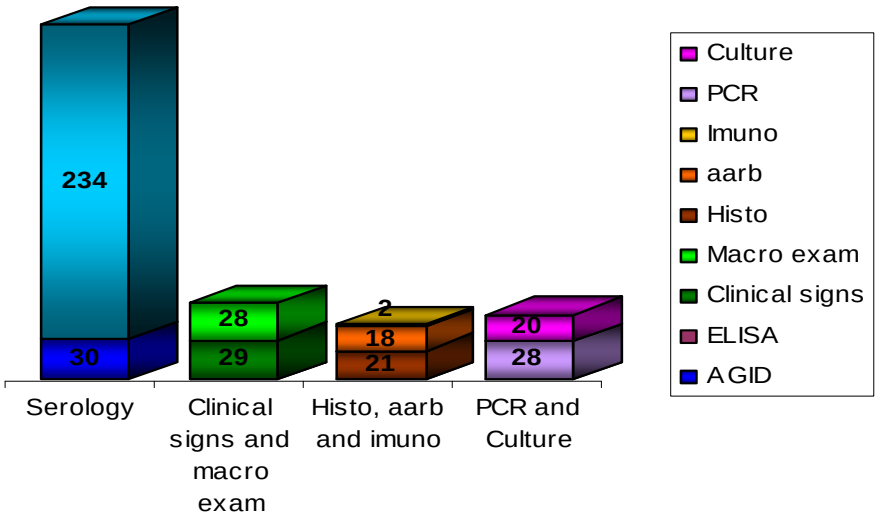

Fig. 5. Number of samples tested positive in the nine diagnostic tests used for paratuberculosis

DISCUSSION

The fact that the herds are large sized, implies higher housing density and, consequently, a higher level of exposure to the etiologic agent (Berghaus *et al*., 2005). Only in three herds beddings were removed in a weekly base and other three do that only twice or three times a year which increases the risk of disease spreading.

Transhumance and route sharing is practiced in eight herds, which promotes infection transmission between herds (Collins & Manning, 2001; Navneet *at al*., 2007). None of the herds had maternity pens and, in seven of them, lambing ewes were not placed in lambing pens, a fact that is particularly important since the animal's age is probably the parameter that most affects the infection transmission (Badiola *et al*., 2000). In seven herds, suspected animals were not isolated from other animals, increasing the risk of infection (Badiola *et al*., 2000).

ELISA technique showed higher sensibility and lower specificity than AGID, fact that has been previously referred (Badiola *et al*., 2000; Garrido, 2002). All animals submitted to necropsies were aged between two and six years, which is in agreement with what is known about this disease (Radostits *et al*., 1994; Sweeney, 1996).

The majority (76.5%) of the animals submitted to necropsy presented clinical symptoms compatible with paratuberculosis (Jubb *et al.*, 1991; Wilson, 1998; Martin & Aitken, 2000; McGavin *et al*., 2000). In most of them, lymph nodes lesions were evident and

corresponded to microscopic lesions of granulomatous lymphadenitis. In agreement with other authors (Jubb *et al*., 1991; Wilson, 1998; Gracey *et al*., 1999; McGavin *et al*., 2000; Verna, 2005), diffuse thickening of the mucosa was also evident, with folds that couldn't be smoothed out by stretching, corresponding to a diffuse granulomatous cell infiltrate in the intestine and ileocecal valve mucosa and submucosa,

CONCLUSIONS

The epidemiological inquiry revealed that factors such as: the great size of the herds, poor herd management and sanitary conditions, type of bedding, the absence of suspected animals sequester, as well as common transhumance routes, favoured the spread of the disease.

All the animals submitted to necropsy had equal or superior age required for the disease incubation period to occur. It was noticed a high correspondence between the diagnosis methods of histopathology and Ziehl-Neelsen. All Ziehl-Neelsen positive cases were multibacillary lepromatous type.

A low correspondence between sero-positive animals and direct Ziehl-Neelsen stained and culture of faeces was observed.

All the activities developed in the frame of Project AGRO 786, improved the knowledge of the producers towards this disease and contributed to a better control and awareness of paratuberculosis in Portugal.

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

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INTRODUCTION

Detection of DNA of MAP by PCR in samples easily obtained, such as blood (Bhide et al., 2006), provides a rapid diagnosis. Until now there have been few published reports on the prevalence and epidemiology of ovine paratuberculosis in North East of Portugal (Coelho et al., 2007). Very little was known on the epidemiology of ovine paratuberculosis in the North-East of Portugal until a recent serological study in the Trás-os-Montes e Alto Douro (TMAD) region showed a high flock and moderate sheep prevalence. In order to improve and confirm the sero-prevalence data, and to explore other diagnostic alternatives, a study based on detection of MAP DNA in blood was carried out.

The aim of this study was to assess the prevalence of bacteraemia in ovine paratuberculosis using a PCR blood pool screening approach as a direct measure for estimating the spread of *Map* in the region of TMAD in Portugal.

MATERIAL AND METHODS

Sample size was calculated based on the list of 3162 flocks registered in the region of Trás-os-Montes, Portugal. A total of 150 sheep flocks were randomly selected using the programme WinEpiscope 2.0®. This sample size provides a 95% confidence level for an expected prevalence of 10%. A total of 150 flocks were included in this survey. In order to limit the costs for each flock, a set of five clinically healthy sheep and another one of five clinically suspect paratuberculosis sheep were sampled. In healthy sheep only blood was taken to pooled PCR and individual ELISA. From clinically suspected sheep, both faeces and blood were taken. Faeces were submitted to pooled PCR and isolation on LJ and Middlebrook® 7H11 and blood to absorbed individual ELISA and pool PCR. A total of one thousand and five hundred sheep were examined in total. Processing of faeces for culture was based on the method of Juste *et al*. (1991) and Aduriz et al. (1995). Positive colonies were confirmed by IS*900* PCR, and PCR-REA IS1311 confirmed that they were S strains. Specific Map DNA was detected following the extraction of genomic DNA from frozen samples of faeces, tissues and blood. The PCR analysis procedure for colonies, faeces, tissues and blood has been previously described in detail by Garrido et al. (2000) and Juste et al. (2003). ELISA were performed with a commercial ELISA Kit according to the manufacturers instructions (ELISA paratuberculosis screening test®, Institut Pourquier). Results with each method were compared to each other in order to estimate complementary sensitivity (C.S.), agreement and cost efficiency.

Animal level prevalence for each sheep, based on pooled blood results, was calculated by a method for fixed pool size using Pooled Prevalence Calculator available online (Sergeant, 2004).

RESULTS

PCR detected MAP DNA in 56 (18.7%) of pooled samples. MAP was found in 20.7% of pooled samples from apparently healthy animals and in 16.7% pooled samples from suspect animals. The overall individual prevalence of MAP was calculated to range from 6.4 to 15.4%. Faecal isolation and PCR showed an overall prevalence of 0.9% and 1.9% respectively (Table. 1).

Table 1. Individual prevalence according different sensitivity values in PCR technique**.**

	Pool of healthy			Pool of suspect		
No. pools sampled	150			150		
No. positive pools	31			25		
Sensitivity	0.33[a]	0.66[b]	0.5[c]	0.33[a]	0.66[b]	0.5[c]
Specificity	1	1	1	1	1	1
Prevalence (%)	17.9	7.2	10.1	13.1	5.7	7.8
95% CI (%)	4.0; 31.8	4.1; 10.4	4.8; 15.4	3.7; 22.6	3.0; 8.3	3.6; 12.0
	Total of pools sampled					
No. pools sampled	300					
No. positive pools	56					
Sensitivity	0.33[a]		0.66[b]		0.5[c]	
Specificity	1		1		1	
Prevalence (%)	15.4		6.4		8.9	
95% IC (%)	5.2; 25.5		4.2 ; 8.7		4.9 ; 12.9	
References	[a]Gwózdz et al., 1997; [b]Gwózdz et al., 1997; [c]Juste et al., 2003					

Agreement between pooled methods was generally low (Tables 2 and 3). The highest value agreement beyond chance (κ value) was observed between ELISA and blood PCR in healthy animals.

Table 2. Comparison between fecal culture and fecal PCR with blood PCR

	Fecal culture/Blood PCR		Fecal PCR/Blood PCR	
Pos/Pos	2	(0.7%)	4	(1.3%)
Pos/Neg	4	(1.3%)	11	(3.7%)
Neg/Pos	53	(17.7%)	51	(17.0%)
Neg/Neg	241	(80.3%)	234	(78.0%)
Total	300		300	
Agreement	81.0%		79.3%	
Kappa	0.03		0.04	
C.S.	7.3%		20.0%	

Table 3. Comparison between ELISA and blood PCR in healthy and suspected animals.

	ELISA/Blood PCR Healthy		ELISA/Blood PCR Suspected	
Pos/Pos	7	(4.7%)	5	(3.3%)
Pos/Neg	14	(9.3%)	23	(15.4%)
Neg/Pos	17	(11.3%)	20	(13.3%)
Neg/Neg	112	(74.7%)	102	(68%)
Total	150		150	
Agreement	74.5%		70.9%	
Kappa	0.19		0.015	
C.S.	7.3%		92%	

DISCUSSION

Agreement between pooled methods was generally low, but for healthy versus suspect blood PCR which was moderate. Healthy and suspect pooled ELISA showed a similar average estimate for flock and individual prevalence but costs were much lower.

The lack of significant differences between healthy and suspect groups observed in this study is in contrast with a previous study of Bhide et al. (2006) that detected *Map* in 5.9% apparently healthy animals and in 17.5% suspect cattle. One possible explanation for this result was that the species included in the study was different. Another explanation could be that MAP bacteraemia (actually DNA-hemia) is an indication of infection, but not of progress to clinical disease.

The results of this survey based in PCR assay in blood with pool samples suggests that this technique is easy to perform and soon will become a technically feasible approach

for the diagnostic of ovine paratuberculosis in apparently healthy animals and in clinical cases. The results of this study suggest that the health status is not closely related to the true level of infection with MAP.

CONCLUSION

The results of this study also suggest that the health status is not closely related to the true level of infection with MAP.

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

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ABSTRACT

The aim of this study was to investigate the risk factors for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) sero-prevalence in sheep in the Trás-os-Montes e Alto Douro region, Portugal. A structured questionnaire was used to collect information. The effects on sero-prevalence of several variables such as: individual characteristics; farm management practices; farm characteristics; animal health; and available veterinary services were evaluated. This information was used in a multivariable logistic regression model to identify risk factors for MAP sero-positivity. Univariable analysis was used to screen the variables used in the logistic regression model. Variables that showed $p<0.15$ were retained for the multivariable analysis. Multivariable logistic regression model identified a number of variables as risk factors: when the sheep were a pure local breed and/or a cross of a local breed (OR=2.02; 95% CI: 1.28, 3.18); a herd size of between 31-60 head (OR=2.14; 95% CI: 1.34, 3.41); culling during the Spring-Summer season (OR=1.69; 95% CI: 0.93, 3.08); the use of an anti-parasitic treatment such as Ivermectin as the only anti-parasitic medication (OR=5.60; 95% CI: 1.85, 16.99); use of Albendazole with other anti-parasitic treatment (OR=3.89; 95% CI: 1.71, 8.89); use of associations of Closantel and Mebendazol (OR=2.83; 95% CI: 1.13, 7.09). Considering the paucity of epidemiological reports in the region our results could make a useful contribution towards the prevention of ovine paratuberculosis in the area.

INTRODUCTION

Many factors can influence the prevalence and spread of paratuberculosis. Several individual and management practices have been identified as potential risk factors for the introduction and spread of the disease (Mainar-Jaime and Vázquez-Boland, 1998). There are a number of studies available which evaluate the potential risk factors associated with the transmission of paratuberculosis in cattle (Collins et al., 1994; Johnson-Ifearulundu and Kaneene, 1998; Daniels et al., 2002; Raizman et al., 2005). However, less is known about the transmission and spread of *Mycobacterium avium* subsp. *paratuberculosis* in sheep (Whittington and Sergeant, 2001). Only a small number of studies have been conducted to investigate epidemiological factors in this species (Mainar-Jaime and Vázquez-Boland, 1998; Lugton, 2004). In Portugal, risk factors for ovine paratuberculosis have not been previously defined. The evaluation of potential risk factors should contribute vital information towards planning and developing programs to prevent and control ovine paratuberculosis in the Trás-os-Montes e Alto Douro region.

MATERIAL AND METHODS

Study design

A cross-sectional study was carried out between September 2003 and May 2004. Sample size was determined using the program WinEpiscope 2.0®. Sample size (3900 sheep from 150 flocks) was calculated based on the list of flocks registered on the Ministry of Agriculture's official records for the Trás-os-Montes region. The population data from 2001,

which listed 3162 flocks of sheep, was used. Sample size was calculated using an expected prevalence of 10% and a confidence interval of 95% meaning that a total of 150 sheep flocks were randomly selected using the program WinEpiscope 2.0®. To simplify the sample collection process, the number of samples per flock was fixed at 26, and therefore only flocks of 26 head or more were taken into account. General information about individual characteristics, farm management practices, farm characteristics, animal health and veterinary services were obtained via a questionnaire that was administered to the managers of the farms.

Serological procedure

The collection and processing of sera samples and their identification have been described previously (Coelho et al., 2007). Antibodies for *Mycobacterium avium* subsp. *paratuberculosis* were detected through a commercial enzyme-linked immunosorbent assay (ELISA paratuberculosis screening test®, Institut Pourquier) (Coelho et al., 2007).

Statistical analysis

Statistical analyses were performed using SPSS software version 10 (SPSS Inc., Chicago III, USA, 2000). Univariable analysis was carried out using Qui-square test (χ^2) analysis. All variables from the questionnaire which showed $P<0.15$ at the 95% confidence level in the univariable analysis were subsequently introduced into a multivariable model (Hosmer and Lemeshow, 1989). The logistic regression coefficients (β) and their standard errors (S.E. β) obtained from the chosen model were used to calculate the adjusted odds ratios (OR) and their corresponding 95% confidence intervals. For logistic regression purposes, the status of each animal (positive/negative) was used as a dependent variable in order to identify any risk factors associated with the prevalence of the disease.

RESULTS

Univariable analysis

One hundred and forty-four sheep were found to be ELISA sero-positive (3.7%; 95% CI: 3.1, 4.3) (Coelho et al., 2007). Fifteen variables were associated ($P<0.15$) with sero-positivity in the univariable analysis. All of them were included in the logistic regression model.

Multivariable logistic regression analysis

Table 1 shows the factors associated with sero-prevalence to *Mycobacterium avium* subsp. *paratuberculosis* in the logistic regression model analysis.

DISCUSSION

In this study we report the results of a cross-sectional epidemiological survey conducted in the Portuguese region of Trás-os-Montes e Alto Douro. Results from the logistic regression model used to evaluate risk factors link sero-positivity to *Mycobacterium avium* subsp. *paratuberculosis* in local breeds and their crosses. Nevertheless, other authors have reported that sero-prevalence was higher in foreign breeds and their crosses (Mainar-Jaime and Vásquez-Boland, 1998). This observation, taken from the present study, could be explained by the differing management practices between ovine breeds since foreign-purchased animals are usually managed with more care, therefore reducing the spread of infection. The present study indicates higher levels of *Map* sero-prevalence among herd sizes of between 31-60 head than in smaller or larger herds. These findings are in contrast to those observed by Mainar-Jaime and Vázquez-Boland (1998), who reported that sero-positivity was higher in larger flocks of more than 200 head. These flocks were usually associated with mass management practices which are typically more difficult to control and allow for closer contact between animals and their environment. The reasons behind our results might be that smaller flocks are usually found on private farms. These farms are often found to favour traditional animal management procedures that can favour transmission of infection. However, this also applies to smaller flocks with less than 30 sheep. On the other hand, on large farms, infection is often prevented by the widespread use of measures which

promote hygiene, such as organized feeding and cleaning. In all probability, the results are related to other farming factors not identified in this study.

Table 1. Factors associated with sero-prevalence to *Mycobacterium avium* subsp. *paratuberculosis* in the logistic regression model analysis

variable	*β*	S.E.*β*	P	OR	95% CI[a] (OR[b])
Breed					
Foreign and their crosses				1.00	
Local and their crosses	0.73	0.23	0.002	2.02	1.28, 3.18
Herd size					
> 60 head				1.00	
31-60 head	0.76	0.24	0.001	2.14	1.34, 3.41
< 30 head	-0.70	0.44	0.108	0.50	0.21, 1.17
Duration of winter housing					
> 6 months				1.00	
3 a 6 months	-1.23	0.40	0.002	0.29	0.13, 0.64
> 3 months	-1.51	0.49	0.002	0.22	$8.5x10^{-2}$, 0.68
Season when culling takes place					
Any season				1.00	
Spring-Summer	0.53	0.31	0.086	1.69	0.93, 3.08
Autumm-Winter	$9.6x10^{-2}$	0.43	0.829	1.09	0.47, 2.54
Anti-parasitic treatment used					
Combination of Ivermectin				1.00	
Combination of Albendazole	1.36	0.42	0.001	3.89	1.71, 8.89
Ivermectin	1.72	0.57	0.002	5.60	1.85, 16.99
Closantel+Mebendazole	1.04	0.47	0.027	2.83	1.13, 7.09
Animals grazing on pasture in the same season manure was spread					
Yes				1.00	
No	-0.89	0.49	0.067	0.41	0.16, 1.06
Access to manure storage area					
Yes				1,00	
No	-0.57	0.35	0.109	0.57	0.28, 1.13

β: logistic regression coefficients; S.E.*β*: standads error; [a]odds ratio; [b]confidence interval

Another interesting result of our study is that there was a greater association with sero-positivity when culling was carried out in the Spring-Summer period. The season when sheep parturition is carried out also overlaps the Spring-Summer period. Although it is difficult to be certain, the rise in antibodies associated with parturition may account for our findings. These results could also be put down to cold winter conditions, poorer quality pastures, loss of fleece protection that may cause additional stress, and clinical diseases during Spring-Summer (Lugton, 2004).

One of the clearest predictors of paratuberculosis sero-prevalence in this study was the use of Ivermectin as the only anti-parasitic treatment. Ivermectin is a widely used macrocyclic lactone which acts against a wide variety of nematode and arthropod parasites. It is used for the treatment and control of parasites in cattle, horses, and sheep. The strong association between the use of Ivermectin and a higher sero-prevalence of paratuberculosis has not been previously reported. This association remained very strong even after controlling for potentially confounding variables in multivariable analysis. Lugton (2004) suggests that factors associated with the control of gastrointestinal parasites do not influence the disease. The reasons for this are unclear, but may be related to the fact that Ivermectin's anti-parasitic spectrum fails to include all types of parasites, or to an increase in anti-parasitic resistance (Köhler, 2001). This would increase the probability of sheep enduring increased exposure to parasitic infections, which could cause a sheep's body condition to deteriorate further than other animals and contribute to a higher sero-positivity. In our study, not

spreading manure on pasture which is to be grazed in the same season was a preventative factor. This is perhaps to be expected, given that the practice of spreading manure on pasture to be grazed in the same season is a management factor that has been associated with infection in previous studies (Raizman et al., 2005). One of the most important preventive factors for paratuberculosis was the prevention of access to manure storage areas. This discovery reinforces and extends the reports of several authors concerning sanitary strategies which benefit the flock. Limiting the exposure of animals to contaminated manure is an important measure, since *Map* is transmitted horizontally with the spread of manure (Sweeney, 1996; Daniels et al., 2002).

CONCLUSION

The risk and preventive factors detected in this study are closely associated with the traditional management systems used for small ruminants in the Trás-os-Montes e Alto Douro region, and proven management practices universally recommended for disease control.

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PCR detection of *Mycobacterium avium* subspecies *paratuberculosis* and its use on a limited survey of environmental water samples

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Mycobacterium avium subspecies *paratuberculosis* (Map) is the known cause of Johne's disease of ruminants and has been implicated as a cause of Crohn's disease in humans. Previous work has shown that Map is present in untreated water entering water treatment works (WTWs) in Northern Ireland. The work reported here was directed at extending this study by developing both molecular and culture methods for detecting Map and using them to conduct a limited survey of two local WTWs. These have the same source water, Lough Neagh, but have different water treatment systems viz. WTW1 is based primarily on slow sand filtration (SSF) while WTW2 is based on dissolved air floatation (DAF). The SSF process incorporates a schmutzdecke which is a biologically active 'dirty layer' responsible for most of the bactericidal effects while DAF causes particulate matter, including microorganisms, to flocculate and rise to the surface where they are physically removed. This work not only allowed the efficiency of the water treatment processes to kill or remove Map to be determined but also compare the two respective water treatment systems. The survey was carried out over a 9-month period to take account of seasonal effects and husbandry practices. The molecular method used was based on centrifugation, filtration and in-house immunomagnetic separation (IMS) followed by conventional and real-time PCR, the latter based principally on the IS*900* insertion element. The method was calculated to have a sensitivity of 10 Map cells ml^{-1}. Map was found throughout both WTW processes from source water to final treated water. No definite concentration of the organism was found at any particular stage. It is recognized that the PCR method employed does not distinguish between viable and non-viable cells. It is hoped that the culture methods that have been performed in parallel with these PCR assays will shed light on this question. It is also hoped that laboratory biofilm studies will provide more fundamental information on the behaviour of Map during both water treatment processes. Since water is one of the possible routes of transmission, the outcome of this work should contribute to a more meaningful risk assessment of the public's exposure to Map and hence inform on possible intervention strategies.

Association of farm management and soil risk factors with ovine Johne's disease in Australia

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Farm management and soil risk factors are known to effect ovine Johne's disease (OJD) prevalence, but little is known about their impact after adjusting for each other. This study aimed to evaluate both sets of factors simultaneously in 92 sheep flocks in Australia in 2004-05.

Pooled faecal samples were collected from an identified cohort of sheep in every flock to estimate OJD infection status and soil samples from the paddocks grazed by this cohort of sheep to measure soil characteristics. A questionnaire was administered to farmers by face-to-face interview to obtain information about husbandry and management factors. Multivariable ordinal logistic regression, generalised- and general-linear mixed model analyses were conducted to test the simultaneous association of management and soil factors with OJD.

Both farm management and soil risk factors were significant in the final models. OJD prevalence was higher in sheep whose dams were maintained at a higher stocking rate and had lower condition scores during lambing. Prevalence was also higher in flocks that grazed sheep along the roads shared by neighbours and that had adopted some OJD control practices. Soil organic carbon%, an indicator of soil organic matter content, had a positive linear association with OJD prevalence.

Our results suggest that both farm management and environmental factors are important in the epidemiology of OJD. Implications of the study results for OJD control programmes will be discussed.

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

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

Reduced incidence of Johne's disease in dairy cattle herds on a long-term herd management program

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

Mycobacterium avium subsp. *paratuberculosis* (MAP) infection in cull cows from Johne's herds

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INTRODUCTION

The distribution of MAP in tissues of cattle culled from known infected herds has not been well characterized especially relating the MAP tissue burden to previous fecal cultures over time. The objective of this project was to better correlate fecal shedding to MAP tissue bio-burden and to determine what proportion of fecal culture negative cows would have culture positive fecals and tissues at slaughter.

MATERIALS AND METHODS

Tissues and fecal samples were harvested from cows culled from 3 dairy herds. Samples were collected from a total of 174 animals. Five samples were collected from each animal; 2 intestinal lymph nodes, ileum, IC valve and a fecal sample. Fecal samples were process using the 3 day incubation method with HPC/BHI and tissues were processed using 2 day method in HPC. All samples were plated on solid media – Herrold's egg yolk with mycobactin J.

DISCUSSION

Of the 174 cattle with harvested tissues, 17 (9.8%) were previously fecal culture positive for MAP. Fourteen of 17 animals were positive at slaughter (82%) and 3/17 (18%) cattle had all 5 samples culture negative. Each of these three cows with negative tissues was previously fecal culture positive with only one cfu of MAP on one of four tubes of HEYM. These three positive fecal samples may have represented transient "pass-through" MAP from other cattle in the herd.

Of the 20 fecal culture positive cattle at the time of slaughter, 9 were characterized as heavy shedders in the fecal samples with over 100 cfu MAP/tube and the same 9 cows massively infected in each of the four tissues examined with more than 300 cfu MAP/tube. No other fecal culture positive or negative cows had such massive tissue infection with MAP. Interestingly 5/20 (25%) fecal culture positive cows at slaughter had negative cultures on all four tissues. Three positive fecal culture cows (very low shedders) had only one colony on the four tissues cultured, suggesting infections as adults. The other 3 cows had modest levels of MAP in several tissues.

Of the 156 cattle with all negative fecal cultures prior to culling, 58/156 (37%) had at least one positive sample at slaughter. Of these 58 cattle, 25 were culture positive only on one sample, 11 on two samples, 12 on three samples, 6 and 4 samples and 4 cows positive on all five samples. An intestinal lnn was positive most frequently, followed closely by ileum and IC valve. 26/58 (45%) cattle had less than 10 total cfu of MAP on 20 tubes of HEYM for the five samples suggests a more recent infection. While 18/58 (31%) cattle had the next higher tissue level of MAP ranged between 10 and 100 total cfu MAP for the 20 tubes, suggesting a heavier and or repeated doses over time as adults.

CONCLUSIONS

Cattle shedding more than a few colonies of MAP or had multiple positive fecal cultures had multiple tissues positive at high MAP concentrations. An estimated 35% of always culture negative cattle will have positive tissues at slaughter with a wide spectrum of MAP concentrations suggesting a moderate level of adult infections.

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A multi-species approach to control Johne's disease in New Zealand

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Johne's disease (JD) has significant economic impact on deer and to some extent on dairy cattle production while little is known about its effect on beef, sheep and dairy goat enterprises in New Zealand. Since *Mycobacterium avium* subtype *para-tuberculosis* (MAP) causes considerable economic loss and is a hypothesised human health hazard, considerable development efforts focus on the control of Johne's disease in New Zealand. This presentation summarises current research findings and evidence of inter-species transmission including domestic livestock and wildlife species. Options for control involving multi-species farming practices are evaluated leading to major new research objectives for the next five years.

Highlights of current evidence include a quantification of production loss and risk factors for clinical JD in dairy cattle, the impact of co-grazing with sheep and cattle and other risk factors on clinical JD in deer, evidence for a virulence difference between sheep and cattle strains, and performances of current and new diagnostic tests to detect sub-clinical infection with MAP.

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

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

Time from shedding of *Mycobacterium avium* subsp. *paratuberculosis* to occurrence of ELISA-positive cows

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Infections with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are characterised by long incubation periods. Diagnosis using repeated ELISA testing is preferred in the Danish control programme to detect infectious animals. Therefore, the time from testing positive by ELISA to MAP shedding was studied.

Repeated ELISA and FC results were available from 1892 dairy cows. The cows were divided into five shedding groups based on the FC results: Non-shedders (NS, n_{NS}=1507); potential transient shedders (TS, n_{TS}=40); intermittent shedders (IS, n_{IS}=116); low shedders (LS, n_{LS}=142); and high shedders" (HS, n_{HS}=87). Cows were defined as TS if a positive FC was followed by three negative tests; IS if had more than one positive FC, but no successive positive samples and the last sample was negative; LS and HS if had a series of positive samples, and FS if had a minimum of one sample with > 50 CFU / g faeces..

The time between entering the shedding group to testing positive by ELISA was assessed by a generalised additive model.

The results showed that 15 to 20% of cows in the three shedding groups IS, LS and HS were positive by ELISA one year prior to entering the shedding group. Among IS, 50% were ELISA positive when shedding was detected, whereas among low and high shedders 60% were ELISA positive when entering the shedding group. One year after detection of MAP shedding, 80 to 90% in the three groups had positive ELISA results. Only 9 (10%) of the HS cows were shedding > 50 CFU prior to being ELISA-positive. Among the TS-group, 40% had positive ELISA-reactions, of which most occurred 0 to 3 years after entering the TS-group. The latter result indicates that many cows that were classified as transient shedders were probably infected and should have been classified as intermittent shedders. In conclusion, although many shedding cows are detected by ELISA prior to shedding, a large proportion may only become ELISA-positive after shedding has started, but the amount of bacterial shedding is at low levels until the animal become ELISA-positive.

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

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Consecutive semen samples of a breeding bull suffering from paratuberculosis were collected over a period of one year and tested for the presence of *MAP*. Furthermore blood, faecal and after culling organ samples were collected. Semen was tested for *MAP* by IS*900* and *F57* Real – Time PCR, ELISA was used for the detection of specific antibodies in blood, faecal and organ samples were tested by culture on Herrold's Egg Yolk Medium and Ziehl-Neelsen staining.

All faecal samples taken during the trial were positive for *MAP* by culture, blood ELISA also gave a positive result at all times. At necropsy the bull showed typical signs of advanced paratuberculosis. *MAP* was detectable in multiple organs including the intestine, intestinal lymph nodes and reproductive organs. *MAP* was detected by IS*900* Real – Time PCR in all 9 (100%) semen samples and in 6 of 9 (66.6%) samples using the *F57* Real – Time PCR. Semen quality, especially viability of the sperm, was poor in collected samples. The finding that all semen samples were positive for the period of one year in the described case, underlines the potential risk for the spread of *MAP* by natural or artificial insemination.

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

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In February 2006 the Danish Cattle Federation initiated a national voluntary control programme “Operation Paratuberculosis” (“Operation PTB”). The programme focuses on closing transmission routes, because it has been demonstrated to be essential for the control of paratuberculosis in cattle herds. However, closing transmission routes is also difficult to practice, because persistence and much extra labour is needed. This amount of labour is reduced in “Operation PTB” which implies diagnostics of all cows 3-4 times per year by milk-ELISA, and – in cooperation with the herd health advisor – a contingency plan for changes in management and housing systems is made in order to reduce transmission of PTB. Transmission routes between calves and *all cows being diagnosed as infectious* should be broken effectively, and the most infectious cows culled before the next calving. The improvement of management is thus focused only on cows having been diagnosed as infectious instead of on the whole herd. Thereby the labour and time needed for improving management are reduced considerably.

The expected long-term effects of this strategy compared to alternative strategies were evaluated by simulation studies with the herd-simulation model PTB-Simherd. Scenarios were simulated in a herd with 200 cows (500 replications), an initial herd prevalence of 25% and an otherwise typical Danish herd management.

The simulated results of following “Operation PTB” were a reduction of prevalence from 25% to 5% after 5 years and less than 1% after 8 years, which makes this strategy just as effective in reducing prevalence as if management had been optimized for *all* cows in the herd. If no action was taken to control PTB, the prevalence would increase to 75% after 10 years. If transmission routes were not broken by improving management, but infectious cows were still culled within the next calving, the prevalence continued to increase to 26-39% depending on how quickly the infectious cows were culled after having the diagnosis. The economy of “operation PTB” compared to the economy of optimizing the management of *all* cows (which implies more labour hours, but no costs for tests) depends directly on the hourly rate and the time spent on optimizing management. If the extra workload per calving is assumed to be 1 hour, “Operation PTB” is the economically most attractive option in Denmark whenever the hourly rate exceeds 7 € (9 $).

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

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

Johne's Disease in a free-ranging White-tailed Deer from Virginia and subsequent surveillance for *Mycobacterium avium* subspecies *paratuberculosis*

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

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

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A panel was assembled of 168 field isolates of *Mycobacterium avium* subspecies *paratuberculosis* isolated from 19 different host species from the Czech Republic, Finland, Germany, Greece, The Netherlands, Norway, Spain and the United Kingdom. The panel was typed by pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism and hybridisation to IS*900* (RFLP-IS*900*), mycobacterial interspersed repetitive repeats and variable number tandem repeat (MIRU-VNTR) and amplified fragment length polymorphism. A total of 17 BstEII profiles were detected by RFLP-IS*900* analysis and the C1 profile was found to be the most predominant in Europe. Thirty one different multiplex PFGE profiles were detected using SnaBI and SpeI and the most widely distributed profile was [2-1]. Twenty five different MIRU-VNTR types were detected with INMV1 and 2 being the most widely disseminated. A few strains were found to be restricted to specific geographic locations although larger numbers are required to determine if this is significant. No evidence was found for species-specific strains and where details were available, wildlife isolates on a single farm were found to be identical to those of cattle on the same farm suggesting interspecies transmission. A comparison of the discriminatory power of the various techniques indicated that PFGE was the most discriminatory followed by MIRU-VNTR and if both of these techniques were combined the discriminatory power was sufficient for epidemiological surveys.

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

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

Review of prevalences of paratuberculosis in farmed animals in Europe

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Multiple studies have been carried out to assess the prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in farmed animals. However, most studies are not directly comparable because different diagnostic tests were used. True prevalences can be calculated from apparent prevalences if test-accuracy estimates for the diagnostic test used are available. The objective of the present study was to conduct a review of MAP prevalences among farmed animals in Europe.

Data about prevalence of MAP in all farmed animal species were included from a variety of literature databases. Information on target population and study design, tests used and apparent prevalences was recorded, and subsequently true prevalences were calculated when possible. A full critical review of the included studies indicated that although a wide range of studies have been conducted, credible true prevalences could often not be calculated. Based on a few studies in which the prevalences appeared plausible, it was concluded that prevalences of MAP would have to be guesstimates based on available data. Among cattle, approximately 20%, or a minimum of 3 to 7% were infected in several countries. Between-herd prevalence estimates appeared to be >50%. No countries appear to have published sufficient information to state that they have a low or a zero prevalence of MAP infections. In goats and sheep the only within-herd prevalence guesstimates were 14% and 2%, respectively, but these figures were based on Norwegian populations only. The between-herd prevalence guesses were >23%, based only on figures from Switzerland.

Variable Numbers of Tandem Repeats (VNTR) Typing of *Mycobacterium avium* subsp. *paratuberculosis* isolates in Japan

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Variable Numbers of Tandem Repeats (VNTR) typing is a nominee of new PCR-based molecular genotyping methods that are more rapid and discriminatory procedures than the most widely used IS900-RFLP. We have been developing the VNTR typing using 17 loci of tandem repeats for *Mycobacterium avium* subsp. *paratuberculosis* (Map). In this study, 678 strains of Map that were isolated in 203 farms (daily or beef cattle) in Japan were differentiated into 12 VNTR allele profiles that were named as Map-1 to 10, -14, and -15. Map-1 and Map-2 were 2 major profiles; 203 isolates of Map-1 (29.9%) and 361 isolates of Map-2 (53.2%) were detected in 74 farms (36.5%) and 118 farms (58.1%), respectively, but more than 2 types were detected in 20 farms. It was suggested that VNTR typing could be the first choice for molecular epidemiology of Map in Japan.

Bayesian methods for assessing genetic similarity and familial aggregation of paratuberculosis in beef cattle of unknown pedigree

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

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

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

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

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INTRODUCTION

The National Animal Health Monitoring System (NAHMS, 1996) has estimated that the annual cost to infected U.S. dairy operations is over $100 per cow in inventory, with higher costs of more than $200 per cow in inventory per year in herds with high infection levels. These estimations however were performed more than 10 years ago and were based on serum ELISA test results, which is known to have less sensitivity than bacterial fecal culture, and therefore can bias any prevalence estimation. The scientific literature provides limited information about the economical impact of *Mycobacterium paratuberculosis* (Map) fecal shedding in dairy cattle on lactation performance. Quantification of the monetary impact of Map fecal shedding or clinical JD on lactation performance is critical to participation by dairy cattle producers in JD control programs, because it enhances the relationship between stage of disease and economic loss. This information will allow dairy producers to make appropriate management decisions within their operations regarding implementation of control measures to decrease herd JD prevalence. The objective of this study was to evaluate the economical cost of *Mycobacterium paratuberculosis* (Map) fecal shedding prior to calving and of cows that were culled due to clinical Johne's disease (CCJD) during the subsequent lactation.

MATERIALS AND METHODS

1,050 cows from 2 Minnesota dairies were enrolled where fecal samples were obtained during the close-up period. Milk production, clinical diseases (other than CCJD), and reproductive performance data were recorded for each cow. The model was built in an Excel ® spreadsheet where we took into consideration the following parameters: Loss of value across lifetime of a cow: average loss in value per lactation (US$105), average slaughter value ($450), etc; Income over feed cost during the lactation (IOFC): milk price per pound ($0.14 milk price/lb), dry matter intake feed cost feed ($0.08/lb), cost to support maintenance in a milking cow/d ($1.76) and cost of feed/lb of milk/d above maintenance ($0.03); Reproduction managements costs: cost of an extra day open above a baseline of 85 days ($2.5), and cost of an insemination ($12). For diseases costs we considered only treatment, labor, and milk discard costs.

RESULTS

Among culled cows, mean culling loss of fecal negative and positive cows was US$779 and $727 ($p>0.05$). The cost per cow in the herd, however, was $265 and $545 for fecal negative and positive cows, respectively. Among culled cows mean IOFC for negative and positive cows was $1200 and $840 ($p<0.01$) and among non-culled cow IOFC for fecal negative and positive cows was $1960 and $1680, respectively ($p<0.05$). Mean IOFC for CCJD was $1075 and for light, moderate and heavy fecal shedding cows $1460, $960, and $370, respectively. Cost of disease for culture negative and positive cows was not significantly different. Mean reproduction cost for fecal negative cows was significantly higher than for fecal positive cows ($145 vs. $43; $p<0.001$), probably because of early culling of culture positive cows.

CONCLUSIONS

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

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

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Paratuberculosis (Johne's disease) is a chronic granulomatous enteropathy of ruminants. It affects primarily cattle, sheep, and goats and is caused by *Mycobacterium avium s*ubsp*. paratuberculosis (Map).* Paratuberculosis is a common disease in many countries worldwide, and its effects on production can be economically significant. In Chile paratuberculosis has been reported in cattle, sheep and more recently it has been described in goats. Preliminary data suggests that the infection is also present in Chilean wild animals such as guanacos, as well as in some other introduced wild animal species. Nevertheless, there is a lack of molecular epidemiological data on the type of strains existing in Chile. A deep knowledge of paratuberculosis infection transmission between and within both domestic and wildlife host species should be the basis to set up a national control programme. The classification of *Map* isolates using genomic typing methods provides some understanding of the infection. A method that detects a stable variation at base pair 223 in the *IS*1311 using polymerase chain reaction with restriction endonuclease analysis (PCR-REA) provides a fast and easy way to differentiate between cattle and sheep *Map* strains. This *IS*1311 PCR-REA analysis was used to detect genetic differences among 28 *Map* isolates from cattle (19), goats (9), guanacos (3) and deer (1) from different regions of Chile. All isolates were C-type and probably of bovine origin. These results showed no genetic differences between Chilean *Map* isolates from different geographic and host sources. Tracing with regard to these factors are epidemiologically important. The study described isolates of *Map* from different Chilean animal host species using *IS*1311. The typing result for the guanaco isolates indicates that these animals have not been infected from the sheep, more likely from cattle or goats. PCR-REA as a simple and rapid test that can be used on a range of diagnostic samples for the confirmation of paratuberculosis and will be of benefit in control and eradication programmes for this disease.

A serological survey of paratuberculosis in Finnish suckler Herds

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In Finland, symptomatic paratuberculosis was recorded for the first time in 1992 since the early 1900s. During the years 1992–2000 paratuberculosis was detected in five beef herds. After that paratuberculosis was not found in beef cattle, and it has never been detected in dairy herds.

This serological survey will give information on the spread of paratuberculosis among beef cattle in Finland. The results will be used in risk analysis of paratuberculosis: to evaluate the risk of beef cattle for spreading paratuberculosis to dairy herds, to plan control measures to combat paratuberculosis, and to assess the need of a specific control program.

About 14 500 blood samples from about 1450 suckler herds were collected at slaughter in 2003 – 2005 to survey bovine viral diarrhoea virus. From these samples, those from over three-year old animals are selected to determine antibodies to paratuberculosis. With the help of the identification number, we can find the age, breed, sex and potential foreign origin of the animal in the national data system Elite. About one third of the samples are from cattle over three years old. The serum samples are examined with ELISA test (Parachek) and the results will be presented in the poster.

This study is supported by the Walter Ehrström Foundation.

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

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Poor reporting of Johne's disease (JD) in India is mainly due to lack of diagnostic kits. Information on prevalence of JD in domestic ruminants was extracted from literature, compiled and presented here. Part A reports prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in domestic livestock in North India, using sensitive culture, ELISA kit and PCR tests and includes the work on Johne's disease by us (1985 – 2007), at Central Institute for Research on Goats (CIRG). Part B includes works of other Indian workers from 1940. Retrospective information (Part A and B), was collected from published works and less accessible records like thesis/dissertation, project reports and annual reports of institutes. Published data includes serum samples submitted to Microbiology lab (CIRG), for screening of suspected cattle (26) and buffaloes (15), from Indian Veterinary Research Institute, Bareilly, UP and on 69 samples from prospective cattle bulls (24, Sahiwal and Hariana breed) and Buffalo bulls (45 Murrah breed), earmarked for purchase from Rohtak district of Hariana from farmer's herds. These animals were screened using indigenous serum ELISA kit. Using Milk culture the presence of MAP in milk of lactating goats and cows (including commercial milk and milk products) in South UP and Punjab was 5.0–91.6%, Using milk ELISA kit, prevalence was 37.3-54.7% and 18.1-88.4%, in goats and dairy cattle, respectively from South UP and Punjab. Sero-prevalence of MAP on the basis of serum ELISA kit was 8.5–65.2%, 11.1-61.9%, 17.3–42.6% and 8.6-46.7% in goats, sheep, cattle and bulls and buffaloes, respectively in South UP, West UP, Punjab, Rajasthan and Tamil Nadu states of country. Prevalence on the basis of fecal culture, was 52.1-80.0%, 13,3-37.5%, 28.3-96.1%, 23.8% and a case report in goats, sheep, cattle, blue bulls and Hog deer, respectively in South UP, Punjab and North India. On the basis of tissues culture in South UP, recorded presence of MAP was 22.2-65.2%, 42.3-58.9% and 48.0% in goats, sheep and buffaloes, respectively. Using IS900 PCR, the presence of MAP in different samples and animal species was recorded..By using colony PCR, the DNA from different sources was characterized as MAP. Vohra (2005), reported 37.9% prevalence of MAP in vaginal secretions by culture from post parturient farm goats in South UP. Singh (1998, reported 11.3% prevalence of MAP, by fecal culture, in 12 farms of goats in North India in 1998. The prevalence was highest in Jamunapari farm of Etawah (13.4%). Using microscopic examination of fecal samples of farm goats, variable prevalence has been reported by authors (10.6-82.6%) in South UP and North India. Microscopic examination of target tissues of farm young goats in South UP by Hajra (2003), recorded 31.0% (74) samples positive. In fecal samples of farm sheep, 10.4% animals were reported positive. Reported prevalence was 17.3-96.1% and 8.6–50.0% in cattle and buffaloes, respectively in North India. Part B: prevalence in cattle and buffaloes reported by other workers in India was 1.4-66.6% and 4.9-8.5%, respectively. Similarly in goats and sheep, reported prevalence of Johne's disease by other workers in India and Nepal was 2.0-14.1% and 9.7-24.0%, respectively.

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

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

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

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

Association between Johne's Disease milk ELISA test result and milk production and breed in Canadian dairy cows

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Various studies have shown that cows with clinical and subclinical Johne's disease (JD) are likely to produce between 4 and 24% less milk than test negative cows. The objective of this study was to quantify the difference between the milk production of JD milk ELISA positive cows and test negative cows, as well as to evaluate variations in the test results due to breed.

The data included the test day information from 47,418 cows from 817 CanWest DHI herds in Ontario, Manitoba, Saskatchewan, Alberta and British Columbia. The cows were tested between March 2005 and April 2007 with the AntelBio Johne's Milk ELISA. The optical density (OD) cut points for a suspect or positive test result were set at > 0.065 and > 0.1, respectively. A mixed model was fitted to investigate the association of a positive milk ELISA test with the estimated cumulative 305 day milk production. Only factors with a p-value < 0.01 were retained in the model. A second model was fitted to examine the relationship between breed and observed test result. The SAS Glimmix procedure was used, including only herds with at least one positive cow. Herd was included in both models as a random effect.

The number of tested cows per herd varied between 1 and 342 (Interquartile range: 35 -71). The breeds were Holstein (n= 44,420), Jersey (n= 2,078), Brown Swiss (n= 361), Ayreshire (n= 308), Guernsey (n=141), and Milking Shorthorn (n= 110). The majority of the cows (n= 46,515; 98.1%) tested negative, while 753 tested positive (1.59%) and only 150 cows (0.32%) had suspect test results. Therefore, positive and suspect cows were combined into one JD positive group. The percent of positive cows by province varied from 0.74% to 2.27% ($p < 0.0001$).

The average 305 day milk production was 9,696 kg/ cow. After adjusting for breed, lactation number, season of calving, somatic cell count, days in milk on test day as well as several interactions among these factors, cows that tested positive for JD produced 748 kg or 7.7% less milk over the estimated 305 day lactation compared to milk ELISA test negative cows ($p < 0.001$).

The between breed comparison was adjusted for the lactation number, province, days in milk on test day and somatic cell count. Guernsey and Jersey cows were 3.9 and 2.3 times as likely to test positive for JD as Holstein cows ($p \leq 0.0001$).

These findings indicate differences in JD prevalence across the 5 western most provinces in Canada. Furthermore, cows testing suspect or positive with the milk ELISA test produced significantly less milk than their negative herd mates and cows of the Channel breeds were more likely to test positive than Holsteins. Further research is needed to examine the differences in dairy herd management practices among the provinces that might explain the variations in the JD prevalence, and the cause for the variation in the test results among the different dairy breeds.

Safety and efficacy of Silirum® Bovine Johne's Disease Vaccine in an experimental bovine challenge model

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The objective of this study was to evaluate the safety and efficacy of a killed *Mycobacterium paratuberculosis* vaccine (Silirum®, Pfizer Animal Health) using a bovine experimental infection model. Twelve newborn Holstein male calves were randomly assigned to one of two groups. The vaccinated calves (n=6) received a single dose of vaccine, administered subcutaneously in the side of the neck, at 14 days of age. Control calves were sham vaccinated with placebo. All calves were given an oral challenge of 10^9 CFU live field strain *Mycobacterium paratuberculosis* (MAP), administered on days 35 and 36 of age. Body temperature and injection site diameter were measured periodically following vaccination. Blood samples were collected at various time points for measurement of antigen specific release of Interferon-gamma by peripheral blood cells (Bovigam®, Prionics) and for ELISA testing for detection of serum antibodies against MAP (Paracheck®, Biocor). Calves were euthanized at 98 days of age and 32 tissues collected for culture of MAP, using both solid media (Herrold's Egg Yolk Media) and liquid media (MGIT®, Becton Dickinson). Total number of tissues that were culture positive, as well as the total number of CFU/calf on HEYM were compared for the two groups. For liquid culture, time to signal positive (TTP) was compared for the two groups, with a more rapid TTP indicating a higher concentration of MAP in the original sample. MAP-induced IFN-gamma release by prescapular lymph node cells cultured in-vitro was measured.

Following vaccination, there was a transient rise in body temperature (approximately 1 degree F), significantly different from control, on Days 1 and 2 following vaccination, with a return to baseline on Day 3. Vaccinated calves had visible swelling at the injection site that persisted throughout the study, but swellings were not painful and did not develop drainage. Vaccination sites were culture-negative for MAP at the conclusion of the study.

ELISA testing for serum antibodies to MAP gave negative results for all calves in both groups. Vaccinated calves had significantly higher IFN-gamma release by peripheral blood cells, and by prescapular lymph node cells, compared with controls.

Vaccinated calves had significantly reduced colonization of tissues by MAP, compared with control calves, whether measured by CFU/calf in the HEYM system or TTP in the liquid media system. There were on average, 22 tissues positive per calf in the vaccinated group compared with 28 per calf in the control group. There was a greater than seven-fold reduction in CFU/gram tissue in vaccinated calves compared with control, and TTP in liquid culture was 10 days longer on average for vaccinated calves. All of the above differences were significant at $P<0.05$. We conclude that Silirum® vaccine was associated with reduced tissue colonization by MAP when administered to calves.

In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*

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

Industry and government partnerships - an alternate model for national disease control programs

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INTRODUCTION

Johne's disease occurs at a very low prevalence in the Australian beef cattle population with known infected herds accounting for less than 0.1% of the nation's herds. About two-thirds of the beef herds are located in Free and Protected Zones. Control in most areas has primarily been through a highly regulated approach involving herd testing, preferential culling and quarantine of known infected herds and zoning to prevent the spread of disease.

Fig. 1. Zones for the control of bovine Johne's disease in Australia, October 2007

OBJECTIVES

To improve control of Johne's disease, principal stakeholders in the Australian national program aimed to establish a strong partnership to provide a broad range of support to beef producers, whether their herds were affected by Johne's disease or likely to be free of infection. This program encourages individual producers to become more aware of Johne's disease, understand how infection spreads and manage the risks associated with the purchase of stock.

APPROACH

The national cattle industry, through the Cattle Council of Australia (CCA), recognised the negative social and economic impacts of regulatory disease control programs on known infected and suspect beef herds and on sales of cattle from beef herds in zones in south-eastern Australia (where Johne's disease is more common in the dairy industry).

This has been the catalyst to develop new approaches to managing and controlling Johne's disease based on

- a standardised industry risk assessment called *Beef Only*
- voluntary herd testing
- support for disease identification through field investigations
- assistance to resolve Infected and Suspect status.

Standardised industry risk assessment called Beef Only

Herd owners who complete a written declaration that their cattle satisfy the nationally standardised criteria to be given the low risk status, *Beef Only*, enjoy improved trading access and market prices.

A herd can qualify for *Beef Only* if all the animals in the herd:

1. Have not been part of a herd which is classified as Infected Suspect or Restricted according to the National Johne's Disease Standard Definitions and Rules for Cattle, and
2. Are from a beef herd which has had no contact with dairy cattle or dairy-cross cattle at any time during the previous five (5) years, unless those dairy cattle were from a herd enrolled in the Australian Johne's Disease Market Assurance Program for Cattle (Cattle MAP), at the time, and,
3. Have not grazed on land that, in the previous 12 months, had been grazed by adult dairy cattle (2 years old or older) except where those dairy cattle were from a herd enrolled in the CattleMAP at the time and,
4. Are individually identified under the National Livestock Identification Scheme, as they enter (other than by birth) or leave the herd.
5. Any animals that were not born in the herd must have been introduced into the herd or onto the property from herds, which are of the same (*Beef Only)* or higher status for Bovine Johne's Disease (BJD) and came with a BJD vendor declaration to that effect.
6. All animals must be identified on, and accompanied by, an approved vendor declaration issued by the owner/person in charge of the animals, which declares that all the above criteria have been met.

Governments provide the regulatory framework for auditing *Beef Only* sales and approving the movement of *Beef Only* cattle to Protected Zones. To date in 2007, 702 lots representing 348 owners across Australia were audited at saleyards, with a compliance rate greater than 95%. Governments also provide penalties through legislative support if producers provide misleading information about the status of their herd. Table 1 provides detail on the audits that have been conducted in 2007.

Table 1. *Beef Only* Audits conducted for the period January – September 2007

	Routine audits		**Intensive audits**		**On farm audits**
	No of lots	**No of vendors audited**	**No of lots**	**No of vendors audited**	
Victoria	291	137	330	173	12
South Australia	63	20	18	18	3
TOTAL	354	157	348	191	15

Table 2. Number of herds and cattle tested May 2001 to 31 December 2006

State	Total No. of Herds Tested	No. of Herds ELISA +ve	Total No. of Cattle Tested	No. of Cattle ELISA +ve	No. of Cattle Infected
Victoria	257	13	15,696	25	1
South Australia	363	17	24,515	27	0
New South Wales	832	26	9,144	8	0
Tasmania	146	2	41,817	26	1
Total	**1598**	**58**	**91,172**	**86**	**2**

Voluntary herd testing
Cattle Council provides a rebate on voluntary herd testing undertaken to confirm the low risk of individual beef herds. Table 2 confirms the low prevalence of BJD in the Australian beef herd. All beef producers, even those who are ineligible for the *Beef Only* status, or wish to achieve an even higher level of assurance by participating in the CattleMAP, a quality assurance program based on testing and farm biosecurity, can access this rebate. Cattle that are identified as ELISA positive routinely undergo further investigation by either individual faecal culture or slaughter and histopathology.

Support for disease identification through field investigations
Subsidies are available for private veterinarians who conduct field examinations of higher risk beef animals that present with weight loss and scouring.

Assistance to resolve Infected and Suspect status
CCA has also worked with Animal Health Australia and state governments on improved strategies for infected and suspect beef herds. The beef cattle industry funds two special counsellors as part of a National BJD Financial and Non Financial Assistance Package (the Package). The counsellors, who coordinate both social and financial support for *Beef Only* producers who voluntarily enter into programs to eliminate Johne's disease from their herds, are the first point of contact for producers who wish to access the benefits of the Package. The assistance package is available to all beef producers whose beef herds have been confirmed as infected with BJD or are classified as suspect for BJD.

Suspect herds
Owners of herds that are officially suspected of being infected with BJD are now eligible for assistance to investigate and resolve the status of their herds. This may involve screening the herd by blood test and then investigating the infection status of cattle that react to the test. Assistance is provided for testing and any necessary slaughter of reactors to resolve their status and is provided at the same assistance levels as outlined below for infected herds. If a suspect herd is confirmed to be infected, it will then be eligible for the assistance that is available to infected herds.

Infected herds
There are two components to the assistance package for owners of infected herds; a non-financial component that includes access to a BJD counsellor to assist the producer consider management and trading options, develop a business plan and liaise with the supervising veterinarian. The second is a financial component that is available to producers who meet specific criteria and who are willing to implement an Enhanced Property Disease Management Plan with the long-term goal of eliminating BJD from their herd. The financial support is not a compensation package. It provides assistance to producers to identify and remove for slaughter high risk animals from within the herd.

Since its inception in July 2004, 105 producers have accessed the Financial and Non Financial Assistance Package and in excess of A$2 million has been allocated to eligible producers. Government veterinarians work in partnership with the counsellors by providing sound technical advice upon which to base the disease eradication program in each herd.

CONCLUSION
The *Beef Only* status has improved risk awareness of Johne's disease resulting in increasing numbers of beef producers sourcing replacement breeding cattle from these herds. For those producers whose herds are identified with BJD Assistance is available for them to resolve their status. A recent external review of the Package has confirmed the program is addressing most of the earlier negative impacts on owners of infected and suspect herds, by reducing the social impact of BJD on herd owners, the economic impact, and following eradication reduces the trade impacts associated with regulation. Participation in the Package is also improving producer understanding about animal health risks.

Paratuberculosis diagnosis and control in Thailand

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ABSTRACT
Paratuberculosis in Thailand was first observed in an imported bull in 1981 and the diagnosis was made from the observation of chronic diarrhea and pathological examination. Since 1987, serological tests such as complement fixation test (CFT) have been carried out for detecting antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (MAP). From 1987 to 1996, infected dairy herds were tested, and the results showed that the percentage of sero-positive results ranged from 0.58 to 13.2%. During 1997-2006, serological surveys were conducted on serum samples of both of dairy and beef cattle collected nationwide. The results of sero-positive animals were from 0.37 to 1.53%. Thirty sero-suspected cattle that had been tested during 1991-2005 were re-examined. Fourteen of these 30 cattle were diagnosed as paratuberculosis using serological tests, bacterial culture and pathological examination. Among those confirmed infected animals, 6 of 14 (43%) cattle were imported from foreign countries. In addition to the serological routine diagnosis, the fecal samples were also collected for bacterial detection by acid fast staining and isolation. The results showed that 24 out of 3,970 fecal samples were positive for paratuberculosis, and they included 12 fecal samples from the infected cattle. For a successful control of paratuberculosis, it is advised that the field veterinary official be trained, as they will play an important role by giving advices to farmers who will eventually accomplish the program. At present, control of paratuberculosis has been accomplished by serological survey throughout the country; this also allows the evaluation for the disease status. For sero-positive cattle, it is imperative that fecal samples are collected for the confirmations by acid-fast staining and cultivation. Since paratuberculosis is found throughout the country, it is included to the National Control Program.

Key words: control, diagnosis, paratuberculosis.

Introduction
Paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Diagnosis is based on clinical signs and serological test, and definitive diagnosis is carried out by cultivation and identification of the causative organism. In Thailand, the first case was reported from an imported bull which developed chronic diarrhea (Intarachote et al., 1981). Paratuberculosis was selected as 1 of 5 major animal diseases for epidemiological survey, research activities, and the effective control program granted by Japan International Cooperation Agency (JICA) to Department of Livestock Development (DLD).

Diagnosis of paratuberculosis by complement fixation test (CFT) and Johnin test were carried out in the central part of Thailand (Ekgatat et al., 1991). Pathological observation and bacteriological cultivation were performed for MAP identification (Ekgatat et al., 1997). Development of the ELISA was carried out for potential application for survey and control of the disease in Thailand (Ekgatat et al., 2001). Nowadays, CFT in parallel with an indirect ELISA (iELISA) system is used as routine serological diagnosis in dairy cattle.

MATERIALS AND METHODS
Serum samples
Sera from a total of 86,259 cattle were collected from several projects during 1987-1996, and infected cattle from many herds were identified by CFT. In addition, 649,503 samples were collected during 1997–2006 from all parts of country under the national control program for brucellosis, tuberculosis and paratuberculosis.

CFT

The antigen was prepared from MAP Teps strain (provided from NIAH, Japan) and optimized with positive serum for CFT cold-system (personal communication, NIAH,Japan, Ekgatat et al., 1993).

ELISA

The ELISA was used as described by Yokomizo et al. (1991) and modified by Ekgatat et al. (2001).

Bacteriological and Pathological investigation

The 3,970 fecal samples were collected from sero-positive and suspected animals with no data of bacterial isolation and identification. The feces were inoculated on Herrold's Egg Yolk medium (HEYM) with and without Mycobactin J and observed weekly for 20 weeks. Identification criteria were based upon acid-fast staining, size and morphology of the colony, and dependence of the colony on mycobactin. For pathological investigation, 30 suspected cattle were carried out for necropsy, and gross lesions of MAP infection were observed. Mesenteric lymph nodes and intestines were also taken for histopathological examination and bacteriological isolation.

Control measures

The disease control strategy is the identification of the causative agent of MAP followed by a series of effective biosecurity procedures. Following the test results, infected animals should be culled immediately as economic consideration permits. It is also important that the information and knowledge for the control strategy be transferred to regional veterinary officials in order to establish the network of national control program.

RESULTS

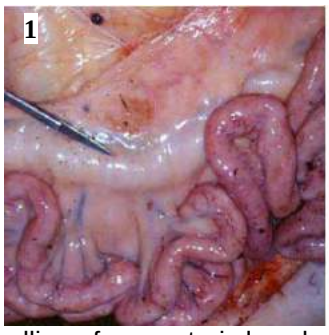
Serological testing

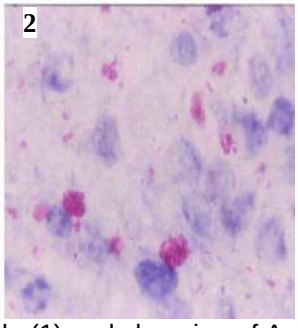
Samples from suspicious cattle herds were collected during 1987 to 1996, and the results showed that the infected cattle were from 0.58 to 13.17%.

During 1997 to 2006, serological surveys were conducted on serum samples of both of dairy and beef cattle collected nationwide. The results showed that sero-positive animals were from 0.37 to 1.53% (Table 1).

Bacteriological and Pathological investigation

From 1991 to 2005, necropsy of 30 cattle suspicious for MAP by CFT or ELISA was performed for MAP identification. Macroscopic examination of 17 out of 30 cattle showed thickening and/or hemorrhage of intestinal mucosa**.** Fourteen out of these 30 sero-positive cattle were also diagnosed as paratuberculosis based on bacterial culture and/or pathological examination (Table 2). In addition, feces of all suspected cattle were collected for MAP isolation, and the result showed that 24 out of 3,970 fecal samples were positive for MAP with 12 fecal samples from the post-mortem being included (Table 3).





Pictures 1-2. Swelling of mesenteric lymph node (1) and clumping of Acid Fast Bacilli (2)

Control measures

The information and knowledge of the disease and control measurement were transferred to 7 Regional Veterinary Research and Development Centers and regional veterinary officials. Consequently, the disease-related knowledge was also conveyed to farmers in an attempt to promote sound animal husbandry and management and to increase public awareness regarding the economic loss potentially caused by paratuberculosis.

Table 1. Sero-prevalence of bovine paratuberculosis during 1987 - 2006

Year	Type	Tested	Positive	Pct.
1987*	Dairy	357	47	13.17
1988*	Dairy	386	30	7.70
1989	Dairy	1540	74	4.80
1990	Dairy	5134	279	5.40
1991	Dairy	15669	165	1.00
1992	Dairy	6515	52	0.79
1993	Dairy	12168	94	0.77
1994	Dairy	21088	171	0.81
1995	Dairy	21517	241	1.12
1996	Dairy	1885	110	0.58
1997	Dairy	12342	52	0.42
	Beef	12368	70	0.56
1998	Dairy	11211	77	0.69
	Beef	5973	19	0.31
1999	Dairy	10337	123	1.19
	Beef	3398	20	0.58
2000	Dairy	20766	79	0.38
	Beef	9936	35	0.35
2001	Dairy	49234	460	0.93
	Beef	17236	113	0.65
2002	Dairy	51311	689	1.34
	Beef	7528	42	0.55
2003	Dairy	58608	1008	1.71
	Beef	15591	133	0.85
2004	Dairy	113768	1166	1.02
	Beef	20052	138	0.68
2005	Dairy	114440	1482	1.29
	Beef	15824	67	0.42
2006	Dairy	46206	846	1.83
	Beef	258	53375	0.48

*Infected herds

Table 2. Results of serological, bacteriological and pathological examination of 30 cattle

Types	Clinical	Gross	Serology		Bacteriology		Pathology	Dx		No. of animals	Imported animals
			CFT	iELISA	AFB	Isolate	Histo	AFB			
A	+	+	+	+	+	+	+	+	+	3	3
B	+	+	+	+	+	+	+	+	+	6	0
C	-	+	+	+	+	+	+	+	+	2	0
D	-	+	+	+	+	+	+	+	+	1	1
E	+	+	+	+	-	-	+	-	-	2	2
F	-	-	+	+	-	-	#	-	-	1	1
G	-	+	+	+	-	-	-	-	-	3	0
H	-	-	s	+	-	-	-	-	-	3	0
I	-	-	+	-	-	-	-	-	-	4	0
J	-	-	s	-	-	-	-	-	-	5	0
	11	17	30	21	12	12	14	14	14	30	7

CFT = complement fixation test AFB = acid fast bacilli Dx = diagnosis Histo = histopathology
# mast cell +4

Table 3. Comparison of CFT and MAP isolation from feces

Serology CFT	Samples	Isolation	
		Positive	Negative
+	348	20	337
+	3373	1	3372
-	161	0	161
No data	88	3	85
Total	3970	24	3955

DISCUSSION

From 30 cattle examined by necropsy, 14 suspected cattle were identified as paratuberculosis (Table 2). The negative result may due to (1) MAP shed small numbers of organisms into feces or (2) observation was carried out only one time of investigation at post–mortem. Sampling of the ileal and caudal jejunal lymph nodes was very important for histological analysis (Gonzalez et al., 2005). However, only 24 samples showed positive results by fecal culture from 3,970 samples; this is partly because of the limitation of fecal culture. The single culture using HEYM may not be able to identify specimens loaded with low number of bacteria (Harris et al., 2005). The serological test could, however, be used to identify these suspicious specimens which may be infected with MAP as at an early state, whereas the pathological examination revealed well developed lesions or pathological changes at later stage or in chronic infections. The results of bacterial isolation and pathology were compatible. The amount of bacteria detected by isolation was apparently sufficient for the progressiveness of significant pathological lesions (Collins, 2003). Thus, the selective criteria for diagnosis of paratuberculosis in animals in Thailand were based on bacterial isolation and identification, acid fast staining, and serological analysis.

From the surveys conducted for more than 20 years, it is clear that paratuberculosis and the infected animals can be found throughout the country (Table 1). However, the prevalence was generally low, obliging that the surveillance be continuously conducted for regular and early detection of the sero-positive animals before shedding of bacteria occurred. Calves are more susceptible to infection than adult cattle (Collins, 2004). Although the disease resistance may vary within animals of different ages, infection by MAP can be found in cattle at any ages particularly when susceptible, naïve animals are introduced into an infected areas. The control strategies must include managements at 3 levels, consisting of managements of newborn cattle, managements of infected animals, and biosecurity management. However, the control program can only successfully be accomplished if good cooperation between livestock officials and farmers is carefully established. The knowledge transferred from officials should focus on farmer's awareness regarding the economic loss potentially caused by paratuberculosis.

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

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ABSTRACT

Due to the documented increase of *MAP* (*Mycobacterium avium* subsp. *paratuberculosis*) in Austria (Baumgartner et al., 2005) the Regulation of the Austrian Federal Ministry of Health and Women on monitoring and abatement of clinical paratuberculosis in ruminants (Paratuberculosis-Regulation) came in force in April 2006. Animals showing clinical signs of paratuberculosis have to be notified followed by culling of the effected animal if the disease is confirmed by laboratory testing.

A survey of the current situation concerning paratuberculosis in ruminants in Austria, the most important parts of the new compulsory measures and their practical implementation are presented in this paper.

PARATUBERCULOSIS IN AUSTRIA

More than 2 million cattle on almost 83.000 farms have been counted in Austria in 2005 (Kalcher et al., 2006). Milk production, beef production and sale of breeding cattle are the main branches of the Austrian cattle industry.

Two nationwide studies, performed in the years 1995 to 1997 and 2002/2003 revealed a significant increase in cattle herds showing specific antibodies against *MAP* from 6.97% to 19.05 % (Baumgartner et al., 2005). In contrast to the cattle population *MAP* does not seem as widespread in sheep and goats (Khol et al., 2006).

LEGAL REGULATIONS

Due to the documented increase of *MAP* in Austria and the ongoing discussion about a possible link between paratuberculosis and Crohns disease in humans (Nakase et al., 2006; Pickup et al., 2004) the “Regulation of the Austrian Federal Ministry of Health and Women on monitoring and abatement of clinical paratuberculosis in ruminants (Paratuberculosis-Regulation)” came in force in April 2006.

The regulation affects cattle, sheep, goats and farmed deer. Animals showing clinical signs of paratuberculosis have to be notified to the district veterinarian and separated. Slaughtering and use of milk of these animals is prohibited. The major symptoms leading to the suspicion of clinical paratuberculosis are listed in the regulation. Blood and faeces of suspicious animals are taken by the district veterinarian and sent to the National Reference Laboratory for Paratuberculosis of the Austrian Agency for Health and Food Safety.

If an individual is showing severe emaciation during slaughtering, which could have been caused by *MAP*, tissue samples including liver and intestinal lymph nodes as well as parts of the small intestine have to be sent to the reference laboratory for testing by Polymerase Chain Reaction (PCR). The same actions have to be taken if signs of clinical paratuberculosis occur in animals that die or are culled.

Samples of suspicious animals are tested and adjudged by ELISA and if necessary also tested with PCR by the National Reference Laboratory for Paratuberculosis. Clinically ill, *MAP* positive animals have to be culled within 3 days. Furthermore, hygienic precautions listed in the Paratuberculosis-Regulation have to be performed. The list of hygienic measures includes hygiene at birth, rearing of young livestock and general actions to prevent the spreading of the infection. Compensation for culled animals depending on age and value of the animal is paid by the government.

Meat of slaughtered animals found *MAP* positive is declared unfit and has to be disposed.

Whenever an animal showing clinical signs or emaciation is diagnosed as MAP positive by the reference laboratory the farm of origin has to be placed under the control of the district veterinarian for further cases of clinical paratuberculosis.

Due to the difficulties in the diagnosis of subclinically infected animals, ruminants with a positive ELISA or PCR result for *MAP* but not showing any clinical signs are not affected by the regulation.

For a more detailed description of the compulsory abatement of paratuberculosis in Austria, diagnostic procedures and first results of implementation please see Khol et al. (2007).

DISCUSSION AND CONCLUSION

Many different countries have established voluntary programs to control paratuberculosis and prevent further spreading of the disease. This helped to reduce clinical disease and spreading of infection for participating farms or areas but due to their voluntarily character could not be applied to the entire livestock population (Kovich et al., 2006; Sockett, 1996). Only in Sweden, which has a very low incidence of paratuberculosis, the disease is compulsory notifiable in all animals, followed by stamping out of infected herds. Beside Sweden, Austria is the second European country to declare clinical paratuberculosis a notifiable disease. Although, due to insufficient way of diagnosing subclinically infected animals, only ruminants showing clinical paratuberculosis are affected by the law, the Austrian regulation on monitoring and abatement of clinical paratuberculosis in ruminants (Paratuberculosis-Regulation) could be an important step in the fight against this disease. It has been reported that animals showing clinical signs of paratuberculosis signify only the tip of the iceberg and that there is always a significantly higher number of subclinically infected animals (Whitlock, 1996). But on the other hand a severely affected animal can shed high amounts of *MAP* to the environment and some so called “super-shedders” can even excrete more bacteria with faeces than 20.000 low shedding animals (Whitlock et al., 2005). The reduction of these “super-shedders” in livestock can cause a significant decrease of *MAP* in farms and environment and might help to prevent further spreading of the disease.

Regulation of an animal disease always introduces social and economic disincentives for farmers and veterinarians to notify the authorities. One incentive, beside the fact that it is an offence not to comply with the regulation, for farmers to participate in the program could be the compensation paid for culled animals by the state. Industrial and public pressure as well as economic impact of the disease for the farmer could work as an additional force and overbalance possible disadvantages for the farmer by being known to have *MAP* positive animals. Another advantage of declaring clinical paratuberculosis a notifiable disease with compulsory culling of affected animals and compensation is that consequently the disease is recognized as important by farmers, veterinarians and consumers.

Paratuberculosis is a worldwide problem that cannot be controlled by one country alone. Actions on a European level are necessary to prevent further spreading of the disease and to protect *MAP* free livestock. Better diagnostic tools for young and subclinically infected animals are needed followed by measures in livestock trading to decrease the prevalence of paratuberculosis in ruminants.

The future will show if the National Paratuberculosis-Regulation can fulfil the expectations of the lawmakers and is a suitable tool for the reduction of paratuberculosis in Austria. For sure a lot of information and awareness training is necessary to persuade those who are keeping and working with ruminants to follow the guidelines of the new regulation.

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Present approach to Johne's disease control in Japan: features and incidence

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INTRODUCTION

Johne's disease, designated as a notifiable disease in Japan since 1971, was detected more frequently in the early 1980s (Yokomizo, 1999). After annual identification reached more than 200 cases in the mid-1990s, the law and regulations were amended in 1997 to include active disease surveillance, in addition to passive surveillance. In active surveillance, the minimal requirement is to have all targeted animals tested at least once every five years (Ministry of Agriculture, Forestry and Fisheries, 1997). However, active surveillance methodology and subsequent measures vary largely depending on the guideline administration of each prefecture. Although more effective nationwide, control of Johne's disease requires an understanding of variation in surveillance methods among prefectures and the epidemiology of incidents, few reports from Japan on this issue have appeared in the international literature (Kobayashi et al., 2006). Here, we briefly introduce the present approach to the control of Johne's disease in Japan and analyze recent disease incidence.

MATERIALS AND METHODS

Between May and November 2005, all 47 prefectures of Japan were requested to provide detailed information on active surveillance and detection of Johne's disease between 1995 and 2004. Data were received from 46 prefectures, and nationwide characteristics of surveillance methods, as well as cases where Johne's disease was detected in cattle and farms, were carefully analyzed. In addition, trends in Hokkaido, the main dairy industry region of Japan, were compared with those of other regions in Japan.

RESULTS

Active surveillance methods and subsequent monitoring in infected farms

Active surveillance targeted dairy and beef cattle in 60% of prefectures and dairy cattle only in the remaining 40% (Fig. 1, upper left). This implies that all dairy cattle (1,690,000 head, based on 2004 national livestock statistics) have been tested at least once for Johne's disease. Beef cattle from the 60% of prefectures represent 26% of the total number of breeding beef cattle in Japan (262,090 head, based on 2004 national livestock statistics). In addition, although active surveillance is required to be carried out at least every five years, 70% of prefectures did so at shorter intervals (Fig. 1, lower left).

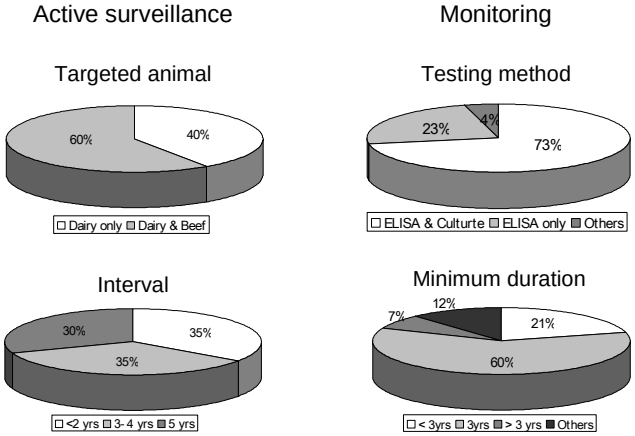

Fig. 1. Proportion of prefectures (n=46) using active surveillance (left) and subsequent monitoring (right)

After Johne's disease was detected, the affected farm was monitored and periodically tested, as described in prefectural guidelines. 73% of prefectures tested affected farms using a combination of ELISA and faecal culture, whereas 23% tested using ELISA only (Fig. 1, upper right). The minimum duration to complete monitoring with no subsequent disease detection was calculated from testing frequency and interval, with 60% of prefectures surveyed carrying out monitoring no earlier than three years (Fig. 1, lower right).

Detection in cattle between 2002-2004

Five diagnostic standards are approved in Japan. Johne's disease was detected in a total of 2,500 cattle between 2002-2004, mainly from positive results from two sequential ELISA tests (56%), agent isolation by faecal culture (41%), or microscopic confirmation of agent presence in faecal samples from animals showing clinical symptoms (3%). These incidents include all detections made by passive and active surveillance, as well as by monitoring of affected farms. In practice, active surveillance is conducted by ELISA, and monitoring by either ELISA only or a combination of ELISA and faecal culture. In cases where animals show clinical symptoms, microscopic inspection of faecal samples is conducted.

Incidence trends in animals and farms

Trends in Johne's disease incidence for animals and farms are described in Fig. 2. On animal level, the total number of cases detected increased from 700-800 head in 2003 to more than 1,000 head in 2004 and 2006. Until 2001, the proportion of cases detected in Hokkaido was larger than that in other prefectures. The numbers in these other prefectures increased after the introduction of active surveillance, with proportions reaching nearly half the total national number in recent years.

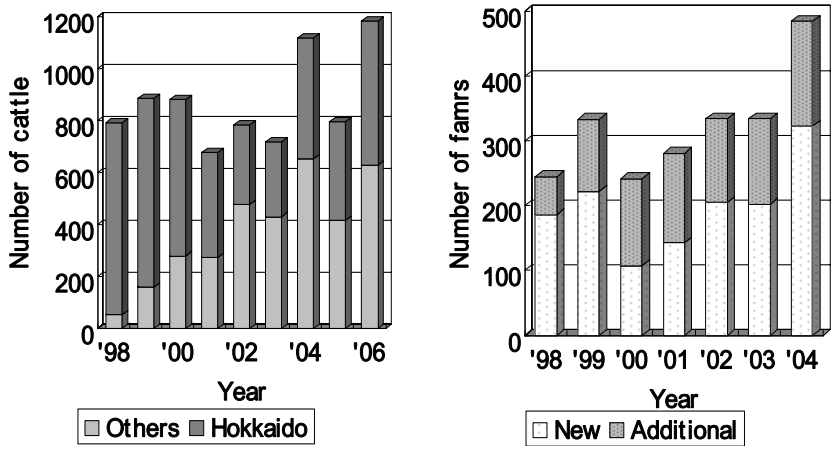

Fig. 2. Incidence trends of bovine Johne's disease in Japan between 1998-2006 for animals (left), and 1998-2004 for farms (right).

In addition to the animal level, the number of farms in which Johne's disease was detected also increased. A few years after the initiation of active surveillance and until 1999, most detection occurred in farms in which no previous cases had been detected, followed later by the increasing detection of additional detections. After 2000, the number of additionally detected farms during the monitoring period was relatively stable, while that of newly affected farms gradually increased up to 2004.

DISCUSSION

In the present study, we analyzed the number of animals and farms in which Johne's disease was detected in 46 prefectures in Japan. Among animals targeted for active surveillance, all dairy cattle were subjected to periodic testing, whereas 70% of breeding beef cattle received no testing. Among prefectures that did not test beef cattle, a number are major beef

producers in Japan. In contrast, the shorter period between surveillance than the minimum required by the Japanese government suggests high motivation among prefectures to control of Johne's disease. Increasing the involvement of beef producers in surveillance is a critical issue for further discussion. In addition, future control measures may be established in consideration of the significant differences in management practices and economical impact of the dairy and beef industries.

Additional critical issues in the establishment of control measures for Johne's disease are testing method and quality. In Japan, two sequential ELISAs are performed, mostly due to the simplicity of this procedure compared to other methods. Although some prefectures use ELISA only, in disease-affected farms faecal culture is the second major monitoring method. In some countries where Johne's disease incidence has been decreased, only faecal culture is performed due to the undesirable aspects of ELISA, including lower sensitivity and specificity (Sternberg and Viske, 2003). In the present study, nearly 60% of total disease cases were detected using ELISA, whereas 40% were detected by faecal culture. Because not all animals were tested by both, however, assessing the degree of agreement between them, as well as their sensitivity and specificity was difficult. As evaluation of diagnostic test performance is essential to assessing the impact of disease control programs, further investigation of test performance is strongly recommended.

Between 1998-1999, the majority of annual Johne's disease cases were observed in Hokkaido prefecture, which is the largest dairy producer and the major cattle distributor in Japan. During this period, intensive testing on cattle was performed in Hokkaido before other regions in Japan, which started active cattle surveillance later, leading to a gradual increase in cases detected. Recently, active surveillance has been carried out throughout Japan, resulting in over 1,000 detections between 2004 and 2006. These results suggest that the number of cases detected depends heavily on the intensity of testing. To date, however, this active surveillance program has produced no tangible decrease in the number of cases.

The number and proportion of new farms detected has also increased over time. This suggests that either early detection has occurred using active surveillance, or that a certain proportion of newly detected farms included more false-positives than expected. These interpretations may explain the lower subsequent detection during a monitoring period. Another possible cause for the increasing number of new disease cases is the nationwide dissemination of Johne's disease in spite of nearly ten years of active surveillance.

In the present study, we introduced the current approach used for the control of Johne's disease in Japan, and describe a number of features of disease incidence in cattle and farms. A better understanding of this disease and its rational and effective control nationwide requires further epidemiological investigation.

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Abattoir surveillance for Ovine Johnes Disease (OJD) in New South Wales

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ABSTRACT

Monitoring of adult sheep for Ovine Johne's Disease (OJD) in NSW and interstate abattoirs confirmed that 48% of direct consignments from the High Prevalence Area and 30% from the Medium Prevalence Area were positive in 2006 compared with 0.5% positive in the Very Low Prevalence Area of NSW. The origin of 3796 sheep consignments monitored in 2006 and 17436 consignments from 1999-2006 were mapped based on locality derived from their unique property identification code (PIC) or from abattoir records. Mapping was confirmed as an important communication tool for use in control programs.

INTRODUCTION

OJD was first detected in sheep in Australia in the Central Tablelands of NSW in 1980. Monitoring of adult sheep for OJD commenced in NSW abattoirs in late 1999 and subsequently in other states under a National Ovine Johne's Disease Control and Evaluation Program (1998). Abattoir monitoring has been estimated to have an individual animal sensitivity of approximately 70% in heavily infected flocks (Bradley & Cannon, 2005) and 50% in low prevalence flocks (OJD Technical Advisory Group, unpublished). Revised Prevalence Areas were introduced across Australia in July 2004 (Figs. 1 & 2). Gudair® OJD vaccine (CZ Veterinaria, Spain) was allowed under special permit as an aid in the control of OJD in heavily infected flocks (5% or more annual mortality) in NSW from January 2000 and was registered nationally for wider use in April 2002. The distribution of OJD in NSW determined by monitoring adult sheep slaughtered from 1999-2006 is reported in this study.

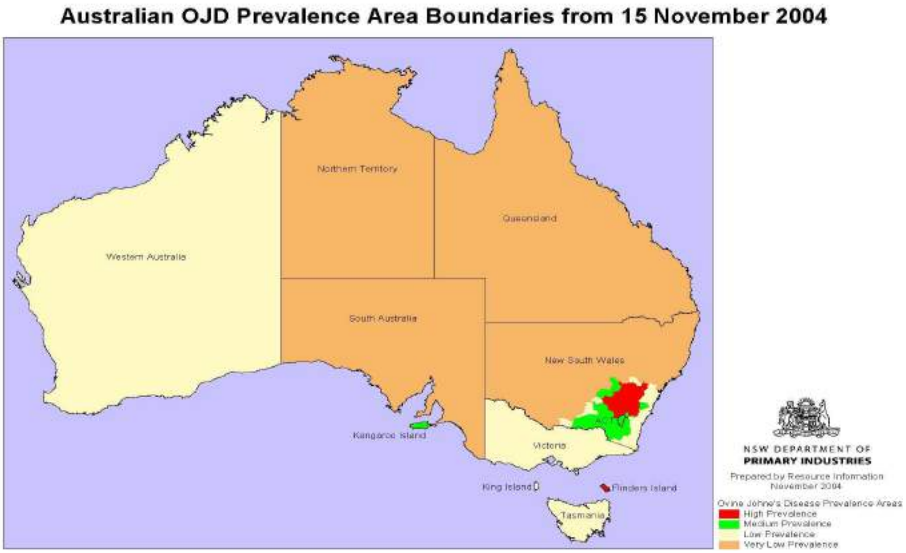

Fig.1: OJD Prevalence areas implemented across Australia from 1 July 2004 (modified 15 November 2004)

MATERIALS AND METHODS

The lower small intestine of each sheep was visually inspected and palpated for signs of thickening with confirmation of OJD by histopathology on up to 3 sheep with lesions sampled per consignment. A consignment was determined positive if 1 or more sheep were confirmed with OJD. More than 4.13m adult sheep were individually inspected (1999-2006) derived from all sheep producing areas of NSW. The origin of each consignment was determined

from abattoir records. The introduction of the National Livestock Identification System (NLIS) in 2002 required sheep consignments to be accompanied by a National Vendor Declaration (NVD). Recording of the Property Identification Code (PIC) on the NVD became compulsory from January 2006. This enabled consignment to be precisely allocated to a property, locality, local government area or OJD prevalence area.

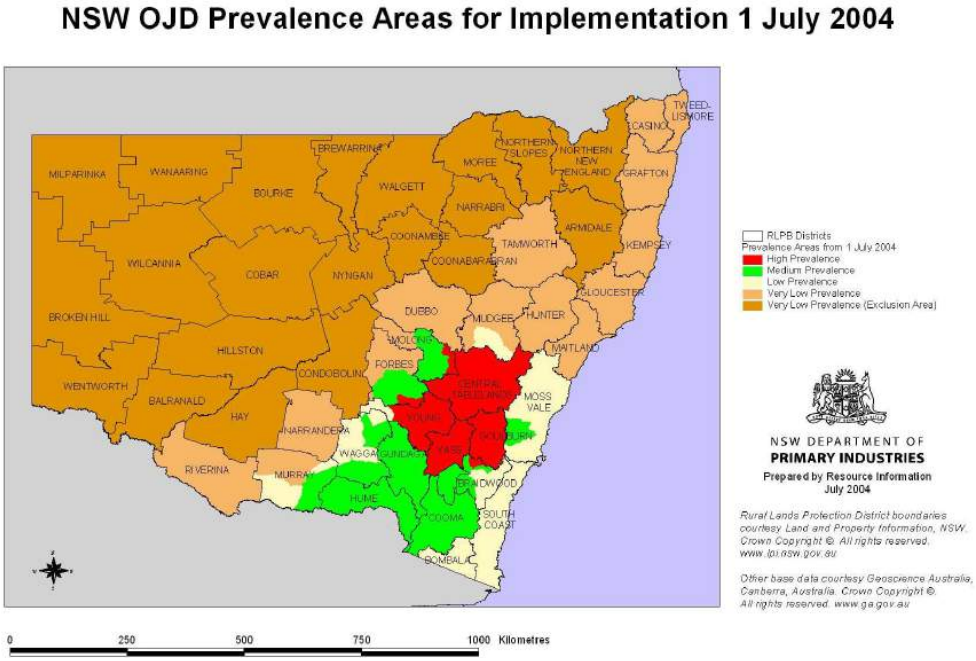

Fig.2. OJD Prevalence areas implemented in NSW from 1 July 2004.

RESULTS

OJD was confirmed in 48% of direct consignments from the High Prevalence Area of NSW (Fig 2) and in 30% from the Medium Prevalence Area in 2006. In contrast 0.7% of the consignments from the Very Low Prevalence Area of NSW were positive in 2006 (0.5% when corrected for multiple positive consignments from individual properties destocking). Figs 3 & 4 show the origin of the 3796 total consignments (green discs) and the 756 positive consignments (red discs superimposed) in 2006. Fig 5 shows the distribution of the cumulative monitoring results for NSW 1999 – 2006 (17,436 consignments, 2,444 positive).

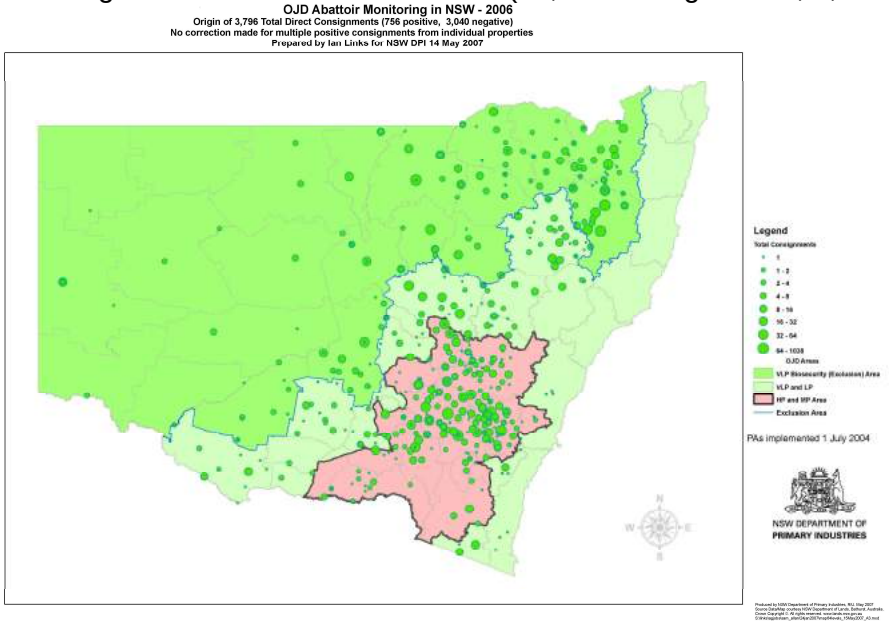

Fig.3: Origin of 3796 direct consignments of adult sheep monitored in NSW for OJD in 2006 (size of each green disc proportional to the number of consignments from that locality)

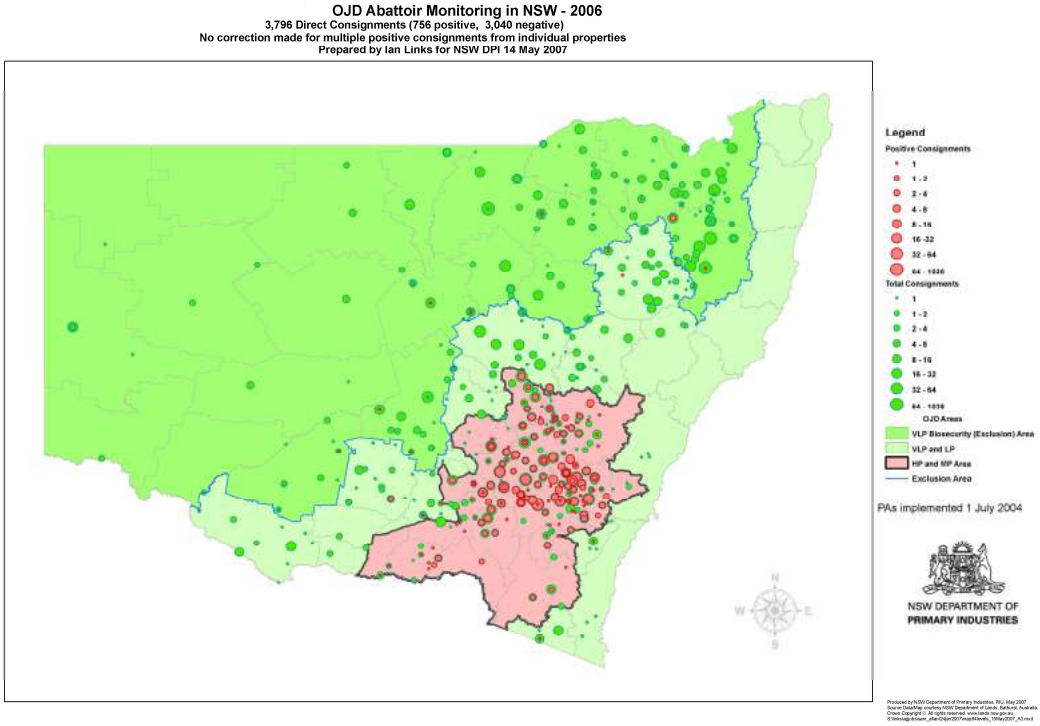

Fig. 4: Origin of 756 positive (red discs) & 3796 total direct consignments (green discs) of adult sheep monitored in NSW for OJD in 2006 (size of each disc proportional to the number of consignments from that locality)

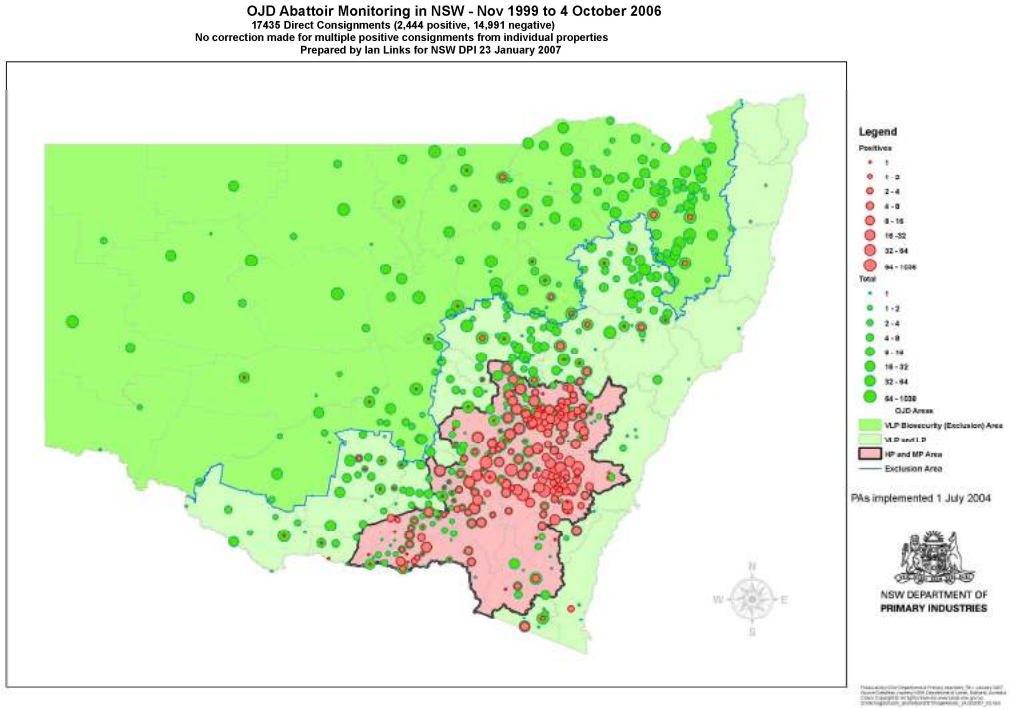

Fig. 5. Origin of 2,444 positive (red discs) & 17,435 total direct consignments (green discs) of adult sheep monitored in NSW for OJD 1999 - 2006 (size of each disc proportional to the number of consignments from that locality)

DISCUSSION

Since 1999, OJD has spread to infect more flocks in the High Prevalence and Medium Prevalence Areas of NSW. However around half of the sheep flocks in NSW are located in the Very Low Prevalence Area where infection remains at a low level. The monitoring results

correlate closely with the prevalence areas implemented in July 2004. Early detection in the Very Low Prevalence Area is critical in minimising the risk of further spread.

Property Disease Management Programs are implemented in infected flocks in the Very Low Prevalence Area and are strongly encouraged in other prevalence areas. Biosecurity programs (property & regional), on-farm management strategies and Gudair® vaccination are the primary control tools. Field reports indicate that, despite the increase in the proportion of infected flocks, high levels of vaccination in the High and Medium Prevalence Areas have markedly reduced the regional incidence of clinical OJD. Concurrently, the level of sub-clinical infection evidenced by abattoir monitoring has also declined, particularly in the High Prevalence Area (Links et al, 2007).

Mapping provides an excellent visual communication tool that has markedly improved the capacity to review progress with the national OJD program. This has been particularly important in consultation with sheep industry representatives regarding the location of prevalence area boundaries at the regional, state and national level.

ACKNOWLEDGEMENTS

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia, Commonwealth of Australia & managed through Animal Health Australia. The support of Abattoir Management (Dubbo, Goulburn, Deniliquin, Wallangarra, Cowra, Cootamundra, Young, West Wyalong, Junee, Mudgee and interstate abattoirs) & the Australian Quarantine Inspection Service was essential. The contribution from the OJD Inspectors, the data entry team, Regional Veterinary Laboratory Orange pathologists, the NSW DPI mapping section and interstate abattoir surveillance coordinators is gratefully acknowledged.

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A comparison of vaccination for Ovine Johne's Disease (OJD) and prevalence of lesions detected by abattoir monitoring in New South Wales (NSW)

Links I[a], Denholm L[b], Kingham L[c], Evers M[d]

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ABSTRACT

The prevalence of sub-clinical ovine Johne's disease (OJD) in adult sheep populations in NSW was determined by inspection of the terminal ileum of 4.13m sheep at slaughter from late-1999 to 2006. The prevalence of lesions within the sheep population in the NSW High Prevalence Area progressively declined from >4% in 1999-2000 to <1% in 2005-2006. This coincided with the implementation of Property Disease Management Plans and widespread uptake of vaccination against OJD.

INTRODUCTION

OJD was first detected in sheep in Australia in the Central Tablelands of NSW in 1980. Monitoring of adult sheep for OJD commenced in NSW abattoirs in late 1999 and subsequently in other states under a National Ovine Johne's Disease Control and Evaluation Program (1998). Abattoir monitoring has been estimated to have an individual animal sensitivity of approximately 70% in heavily infected flocks (Bradley & Cannon, 2005) and 50% in low prevalence flocks (OJD Technical Advisory Group, unpublished). Revised Prevalence Areas were introduced across Australia in July 2004 (Fig. 1). Abattoir monitoring provides a direct measure of the level of OJD sub-clinical infection present in the sheep population of NSW.

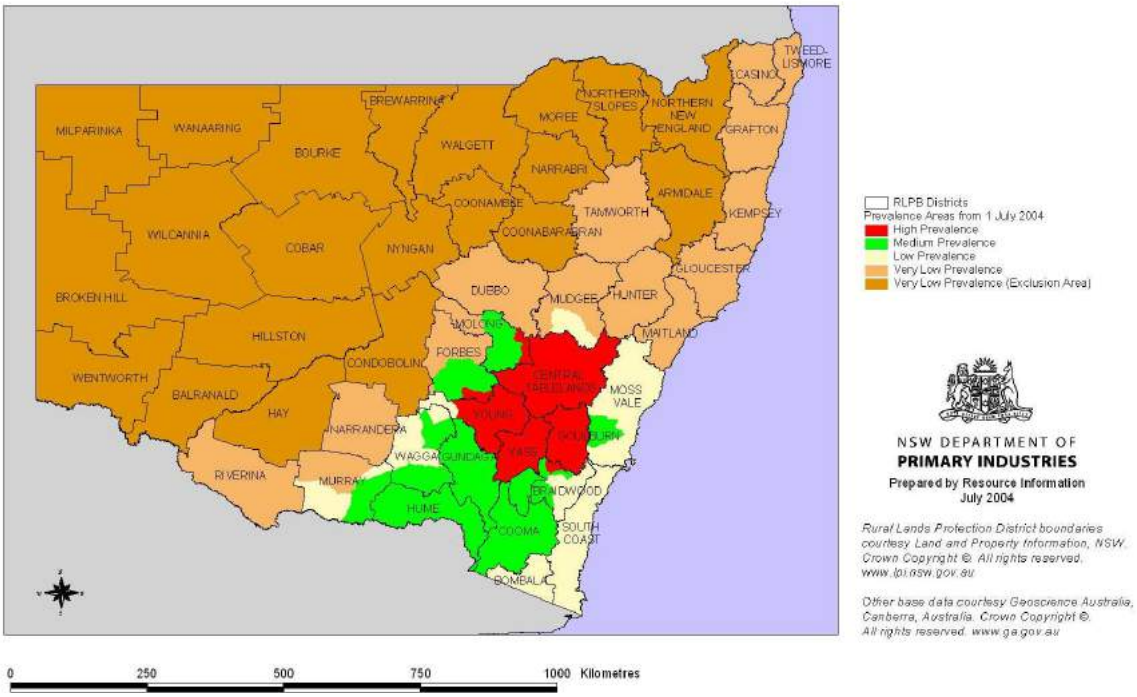

Fig.1: OJD Prevalence areas implemented in NSW from 1 July 2004

In January 2000, use of Gudair® OJD vaccine (killed *M. paratuberculosis* with adjuvant, CZ Veterinaria, Porrino, Spain) was allowed under special permit in heavily infected flocks (5% or more annual mortality) in the Residual Zone (now part of the High Prevalence Area) of NSW. Following the results of research trials in NSW, Gudair® was approved in April

2002 by the National Registration Authority for wider use as an aid in the control of OJD. Revised Prevalence Areas were introduced in NSW in July 2004 (Fig. 1).

The results of monitoring adult sheep at slaughter from the High and Medium Prevalence Areas of NSW and comparison with vaccine uptake are the subject of this report.

MATERIALS AND METHODS

The lower small intestine of each sheep was visually inspected and palpated for signs of thickening at slaughter with confirmation of OJD by histopathology on up to 3 sheep sampled per consignment. A consignment was determined positive if 1 or more sheep were confirmed with OJD. Consignments were allocated to a prevalence area after reviewing all available information on locality and local government area (Rural Lands Protection Board).

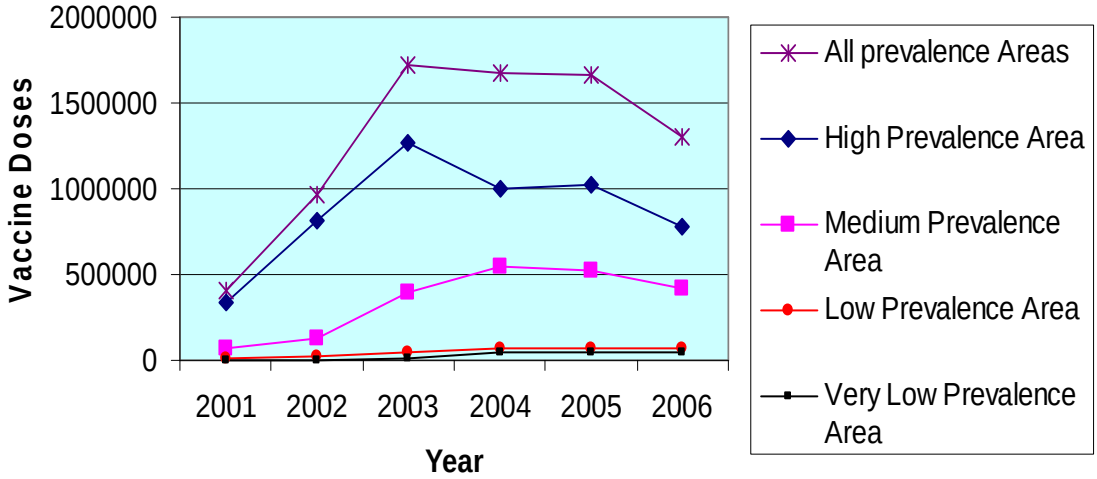

Fig. 2. Annual Gudair OJD vaccine sales in NSW by Prevalence Area – 2001-2006 (data courtesy of Pfizer Animal Health

RESULTS

From January 2001 to December 2006 more than 7.7 million doses of vaccine were sold in NSW. Vaccine sales for NSW are shown in Fig. 2 (data courtesy of Pfizer Animal Health, Australia). More than 4.13m adult sheep were individually inspected for OJD (1999-2006) from 19,838 direct consignments derived from all sheep producing areas of NSW. The percentage of sheep detected with lesions consistent with OJD (number of sheep with lesions from positive consignments/total sheep inspected from all consignments) from the High and Medium Prevalence Areas are shown in Table 1 & Fig 3. The total number of direct lines (consignments) monitored and the percentage of lines positive in the High and Medium Prevalence Areas is also shown in Table 1. No correction has been made for multiple consignments from individual properties.

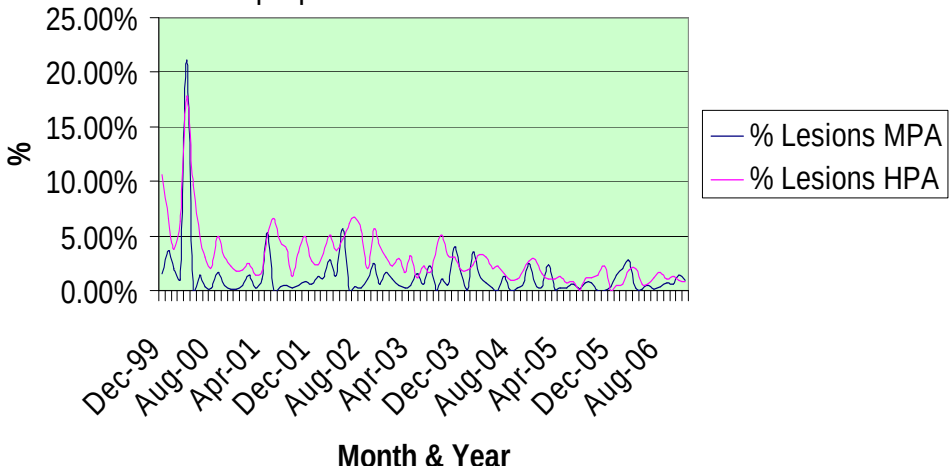

Fig. 3. NSW High and Medium Prevalence Area. Sheep with OJD lesions as a percentage of the monitored kill December 1999 to December 2006.

Table 1. Annual Monitoring Results for the High and Medium Prevalence Areas in NSW (1999 -2006)

	High Prevalence Area					Medium Prevalence Area				
Year	No. of Sheep	No. of Lesions	% Lesions	No. of Direct Lines	% +ve Lines	No. of Sheep	No. of Lesions	% Lesions	No. of Direct Lines	% +ve Lines
1999	13,067	1,381	10.57%	49	65.3%	6,325	97	1.53%	16	31.3%
2000	213,207	9,507	4.46%	984	38.2%	106,749	1,071	1.00%	516	9.7%
2001	176,724	5,919	3.35%	737	55.4%	69,738	416	0.60%	274	23.0%
2002	221,831	9,211	4.15%	962	59.5%	84,083	1,279	1.52%	368	33.2%
2003	138,478	3,573	2.58%	701	39.9%	52,913	686	1.30%	248	27.4%
2004	183,801	4,326	2.35%	829	43.6%	77,858	917	1.18%	350	22.3%
2005	119,209	1,182	0.99%	573	29.0%	65,234	402	0.62%	231	15.2%
2006	395,392	3,816	0.97%	1,330	49.4%	118,924	922	0.78%	394	32.0%
Total	**1,461,709**	**38,915**	**2.66%**	**6,165**	**46.3%**	**581,824**	**5,790**	**1.00%**	**2,397**	**22.8%**

DISCUSSION

The percentage of direct lines positive for OJD in the High Prevalence Area in 2006 (49%) is similar to the average (46%) for the period 1999-2006 although there have been fluctuations year by year. However, in the same period there is evidence from the percentage of animals detected with lesions that the regional prevalence of sub-clinical OJD has progressively declined (Table 1, Fig. 3). It is likely, nonetheless, that recruitment of new infected flocks is continuing while the implementation of PDMPs is reducing the overall level of infection in the sheep population. Targeted vaccination and culling of heavily infected flocks occurred from 2000 while widespread adoption of vaccination commenced in 2002.

In contrast, the percentage of positive lines in the Medium Prevalence Area rose in 2006 (32%, average 1999-2006 of 23%) although it had dropped in 2005 (15%). Concurrently, the percentage of sheep with lesions (0.8%) dropped below the 1999-2006 average (1.0%). This would suggest an increasing percentage of infected flocks over time implementing PDMPs (including vaccination) and helping prevent amplification of infection within those flocks.

The availability of OJD vaccine changed the emphasis of Property Disease Management Programs (PDMPs) from reliance solely on management strategies. The major emphasis was on vaccination of lambs at less than 16 weeks of age in infected and at-risk flocks. However, research demonstrating no untoward outcomes following vaccination of adult sheep in a heavily infected flock was pivotal in encouraging many producers to undertake whole of flock vaccination.

It is estimated from 2003 to 2006 around 70% of replacement sheep were being vaccinated annually in the High and 30% in the Medium Prevalence Area. Field officers report that clinical disease is now very rare in vaccinated flocks that were previously suffering significant mortalities from OJD in the High Prevalence Area. While this is generally considered due to vaccination, the role of better management strategies, including strategic culling of heavily infected mobs or age groups, and environmental factors (including drought) remains unclear.

The complex relationship between the percentage of vaccinated sheep (presumed immune) in the population, the level of infective challenge (presumed decreasing), and the long incubation period of the disease necessarily make these analyses and interpretation preliminary.

ACKNOWLEDGEMNTS

Funding was provided by NSW DPI, Sheepmeat Council of Australia, WoolProducers Australia, Commonwealth of Australia & managed through Animal Health Australia. The support of Abattoir Management (Dubbo, Goulburn, Deniliquin, Wallangarra, Cowra, Cootamundra, Young, West Wyalong, Junee, Mudgee and interstate abattoirs) & the Australian Quarantine Inspection Service was essential. The contribution from the OJD Inspectors, the data entry team, Regional Veterinary Laboratory Orange pathologists, the

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Cattle movements in to and out of Johne's infected suckler herds in Ireland

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INTRODUCTION AND OBJECTIVES

In the period 2002 to 2006 a total of 96 beef suckler herds submitted faecal samples to the Central Veterinary Research Laboratory, which yielded a culture positive result for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Movements in to and out of these herds were analysed and compared with those from a control group of 244 suckler herds, which gave ELISA negative results as part of a survey which was conducted to estimate the prevalence of paratuberculosis (Johne's disease (JD)) in the Irish cattle population.

We had expected that infected herds would have a higher percentage of bought in animals than control herds, which might help to explain why the herd had become infected in the first place. With herds in which animals show clinical symptoms there are usually a larger number of animals that show sub clinical signs and are often culled earlier. We therefore expected a higher percentage of animals sold out of infected herds than control herds.

Statistical analysis was performed by the use of EpiInfo Version 3.4 obtained from http://www.cdc.gov/epiinfo/. Data was analysed using Unconditional Logistical Regression.

Table1. Max, min and average profile of 96 infected and 244 control suckler herds.

	Infected: Range (Average in brackets)	Control	Significant difference
Male calves under 6 mo	0 - 37 (4)	0 - 16 (2)	P= 0.0010
Female calves under 6 mo	0 - 31 (4)	0 - 13 (2)	P= 0.0005
Males over 6 months	0 - 89 (19)	0 - 72 (10)	P= 0.0101
Females 6mo to 1 year	0 - 73 (11)	0 - 20 (2)	P= 0.0000
Females 1 to 2 year	0 - 55 (11)	0 - 31 (6)	P= 0.0000
Cows	4 - 267 (47)	4 - 110 (23)	P= 0.0000
Total	4 - 495 (95)	7 - 178 (45)	P= 0.0000
Cows as % of herd	31 - 100%(48)	30 - 100%(51)	P= 0.1462

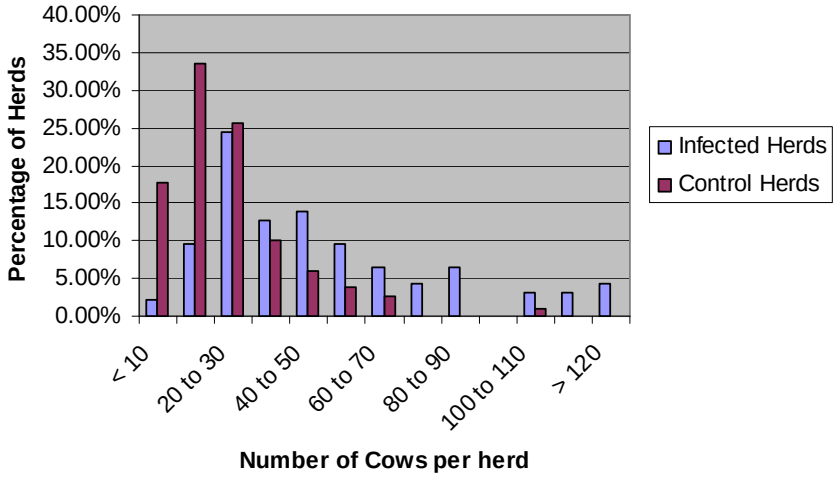
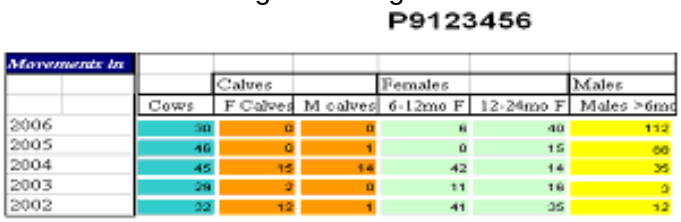


Fig. 1. Number of cows per herd in 96 case herds and 244 control herds.

Movements In

Movements into the herds were also obtained and analysed with MS Access. An example of the output from the Access database is given in Figure 2.



P9123456

Movements In						
		Calves		Females		Males
	Cows	F Calves	M calves	6-12mo F	12-24mo F	Males >6mo
2006	30	0	0	6	40	112
2005	46	0	1	0	15	66
2004	45	15	14	42	14	35
2003	29	2	0	11	16	3
2002	32	12	1	41	35	12

Fig. 2. Movements in to Herd Number P9123456 between Jan 1st 2002 and December 31st 2006.

Inward movements over the five-year period in the ninety-four infected herds varied from a low of no animals introduced to a high of five hundred and nine. Expressed as a percentage of the number of animals on the herd profile this ranged from 0 % to 1018 % with a mean of 97 % over the five-year period or roughly 20 % of the herd each year.

In the control herds, inward movement varied from a low of no animals introduced to a high of one thousand and thirty one. Expressed as a percentage of the number of animals on the herd profile this ranged from 0 % to 2730 % with a mean of 126 % over the five-year period or roughly 25 % of the herd each year.

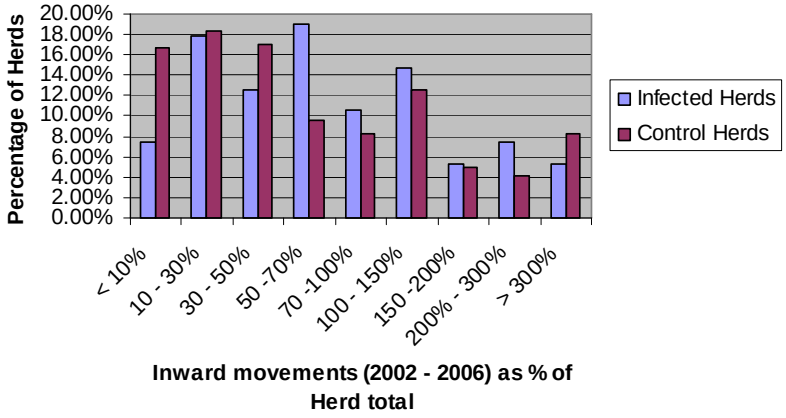


Fig. 4. Inward movements in to JD Infected and Control Herds.

Table 2. Buying in (2002 – 2006) relative to herd size.

Number of Animals in herd	Non-infected (Min, Max, Average)	Infected (Min , Max, Average)
< 20	0 - 153 (14.9)	1 - 112 (33.3)
20 to 30	0 - 85 (15.2)	4 - 29 (12.5)
30 to 40	0 - 950 (65.4)	2 - 118 (27.9)
40 to 50	2 - 950 (80)	0 - 103 (41.8)
50 to 70	0 - 250 (51.9)	2 - 84 (44)
70 to 100	11 - 505 (71.2)	2 - 507 (83.7)
> 100	7 - 312 (103.9)	3 - 387 (88.2)

Significant differences were only seen between the two groups of herds in inward movements of heifer calves in 2003 ($p = 0.0476$), and six-month heifers in 2005 ($p = 0.0300$) and 2006 ($p = 0.0241$), which were all greater in the control herds. When inward movements were totalled over the five-year study period there were no significant differences between case and control herds. It could be inferred from this that inward movements was not a risk factor in the introduction of JD in to the herd. However if the prevalence of the disease was higher than it was in the period 2002 – 2006, different results might have been obtained. It would have been helpful to be able to analyse inward movements from the time of infection of the herd. However herds were defined as infected when one positive faecal sample had

been obtained. There is no way of knowing if this was the first clinical case or how long the incubation period had been in the affected animal.

Movements Out

Similarly movements out of the herds were also analysed with MS Access. An example of the output from the Access database is given in Figure 2.

P9123456

Movements out

		Cows	Calves		Females		Males	Males
			F Calves	M calves	6-12mo F	12-24mo F	Bulls	Steers
2006	Sale	0	0	0	29	0		34
	Factory	0	0	0	0	0		0
	Died	5	11	13	1	0		2
2005	Sale	9	6	5	136	34		141
	Factory	30	0	0	0	0		1
	Died	14	15	8	0	0		4

Fig. 4. Movements out of Herd Number P9123456 between Jan 1st 2005 and December 31st 2006.

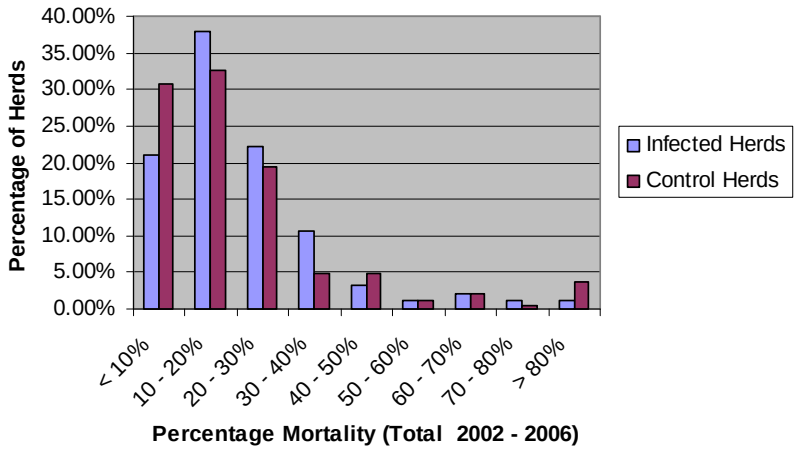


Fig. 5. Cow On Farm deaths % over a five-year period in Infected and Control Herds (2002 – 2006)

Significant differences were seen in males sold in each of the five years, males died in 2002, 2003 and 2005, cows died in each of the five years, cows slaughtered in 2004 and cows sold in four of the five years. Significant differences were also seen between the two groups in female calves over six months slaughtered in 2002 and 2003, female calves over six months sold in each of the five years, female calves under six months that died in each of the five years and female calves under six months that were sold in 2004. Differences were also seen in heifers that were slaughtered in 2002, heifers sold in four of the five years and heifers that died in 2005, male calves under six months that were sold in 2002, 2003 and 2004 and male calves under six months that died in each of the five years. These were all higher in the infected herds than in the control herds.

The greater herd size of infected herds was thought to be a confounding factor in this analysis so analysis was repeated comparing each of these factors as a percentage of animal groups on the herd profile and the only significant differences in movements were greater in the infected herds than control herds (Table 3)

Table 3. Significant differences between movements out as % animals on herd profile.

Term	P-Value
Cows_Sold_2004	0.0036
Heifers_sold_2003	0.0047
malecalvesunder6modied_2003	0.0006
malecalvesunder6modied_2004	0.0122

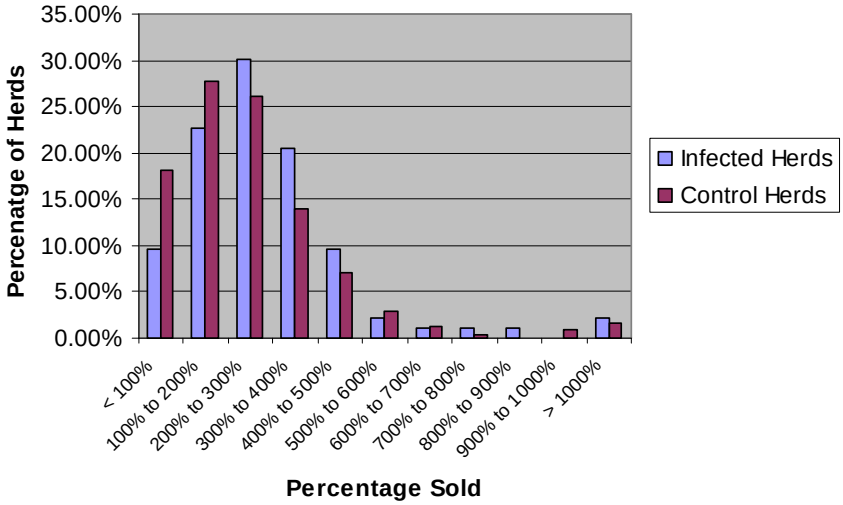


Fig. 7. Animals % moved from Infected and Control Herds (2002 – 2006).

DISCUSSION

Infected herds had significantly more cattle in all age and sex groups. The numbers of closed herds, where no animals were brought in to the herd in the five-year study period, was greater in the control herds (18 closed herds (7%)) than in the infected group (1 closed herd (1%)). There was only one herd that bought in more than ten animals that did not buy in females. Seventy two of the two hundred and forty three control herds (37%) had less than ten bought in animals in the five year study period while only nineteen of the hundred and seven infected herds (19%) had bought in ten or less animals. Of these herds seven of the control herds and one of the infected herds had only bought in bulls. Buying in relative to herd size is shown in Table 2. The risk of introducing infection into a herd increases in proportion to the number of animals purchased.

The large number of animal movements in both groups of animals would need to be markedly reduced if a JD control programme had any possibility of working. The only sure way of preventing infection with JD is by not buying in infected animals and once a herd is infected animals should only move out of the herd directly to slaughter. There is a risk of an increasing prevalence of JD in Ireland if unrestricted movement from JD infected herds is allowed to continue. If movement directly to slaughter only was encouraged this would greatly reduce the potential for spread.

Experimental control program for paratuberculosis in dairy cattle in the Veneto region

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INTRODUCTION

In 2001, a survey to estimate the seroprevalence of paratuberculosis in dairy herds was carried out in the Veneto region (North Eastern Italy). This region has about 220,000 dairy cattle and is the 3rd milk producing area in Italy. The results of the survey showed 27% of the herds positive, and in those with 100 heads or more, the prevalence of infected animals reached 50% (Robbi et al, 2002).

For this reason, a three-year experimental control program for paratuberculosis was implemented in 2004 and was focused on two issues: application of biocontainment and biosecurity measures in order to prevent the introduction and spread of the disease; in-farm management of the animals tested positive on the basis of a semi-quantitative risk assessment taking farm productivity into account as well.

MATERIALS AND METHODS

Five infected herds from 70 to 200 cows were selected on the basis of the farmers' willingness to participate. All the farms were visited at the beginning of the study and evaluated for the risk of disease diffusion within the herd: a questionnaire derived from the one developed by the National Johne's Working Group, US, was used to inquiry each farmer.

All the >24 month old cows were tested serologically every six months and at drying-off, and whenever positive or doubtful results were observed, faecal culture and IS*900*-PCR were performed. All the farms but one were tested the first time in spring 2004. Data on animal movements during the study period were also collected.

Nonparametric statistical test on the equality of medians and Cochran Armitage Test for Trend of prevalences were performed using Stata v.9.2.

RESULTS

The farmers enrolled were asked to avoid the introduction of new animals during the study period, to apply the management measures agreed, and to provide information for the program evaluation when requested. The proposed measures were: definition of two separated calving areas for positive and negative cows detected by serological testing at drying, early separation of newborn calves from dams, use of colostrum from negative cows in calves born from positive dams, and separate grazing areas for young calves and cows.

Data analysis showed that almost 88% of the animals belonged to Holstein breeds. No differences were detected among herds in regard of the age of culling of >24 months cattle (median= 58.7 months, 1Q= 42.0, 3Q= 86.5; p= 0.07), neither for the age of the first microbiological or PCR positive result (median= 44.7 months, 1Q= 39.2, 3Q= 57.0; p= 0.50).

At the beginning of the study, incidence of clinical cases was 2-8% per year. Sero-prevalences in each selected herd (Fig.1) decreased with time. This picture was confirmed for farms B, C and E (p< 0.01). Median time lag between the first positive result at faecal culture or PCR and culling was (median=7.0 months; 1Q=2.7; 3Q=13.8; p= 0.64).

Although not all the farmers strictly applied all the measures specified in the program, after 3.5 years, only one clinical case was observed in the last year and average herd sero-prevalence decreased from 14.8% (range 6.2%-26.0%) to 4.4% (range 1.3%-8.1%).

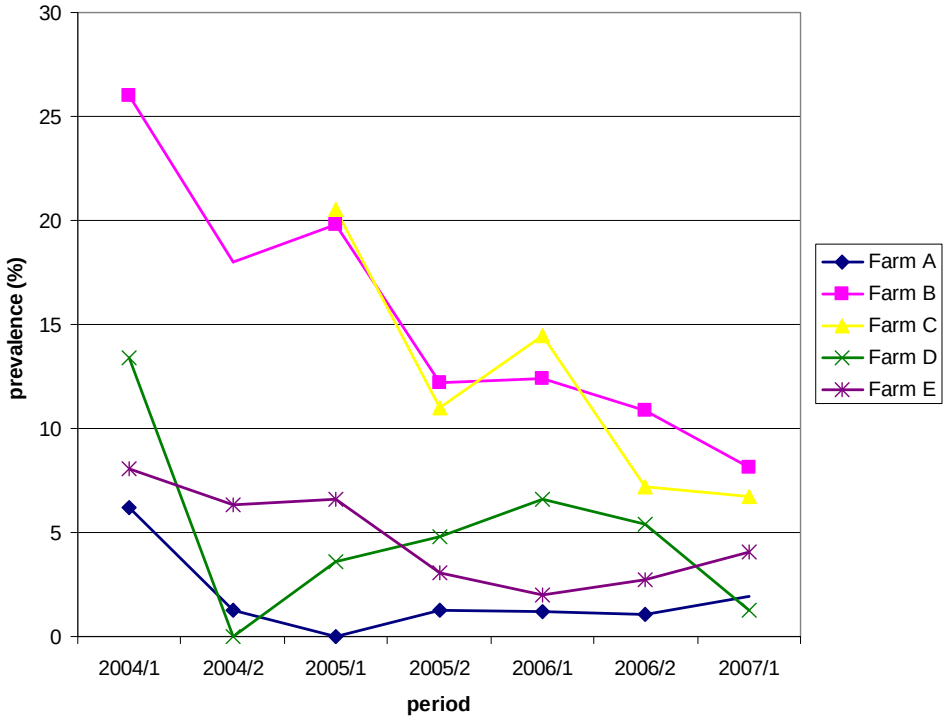

Fig. 1. Johne's disease prevalence per herd at biannual whole herd testing

DISCUSSION

Johne's disease is widespread in Northern Italy and, in the Veneto region almost half of the herds with >100 heads are affected. The experimental control program implemented in 5 pilot herds of the Veneto region provided interesting results but also showed critical points.

Despite the advice of timely culling culture or PCR-positive animals, the time-lag between test results and culling varied considerably both among herds and within the same herd depending on farmer's attitude and cow values.

The timely adoption of proper management practices can reduce in a couple of years clinical and subclinical cases and the spread of the diseases in the dairy farms.

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Study design and application of a paratuberculosis assurance program in Brown breeders' herds in the Italian central Alps

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INTRODUCTION

Health status in regard to Johne's disease is a major concern in the selection of bull calves for reproduction purposes. This has been emphasized by the EFSA (2004), which recommended that only *Mycobacterium avium* subsp. *paratuberculosis* (MAP) negative cows from herds with low prevalence should provide calves for reproduction.

Considering that rules on semen quality could become more stringent in the future, a program for a group of farms that regularly provide bull calves to the same reproduction centre was developed by the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in 2005. This program was based on annual MAP screening and individual intervention strategies in order to reduce the prevalence in infected herd and to certify negative herds.

MATERIALS AND METHODS

Twenty-three dairy cattle herds (average no. heads = 77; min = 33, max = 173) located in Trentino region, Italy, that regularly provide bull calves to the same reproduction centre were enrolled. All the cows older than 24 months were annually subjected to the Elisa Pourquier® screening test. Confirmation of positive or doubtful results was carried out by fecal culture and IS*900* PCR tests.

In spring 2007 a questionnaire derived from the one developed by the National Johne's Working Group, US, was administered to each farmer. Negative farms were evaluated for the risk of disease introduction while the infected ones for the biocontainment measures implemented. The farmers were asked to provide information on the management of each production phase, restocking rates, production performances, and animal movements.

RESULTS

In 2005, 15 herds resulted negative, 5 had only few sero-positive results that were not confirmed by PCR or faecal culture, and 3 were infected, with sero-prevalence of 2.1%, 5.1% and 6.4% respectively. Clinical cases were observed only in the two latter farms.

Based on these results, three mid-term (five-year) programs were developed in order to certify herd health status, eliminate the disease in low-prevalence herds, and reduce the sero-prevalence under 5% in highly infected herds. In 2006, negative herds dropped to 9 by ELISA assay with the detection of Singleton reactors in all cases but one, in which the infection was confirmed by faecal culture. Another three herds were confirmed within seropositive farms. In 2007, an increase in seroprevalence was detected in the two highly infected herds, reaching 10.4% and 13.5% respectively. Low prevalence remained constant in slightly infected herds and confirmatory results are pending.

Result of the ELISA screening by year of study is presented in Table 1. Data analysis showed that median age of sero-positive cows was 47.9 months (1Q= 40.7, 3Q= 55.7), and the median time-lag between test-positive and culling was 7.5 months (1Q= 6.3, 3Q= 19.0).

Table 1. Results of the ELISA screening by year (confirmed cases)

Prevalence	**2005**	**2006**	**2007**
>5%	2 (2)	2 (2)	2 (*)
<5%	6 (1)	12 (4)	13 (*)
Negative herds	15	9	8

* results pending

Data collected by questionnaires revealed that all the farms practice free rearing and summer transfer to alpine pastures. Usually heifers of 6-9 months or older and dry cows are brought to pastures, but in less than 30% of the herds also lactating cows. Pasture sharing among animals from farms of unknown health status was recorded in all cases but one. This is a well known risk factor for spred of Johne's disease (Pozzato et al., 2005). Frequency of infection resulted higher in bigger farms (Table 2). 18 farms out of 23 introduced animals during the previous 5 years, although the quantities differed.

Control measures for the calving phase were not sufficient in most cases. E.g. absence of calving box coupled with high animal density and poor hygiene was recorded in 7 out of 23 farms. The other activities (calves rearing, heifers and cows) were adequately conducted in almost all farms. Differences between infected and non-infected herds raised from the questionnaire's data analysis were not statistically significant due to the limited number of farms.

Table 2. Risk factors distribution among infected and non-infected herds

Variable/risk factor	Infected herds	Non-infected herds
>70 heads (over 24 months)	5/6	7/17
introduction from other herds	5/6	13/17
proportion of Holstein breed >40%	3/6	1/17

DISCUSSION

Sensitivity of diagnostic tests for paratuberculosis is quite low, in particular in pre-clinical phases; repeated testing of the same animals along time increase test sensitivity. This is clearly shown in this study where some farms resulted infected in the second or third year of testing.

On the basis of the health status and of the information collected by the inspection and the questionnaire, a set of biosecurity and biocontainment measures tailored to individual farms was agreed between farmers and veterinarians, and a reduction of the prevalence in infected herds and of the risk of introduction of paratuberculosis in negative ones are expected. The program applied in these farms is an extension of the protocols already implemented by the "ASSOGENE", the Italian association for bovine semen, considering that adoption of control and eradication programs for infectious and contagious disease in cattle is more stringent for farms that provide young bulls for reproduction. Finally, the application of these management practices was expected to lead to a general improvement in the health status of the considered farms.

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Control of paratuberculosis in Sweden

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INTRODUCTION

The control of paratuberculosis has been an ambition of the Swedish authorities for more than half a century. The disease was included in the Swedish Epizootic Act (SFS 1999:657) already in 1952. According to this legislation any suspicion of *Mycobacterium avium* subsp. *paratuberculosis* is notifiable for animal owners, veterinarians or other professionals with animal contact. This applies regardless of what animal species is suspected to be infected. Moreover, the Swedish Board of Agriculture (the responsible authority under the Department of Agriculture) must investigate all suspect cases and take all necessary means to eradicate and prevent the spread of the infection, if confirmed.

Due to the nature of the disease and insufficiency of diagnostic tests it is virtually impossible to formally declare freedom from paratuberculosis on a national level. However, the aim in Sweden remains to eradicate the infection whenever detected and thus keep the national prevalence at a very low level, preventing the spread of the disease. This is achieved by a combination of passive and active surveillance, and legislation that makes the eradication of the infection mandatory, whenever detected.

A stamping out policy is applied if infected herds are detected, followed by extensive tracing of all contact herds. After thorough cleaning and disinfection of the entire premises where the infected animals have been held, a holding period is applied on buildings, pastures and farmland where infected animals have grazed or infected manure has been spread.
In 1993, paratuberculosis was detected in an imported beef cow. This prompted a thorough tracing that led to the discovery of a few more infected herds (Engvall et al., 1994; Viske et al., 1996) and the above-mentioned measures were applied accordingly. Since then, a series of investigations have been undertaken to detect and take measures in any infected herds. The aim of this paper is to describe the extent of the overall investigations and control of paratuberculosis in Sweden.

Investigations in cattle

Passive surveillance, based on the legislation that enforces veterinarians to sample suspect cases of paratuberculosis, has been in place for more than half a century. For the past decade, a number of information campaigns have been conducted, directed at veterinarians and aiming to remind them of the clinical symptoms that should prompt a suspicion as well as what the legislation states in such cases.

A number of surveys has been conducted, and shown a very low prevalence of paratuberculosis (Sternberg and Viske, 2003) (Table 1).

In the 1990s, directed surveys by faecal culture and serology on all imported cattle and all herds with cattle of the Limousin breed were carried out, as well as a post-mortem culture investigation of a random sample from abattoirs. The targeting of the Limousin breed and imported cattle was based on the fact that most cases had a direct link to imported animals, and the discovery of a domestic chain of infection in the Limousin breed, originating from imported animals (Viske et al., 1996).

Also, a voluntary control programme including the majority of all pedigree beef herds has been in place since 1998. This programme is based on regular faecal sampling of all adult animals and trade is restricted to herds with the same status in the programme (Sternberg et al., 2002b).

Table 1. Summary of surveillance activities in cattle.

Year	Activity	Sample size	Diagnostic method	Result
1993	Extended examination at slaughter	273,000	Macroscopic inspection. Culture from intestinal wall, lymph nodes and content on suspect cases	No case detected
1993	Serological survey of cattle (collected within the EBL programme)	4,000 animals in 1,576 herds	Serology (culling and culture from positive animals)	48 sero-positive, none confirmed
1993-1994	Survey of imported cattle from Denmark	217 animals in 99 herds	Serology and faecal culture	7 cases in 6 herds
1995-1996	Examination of all animals imported 1980-1994	500 animals (culture) 120 herds (serology)	Faecal culture from imported animals, serology on herd if animal no longer present. Repeated twice, at least three months apart.	2 cases detected
1995-1996	Examination of emergency slaughtered animals	3,166	Macroscopic inspection. Culture from intestinal wall, lymph nodes and content on all animals	1 case detected
1996	Survey within Limousin breed	130 herds	Faecal culture and serology. Culture was repeated twice with sampling at least three months apart.	2 cases detected
1998 onwards	Control programme in pedigree beef herds	>600 herds	Faecal culture	1 case detected year 2000
2001	Survey of dairy cattle	4,000 animals in 200 herds	Faecal culture	No case detected
2004	Survey of dairy cattle	4,000 animals in 200 herds	Faecal culture	No case detected
2004 onwards	All adult cattle submitted for necropsy	Average 215 animals/year	Culture from intestinal wall, lymph nodes and content.	1 case detected year 2005
2005	Survey of dairy cattle	4,000 animals in 200 herds	Faecal culture	No case detected
2006-2007	Survey of herds having imported since 1995	687 animals in 38 herds	Faecal culture	No case detected

So far, all cases have been found in beef herds, the status of the dairy herds has not been extensively investigated. Investigations of clinical suspicions in dairy herds have never detected paratuberculosis, but the need for active surveillance in dairy herds has prompted a series of surveys targeted towards this part of the cattle population. Since 2000, three faecal culture surveys on a stratified random sample of the dairy cattle population have been conducted (Sternberg et al., 2002a). These each included faecal cultures from the 20 oldest cattle in 200 randomly selected herds, stratified by geographic location.

Sick animals that die or are culled are regarded as having a higher risk of paratuberculosis infection. Thus, since 2004, culture samples are taken from all adult cattle submitted for necropsy.

Table 2. Total cattle and sheep population in Sweden 1990-2006 (Yearbook of Agricultural Statistics, 2007)

Year	No. of cattle	No. of cattle herds	No. of sheep	No. of sheep herds
1990	1,718,400	47,300	404,800	9,700
1995	1,777,100	42,000	461,800	10,000
2000	1,683,800	32,100	431,900	8,100
2005	1,604,900	26,200	471,300	7,700
2006	1,590,400	25,100	505,500	9,200

As all cases have been directly or indirectly linked to imported animals, herds having imported animals are also regarded as having a higher risk of paratuberculosis infection. Another directed survey being conducted in 2006-2007 includes cattle herds that have

imported animals since 1995. The size of the total cattle population during the relevant period is shown in Table 2.

Investigations in sheep and other animals

Apart from the passive surveillance in sheep, annual surveys have been conducted since 1993, until 2003 based on serology and since 2004 based on faecal culture (Sternberg Lewerin et al., 2005). The serologic surveys where conducted on blood samples from the Maedi-Visna control programme, including the majority of pedigree herds. The faecal surveys also target herds within the Maedi-Visna control programme. This is both for practical reasons, as a veterinarian visits these herds anyway, but also because they include the largest herds that sell most animals. The surveys conducted in sheep are summarised in Table 3.

Table 3. Surveys conducted in sheep

Year	Analysis	Samples	Positive	Confirmed
1993	Serology	3850	1	0
1995	Serology	43	0	0
1996	Serology	22	0	0
1997	Serology	1517	2	0
1998	Serology	1614	0	0
1999	Serology	3200	1	0
2000	Serology	3000	0	0
2001	Serology	3000	1	0
2002	Serology	2813	0	0
2003	Serology	2732	2	0
2004-onwards**	Faecal culture	2164	0	0

*Yearbook of Agricultural Statistics, ** October 2007

In addition, since 2004, culture samples are taken from all adult sheep (> 1 year) submitted for necropsy, on average 63 samples yearly. The size of the total sheep population in Sweden during the relevant period is shown in Table 2. Moreover, Sweden has a long-standing programme for the surveillance of wildlife diseases and numerous post-mortem investigations are conducted in wildlife every year (Pedersen Mörner and Mörner, 1990; Bölske et al., 2001). If paratuberculosis is suspected in any of these cases, culture samples are taken.

Laboratory methods

For serology in cattle, an ELISA test (Johne's Absorbed EIA kit, CSL Ltd, Australia) was used. The method used for serology in sheep was the agar gel immunodiffusion test, AGID (*M. paratuberculosis* protoplasmic antigen, Allied monitor, Missouri, USA). Faecal culture of bovine samples was performed on modified Löwenstein Jensen medium, with and without mycobactin, and from year 2000 also on Herrolds Egg Yolk medium, for up to four months. (Beerwerth, 1967; Jørgensen, 1982). Culture of ovine samples was performed on modified Löwenstein Jensen medium with mycobactin and from year 2004 also on modified Middlebrook 7H10 (Whittington et al. 1999) up to six months. PCR using the IS*900* sequence was used to confirm the identification of isolated strains (Englund et al., 1999; 2001). An evaluation of the detection capabilities of the tests used in cattle was made in 1999 (Bölske et al., 1999), resulting in the cessation of the application of serology in cattle.

RESULTS

A total of 53 infected herds have been identified since 1993, the two most recent in 2000 and 2005. The detailed results of each investigation in cattle are shown in Table 1. In these investigations 14 cases were detected, the remainder of cases were found at tracing from infected animals. All cases have been linked to imported animals and none have been in dairy herds.

In sheep, no domestic case of paratuberculosis has ever been detected. A total of seven sero-positive were detected during the ten years of sero-surveillance in sheep. All these animals were culled and cultured post-mortem. Faecal culture was also conducted on all adult animals in each herd. Based on this, all reactors were concluded to be false positives.

One case in quarantine, an imported ram, was detected in 1999. The infected animal was culled and the entire consignment was refused entry into Sweden. Paratuberculosis has never been detected in the passive surveillance in wildlife.

Research

Studies on diagnostic methods, as well as epidemiological studies help form the basis of the Swedish control policy. In addition to the vast amount of international research, aspects relating to the Swedish situation must be included. PCR systems to be used in combination with, and sometimes instead of, routine culture have been developed (Herthnek, 2006, Herthnek et al., 2006). Epidemiological investigations have been supported by molecular techniques (Englund, 2002). The aspect of test specificity is especially important for diagnostics in Sweden, as a positive test always leads to severe consequences for the herd. Other aspects of disease epidemiology and test sensitivity are, however, important for tracing of the infection from positive herds and optimising eradication measures. Studies covering some of these aspects are planned or underway. In addition, a project including an effort to estimate the probability of disease freedom based on the major surveillance components as described by Martin et al. *(*2007) is currently ongoing.

Import control

Live cattle and sheep that are imported to Sweden are checked for paratuberculosis, either by sampling the imported individuals or by investigations in the herd of origin (Holmström and Stenlund, 2005). Swedish Farmers' Disease Control Program, an industry-based voluntary import control organisation, requests additional samples for paratuberculosis on imported animals and in the exporting herd.

Although mandatory sampling of imported animals has not prevented the introduction of paratuberculosis to Sweden in the past, it has undoubtedly helped keep the prevalence at a low level. However, trade aspects may lead to the cessation of this import control. It is of vital importance that some kind of import control remains in place unless exporting herds can be certified as having the same status regarding paratuberculosis as Swedish herds. Otherwise, the money and efforts spent on eradication measures so far will soon be wasted.

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Results of the Dutch bulk milk quality assurance programme for paratuberculosis

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ABSTRACT

In January 2006, a bulk milk quality assurance programme (BMQAP) for paratuberculosis in Dutch dairy herds was initiated. The aim of the BMQAP is to reduce the concentration of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in milk delivered to the milk factories. The aim of this paper is to summarise progress within the BMQAP during its first 15 months.

Initiation of the BMQAP increased the total number of participating herds from 1071 in December 2006 (IPP only), to 1720 herds at April 1st, 2007 (including 494 herds in the IPP and 1226 in the BMQAP). Results of the initial assessment of all 670 dairy herds that newly joined the BMQAP (i.e. did not shift from IPP to BMQAP) were analysed in detail. In 363 of these 670 herds, ≥1 cattle were ELISA-positive. In 202 of these 363 herds, confirmatory faecal culture of ELISA-positives was confirmed. In 107 of these 202 herds, ≥1 cattle were FC-positive.

Based on the results of and experiences with the BMQAP, it is concluded that the programme is attractive to farmers and a practical and cost-effective way to assign a herd-status. However, in absence of consequences of a herd-status, the number of participating herds is still limited.

INTRODUCTION

Paratuberculosis is of concern to the cattle industry world-wide because of the unresolved issue of a potential role of *Mycobacterium avium* subsp*. paratuberculosis* (Map) in the pathogenesis of Crohn's disease in humans (Grant, 2005). In addition to pasteurisation, the most effective way to reduce any potential human health risk of exposure to Map through consumption of dairy products is to control and prevent paratuberculosis in the national dairy herd (O'Reilly et al., 2004). Therefore, a bulk milk quality assurance programme for paratuberculosis (BMQAP) in Dutch dairy herds was developed (Weber et al., 2005; van Roermund et al., 2005; Velthuis et al., 2006) and initiated in January 2006. The aim of the BMQAP is to reduce the concentration of Map in milk delivered to the milk factories (Franken, 2005). The BMQAP is run alongside the pre-existing 'Intensive Paratuberculosis Programme' (IPP), which aims at low-risk trade of cattle. Prior to its start, the BMQAP was expected to be cost-effective, farmer friendly, simple to manage and easy to communicate (Franken, 2005). The aim of this paper was to summarise progress within the BMQAP during its first 15 months of existence.

BULK MILK QUALITY ASSURANCE PROGRAMME

The BMQAP starts with an initial assessment consisting of a single herd examination. Test-negative herds enter a surveillance procedure consisting of biennial herd examinations. Test-positive herds enter a control procedure consisting of annual herd examinations and culling of test-positives. All herd examinations include testing milk samples of all lactating cattle or serum samples of all cattle ≥3 years of age by ELISA (ELISA Paratuberculosis Antibody Screening; Institut Pourquier, Montpellier, France). To reduce costs of sampling, milk samples are obtained at regular samplings for Milk Production Registration. ELISA results may be confirmed by individual faecal culture (IFC) of the animal concerned; if culture-negative, the animal is considered test-negative. Herds testing positive in the surveillance procedure shift to the control procedure; herds in the control procedure shift to the surveillance procedure if all results of a herd examination are negative. Herds in the surveillance procedure (i.e. 'green herds' in our previous modelling studies; Weber et al., 2005; van Roermund et al., 2005) are assigned 'Status A'. Herds in the control procedure (i.e. 'red herds' in our previous modelling studies) are assigned 'Status B' (if all test-positive

cattle have been removed from the herd) or 'Status C' (if any test-positive cattle are still in the herd).

RESULTS

Initiation of the BMQAP increased the total number of participating herds from 1071 in December 2006 (IPP only), to 1720 herds at April 1st, 2007 (including 494 herds in the IPP and 1226 in the BMQAP; Fig 1). Prior to the initiation of the BMQAP the number of herds participating in the IPP steadily decreased over time, which is related to the decreasing number of dairy herds in the Netherlands. After initiation of the BMQAP, a number of herds in the IPP shifted to the BMQAP. However, in addition to those herds, the BMQAP attracted 670 newly participating dairy herds between January 2006 and March 2007.

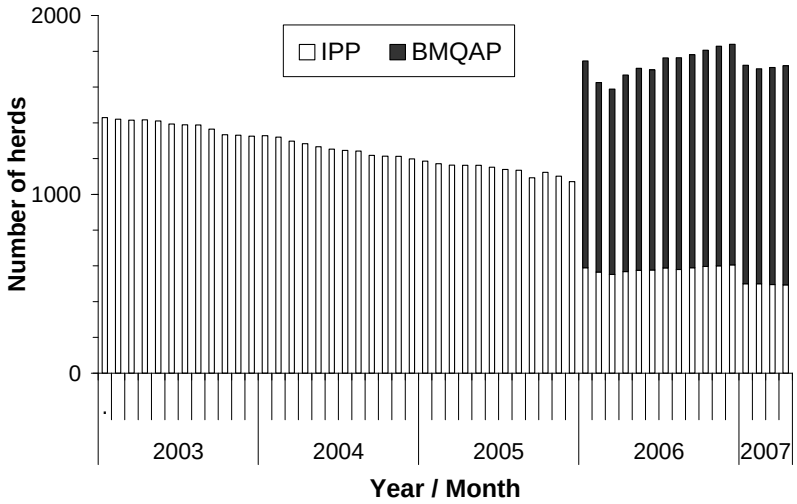

Fig. 1. Number of participating herds in the bulk milk quality assurance programme for paratuberculosis (BMQAP) and the pre-existing 'Intensive Paratuberculosis Programme' (IPP).

Results of the initial assessment of all 670 dairy herds that newly joined the BMQAP were analysed in detail (Fig 2). In 363 (54%) of these herds, ≥1 cattle were ELISA-positive. In 202 of these 363 herds, confirmatory IFC of ELISA-positives was performed. In 107 (53%) of these 202 herds, ≥1 cattle were FC-positive. If confirmatory IFC was performed, the probability (95% CI) of ≥1 IFC-positive individual in the herd increased with the number of ELISA positives from 27% (17%, 38%) in case of one ELISA-positive individual to 97% (86%, 100%) in case of ≥6 ELISA-positive cattle (Fig. 3). In a logistic regression, the probability for an ELISA-positive individual to be IFC-positive (if IFC was performed), much increased with the sample-to-positive ratio of the ELISA (Fig. 4).

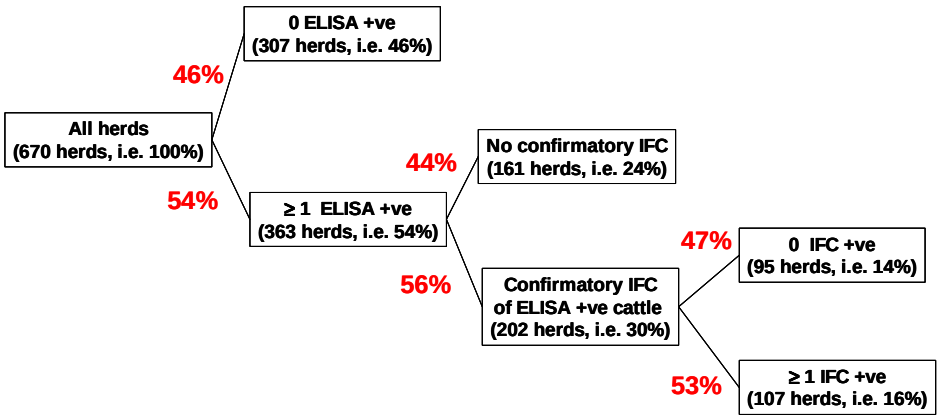

Fig. 2. Results of the initial assessment of 670 dairy herds that newly joined the BMQAP (i.e. did not shift from IPP to BMQAP) between January 2006 and March 2007.

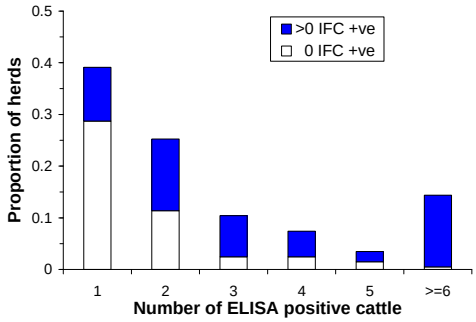

Fig. 3. Number of ELISA-positive cattle in 202 herds in which confirmatory individual faecal culture (IFC) was performed (in all or part of ELISA-positive cattle) and resulted in 0 or >0 IFC-positive cattle.

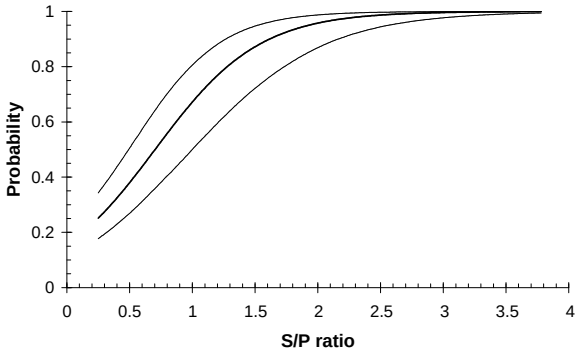

Fig. 4. Median and 95% CI of the predicted probabilities of a positive faecal culture result in dependence of the S/P ratio in a logistic regression on the results of a subset of 411 cattle with a positive milk ELISA (i.e. S/P ratio ≥0.25).

DISCUSSION

The results of the BMQAP indicate that the expectations of the programme as presented at 8ICP (Franken, 2005) have been met. Initiation of the programme resulted in an increase of the total number of participating herds in the paratuberculosis programmes, even in absence of incentives to participate or consequences of the herd status 'A', 'B' or 'C'. This indicates that differentiation of paratuberculosis programmes towards needs of various groups of farmers (such as milk quality assurance, enabling low-risk trade of cattle, or elimination of Map from known infected herds) is a key to success of such programmes. However, participation in the programmes is still restricted to a minority of today's ~ 21.000 Dutch dairy herds. Incentives are needed for farmers to justify the costs of participation in a BMQAP (Velthuis et al., 2006).

The observed proportion of ELISA-positive herds in the initial assessment (54%) was larger than estimated in our previous modelling studies (31%; Weber et al., 2005). Also, in the subset of herds where confirmatory culture of ELISA positives was performed, the proportion of herds with at least one faecal culture positive individual was larger than expected (53% versus 32%; Weber et al., 2005). Probably, the high proportion of test-positive herds is related to a selection bias of participating herds. Participating herds are larger than average (data not shown), and therefore the herd-level sensitivity of the initial assessment to detect infected herds is increased. Moreover, farmers familiar with paratuberculosis are more likely to enter the bulk milk quality assurance programme than farmers that are not familiar with the disease.

Based on the results of this study, farmers can be advised on the probability of a positive faecal culture of ELISA-positive individuals. Confirmatory faecal culture appears not to be cost-effective in herds with ≥6 ELISA-positive cattle (Fig 3). Currently, S/P ratio's of ELISA-positive cattle are not used in the programme. However, as the probability of a positive faecal culture result of an individual increases with its S/P ratio (Fig 4), S/P ratio's

might be used in the future to advise farmers on cost-effectiveness of confirmatory faecal culture and the probability that the individual is shedding Map.

CONCLUSION

We conclude that the Dutch bulk milk quality assurance programme does meet the expectations presented at 8ICP and is indeed attractive to farmers. The programme offers a practical way to assign a herd-status related to the objectives of the programme. Tailoring the Dutch paratuberculosis programmes towards the needs of various groups of farmers, by initiating the BMQAP alongside the IPP, increased the total number of participating herds. However, in absence of incentives to participate and consequences of a herd-status, participation is still limited to a minority of Dutch dairy herds.

ACKNOWLEDGEMENTS

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A risk assessment approach to the control of Johne's Disease

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In the UK, eradication and control of Johne's disease in cattle has been at the individual herd level, and a number of herds are accredited free of the disease as defined by Cattle Health Certification Standards (CHeCS). By necessity, the requirements to reach and maintain accreditation are onerous and involve the testing of all cattle in the herd over 2 years of age for antibodies to *Map* annually. Consequently, the majority of members of CHeCS programmes are pedigree herds selling breeding stock where a premium is commanded by high health status. We have developed a Johne's risk assessment (JRA) scheme, particularly directed to commercial herds where the burden of whole herd testing is not financially viable, as a rational approach towards investigating a herd's Johne's status. The JRA scheme is consistent with advice from the UK government and offers a logical classification of herd status according to the risk of presence of *Map* based on the purchase and clinical history over the preceding 10 years and the results of annual targeted sampling. Green, amber and red colour coding is used respectively to indicate herds with a low, medium or high risk of having cattle infected with *Map* and over time; herds have the opportunity to progress to a lower risk category if they purchase replacement stock solely from sources with a higher health status and have consistently negative targeted sampling results. The JRA scheme provides a framework for vets and farmers to investigate the Johne's status of a farm, and emphasises the impact of buying in replacement stock from herds with an unknown health status. If all laboratory results are clear, the farmer can work towards improving the herd's risk status in future years, however if targeted testing reveals evidence of *Map* infection, there is an impetus for vet and farmer to develop an appropriate control programme.

Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* infection over the first three years of the U.S. National Johne's Disease Demonstration Herd Project

Fossler CP, Lombard JE, Carter ME

United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services

The National Johne's Demonstration Herd Project (NJDDHP) in the United States was initiated to evaluate the long-term feasibility and effectiveness of management-related practices designed to control Johne's disease on dairy and beef cattle operations. The NJDDHP was started in 2003, but a few States had demonstration herds prior to the start of the National Project. The NJDDHP includes 67 dairy herds and 21 beef herds in 17 states. Adult populations in these herds range from 35 to more than 4,000 animals. All enrolled herds began with culture-confirmed *Mycobacterium avium* subspecies *paratuberculosis* (MAP) on the operation. Sufficient time since the start of the NJDDHP has now elapsed such that Johne's testing information on cattle born since the beginning of the project are becoming available. Using a generalized estimating equations approach to adjust for effects of herd and after adjustment for cow age, odds for fecal shedding at moderate to high levels were significantly less in the third year of the project compared to the first year (3rd Year OR=0.73 95% CI 0.56,0.96). Compared with the first year, significant differences were also noted for both dairy and beef herds by ELISA test results (Dairy: 2nd Year OR=0.80, 95% CI 0.65,0.97; 3rd Year OR=0.75 95% CI 0.62,0.91 Beef: 2nd Year OR=0.85, 95% CI 0.59,1.25; 3rd Year OR=0.36, 95% CI 0.24,0.55). These results to date suggest that herd prevalence has decreased since the beginning of the project.

Incidence of *Mycobacterium avium* subspecies *paratuberculosis* infection over four years of the U.S. National Johne's Disease Demonstration Herd Project

Fossler CP, Lombard JE, Carter MA

United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services

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

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

Koren O

Israel Dairy Board

The Israel Johne's disease control program (IJDCP) is operating since 2003. The program is voluntary and consist a herd management program and a whole herd testing by milk and/or serum Elisa and faecal culture of the sero-positive or doubtful cows. In the last 4 years 224 dairy herds were tested (42000 cows) for at least once.

The results of first testing indicate that half of the herds are lightly infected or not at all (0 to 2% sero-prevalence). Some of these herds suffer sporadic clinical cases and had some positive cultures. 18% of the herds from the other half are considered heavily infected with 5% or higher sero-positive cows, higher incidence of clinical cases and faecal culture positives.

Of the above mentioned herds 121 were tested twice or more in one year intervals. Most Herds with low (0-2%) prevalence in the first test maintained their status on the second test. Herds with medium (2-5%) prevalence didn't show marked improvement in the 2nd test. A few High (5%<) prevalent Herds showed some improvement which thought to be technical due to selection of positive cows, but the true picture was seen in the third (or higher) testing.

Herds that used a proper maternity pen, showed reduction from average of 4.6% in the first two tests to 3.4% positive sero-prevalence in the 3rd test while herds, which didn't do so increased their prevalence to 6.3%.

On national level there is some improvement concerning herd sero-prevalece. There are 13% heavily (5%<) infected herds in comparison to 19% at start point.

These Heavily infected herds produced an average of 11423 L of milk in 2005 with 3.57% fat and 3.12% protein which is not different from the country average on the same year.

Education for veterinarians and producers as part of the national control program

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Successful control programs depend on knowledgeable veterinarians and producers. In the United States we developed a multi-pronged approach to Johne's disease education, targeting regulatory veterinarians, as well as veterinarians in private practice, and producers of multiple species.

For veterinarians, we developed the Online Johne's Disease Veterinary Certificate Program, consisting of seven modules covering the basics of Johne's disease pathobiology and epidemiology, diagnostics and test interpretations, risk assessment, and management and control in dairy and beef operations. For practical application we created four virtual farm visits (dairy and beef) so that veterinarians can practice assessing the risk of Johne's disease occurrence and developing management plans for different types and sizes of operations with varying levels of disease prevalence. We also have modules that address Johne's disease in other species: goats, sheep, cervidae, camelids, and bison.

For producers, we have revised the modules for the certificate program to specifically provide relevant information. These modules are organized by type of operation (dairy or beef) and species (goats, sheep, cervidae, camelids, and bison.) In addition, we are developing a series of four modules where producers talk to producers about the economic impact of Johne's disease and control efforts on their businesses.

We will also report on the evaluation studies underway of both the certificate program and the Dairy Producer module. The purpose of the studies is to gain further insights into the impact of the respective education programs on veterinarians' and producers' knowledge and practice. In addition, we are assessing veterinarians' and producers' individual learning preferences, strategies, and activities, during and after their participation in the online education programs.

Danish Control Programme for Bovine Paratuberculosis

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Paratuberculosis is widespread in Denmark and a voluntary control programme was established in 2006, aiming at providing tools for farmers to control infections, and to ultimately reduce the prevalence in the country. Approximately 1150 (24%) of dairy farmers were enrolled in the programme by September 2007. Participating herds test all lactating cows four times / year by use of a milk antibody ELISA. The test-results are recommended to be used for risk-based management of infectious animals. This risk-based approach is aimed to reduce the workload of herd managers, thereby making implementation of changes more feasible than if all cows had to be managed with increased awareness. The test results are also used for communication to farmers, which is a central part of the programme. Communication between farmers and advisors also takes place via risk assessments, which helps the farmers identify risk areas of transmission. The end result is that farmers are well informed and play a role in management of their herd health.

Farmers are informed that the control programme in their herd is expected to last 6 to 8 years. Therefore, there is a continued need from farmers, their advisors and the central administration to identify tools and methods to ensure ongoing enthusiasm.

A surveillance component may be added to the programme at a later stage, but currently no officially recognised recommendations are available related to trade of live animals. The surveillance component may be a next step to maintain farmers in the programme and encourage more farmers to join.

Paratuberculosis control programme by faecal culture in cattle and other ruminants in the Czech Republic in the years 1992-2006

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Faecal culture examination using the 0.75% HPC method was used for the control of paratuberculosis in the Czech Republic in cattle, sheep, goats, wild capricorn, antelope, moufflon, fallow and red deer. A total of 117 210 cultures from cattle and 12 968 cultures from wild ruminants were examined. The introduction of infected animals from other farms (in the main cases, the importation of highly pregnant heifers and the purchase of their progenies) was the main reason for the spread of paratuberculosis in the Czech Republic with 54 officially registered cattle outbreaks in 2006. Successful control was adopted in 19.3% of cattle herds, in one herd of Capricorn and one herd of moufflon. The main reason for the achievement of the successful outcome was the removal of all faecal culture positive animals from the herd, including their progenies, separate rearing of calves from old animals and stringent hygienic management. However, the control programme is not yet completed in 45.0% of herds. The cause of the prolonged and unsuccessful control programme was failure to remove all progenies of infected animals and feeding calves with mixed colostrum and/or unpasteurised milk. A radical control programme was applied in 20% of cattle herds and a few herds of wild ruminants (three farmed red deer and fallow deer herds, one antelope herd and three herds of moufflon). In the rest of the outbreaks the control programme was suspended for financial reasons and due to low motivation of farmers. Certification program based on milk examination by PCR is now under discussion and preparation.

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MAP in retail pasteurised cow's milk. First report in Italy

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INTRODUCTION

To confirm the presence of MAP in commercially pasteurised cow's milk, several studies have been carried out in different European and American countries and mainly in the United Kingdom, where 1.8% pasteurized milk samples tested was MAP culture positive (Grant et al., 2002). In Germany MAP survival in HTST pasteurized cow's milk has been detected by Hammer et al., 2002. Since then, viable MAP has been cultured from commercially pasteurised milk in California, Minnesota and Wisconsin (USA) in 2005 (Ellingson et al., 2005). Mc Donald et al. (2005) has found the same results in Australia so has Ayele et al. in Czech Republic and Paolicchi et al. in Argentina in 2005. Gao et al. (2002) in Canada and O'Reilly *et al*. (2004) in Republic of Ireland found in commercial milk IS*900* PCR positive without culture isolation of MAP.

MATERIALS AND METHODS

To isolation and detection of MAP, 22 samples of pasteurised cow's milk have been collected from supermarkets in 3 Italian regions: Lombardia, Emilia Romagna and Latium (Fig. 1).

Fig. 1. Regions of Italy were has been collected cow's milk pasteurised tetrapack

Cultural method

For isolation of MAP from milk samples, 50 ml milk was centrifuged at 2000 rpm for 30 minutes at room temperature and the surnatant (whey) was removed. Different methodologies can be adopted to decontaminate samples. We used 30 ml of HPC 0.75% - BHI and the pellet was decontaminated for 4 hours at 37℃. After the decontamination, the samples were centrifuged at 2500 rpm for 20 minutes and the supernatant was removed. The pellets were resuspended in 1 ml of antibiotic solution constituting of 100 µg/ml nalidixic acid and 100 µg/ml vancomycin, and 0.1 ml of sample suspension was inoculated. We use Herrold's medium added with mycobactin J and natrium piruvate. The flasks were incubated at 37℃ for 12 weeks.

DNA extraction

To extract DNA ADIAPURE® ParaTB Milk Kit (Adiagene) was used.
1st step: Mycobacteria capture: add 1 ml of LO Buffer at 10 ml of milk, vortex, add 50 µl of magnetic beads suspension and gently mix 30 minutes for capture the bacteria. After 10 minutes to catch all beads on one side of the tube, the liquid is carefully discarded. The beads pellet is suspeded again in 600 µl of Lysis Buffer L1.
2nd step: Grinding with the Q-biogene Fast Prep.Put the whole mix into a microtube containing 300 mg of glass beads (Q biogene beads) and disrupt the bacteria at 4 m/sec for

3 X 45 seconds. Centrifuge the sample for 5 minutes at 7500 g, place 300 µl of the supernatant into a new 1.5 ml microtube and add 20 µl of L2 buffer. Afterwards, incubate 10 minutes at 70℃, then 15 minutes at 95℃.

DNA amplification

The DNA amplification of MAP was carried out with the PCR Kit ADIAVET Paratub ® Adiagene. This method is based on the amplification of a DNA fragment insertion sequence IS*900* specific for MAP strains. The home-made PCR was employed by PCR 1 and PCR 2. The target of PCR 1 primers (p 90 / p 91) was 400 bp fragment localized within the IS*900* insertion element. To generate a specific internal probe sequence, a second pair of oligonucleotides (p 25 / p 26) for a Nested PCR application was designed; these primers amplify a 229 bp using the product of PCR 1 as DNA template.

RESULTS

All 22 cartons samples of pasteurised cow's milk,produced in Italy and purchased in different markets were found negative by the cultural method. Only one sample of pasteurised and semi-skimmed milk from a local cooperative, in the Rieti province (Latiun region), was positive in the Nested-PCR reaction and in the kit PCR Kit also (Figs. 2 and 3). This milk sample was collected from small dairy farms in the province, which have had one of highest herd prevalences of paratubercolosis in Latium (52%, +/- 5%-CI 95%).This result was reported in our study in 2003 (Fig. 4).

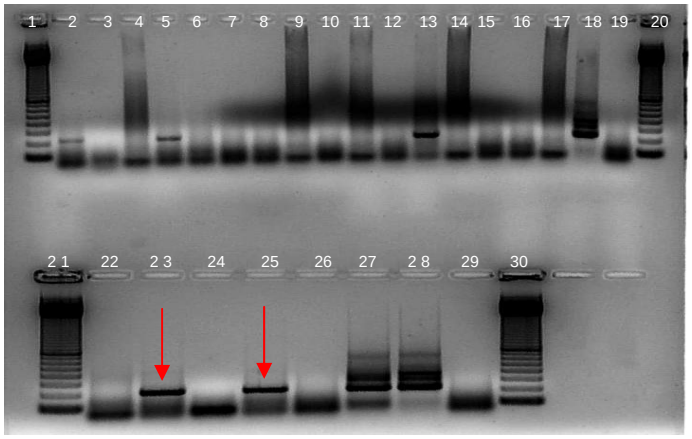

Fig. 2. PCR KIT Commercial
1, 16, 17, 24: Ladder 100bp
4, 5: positive controls
9, 21: positive samples from one pasteurised cow's milk
23 : negative control

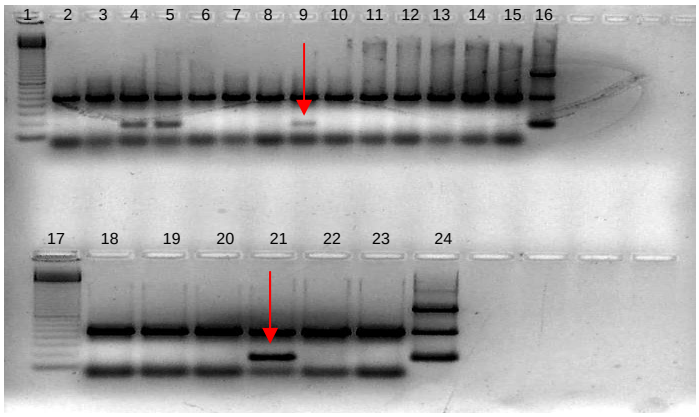

Fig. 3. NESTED PCR
1, 20, 21, 30: ladder 100 bp
23,25: positive samples from one pasteurised cow's milk
2,5: positive controls
19 : negative control

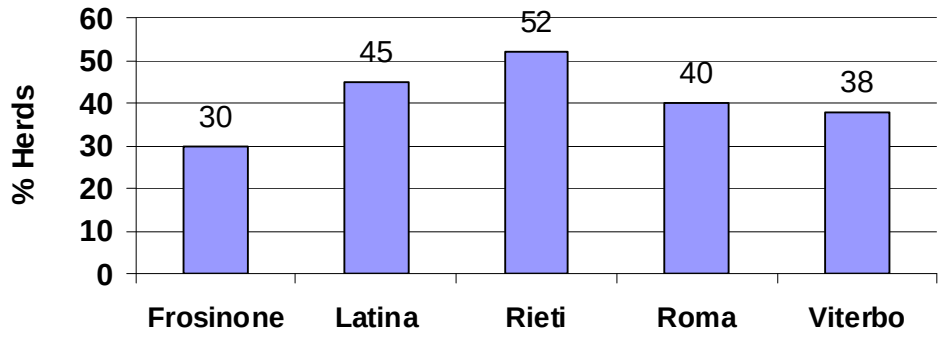

Fig. 4. Percentage of positive herds examined in 5 Latium provinces

DISCUSSION

The results of the study show that Map can be detected in pasteurised cow's milk in Italy too. The pasteurisation process does not inactivate 100% of the bacterial cells, even if it can reduce the microbic charge (from 4 to 7 log).The cultural medium does not allow an accurate MAP numbering in milk. We were not able to verify whether milk contains "x" CFU / ml after pasteuritation or not. The method adopted has proved to be more sensitive, if compared to the previous ones, but not yet so reliable to avoid false negative results. Indeed, they instil a false sense of safety in the end consumers as far as these products are concerned.

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

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INTRODUCTION

Mycobacterium avium subsp *paratuberculosis* (MAP) is an important veterinary pathogen, implicated in Sarcodosis and Crohn's disease in human beings. Recently, Sechi et al. (2007) reported involvement of MAP in T1 Diabetes Mellitus. MAP causes systemic paratuberculosis in animals and has been recovered from breast milk of lactating mothers and from blood of Crohn's disease patients (Naser et al., 2000; 2004).

Bacilli can be mainly found in the endothelium and Peyers patches of intestines and mesenteric lymph nodes of clinical and sub-clinical animals. Bacilli have been isolated from udder, supra-mammary lymph nodes (SMLN), uterus and testis of clinical animals (Sweeney et al., 1992; Vohra et al., 2007). In-utero transmission of MAP has been documented (Buergelt et al., 2004). MAP infection of uterus and placenta may lead to congenital infection and abortion in animals (USDA 2006).

In India, MAP infections are endemic in goats and sheep (Singh et al. 1996). High prevalences of MAP were recorded in dairy cattle (Sharma et al., 2007) and buffaloes (Yadav et al., 2007). Recently, Singh et al. (2007a) reported a high presence of live MAP in milk and milk products in commercial brands indicating increased presence in food chain and risk of exposure of MAP to human population in India. High rate of miscarriage or spontaneous abortions in women (>10) have been reported from most states in India (NFHS, 1999).

The present study estimated sero-prevalence of MAP specific antibodies in spontaneous cases of human abortions in North India to seek association between MAP and spontaneous cases of abortions.

MATERIALS AND METHODS

Cases of spontaneous abortions in human patients

Fifty serum samples (20 from Bareilly region of Uttar Pradesh and 30 from Punjab) from spontaneous cases of abortions in human beings in North India were screened for specific MAP antibodies using indigenous ELISA kits. Twenty samples from Bareilly region were collected from 4 different centers (7 from District female hospital, 8 from Nildev hospital, and 5 from Suvidha hospital). While 30 samples from Punjab were collected from Gurpreet Nursing Home, Ludhiana. Serum samples were collected aseptically from these patients and were transferred to Central Institute for Research on Goats, Mathura for screening under ice.

ELISA kits

Two ELISA kits, using native semi-purified protoplasmic antigen (NPA) from indigenous MAP 'Bison Type' strain of goat origin (Kit 1 – 'Indigenous ELISA kit'), and another kit using commercial purified protoplasmic antigen (PPA) of MAP 'Bovine' origin (Kit 2 – ''Commercial ELISA kit') were used. ELISAs, initially developed for the screening of goats (Singh et al., 2007b) and adapted for use in human beings (Singh et al 2007c), were used for the screening of cases of spontaneous abortions against MAP infection. Each of the 50 serum samples was screened using the two ELISAs. Serum samples from culture positive CD patient and culture negative healthy person were used as positive and negative serum controls, respectively. OD values were transformed to S/P ratios as per Collins (2002).

RESULTS

Sero-prevalence of MAP antibodies

Screening of serum samples from 50 women with history of abortion from North India by ELISA 1 and ELISA 2 detected, 34 and 30% sero-positives, respectively. Of the 20 patients from Bareilly region, 30% were positive by each ELISA 1 and ELISA 2. Individually, 14, 50, 20% and 14, 38, 40% patients were positive in District female, Nildev and Suvidha hospitals from Bareilly region using ELISA 1 and ELISA 2, respectively. Of the 30 patients from Punjab (Gurpreet nursing home), 37 and 30% were positive by ELISA 1 and ELISA 2, respectively (Table 1).

Table 1. Screening of spontaneous abortion patient's from North India for anti MAP antibodies by ELISA

State/ Region	Hospital	Patients	Percent Positives	
			ELISA 1	ELISA 2
Utter Pradesh /	Nildev	8	50	38
Bareilly	Suvidha	5	20	40
	District Female	7	14	14
Punjab / Ludhiana	Sub Total	20	30	30
Total		50	34	30

Comparison of ELISAs

The two ELISAs together detected 42% sero-positive among patients with abortions. Only 22% of the samples were found positive in both the kits. There was 80% agreement (positive and negative) between the two ELISA, whereas, 12 and 8% patients were detected independently by ELISA 1 and ELISA 2 respectively (Table 2).

Table 2. Comparative evaluation of 2 ELISA kits

	ELISA 2 Positives	ELISA 2 Negatives
ELISA 1 Positive	22%	8%
ELISA 1 Negatives	12%	58%

DISCUSSION

Earlier studies led to rising interest concerning fertility and outcome of pregnancy in patients suffering from inflammatory bowel disease (Mogadam et al., 1981; Nielson et al., 1984;). In this study, the status of MAP specific antibodies was measured in cases of spontaneous abortions from North India. The status of patients with respect to inflammatory bowel disease was not known. A commercial ELISA kit used in cattle has been adapted for screening of human beings by Bernstein et al. (2004). Using a commercial ELISA kit, Collins et al. (2000) reported that MAP sero-positivity rate was approximately 35.0% for all the groups and there was no difference between Crohn's disease (CD) and Ulcerative colitis (UC) patients and healthy controls or non-affected sibling.

Presently country lacks indigenous ELISA kits for the diagnosis of MAP both in animals and human beings. Therefore the two ELISA kits (ELISAs 1 and 2), initially developed for the screening of goats (Singh et al., 2007b) were adapted for use in human beings (Singh et al., 2007c) and employed in the present study for the screening of cases of spontaneous abortions. Comparison of 'Indigenous ELISA kit' using NPA with commercial kit (PPA) in goats, showed that the 'Indigenous kit' had significantly higher sensitivity (Singh et al., 2007d).

Among patients from North India, 34 and 30% were sero-positive for specific MAP antibodies using ELISA 1 and ELISA 2 respectively (Table 1). In another study of Singh et al. (2007c), ELISA 1 and ELISA 2 detected 100 and 40% serum samples positive from CD patients. The 80% patients were positive for MAP in their stool culture. MAP colonies were characterized using IS*900* PCR. In the same study 38 and 41% of the healthy population of human beings from North India were positive for specific MAP antibodies using ELISA 1 and ELISA 2, respectively. The high prevalence of specific MAP antibodies in the healthy population may be due to the continuous exposure of human population to MAP via the food

chain. Lower sero-positivity to ELISA kits (34% for NPA and 30% for PPA) in the present study as compared to healthy population may be due to immuno-suppression during pregnancy (cases of spontaneous abortions). On comparative evaluation, the 2 ELISAs together detected 42% patients positive for MAP antibodies. The 22% patients were detected positive by two ELISAs. Agreement (positive and negative) between the two ELISAs was 80%. The 12% and 8% patients were detected independently by ELISA 1 and ELISA 2, respectively. The study indicated exposure of human population to MAP of animal origin. The rates of sero-positivity for specific MAP antibodies in spontaneous cases of abortion in North India were not more than the positivity rates in healthy population. However, the rates were high in regions where rates of spontaneous abortions were low (Punjab). This indicated that other factors may be responsible for spontaneous abortions.

CONCLUSION

The present study indicated that MAP may not be associated with the cases of spontaneous abortions. ELISA 1 was superior to ELISA 2 in detecting patients with MAP infection. This may be due to predominance of 'Bison type' genotype of MAP in human population. The study showed the need for much wider study and MAP may be more prevalent in human beings and associated with many health problems than found by us.

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

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Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is considered as a highly resistant species among mycobacteria towards various stress factors. The purpose of this study is to assess a resistance profile for *MAP* towards inactivation factors relevant in food processing, such as an increased temperature, UV-light pulses, chlorine dioxide and lactic acid. Two strains of *MAP*, a reference strain and a bovine isolate from faeces, have been subjected to the heat treatment (60-90°C at 10s, 20s and 30s intervals), the chlorine dioxide treatment (6-12 ppm, 2 min. interval), and the lactic acid treatment (25, 50, 75 and 100 mg/ml, 2 min. interval). Two repeated cycles of inactivation by UV-light pulses with the same strains have been performed in order to induce resistant variants (number of pulses: 10-120). Controls are included in all experiments. The results of inactivation have been measured by the time to detection in liquid MGIT media and by the log mean CFU/ml on solid HEYM media. A not so clear effect of an increased temperature on *MAP* has been observed within the time intervals, even some kind of activation has been shown from the curves at 60-80°C. With UV-light pulses, only mild inactivation has been observed for both the first and the second repeated experiments, depending on the strain. On the other hand, when using chlorine dioxide, a strong effect of inactivation for the chosen range of concentrations has been demonstrated. Similarly, a strong effect of lactic acid on *MAP* has been documented. The reference strain, probably more sensitive than the bovine isolate, does not grow after some treatments on solid HEYM, but it grows in liquid MGIT. Thus, the use of the MGIT culture system may be promising for such studies or perhaps for primary isolation of the etiological agent of paratuberculosis.

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

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

The coupling of culture enrichment with real time quantitative PCR (qPCR) to detect viable *Mycobacterium avium* subsp. *paratuberculosis*

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Considerable attention has been given to milk and dairy products as potential vehicles of *Mycobacterium avium* subsp. *paratuberculosis* (Map) transmission to the food chain. Surveillance of such products for the presence of Map has been hindered by difficulties in culture methodology. Molecular detection methods for Map, including endpoint PCR (duplex, multiplex and nested) and real time quantitative PCR (qPCR) have been widely reported. However, these methods cannot differentiate between DNA recovered from dead and viable cells.

The method assessed in this study involved addition of test sample to enrichment broth, removal of initial sub-sample for Map-DNA extraction/qPCR followed by enrichment and a subsequent DNA extraction/qPCR assay. A significant reduction in the qPCR Ct value following enrichment is indicative of Map growth/recovery.

Samples containing < 2 cfu/ml Map were enriched into Middlebrook 7H9 broth, Dubos broth and a customised enrichment broth. Following Map-DNA extraction using the Adiapure® kit and real time PCR (IS900 and F57), Map was undetected at time zero but was detected in 7H9 enrichment medium after 7 d (Ct: 36); 14 d (Ct: 25); 21 d (Ct: 20). Enrichment in Dubos medium and the customised broth resulted in slower rates of growth and qPCR detection. Raw milk cheeses (n = 14) were subject to PANTA-supplemented 7H9 enrichment/qPCR. No viable Map were detected (enrichment (>100 d)/qPCR) despite a number of the samples revealing Map presence prior to enrichment. All samples were negative by culture (7H10 and HEYM medium) after 20 wk incubation suggesting no viable Map present in cheese samples. Map- spiked pasteurised and raw milk samples were heat treated and subsequently enriched. Decreases in Ct values (qPCR) in the enriched pasteurised samples after 5 wk was indicative of recovery and growth of heat-treated Map cells whereas enriched raw milk samples presented problems for the PCR assay.

This enrichment-qPCR strategy may represent a methodological option to screen samples for the presence of viable Map rather than liquid culture which can rely on continual monitoring, subculture and confirmation.

Evaluation of real time and end point PCR for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in artificially-contaminated raw and pasteurised dairy products

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Dairy products, such as cheese and yoghurt, are potential vehicles of transmission of *Mycobacterium avium* subsp. *paratuberculosis* (Map) to the food chain. In this study a method has been developed for the extraction and detection of Map DNA from raw and pasteurised dairy products; Gouda (semi-hard), Danish Blue (semi-soft) and Munster (soft) cheese, along with plain full fat yoghurt, artificially-contaminated with three Map strains:- NCTC 8578, Niebuell and NIZO B2962.

Cheese and yogurt samples were spiked with a range of Map inocula. Map-DNA was recovered from homogenised (sodium citrate-based) cheese and yogurt samples using the Adiapure® kit (Adiagene, France). Template DNA was assayed by real time PCR, targeting both IS*900* and F57genetic elements (ABI 7500) in addition to end point PCR using P90/P91 primers and the Adiavet® kit (Adiagene, France). Initial inoculum levels were determined by plate culturing on Herrold's Egg Yolk and Middlebrook 7H10 media.

In real time PCR assays, higher sensitivities were achieved by probes targeting IS*900* than the f57 target. Using the former, Map was detectable at < 20 cfu g^{-1} in the cheeses Danish Blue and Munster and yogurt whereas Map extraction and subsequent real time detection was less sensitive for the harder Gouda cheese. This may imply a more efficient Map-DNA extraction from the matrices having a higher water content. For all matrices and Map strains assessed the endpoint Adiavet® kit provided a higher rate of detection/improved sensitivity than endpoint derived P90/P91 (both based on IS900) PCR. However, the real time PCR assay represented a more sensitive molecular detection method than either end point PCR assay.

The real time PCR method coupled with the Adiapure Map-DNA extraction kit represents a reproducible, sensitive and convenient assay for the detection of Map-DNA from a range of raw and pasteurised dairy products.

Is *M. avium* subspecies *paratuberculosis* (MAP) the cause of multiple "autoimmune" and "inflammatory" diseases in man? Inferences from the antiMAP activity of methotrexate, 6-MP, 5-ASA and thalidomide, on MAP in culture

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BACKGROUND
We have shown that the "immuno-modulators" methotrexate and 6-MP and the "anti-inflammatory" 5-ASA inhibit MAP growth (www.PLoSONE.org) and concluded that their most plausible mechanism of action in several idiopathic diseases is as antiMAP antibiotics. Thalidomide is an "immunomodulator" used in multiple "auto-immune" and "inflammatory" diseases and the mycobacterial diseases leprosy and tuberculosis. We now test the hypothesis that thalidomide inhibits MAP growth.

METHODS
Thalidomide (±) and (+) and (-) and its two components, phthalimide and 1-hydroxy 2,6 piperidine dione (HPD) were evaluated in culture of two strains each of MAP (ATCC 19698 [bovine] & Dominic [human]) and M. avium subspecies avium (ATCC 25291 & 101.) We used a radiometric ($^{14}CO_2$ Bactec®) detection system. Inhibition is indicated by "percent decrease in cumulative Growth Index" (%-DcGI) from control.

RESULTS
Phthalimide has no dose dependent inhibition on any strain. There was no dose dependent inhibition on either M. avium strain with thalidomide or its components. With the two MAP strains, there is dose dependent inhibition with thalidomide (±); Dominic (31%-DcGI) and ATCC 19698 (26%-DcGI) at 64µg/ml. Thalidomide (+) is more inhibitory than (-). HPD is, on a weight for weight basis, the most inhibitory agent evaluated; Dominic (46%-DcGI) and ATCC 19698 (44%-DcGI at 64µg/ml)

CONCLUSIONS
We show in vitro heretofore-undescribed inhibition of MAP growth by racaemic thalidomide. Thalidomide (+) is more potent than (-). Of thalidomide's two moieties, phthalimide has no antiMAP activity and HPD is the active component in inhibiting MAP growth. We suggest that since 1942, initially with 5-ASA, the medical profession has unknowingly been treating MAP infections. These data are compatible with our concern that MAP is zoonotic. We conclude that all idiopathic "autoimmune" and "inflammatory" diseases, empirically treated with medications that we show are active against MAP, should now be evaluated for MAP as the etiological agent.

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

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BACKGROUND
Although controversial, there is evidence that IBD may be caused by infection with MAP. Recently, three agents (5-ASA, methotrexate and 6-MP) used to treat IBD because of clinical efficacy, but without an accepted mechanism of action, have been shown to inhibit MAP growth in culture. The purpose of this study was to determine the prevalence of MAP DNA in the blood of healthy controls and patients with IBD, to determine the influence of chronic treatment with these antiMAP antibiotics and to evaluate the associations between MAP DNA prevalence, disease activity and patterns of treatment by geographical location.

METHODS
The blood of 100 healthy individuals and 246 patients with IBD was evaluated for MAP DNA using nested PCR. Statistical analysis was by the Fischer Exact Test or Pearson Correlation as necessary.

RESULTS
MAP DNA was detected in 47% (47/100) of the healthy controls and in 16.3% (40/246) of all subjects with IBD ($p<0.0001$). MAP DNA was found in 15%(37/246) IBD patients, who were receiving any antiMAP antibiotic therapy. The lowest MAP DNA frequency was observed with combined methotrexate, sulfasalazine, 6-Mercaptopurine or Ciprofloxacin therapy 3.1%(1/32) ($p<0.02$). The group receiving azathioprine (a precursor of 6-MP) combined with prednisolone was 42%(5/12) MAP DNA+, compared to the group with azathioprine without prednisolone that were 10.5%(4/38) MAP DNA+ ($p<0.03$). MAP DNA prevalence varied by geographical location and showed a correlation with disease activity and pattern of treatment ($p<0.001$).

CONCLUSION
Analogous to leprosy, our data show an unsuspectedly high incidence of MAP DNA in healthy human blood donors. Chronic use of anti-MAP antibiotics is associated with a significantly lower incidence of MAP DNA, possibly an indication of therapeutic efficacy. The use of prednisolone is associated with an increased prevalence of MAP DNA. This may indicate prednisolone immunosuppression, and/or reflect IBD disease activity. The geographical variation may reflect different IBD therapy by local physicians and may become a useful indicator of therapeutic efficacy. These data are compatible with an etiological role of MAP in IBD that could be comparable with leprosy where for every case of clinical leprosy, >100 asymptomatic individuals shed *M. leprae* DNA.

Detection of *Mycobacterium avium* subsp. *paratuberculosis* DNA in blood and immune responses in humans with Inflammatory Bowel Disease

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Inflammatory Bowel Disease (IBD) is generally recognised as a dysregulated immune response to bowel flora in genetically susceptible individuals, although its aetiology has never been fully clarified. The similarities with ruminant paratuberculosis, and the isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from Crohn's Disease (CD) patient tissues suggest Map as an agent in the pathogenesis of CD. This study was aimed at finding microbiological and immunological evidence of an association between Map and IBD. DNA was extracted from blood of 222 patients and 80 healthy donors from the Basque Country (Spain). Nested PCR for the amplification of Map-specific insertion sequence IS900 was performed, as well as measurement of interferon-γ (IFN-γ) production in whole blood stimulated with PPA-3 Map antigen (IFNMap) or with Phosphate Buffered Saline (PBS) (IFNPBS) and antibody absorbed ELISA with PPA-3 Map antigen (ABMap). The analyses showed that 17.1% of IBD patients and 42.5% of controls were positive to IS900. IBD patients showed lower IFNMAP production and higher ABMap (specific response) as well as higher IFNPBS production (non-specific response) compared to controls. Statistical analyses showed significant interactions between Map DNAaemia and disease group for IFNPBS and ABMap. Treatment was associated with decreases in IFNMap and PCR-positive frequency. These results suggest the existence of type I and type II immune responses in blood of both healthy controls and IBD patients related to the presence of Map DNA. Pathogenetic models of other mycobacterial infections such as paratuberculosis, tuberculosis and leprosy, where a large fraction of the population is infected but never become clinical, possibly in relationship with a genetic immune dysregulation, support a Map aetiology hypothesis for CD.

Mycobacterium Paratuberculosis and Crohn's Disease: That's what epidemiology is all about

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Mycobacterium avium subsp *paratuberculosis* (Map) is the causative agent of Johne's disease (JD), a chronic and progressive intestinal disease in ruminants, which imposes large direct and indirect productivity losses on affected farms. Crohn's disease (CD) in humans has been characterized as a chronic, relapsing, and remitting inflammatory process of the digestive tract with protein losing enteropathy, general malabsorption and steatorrhea. Numerous studies have shown that genetic and environmental factors are the basis for the pathogenesis of CD. Twin studies and familial aggregation have provided compelling evidence for the heritable nature of CD, and strong familial pattern has been observed in CD patients. It has been reported that the lifetime risk for developing CD in the sibling of an affected person is approximately 30–40 times greater than that in the general population. To date, several human genes and loci have been identified that may contribute to CD such as the IBD1.

There are several histopathological and clinical similarities between JD and CD. Because of these similarities, a mycobacterial cause of CD has been sought for more than 90 years! Although early studies did not detect Map in tissues from patients with CD by conventional stating and culturing techniques, in the late 80's researchers could isolate Map from tissue samples from patients with CD after many months of incubation, leading to renewed interest in a mycobacterial origin of CD. Recent serologic studies have demonstrated that up to 83% of CD patients showed evidence of serum antibodies to Map. However, molecular mimicry that can serve as target for cross-reactive immunity in CD has been recently described. To date, several antibodies have been identified in the serum from CD patients such as for *Pseudomonas fluorescens, and E. coli*. These antibodies are regarded as signals of abnormal responses to innate or foreign proteins, because they do not usually exist in the healthy population.

The frequent use of the DNA insertion element IS*900* as a tool for accurate identification, in addition to bacterial culture, has yield provocative but inconsistent results, probably because PCR cannot differentiate between viable Map and Map DNA. In these studies, 13%-100% of CD patients tested positive to Map. Other studies, however, have been unable to demonstrate Map DNA in CD tissue. One potential problem with these results is that some primers designed from IS*900* can cross-react with closely related IS*901* and IS*902.* Nevertheless, in addition to CD patients, studies have detected Map also in control subjects. It is possible that low incidence rates in CD patients' tissues may be more of a reflection of the widespread and ubiquitous occurrence of mycobacterial organisms, than an indication that Map is a causative agent in CD. It has been also demonstrated that lactating mothers with CD shed Map in their breast milk. Unlike in JD, where Map can be detected in almost any clinical case, reports are mixed as to the presence or absence of Map at the site of the intestinal lesions.

There is no sound epidemiological evidence that links exposure to Map to an increased incidence of CD disease, even though cows with clinical paratuberculosis do shed viable organisms in their milk at low levels (50 CFU/50 ml milk), and the consumption of inadequately pasteurized dairy products has been a major source of concern for the potential spread of Map to humans. Standard temperatures and times for heat pasteurization of milk, especially either via standard holder methods (63.5°C for 30 minutes) or high-temperature, short-time methods (71.7°C for 15 seconds), have been shown to be insufficient to kill all Map in milk. In Great Britain it has been demonstrated that Map DNA is present in milk samples obtained from retail markets. On the other hand, some studies indicate that through adequately pasteurized dairy products (72°C for 15 seconds), the transmission of viable Map from animals to humans via can be made even less likely,

thus minimizing any remaining potential concern that it may act as a zoonotic agent in CD. The culture, however, of viable Map from pasteurized retail milk samples, raised the concern that milk may become contaminated post pasteurization. Nevertheless, the detection of Map DNA in retailed milk raised the obvious question whether people with lifetime or childhood intense exposure to dairy cows are more susceptible or more resistant to CD?

In cattle, Map infection occurs in a very early age but clinical signs do not develop until at least 2 years of age. If Map were to behave in a similar manner in humans, it would be extremely difficult to associate exposure events occurring during childhood with the subsequent development of CD, perhaps many years later. No clinical evidence has shown an increased incidence of CD in farmers or agricultural workers associated with dairy herds with a high incidence of JD. Nor has eating organs or tissues from infected animals been documented to cause infection of humans with Map. Two studies one from England and another from Israel find no association between exposure to dairy or even to JD cows and CD. Another case-control study from the UK, found that the consumption of pasteurized milk was associated with reduced risk for CD (OR=0.82). Finally a recent study in Ontario, Canada performed a systematic review of the literature on the subject, weighing in on the current evidence for or against a causal relationship. So far, despite the volume of research conducted to address this issue, the evidence for an association between JD and CD has not been strong. Because CD is a rare disease the appropriate epidemiological approach would be a large-population case-control study, looking for potential risk factors with strong association.

These and other study results, even when supporting the presence of Map in CD patients, require us to reflect on two basic concepts in epidemiology: association and causality. An apparent association between Map and Chron's does not imply casual relationship. The issue of causality can only be addressed by examining disease onset in relation to exposure to the putative pathogen. Temporal association, one of the important criteria for causality, makes us wonder what came first? The egg or the hen i.e. Map or CD? Is it possible that Map is only an opportunistic bacteria?

In the absence of suitable animal models, epidemiological studies are the most effective means of determining whether Map plays a causative role in the etiology of CD. As a conclusion it is suggested that although solid evidence insinuate an association between CD and Map, the proclamation of Map as the causal agent of CD and therefore JD as a zoonotic disease requires not only time, but large population epidemiological studies.

Reference available from the author upon request.

Lethality of the milk spray-drying process for *Mycobacterium avium* subsp. *paratuberculosis* using a model system

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This work was undertaken to determine the efficacy of the milk spray-drying process for killing *Mycobacterium avium* subsp. *paratuberculosis* (Map). In the absence of pilot plant equipment with the necessary safety containment facilities a tripartite approach was adopted. Firstly, using *M. smegmatis* as a surrogate for Map and pilot plant equipment the organism was subjected to evaporation heat treatments which are a precursor to the spray-drying stage of milk powder production. *Mycobacterium smegmatis* did not survive evaporation treatments ranging from 75^{o}C for 15s to 85^{o}C for 4 min. Secondly, *M. smegmatis* was found to have a significantly higher ($P<0.05$) D value (D_{63} 8.71 min) than Map (D_{63} 6.78 min) at 63^{o}C using milk concentrate as the heating menstruum. Thirdly, milk powder from 3 plants within Northern Ireland was surveyed over a year period for Map using PCR assays based on the IS*900* insertion element and culture. Although 9.5% (18/190) samples were positive by PCR no culture positives were obtained. This work provides credence for considering spray-drying as one strategy for dealing with milk suspected of containing viable Map and destined for human consumption.

Association of *Mycobacterium avium* subsp. *paratuberculosis* with Type-1 diabetes, a possible trigger

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Mycobacterium avium subspecies *paratuberculosis* (Map) is a zoonotic pathogen whose association with Crohn's disease in humans is under scrutiny. To investigate its association with other chronic diseases where the involvement of a persistent pathogen as Map could be the trigger

Forty-six diabetic patients were recruited along with 50 healthy people as control. Map was searched in the PMBC by a specific PCR targeting IS900. Sequence product confirmed identity by sequencing. Also, all the diabetic patients revealed significant humoral immune responses to two recombinant Map antigens and the whole cell lysate of the Map bacilli.

A total of 29 blood samples out of 46 were found to be positive for Map specific PCR (63%) whereas only 8 out of the 50 healthy control samples (16%) generated a positive signal. Extremely significant humoral responses to recombinant HbHA and GSD proteins and the whole cell lysates of the Map bacilli were recorded in T1DM patients as compared to healthy controls.

We report presence of Map DNA and Map specific antibodies in the blood of Type1 Diabetes Mellitus (T1DM) patients in an endemic setting like Sardinia. Finding evidence of Map involvement in T1DM is perhaps a novel finding that might serve as a foundation stone in establishing an infectious aetiology for T1DM.

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

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

Isolation, identification and characterization of *Mycobacterium avium* subspecies *paratuberculosis* from multiple clinical samples of Crohn's disease patients in India using microscopic examination, culture, ELISA and PCR tests

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

Proposed International Guidelines for Experimental Challenge Models for Johne's Disease

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ABSTRACT

Animal challenge models are critical to evaluate potential vaccine candidates and to study host immune responses to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. Virtually all researchers have developed their MAP challenge model independently of others, resulting in a high degree of variability. The need to standardize challenge models for vaccine efficacy studies was a conclusion reached in August 2005 at the International Colloquium for Paratuberculosis “Role of Vaccination” workshop, held in Copenhagen, Denmark.

An international expert committee of Johne’s Disease (JD) researchers was convened to review and develop guidelines for JD challenge studies in multiple animal species. Members of the committee included Murray E. Hines II, Judith R. Stabel, Raymond W. Sweeney, Frank Griffin, Adel M. Talaat, Douwe Bakker, Geart Benedictus, William C. Davis, Geoffrey W. de Lisle, Ian A. Gardner, Ramon A. Juste, Vivek Kapur, Ad Koets, Jim McNair, Greg Pruitt, Robert H. Whitlock.

Parameters essential for the development of long term and acute infection models were outlined and harmonized to provide a template JD challenge model for cattle, goats, sheep, cervids, and mice. The intent was to develop and propose international standard guidelines for models that would gain acceptance worldwide. The consensus guidelines for models developed by this committee included recommendations for experimental challenge studies listed by animal species for strains of *Mycobacterium avium* subsp. *paratuberculosis* used, challenge dose, dose frequency, age of challenge, route of challenge, preparation of inoculum, method of quantifying MAP in the inoculum, experimental animal selection, quality control and minimal experimental endpoints.

These models will be useful to study host-pathogen interactions, host immunity at the local and systemic level, and for evaluating vaccine candidates and therapeutics. The results of the literature review, and Committee recommendations for experimental model parameters were published in Veterinary Microbiology 2007.

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9ICP Summation

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Colloquia sponsored by the International Association for Paratuberculosis bring together the global community of scientists working on *Mycobacterium paratuberculosis* and related pathogens. The 9th Colloquium in Tsukuba, like those that preceded it, provided the latest cutting edge science in diverse fields of study. From education and national program design to molecular biology and pathogenesis, the breadth and depth of research presented at the 9ICP are impossible to encompass in a single summation. My intent, instead, is to step back from the scientific details and look at the broader issues. I contend that the contemporary reductionist research approach to the paratuberculosis problem obscures an important bigger picture. The old cliché might very well be true: we can't see the forest for the trees.

Dissecting a complex biological problem such as paratuberculosis into its most basic elements is a problem solving approach in vogue today. At its heart is the assumption that by a thorough understanding of the mechanisms and principles underlying complex problems a solution will emerge. Molecular biology has provided novel tools that entice researchers to work at an elemental level of biology, i.e. with the nucleic acids which are assembled in myriad ways to instruct living creatures on what they are and how they behave. Research funding agencies have literally bought into this reductionist research philosophy.

Research presented at the 9ICP, the portfolio of projects contained in the ParaTBTools research consortium in the European Union (>US$ 5.7 million for 3 years), http://www.ucm.es/info/paratbtools/, and the Johne's Disease Integrated Project (JDIP) in the U.S. (>US$8 million for 6 years), http://www.jdip.org/, provide abundant examples of reductionist research on paratuberculosis. However, in spite of the large and growing global effort to understand paratuberculosis, the disease continues to spread affecting ever more domestic animal herds and flocks. In the U.S. for example, the herd prevalence of paratuberculosis has grown from an estimated 22% of U.S. dairy herds in 1996 to over 70% of U.S. dairy herds in 2007. This expanding epidemic is resulting in greater contamination of the environment, wildlife sharing range, foods of animal origin, and humans. Two meta-analyses published this year convincingly demonstrate that *M. paratuberculosis*, as detected by PCR, is consistently associated with Crohn's disease in humans (Abubakar et al., 2007; Feller et al., 2007). The still unresolved substantial issue is whether this is a causal association.

It is my contention that control of paratuberculosis does not require better diagnostic tests or a new vaccine or a more detailed molecular understanding of the organism or the hosts' response to infection. We, the scientific community, understand the epidemiology and pathogenesis of paratuberculosis sufficiently well that we are capable of controlling the infection in most domestic animal populations and thereby restrict contamination of the environment, foods of animal-origin and wildlife. Field trials in multiple countries have proved this to be true.

What is lacking is the motivation to control paratuberculosis. Farmers are apparently not motivated to enroll in paratuberculosis control programs even when governments heavily subsidize those programs. Over the past 6 years the U.S. has invested >US$90 million to support implementation of paratuberculosis control programs but only a relatively small proportion of U.S. farms participate. The reason for this, in my opinion, is that control of paratuberculosis is perceived by producers as more costly than the disease itself.

This observation, one with which other experts may disagree, leads me to my central thesis. There is only one crucial question pertaining to paratuberculosis: Is it a zoonotic infection that results in human disease? If it is not, then paratuberculosis is a veterinary problem of relatively minor economic importance in comparison with other infectious and noninfectious production issues limiting animal health concerns. On the other hand, if it is a

zoonotic disease, paratuberculosis is a problem with enormous and far reaching impact not only on animal health and the economics of animal production systems but also for the food industry, domestic water supply sourcing and distribution system management, as well as human health.

In spite of the fundamental importance of the zoonosis question, it is getting limited funding and therefore minimal research attention. Agriculture-related funding organizations do not consider the zoonosis question their funding responsibility; in fact, they may feel it is not in their best interest to discover that *M. paratuberculosis* is a zoonotic pathogen. Medical, food and water research funding organizations generally do not fund research on animal diseases and seem to be waiting for someone else to decide that *M. paratuberculosis* is a zoonotic agent before investing in research on this pathogen. And so, year after year and colloquium after colloquium we scientists produce data and exchange information on research questions primarily related to the veterinary concerns and not the zoonosis question.

In trying to understand this scientific impasse I borrow a quote from an “almost president” of the U.S. who is now famous for his well-substantiated stance on global warming, Al Gore. I suggest that the human health issue regarding *M. paratuberculosis* is another “inconvenient truth”, that is: “A truth we hold at arm’s length because if we acknowledge it and recognize it, then the moral imperative to make big changes is inescapable”.

I resigned my position as President of the International Association for Paratuberculosis at the conclusion of the 9ICP with a parting recommendation: Our Association should remain the focal point for global scientific exchange on paratuberculosis by expanding its scope of interest and inviting researchers from diverse fields such as dairy processing, meat science, microbial ecology, public water quality management, and medical gastroenterology to bring their unique skills and perspectives on paratuberculosis to our Colloquia. We should establish collaborations with the many scientists in our organization who are experienced in working with this challenging animal pathogen. Like the parable about the blind men and the elephant, it takes many people with many perspectives to fully understand this beast we know as *M. paratuberculosis*.

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