DIAGNOSTIC TOOLS AND GENETIC SUSCEPTIBILITY FACTORS ASSOCIATED WITH BOVINE PARATUBERCULOSIS INFECTION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2008

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To Pilar, Pablito and Santiago

ACKNOWLEDGMENTS

Several people have been instrumental in the completion of this project. Foremost, I desire to express my special gratitude to the members of my advisory committee: Dr. Owen Rae, Chair of my committee, for his constant support and advice and for his permanent willingness to discuss and exchange ideas about this project; Dr. Claus Buergelt for generously sharing with me his invaluable experience and knowledge in Johne's disease and for his permanent help in seeking out opportunities to enrich my training; Dr. Art Donovan for his support as a member of my committee and for his key comments during the analysis of our data; Dr. Pedro Melendez for his support, friendship and help during the research process; and Dr. Laurence Morel for her help as an external member of my committee.

I owe my gratitude to Dr. Louis Archbald for his ongoing interest, strong support and good advice. I thank also Elliot Williams for his friendship and his tremendous help with the laboratory procedures. I am grateful to Dr. Taimour Langaee and Dr. Rongling Wu for their technical support in the genotyping process and in the statistical analysis. I thank also my colleagues and fellow graduate students from FARMS for their friendship and valuable suggestions. I am thankful for the financial support received from the Florida Dairy Farmers Association through the Florida Dairy Check-Off Program and the 2007 University of Florida, College of Veterinary Medicine Spring Consolidated Faculty Research Development Award Grant Competition. I also thank INSECABIO (Chile) for its strong support during this process.

I thank my parents for my education, their support and guidance, and my boys Pablito and Santiago for bringing me back to the real world every evening.

But, above all, I owe my gratitude to my wife Pilar who left her own interests aside and postponed her professional development to live this adventure with me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion
bp	Base pairs
BoIFNG	Bovine Interferon gamma
CARD15	Caspase recruitment domain family, member 15
CS	Complementary sensitivity
CD	Crohn's disease
CFU	Colony-forming units
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	ELISA ratio
IBD	Inflammatory bowel disease
IFN-g	Interferon gamma
IL	Interleukin
IS	Insertion sequence
JD	Johne's disease
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MHC	Major histocompatibility complex
NF-Kb	nuclear factor-kappa B
NOD2	Nucleotide-binding oligomerization domain containing 2
NRAMP1	Natural resistance-associated macrophage protein 1
ORF	Open reading frame
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis

REA	Restriction endonuclease analysis
RNA	Ribonucleic acid
RFLP	Restriction fragment length polymorphism
SLC11A1	Solute carrier family 11, member 1,
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis

Abstract of Dissertation Presented to the Graduate School
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Requirements for the Degree of Doctor of Philosophy

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August 2008

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Major: Veterinary Medical Sciences

Our first objective (Studies 1-2) was to analyze the association among results of five different diagnostic tests for detection of paratuberculosis infection in cattle. Our second objective (Studies 3-4) was to characterize the distribution of polymorphisms in four immune related genes and test their association with susceptibility to paratuberculosis infection in cattle.

In Study 1, results of a serum ELISA, fecal culture, and nested PCR tests on milk, blood, and feces for *Mycobacterium paratuberculosis* (MAP) detection were analyzed to determine associations and levels of agreement between pairs of tests. The agreement between results was poor, slight and fair in two, five and three of the ten possible combinations. Fecal culture and fecal PCR resulted in the highest kappa coefficient (fair agreement). Combined use of ELISA and fecal PCR has the potential to increase the overall sensitivity for the diagnosis of paratuberculosis.

In Study 2, the association between ELISA seroreactivity and MAP presence in milk, as detected by nested PCR was analyzed. An irregular pattern of detection was observed for PCR outcomes along with fluctuations in serial ELISA results. Kappa coefficient indicated a slight agreement between both tests, suggesting that the ability of serum ELISA, as indicator of the likelihood of milk shedding of MAP in dairy cows, is questionable.

In Studies 3 and 4, polymorphisms in four candidate genes related to the immune function; caspase recruitment domain 15 gene *(CARD15)*, interferon gamma *(BoIFNG),* toll-like receptor-4 *(TLR4)*, and solute carrier family 11 member 1 *(SLC11A1)*, were analyzed. Significant differences were found in allelic frequencies between cases and controls for *CARD15*-SNP2197/C733R, *BoIFNG*-SNP2781 and *SLC11A1* microsatellites. In the analysis of genotypes, a significant association was found between infection status and *CARD15*-SNP2197/C733R, *BoIFNG* SNP2781 and *SLC11A1-275-279-281* microsatellites. When variables breed and age were included in the multivariate logistic regression analysis, the only statistically significant effect was for *CARD15*-SNP2197/C733R polymorphism. The estimated odds of infection for heterozygous cows were 3.35 times the odds of infection of cows homozygous for the major genotype. Results suggest a role for *CARD15* gene in the susceptibility of cattle to paratuberculosis infection.

CHAPTER 1
INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic, infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and characterized by progressive weight loss and profuse diarrhea (Chiodini et al., 1984). The disease has a worldwide distribution and is categorized by the Office International Des Epizooties as a list B disease, which is a serious economic or public health concern (OIE, 2004).

Most cattle with Johne's disease are infected as calves by fecal-oral transmission, and *in utero* transmission has also been reported (Seitz et al., 1989; Whitlock and Buergelt, 1996). However, young animals manifest no clinical signs and the incubation period is variable, ranging from 2 to 10 years (Bassey and Collins, 1997; Whitlock et al., 2000; Stabel and Ackerman, 2002).

Diagnosis of paratuberculosis is hampered by a lack of accurate tests. Available methods fail to identify all infected animals (false negative results), and some produce substantial numbers of false positives (Chiodini et al., 1984). Tests for detection of antibodies to MAP, such as enzyme-linked immunosorbent assays (ELISA) present the major disadvantage of moderate to low sensitivity. The usefulness of serological tests is compromised by the variability of the immune response depending on the stage of disease. For this reason, it is generally accepted that their sensitivity in detecting infected animals is only about 30% (Collins et al., 2006), and the ELISA test rarely gives a positive result in animals under 2 years of age, and frequently fails to detect individuals in the early phases of infection (Juste et al., 2005). Despite these disadvantages, ELISA testing of sera is still the method of choice for epidemiological studies and herd-based diagnosis (Bottcher and Gangl, 2004).

Tests based on the detection of the agent likewise present the problem of low sensitivity. The shedding of MAP organisms in feces can be intermittent and detection by culture is imperfect, especially because of contamination, and when few organisms are shed in feces. It has been estimated that fecal culture detects only about 50% of cattle infected with MAP (Stabel, 1997). The introduction of diagnostic methods based on specific bacterial DNA sequences has allowed fastidious microorganisms, such as MAP, to be rapidly identified. Polymerase chain reaction (PCR) tests based on the insertion element IS*900* have been the most widely used for MAP identification (Harris and Barleta, 2001). However, the detection of the etiologic agent is limited by the presence of inhibitory substances, and the frequency and number of organisms that are present in the body fluid or tissue being tested. The isolation of MAP from sites other than the intestinal tract, such as udder, kidney, liver, male reproductive tract and blood, have suggested active dissemination of the bacteria and opens the possibility for detection of the agent by PCR in fluids such as milk and blood of suspicious animals (Buergelt and Williams, 2004).

A combination of independent tests is a common method to improve reliability of laboratory diagnostic tools. As a result of the setbacks of MAP diagnosis, such strategies have already been implemented by using a combination of bacterial fecal culture and PCR or serological screening and bacterial fecal culture (Collins et al., 2006). Moreover, a combination of tests with different sensitivities and specificities allows a classification of animals and herds relative to the probability of MAP infection (Bottcher and Gangl, 2004).

A broader knowledge of the behavior and association between different diagnostic tests is desirable for the implementation of strategies based on the combination of different tests, which could be a useful approach to improve the sensitivity of MAP detection.

In another front, the use of host genetic resistance to disease is an attractive option as a component of livestock disease control. Genetic factors have been associated with differences in host susceptibility to bovine paratuberculosis, and estimations indicate a range of moderate values for heritability of infection (Koets et al., 2000; National Research Council, 2003; Mortensen et al., 2004, Gonda et al., 2006). Research has also been aimed at detecting associations between susceptibility differences and polymorphisms in candidate genes with no definite results (Hinger et al., 2007; Taylor et al., 2006; Gonda et al., 2005, 2007). A candidate gene case-control study aimed at immune related genes is a practical approach for testing the involvement of host genetics in paratuberculosis infection.

The hypothesis of studies 1 and 2 was that different degrees of association exist among tests detecting MAP infection. Our objective was to analyze the association among results of a serum ELISA, fecal culture, and nested PCR on milk, blood, and feces for MAP detection in dairy cows.

The central hypothesis of studies 3 and 4 was that a combination of particular alleles in four candidate genes would be present in higher frequency in case individuals compared to controls, suggesting a role in susceptibility to infection. The objective of this candidate gene case-control study was to characterize the distribution of polymorphisms in the bovine *CARD15, BoIFNG, TLR4* and *SLC11A1* genes and test their association with susceptibility to paratuberculosis infection in Florida dairy and beef cattle.

CHAPTER 2
LITERATURE REVIEW

The Agent

Genus *Mycobacterium*

Mycobacteria are members of the taxonomic group that includes the genera *Corynebacterium, Mycobacterium*, and *Nocardia* (CMN group) and the genus *Rhodococcus* (Cocito et al., 1994).

Mycobacteria are non-motile and non-sporulated rods and are grouped in the supra-generic rank of *Actinobacteria* with a high content (61-71%) of guanine plus cytosine (G+C) in the genomic DNA, and high lipid content in the wall, probably the highest among all bacteria. They also present several mycolic acids in the envelope structure that distinguish the genus (Palomino et al., 2007).

These acid-fast organisms include a number of major human and animal pathogens, comprising over 100 species of obligate parasites, saprophytes, or opportunistic pathogens. Mycobacteria are structurally more closely related to Gram-positive bacteria; however, the genus does not fit into this category given that cell wall molecules are lipids rather than proteins or polysaccharides (Palomino et al., 2007).

Based on genomic analysis, this genus has been divided into two separate clusters, corresponding to the traditional fast-growing mycobacteria, represented by nonpathogenic environmental isolates, and the slow-growing mycobacteria, containing most of the overt pathogens (Harris and Barleta, 2001).

Slow-growing mycobacteria of importance in veterinary medicine are found in two major complexes; *M. tuberculosis* and *M. avium.* The *M. tuberculosis* complex includes *M. tuberculosis, M. bovis,* and *M. microti. M. tuberculosis* causes tuberculosis in man, primates,

dogs and other animals. *M. bovis* causes tuberculosis in cattle, domestic and wild ruminants, man and other primates, and swine and other animals (Eglund, 2002). On the other hand, the *Mycobacterium avium* complex (MAC) consists of genetically similar bacteria including *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominis*, and *M. intracellulare*. MAC organisms are opportunistic pathogens present in multiple locations; although human exposure to MAC is ubiquitous, most individuals rarely develop infection (St. Amand et al., 2005). *M. avium* causes avian tuberculosis, and *M. intracellulare* rarely causes disease in birds, but causes severe pulmonary diseases in man and can be isolated from swine and cattle. *M. silvaticum* (wood-pigeon *Mycobacterium*) is an obligate pathogen for birds, and MAP, a pathogen for ruminants, is an obligate parasite unable to replicate in the environment (Eglund, 2002).

Another classification of mycobacteria relates to its ability to replicate in the environment. Environmental opportunistic mycobacteria are distinguished from the members of the *M. tuberculosis* complex by the fact that they are not obligate pathogens but are true inhabitants of the environment, exhibiting a notorious hardiness, an acid-fast cell wall containing mycolates, and intracellular pathogenicity (National Animal Monitoring System, 1997).

The subspecies designation of *M. avium* is based on DNA-DNA hybridization studies and numerical taxonomy analysis (Biet et al., 2005). At the subspecies level, MAP can be differentiated phenotypically from the other MAC members by its dependence on mycobactin and, genotypically, by the presence of multiple copies of an insertion element, IS*900* (Harris and Barletta, 2001).

An exclusive feature of mycobacteria is the structure and composition of their cell envelope, with an inner layer composed of peptidoglycan linked to arabinogalactan

polysaccharides which are esterified with high-molecular weight mycolic acids, and an outer stratum composed of lipids. A notorious molecule of the cell envelope is a lipopolysaccharide, lipoarabinomannan, with properties comparable to those of the O-antigenic lipopolysaccharide of Gram-negative bacteria (Weigand and Goethe, 1999). The waxy coat confers the distinctive characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to environmental exposure, and distinctive immunological properties. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Palomino et al., 2007).

Mycobacterium Paratuberculosis Characteristics

Mycobacterium avium subsp. *paratuberculosis* is a small, facultative intracellular, acid-fast bacillus, occurring in clumps entangled with one another by a network of intracellular filaments (Chiodini et al., 1984). The main distinguishing feature of this subspecies is its need for exogenous mycobactin for *in vitro* growth. Mycobactin is an iron chelating agent produced by almost all mycobacteria and MAP, in particular, is unable to produce this element in laboratory culture (Chiodini et al., 1984; Li et al., 2005). This siderophore is responsible for the transport of iron into cells, and is a high-molecular-weight complex lipid, containing a core to which Fe is coordinately linked. While a dependence on metals for growth is common to all bacteria, the iron requirement of pathogenic mycobacteria is peculiar in that an organic source of this metal is needed for its uptake and utilization (Cocito et al., 1994). Iron plays a key role in both electron transport and composition of metabolic enzymes, and the ability to acquire iron from various sources is crucial for bacteria (Li et al., 2005). Mycobactin is unique to mycobacteria and nocardiae, but mycobactin dependence is no longer considered pathognomonic for identification of MAP, since mycobactin-dependent *M. silvaticum* and *M. avium* strains have been identified (Cocito et al., 1994).

MAP is an obligate parasite of animals; the only place it can multiply in nature is in a susceptible host, within a macrophage. Out of the host it can survive for extended periods, but it is unable to multiply. Accordingly, the primary source of infection with MAP is an infected animal; though, there is evidence that MAP can exist in vegetative, cell wall deficient and dormant forms (Grant, 2005).

MAP cell wall is acid-fast and resists decolorization with acidified alcohol because of the presence of a waxy material that makes the cells difficult to disrupt. Under the microscope MAP cells appear as rods 1–2 μm in length, which typically occur as clumps of up to several hundred bacterial cells (Grant, 2005).

Environmental Ubiquity and Physical Resiliency

Environmental mycobacteria are normal inhabitants of a wide variety of environmental reservoirs, including water, soil, and aerosols. Water is likely the primary source of MAC infection in humans and environmental mycobacteria are capable of biofilm formation. Environmental mycobacteria also have an extraordinary ability to survive starvation, persisting despite low nutrient levels, and extreme temperature. *M. avium* and *M. intracellulare* have an acidic pH optimum for growth between 4.5 and 5.5, and have been recovered in large numbers from waters and soils of low oxygen levels (National Animal Monitoring System, 1997).

MAP's cell wall enables the organism to persist in the environment and contributes to its resistance to low pH, high temperature, and chemical agents (Manning, 2001).

Resistance to disinfection: Although the effects of chlorine and phenolic compounds on MAP are unknown, *M. avium* is more resistant to free chlorine than are most other bacteria (Manning, 2001).

Survival in water, soil, and manure: As reviewed by Manning (2001), in a 1944 study, MAP survived up to 246 days in manure outdoors. In water of pH 7.0, MAP has been recovered

up to 17 months post-inoculation. A pH above 7.0 and low iron content may reduce viability, as may soil drying and exposure to sunlight. However, in another study survival time was not affected by ultraviolet light. A lower positive serology rate for MAP infection in dairy cattle was associated with the application of lime to pastures, although a direct association between soil pH and MAP survival has not been established. Von Reyn et al. (1993) cultured 91 water samples from environmental sites and piped water supply systems in the United States, Finland, Zaire, and Kenya, and MAC was isolated from all geographic areas and from 22 of 91 (24%) samples.

Whittington et al. (2005), studied the survival of MAP in dam water and sediment in either a semi exposed or in a shaded location, compared to survival in fecal material and soil in a shaded location. Survival of MAP in water and/or sediment in the shade was up to 48 weeks compared to 36 weeks in the semi exposed location. Survival in soil and fecal material in the terrestrial environment in the shaded location was only 12 weeks, suggesting that water may be a significant reservoir of MAP infection.

Finally, in another study (Ward and Perez, 2004), the survival of MAP was analyzed by culture of fecal material sampled from soil and grass in pasture plots and boxes. Survival for up to 55 weeks was observed in a dry fully shaded environment. The organism survived for up to 24 weeks on grass that germinated through infected fecal material in completely shaded boxes and for up to 9 weeks on grass in 70% shade (Ward and Perez, 2004).

Thermal tolerance: MAP is more thermo-resistant than *M. avium*, *M. chelonae*, *M. phlei*, *M. scrofulaceum*, and *M. xenopi*. This heat tolerance decreases the effectiveness of pasteurization for killing organisms in the milk of infected animals.

It has been suggested that the heat-resistance of MAP may be influenced by its tendency to occur as clumps of cells. Bacteria in the center of large clumps may be protected, or

alternatively, because the clumps contain up to 10,000 cells, a proportion of the cells will survive a non-sterilizing heat treatment (Grant et al., 2005).

In testing units of whole pasteurized milk from retail outlets throughout central and southern England, it was found that, over three month periods, up to 25% of commercial units sampled were affected by the presence of MAP DNA (Millar et al., 1996). In a study in the Czech Republic, MAP was cultured from 1.6% of commercially pasteurized retail milk (Ayele et al., 2005). A US' study found viable MAP in 2.8% of milk samples taken from grocery stores in three states (Ellingson et al., 2005). In Britain, viable *M. paratuberculosis* was found in 1.9% of raw and 2.1% of pasteurized milk samples, suggesting that some MAP cells may survive pasteurization and can possibly be consumed by humans (Manning, 2001).

Resistance to ultraviolet light: Most bacteria and viruses are sensitive to ultraviolet (UV) light, but the pH, hardness, turbidity, and biologic oxygen demand in water can significantly alter the UV dose needed for inactivation. Laboratory trials with distilled water show that MAP is as susceptible to UV inactivation as other bacteria. In contrast, UV light may have minimal effect on the organism's viability in MAP spiked soil (Manning, 2001).

Strains

Different strains of MAP have been determined, however, isolates of MAP from different clinical sources have few distinguishing phenotypic characteristics. The main features that differentiate strains of MAP in culture are the rate at which they grow and, sometimes, variations in colony pigmentation. However, several methods have been developed to discriminate closely related strains (National Research Council, 2003).

As presented by the National Research Council (2003), non-molecular methods are based on serology, differences in biochemical properties, antimicrobial susceptibility, and phage typing. Molecular-strain typing has had a great influence on studies of MAP. Among the

techniques used are restriction fragment length polymorphism (RFLP) analysis of DNA, pulsed-field gel electrophoresis of DNA, and multiplex PCR typing. RFLP has been used most extensively. Sequences from IS*900* are the most widely used probe in RFLP analysis of MAP.

At the present, the main MAP strains have been classified into three groups (cattle, sheep and intermediate types), based on RFLP, analysis coupled with hybridization to the insertion sequence IS*900* (IS*900*-RFLP) and culture characteristics. However, other strains affecting bison (*Bison bison*) and differentiated by typing of IS*1311* polymorphisms have also been reported (Whittington et al., 2001),

The cattle type (C), the most common in Europe, has been isolated primarily from cattle and other domestic and wild ruminants, non-ruminant species, and also humans.

The sheep (S) type strains are extremely slow growers and in this group are included: (i) pigmented and non-pigmented strains isolated from sheep in Morocco, Scotland, Iceland, South Africa, Australia and New Zealand; (ii) strains isolated from cattle from Australia and Iceland; (iii) strains isolated from goats from New Zealand. The intermediate group has been described in a few ovine isolates from South Africa, Canada and Iceland as well as caprine isolates from Spain (Stehman, 1996; de Juan et al., 2006).

A similar division of strains has been achieved by characterization by pulsed field gel electrophoresis (PFGE). PFGE allows the division of MAP isolates into three main groups: Types I, II and III, which would correspond with the sheep, cattle and intermediate groups, respectively. PCR-based techniques, requiring lower amounts of high quality DNA divide the MAP strains in two main groups that would correspond with the cattle or Type II, and the sheep or Types I/III (de Juan et al., 2006). The capacity to differentiate individual strains of MAP is essential for evaluating routes of transmission and characteristics of pathogenesis. It is important

also, for livestock producers to be able to identify the source of a new infection because that information often will dictate corrective action. Different control strategies depend on whether a new infection results from introducing livestock from another herd or is attributable to animal contact with something in the farm environment, such as contaminated pasture (National Research Council, 2003). On the other hand, different strains present different exigencies for culture and that is a point that should be considered to evaluate the possibility of false negatives results.

Species specificity among strains has been suggested and separate strains of MAP may be isolated from various ruminant species and may account for some of the differences in culture diagnosis. Observations indicate that the ovine strain is unlikely to be transmitted to cattle. However, the occurrence of a sheep strain in cattle has been reported, indicating that interspecies transmission can not be ruled out (Motiwala et al., 2003) and, probably, most strains can infect across ruminant species lines and should be regarded as infectious to ruminant species other than the species of origin (Stehman, 1996). A final point refers to the hypothesis that different strains of MAP vary in their ability to attach to different regions of the intestinal tract at different rates. However, Schleig et al. (2005) reported significant differences in strains ability to attach, but not in attachment among different regions of intestine.

Mycobacterium Paratuberculosis Genome

The size of the MAP genome has been estimated to be 4.4 to 4.7 Mbp. Compared to other mycobacteria, this is similar to the *M. tuberculosis* genome (4.41 Mbp) and the *M. bovis* genome (4.4 Mbp) but slightly larger than the estimated size of the genome of *M. leprae* (3.3 Mbp). MAP DNA has a base composition of 66 to 67% G+C, similar to *M. tuberculosis* and *M. bovis* (Harris and Barleta, 2001). Recent work in mycobacterial genomics has revealed large sequence polymorphisms as the major contributor of genetic diversity (Sohala et al., 2007).

Genome sequence

Recently, the complete genome sequence of MAP was reported (Li et al., 2005). The strain used was MAP K-10 which is a virulent, low passage clinical strain isolated from a dairy herd in Wisconsin. MAP K-10 has a single circular sequence of 4,829,781 base pairs, with a G+C content of 69.3%. Approximately 1.5% (or 72.2 kb) of the MAP genome is comprised of repetitive DNA like insertion sequences, multigene families, and duplicated housekeeping genes (Li et al., 2005).

Seventeen copies of the insertion sequence IS*900*, seven copies of IS*1311*, and three copies of IS*Mav2*, with a total of 16 additional MAP insertion sequence elements have been identified. Li et al. (2005) determined that K-10 genome contains 4,350 ORFs with lengths ranging from 114 bp to 19,155 bp, which, in sum, account for 91.5% of the entire genome.

A cluster of 10 genes in *Mycobacterium tuberculosis* has been shown to be responsible for the production of mycobactin and the transport of iron. Homologs to this cluster were identified in the MAP genome. However, a direct comparison of the cluster in MAP with those of *Mycobacterium avium* and *Mycobacterium tuberculosis* show significant differences in primary structure of this region (Li et al., 2005).

Insertion sequences

Insertion sequences (IS) are relatively short DNA segments capable of transposing within and between prokaryotic genomes, often causing insertional mutations and chromosomal rearrangements. Use of IS as probes provides discrimination due to the tendency of these transposable elements to insert randomly and occupy multiple sites in the genome. The discovery of insertion sequences in mycobacteria has provided an approach for characterizing MAC isolates (Bhide et al., 2006; Motiwala et al., 2006).

The first MAC insertion sequence, IS*900*, was identified in MAP cultures and was determined to be a unique characteristic of this subspecies (Collins et al., 1989; Green et al., 1989). IS*900* elements are found in multiple copies per genome and provide the diagnostic advantage of improved sensitivity for MAP detection in PCR procedures. The closely related insertion sequences, IS*901* and IS*902* were discovered subsequently, and more recently, IS*1245* and IS*1311* have been identified in MAC isolates (Motiwala et al., 2006).

Insertion sequence *1245* is present in *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*, but was recently demonstrated not to be present in the MAP genome (Johansen et al., 2005). A closely related IS element, IS*1311*, shows 85% sequence identity with IS*1245* at the DNA level. It is present in *M. avium* subsp. *avium* and MAP and has been detected in strains of *M. intracellulare*, *Mycobacterium malmoense*, and *Mycobacterium scrofulaceum*.

Whittington et al. (1998), found 7–10 copies of IS*1311* in strains of MAP. With a given restriction enzyme, the RFLP patterns obtained from isolates of MAP from cattle were all identical, but they differed from those of isolates from sheep. Restriction endonuclease analysis (REA) of the PCR product was used to distinguish isolates of MAP from *M. avium*, in addition to the conventional test for IS*900*. In isolates of MAP from cattle the IS*1311* gene was polymorphic at position 223, which enabled isolates from sheep and cattle to be distinguished by PCR-REA.

Other possible target elements in MAP include the F57, IS*Mav2*, and Hsp X sequences. The F57 and Hsp X sequences are present as single copies, while three or more copies of IS*Mav2* are present in the MAP genome (Tasara and Stephan, 2005). Although these markers may not be as sensitive as the multi-copy IS*900* elements, they are highly specific for MAP.

Insertion sequence *900*

IS*900* was discovered in MAP independently by two groups in 1989 (Collins et al., 1989; Green et al., 1989; Eglund, 2002). This 1,451 bp element lacks inverted terminal repeats and does not generate direct repeats in target DNA. IS*900* is a member of the IS*116* family of insertion sequences present in actinomyces and other bacteria. This group includes IS*901*, IS*902*, IS*1110* and IS*1643* in *M. avium*, all of which share between 60-80% identity with IS*900* (Cousins et al., 1999). IS*900* exists in 14-18 copies in the genome of MAP and encodes a 399 amino acids putative transposase, p43, on one strand and a predicted protein, Hed, of unknown function on the opposite strand. IS*900* inserts in one direction into a consensus target sequence at highly conserved loci within the MAP genome. The insertion sites of different copies of IS*900* are similar and share a common consensus sequence (Bull et al., 2000).

PCR targeting the 5' end of IS*900* has been considered specific for identification of MAP and is frequently applied to confirm the presence of the organism in the diagnosis of Johne's disease (JD). However, the specificity of such procedure has been put into question over the past few years (Green et al., 1989; Cousins et al., 1999; Tasara and Stephan, 2005). Sequences that are highly homologous to MAP IS*900* have been found in other environmental *Mycobacterium* species. Those elements have been described for strains isolated from bovine feces which are positive with most of the current IS*900* PCR systems used for standard *M. avium* subsp. *paratuberculosis* detection (Bull et al., 2000).

Cousins et al. (1999) reported the finding of *Mycobacterium* spp. isolated from the feces of 3 clinically normal animals in Australia that appeared not to be MAP but were positive by IS*900* PCR. The isolates were characterized using mycobactin dependency, biochemical tests, IS*900* and 16 S rRNA sequencing and restriction fragment length polymorphism (RFLP), and showed

between 71% and 79% homology with MAP in the region of IS*900* amplified, appearing to be most related to *Mycobacterium scrofulaceum*.

Antigens

Several antigens have been identified in mycobacteria, particularly in *Mycobacterium tuberculosis*, but few have been identified in MAP. In the early eighties, the use of a protoplasmic antigen for immunoglobulin G1 detection by ELISA was proposed (Yokomizo et al., 1983). This antigen is the basis for some ELISA in use at the present.

Among the more recently reported antigens are the highly antigenic and conserved heat shock proteins GroES and GroEL, 2 alkyl hydroperoxide reductases, a serine protease, superoxide dismutase, and 11 other proteins of unknown function that are named on the basis of their sizes in kilodaltons (Bannantine et al., 2004). Other immunoreactive proteins of MAP include a 32-kDa secreted protein with fibronectin binding properties implicated in protective immunity and a 34-kDa cell wall antigenic protein homologous to a similar protein in *M. leprae*. The *M. paratuberculosis* GroEL protein is homologous to similar proteins of *M. tuberculosis* (93%), *M. leprae* (89%), and *M. avium* (98%) (Bannantine et al., 2004).

The alkyl hydroperoxide reductases C and D (AhpC and AhpD) are recently characterized immunogenic proteins of MAP. AhpC is the larger of the two proteins and appears to exist as a homodimer in its native form since it migrates at both 45 and 24 kDa under denaturing conditions. In contrast, AhpD is a smaller monomer, with a molecular mass of about 19 kDa. Other putative MAP-specific antigenic proteins have been described in the literature. These include a cellular antigen of 34.5 kDa, a 42-kDa protein of unknown function, and a 44.3-kDa antigen (Harris and Barleta, 2001).

Paustian et al. (2004) reported the identification of 13 open reading frames with no identifiable homologs. These MAP genes were cloned into *Escherichia coli* expression vectors,

and nine were successfully expressed as recombinant fusion proteins. Five of these proteins were purified in sufficient amounts to allow immunoblot analyses of their reactivity with sera from naturally infected cattle as well as mice and rabbits exposed to MAP. Fusion proteins representing MAP0862, MAP3732c, and MAP2963c were recognized by nearly all of the sera tested, including those from cattle in the clinical stages of disease, and four proteins were variably recognized by sera from MAP-infected cattle.

In another study, Leroy et al. (2007) presented a post-genomic analysis of MAP proteins where 25 candidate diagnostic antigens were identified as specific antigens that could improve the diagnosis of paratuberculosis.

Some of the antigens recognized at the present are promissory, but none has been incorporated as a routine diagnostic tool according to the published literature and available commercial tests. This may be due, at least in part, to the presence of these antigens in other mycobacterial species (Bannantine et al., 2004).

Johne's Disease

Definition

Paratuberculosis (Johne's disease) is a chronic infectious disease of domesticated and wild ruminants, recognized throughout the world since it was first described in 1895. As presented by Kreeger (1991), at that time, Johne and Frothingham described the disease and identified the presence of acid-fast organisms in the granulomatous lesions of the intestine, considering the disease an atypical form of ruminant tuberculosis. In 1910 the organism was first isolated and received the name *Mycobacterium enteriditis chronicae txeudotuberculosae bovis johne,* which later would be renamed *Mycobacterium avium* subspecies *paratuberculosis*.

The macroscopic and histological lesions of paratuberculosis remain confined to the intestine, mesenteric and ileocecal lymph nodes (Buergelt et al., 1978), and the disease is

characterized by granulomatous enteritis, which leads to chronic, unresponsive diarrhea and progressive emaciation. Most often, the infection is acquired by the young and after a prolonged incubation phase, lasting 2 to 3 years in cattle, the infection results in disease (Chiodini et al., 1984).

Paratuberculosis has a worldwide distribution and is categorized by the Office International Des Epizooties as a list B disease, which is a serious economic or public health concern (OIE, 2004). In spite of efforts directed towards the understanding of the disease, at the present, many questions related to paratuberculosis remain unanswered (Kreeger, 1991).

Spectrum in Animal Species

Paratuberculosis causes enteritis primarily in cattle, goat and sheep, but the infection also occurs in other ruminants and wildlife (Eglund, 2002). MAP also multiplies in horses and mules, which become asymptomatic shedders, and laboratory animals and birds replicate experimentally injected MAP (Cocito et al., 1994). The importance of wildlife reservoirs of MAP in the transmission cycle remains undetermined, and some investigations have examined the role of wildlife in the epidemiology of paratuberculosis (Alifiya et al., 2004; Motiwala, 2004). Among the wild species in which MAP has been reported are ruminants, such as deer (Stehman, 1996), bison (Buergelt et al., 2000, Ellingson et al., 2005a), and elk, as well as non-ruminants, such as wild rabbits (Greig et el., 1997), their predators, including foxes and stoats, and primates, such as mandrills and macaques (Zwick et al., 2002), indicating a wide host range.

A study performed in Scotland (Beard et al., 2001) investigated 18 non-ruminant wildlife species for evidence of paratuberculosis. Using culture and histopathological analysis, fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare, and badger were found to harbor MAP. Similarly, a survey of wild rabbits in Scotland revealed that 67% were infected with MAP,

raising the possibility that rabbits and other wildlife may play a role in the epidemiology of paratuberculosis, with important implications for the control of the disease (Greig et al., 1997).

Surveys for MAP infection in free-ranging mammals and birds were also conducted on nine dairy and beef cattle farms in Wisconsin and Georgia (Corn et al., 2005). Specimens were collected from 774 animals representing 25 mammalian and 22 avian species. MAP was cultured from tissues and feces from 39 samples from 30 animals representing nine mammalian and three avian species. The prevalence of infected wild animals by premises ranged from 2.7 to 8.3% in Wisconsin and from 0 to 6.0% in Georgia, and fecal shedding was documented in seven (0.9%) animals. Finally, a recent study in south central Wisconsin detected MAP specific DNA in 81 of 212 (38%) scavenging mammals, in 98 of the 472 (21%) tissues; viable MAP was cultured from one coyote's ileum and lymph node tissue (Anderson et al., 2007).

Pathology

Entry of MAP in the host mainly occurs via the fecal-oral route, through ingestion of fecal contaminants, milk or colostrum. Viable bacilli have also been isolated from reproductive organs of infected animals and fetuses of infected cows. Thus, intrauterine transmission is possible, though its significance in natural infection and spread of the disease remains to be fully elucidated (Seitz et al., 1989; Valentin-Weigand and Goethe, 1999; Buergelt et al., 2006; Wittington and Winsor, 2007).

Ingested MAP enter the intestinal wall through the small intestinal mucosa, primarily in the region of the ileum, via M cells residing in the Peyer's patches (Momotani et al., 1988). Bacilli are resistant to intracellular degradation, and are eventually phagocytosed by subepithelial macrophages (Valentin-Weigand and Goethe, 1999; Koets et al., 2002; Tiwari et al., 2006). Subsequently, the infected macrophages migrate into local lymphatics spreading the infection to regional lymph nodes, stimulating inflammatory and immunological responses. MAP proliferates

slowly in the ileal mucosa and regional lymph nodes, and stressors such as poor nutrition, transport, parturition, and immunosuppression have been proposed as precipitating the start of the clinical phase of infection (Tiwari et al., 2006).

It takes years from the time of infection until development of clinical signs. Experimental infections carried out in cattle revealed that orally administered MAP were detectable in intestinal macrophages within a few hours after infection. The first granulomatous lesions were seen in the interfollicular regions of Peyer's patches and mesenteric lymph nodes three months after infection, lesions extended into the intestinal mucosa several months later (Valentin-Weigand and Goethe, 1999).

Granulomatous lesions present in the disease have been classified into two types; tuberculoid and lepromatous. Tuberculoid-type lesions, which occur in the early stages of paratuberculosis, consist of small numbers of epithelioid cells and many lymphocytes, plasma cells, eosinophils, and macrophages, with limited numbers of MAP (paucibacillary). These lesions are associated with strong cell-mediated immune responses on which resistance to paratuberculosis is dependent (Tanaka et al., 2005).

In contrast, lepromatous-type lesions, more comon in the terminal stage of the disease, are composed mainly of macrophages and epithelioid cells bearing large numbers of mycobacteria (pluribacillary). Lepromatous-type lesions are associated with strong humoral immune responses in conjunction with weak cell-mediated immunity resulting in progression of the disease, with a reduction in Th1 response and the induction Th2 induced humoral immunity by the anti-inflammatory cytokines interleukin-4 (IL-4), IL-5, IL-6, and IL-10 (Tanaka et al., 2005).

In naturally infected animals, gross lesions typical of chronic enteritis are most notably found in the distal ileum. Histological lesions include macrophages with different amounts of

intracellular mycobacteria. Although the severity of intestinal lesions often does not correlate with occurrence of clinical signs, an association of clinical cases with a high mycobacterial load of macrophages in affected areas has been found in experimental infections. MAP has also been demonstrated in the mononuclear cell fraction of blood and tissue fluid from infected cattle, supporting the idea that macrophages may function as vehicles in dissemination of the organisms from infected sites (Valentin-Weigand and Goethe, 1999, Buergelt and Williams, 2004).

The physiological mechanism for development of diarrhea is thought to be related to antigen-antibody reactions in infected tissue, with subsequent release of histamine, and different cytokines. Macroscopic lesions are found primarily in the intestine and mesenteric lymph nodes, specifically in the region of the ileum, although they can occur throughout the whole length of the intestinal tract. The intestinal wall is thickened and edematous, and the mucosa has transverse folds and the serosal and mesenteric lymphatic vessels are dilated and thickened (Tiwari et al., 2006).

The intracellular destiny of MAP remains unclear, with an intra-phagosome localization of MAP in infected tissues being most likely. A work based on the mouse model demonstrated that MAP can persist in macrophages in vitro for several weeks without significant loss of viability, but the extent of intracellular multiplication under different conditions has not been clarified (Valentin-Weigand and Goethe, 1999).

Some experiments, using a large single oral dose suggested tonsils as the primary portal of entry following oral inoculation, with intestinal lesions developing later. However, other authors using smaller repeated doses showed the small intestine to be the most likely portal of entry, with no evidence for entry of infection via the tonsils (Sweeney et al., 2006). M cells have been considered as an important component in the uptake of MAP after oral inoculation (Momotani et

al., 1988). Sweeney et al. (2006), using small oral doses, produced infection in Holstein calves detectable 3 weeks after infection, with culture positive sites restricted to intestinal or mesenteric lymph nodes, and with only two calves showing culture positive samples of spleen and tonsils.

In another work, Wu et al. (2006) described a surgical approach employed to characterize the early stages of infection of calves with MAP. After inoculation in the ileum, the bacteria were able to cross the intestinal tissues within 1 hour of infection, reaching the liver and lymph nodes. Both the ileum and the mesenteric lymph nodes were persistently infected for months despite a lack of fecal shedding of mycobacteria. During the first 9 months of infection, the levels of cytokines detected in the ileum and the lymph nodes indicated the presence of a Th1-type-associated cellular responses but not Th2-type-associated humoral responses.

A recent study focused on characterizing MAP disseminated infection in dairy cattle and on determining the role of ELISA test in detection of cattle with this condition. Disseminated infection was diagnosed when MAP was isolated in tissues other than the intestines or their associated lymph nodes and was distinguished from infection found only in the gastrointestinal tissues and from absence of infection. Of the 40 cows in the study, 21 had MAP disseminated infection. Results showed that 57% of cows with disseminated infection had average to high body condition and no diarrhea. Cows with disseminated infection had no to minimal gross pathologic evidence of infection in 37% of cases. Only 76% of cows with disseminated infection had positive historical ELISA results and only 62% had a positive ELISA at slaughter (Antognoli et al., 2008).

Another study (Brady et al., 2008) analyzed the relationship between clinical signs, pathological changes and tissue distribution of MAP in 21 cows from herds affected by JD. The bacterium was isolated from 17 individuals, all exhibiting macroscopic lesions. However, with

the exception of diarrhea and lesions in the large intestine, there was little correlation between the presence or absence of clinical signs and the lesions associated with JD. The distribution of MAP in tissue was poorly correlated with either the clinical signs or the lesions and the bacterium was widely distributed in the tissues of some clinically normal animals.

Stages of the Disease

Whitlock and Buergelt (1996) proposed the following four categories of the disease, differentiated according to the severity of clinical signs, shedding of bacteria into the environment, and the possibility of infection to be detected using laboratory methods.

Stage 1, silent infection

In this stage, animals typically exhibit no overt evidence of infection with MAP. Stage 1 is typically found in calves and heifers, most immature young stock, and many adult cattle. No routine or special clinicopathologic tests or serology will detect disease in these animals. Only postmortem tissue culture or, less often, histopathology can detect infection at this early stage of disease.

Stage 2, subclinical disease

Most animals in stage 2 are adults that are carriers of MAP. The animals do not exhibit clinical signs typical of paratuberculosis, but they sometimes have detectable antibodies or exhibit altered cellular immune responses. Many are fecal-culture negative, although they intermittently shed low numbers of organisms in feces. In a small percentage (15–25%), disease can be detected by fecal culture, by altered cellular immune response, by serum antibodies, or by histopathology. An unknown proportion of stage 2 animals progress slowly to clinical disease, but because so many are culled from herds for other reasons and before clinical signs typical of paratuberculosis are recognized, the magnitude of the MAP infection within a herd can be obscured.

Stage 3, clinical disease

The clinical signs characteristic of stage 3 typically develop only after some years of MAP incubation. The initial signs are subtle; they include a drop in milk production, roughening of the hair coat, and gradual weight loss despite an apparently normal appetite. Over a period of several weeks, diarrhea (often intermittent at first) develops. In the absence of a history of herd infection, clinical diagnosis is difficult because other conditions can result in similar signs. Because paratuberculosis diagnosis based on clinical signs is challenging, the first cases in a herd often are misdiagnosed (Whittington and Sergeant, 2001).

Histopathological lesions can occasionally be found in the intestinal tract, with the most common site being the terminal ileum. Serum and plasma biochemical changes are predictable and characteristic of the clinical signs, but they are not specific enough to be of use in diagnosis of JD. Most animals test positive on fecal culture for MAP and have detectable concentrations of antibodies on commercial ELISA and agar gel immunodiffusion tests. A few unusual cases will regress to Stage 2 and remain there for an indeterminate period.

Stage 4, advanced clinical disease

Animals can progress from stage 3 to stage 4 in a few weeks, and their health deteriorates rapidly. They become increasingly lethargic, weak, and emaciated as the disease progresses to Stage IV. Intermandibular edema due to hypoproteinemia, cachexia, and profuse diarrhea characterize stage 4. Dissemination of MAP throughout the tissues can occur. Although the organism can sometimes be cultured from sites distant from the gastrointestinal tract, extra-intestinal lesions are rarely detected. When extra-intestinal lesions are present, the liver, other parts of the intestinal tract and the lymph nodes are the most common sites. At this stage, most animals are culled from the herd because of decreased milk production or severe weight loss.

Death from JD is often the result of the severe dehydration and cachexia (Whitlock and Buergelt, 1996).

The distribution of these four stages in the population is described as "iceberg" effect; for every advanced clinical case of JD in a farm, as many as 25 other animals are expected to be infected. Only 15% to 25% of these infected animals will be detected during one test period (Whitlock and Buergelt, 1996). At any given time in an infected herd, the majority of infected animals will be in stages 1 and 2, with relatively few animals exhibiting clinical signs of disease i.e. stages 3 and 4 (Eglund, 2002; National Research Council, 2003; Whitlock and Buergelt, 1996).

In agreement, in a study presented by Toman et al. (2003), cows in a MAP infected herd were clinically and microbiologically monitored for 4 to 7 years resulting in three groups of animals showing different courses of the infection. One group (non-shedders) included animals negative by fecal culture throughout the monitoring period. A second group (low shedders) shed sporadically small quantities of mycobacteria, remaining clinically healthy throughout the monitoring period. A third group (high shedders) included animals shedding repeatedly large quantities of MAP (≥10 CFU) with a progressive deterioration of the state of health in most of them. Animals with specific antibodies were found in all groups, but the percentage of serologically positive animals was significantly higher in the group of high shedders. Specific cell-mediated immunity was demonstrated in the group of low shedders.

Epidemiology

Prevalence and risk factors

Various surveys have been conducted to establish disease incidence and prevalence in different areas of the United States. In a review presented by Kreeger et al. (1991) results from a 1983 abattoir survey of 1,000 Wisconsin cattle indicated histological lesions compatible with

paratuberculosis in 11% of the animals examined. Subsequent studies across the US reported paratuberculosis seroprevalences among dairy cattle (year 1996) and beef cattle in (1997) of 2.5% and 0.4%, respectively, with at least 22% of dairy herds and 7.8% of beef herds having at least 1 seropositive animal (NAHMS, 1997; Dargatz et al., 2001a).

In an extensive survey which included 32 states and Puerto Rico, lymph nodes were examined from 7,540 animals by culture techniques and an overall prevalence of 1.6% was found. Prevalence rates for 1983–84 in dairy and beef cattle were 2.9% and 0.8%, respectively (Merkal et al., 1987).

Braun et al. (1990), in a survey conducted from 1986 through 1987, found 8.6% and 17.1% prevalence for MAP infection in Florida in beef and dairy cattle, respectively (ELISA test). In a later study, Keller et al. (2004) found, in a population of 32,011 cattle from 75 herds in Florida, an overall prevalence for a commercial ELISA of 6.5% (7.4% and 6.3% on beef and dairy cattle, respectively).

A New England-based study showed a prevalence rate of 18% combining culture techniques and histological evaluation (Chiodini and van Kruiningen, 1986), and in a 16-year survey the National Veterinary Services Laboratory found a disease prevalence rate, as determined by culture techniques, of 7.9% of the 12,917 samples submitted from 44 states, Puerto Rico, and Canada (Kreeger et al. 1991).

An absorbed ELISA (Thorne and Hardin, 1997) was performed on serum samples from 1,954 Missouri cattle, representing 89 herds randomly selected from samples submitted for brucellosis testing. The apparent seroprevalence of paratuberculosis in dairy cattle (8%) was similar to that in beef cattle (5%). When herds were classified as dairy or beef, 74% of dairy herds and 40% of beef herds were positive.

Dargatz et al. (2001a) estimated the prevalence of paratuberculosis infection among cows on beef operations in the US, based on a convenience sample of 380 herds in 21 states. Serum samples were obtained from 10,371 cows and tested with a commercial ELISA. They reported that 7.9% of the herds had 1 or more animals with ELISA positive result; 0.4% of the cow samples yielded positive results.

The prevalence of MAP in culled dairy cattle in Eastern Canada and Maine was determined to be 16.1% based on a systematic random sample of abattoir cattle (McKenna et al., 2004). In total, 8.5% of 984 cows had positive mesenteric lymph node or ileum cultures.

Hirst et al. (2004) estimated the seroprevalence of MAP infection among adult dairy cows in Colorado and determined herd-level factors associated with the risk that individual cows would be seropositive. The study comprised 10,280 adult dairy cows in 15 herds, and the serum samples were tested with a commercial ELISA. Overall, 4.12% cows were seropositive. Within-herd prevalence of seropositive cows ranged from 0% to 7.82%. Infection was confirmed in 11 dairies. Annual importation rate, herd size, and whether cows in the herd had clinical signs typical of MAP infection were associated with the risk that individual cows would be seropositive for MAP infection.

In another study (Adaska et al., 2003), 1,950 serum samples from 65 dairy herds in California, USA were tested for the presence of antibodies to MAP using a commercial ELISA kit. The seroprevalence among cows was 6.9% in the northern region of the state, 3.7% in the central region and 5.2% in the southern region (overall 4.6%).

Beef and dairy cattle serum samples, collected during 2000 at sale barns throughout Georgia, were used to conduct a retrospective epidemiological study (Pence et al., 2003). Statistical samplings of 5,307 sera, from over 200,000 sera, were tested for antibodies to MAP,

using a commercial ELISA kit. An overall period seroprevalence was 4.73%. The period seroprevalence in dairy cattle was 9.58%, in beef cattle it was 3.95%, and in cattle of unknown breed it was 4.72%.

Some studies analyze the association between prevalence of infection and risk factors. Roussel et al. (2005) working with 4,579 purebred cattle from 115 beef ranches in Texas found positive ELISA results for 137 of the 4,579 (3.0%) cattle, and 50 of the 115 (43.8%) herds had at least 1 seropositive animal. Results of mycobacterial culture were positive for 7.3% of seropositive cattle, and 18% of seropositive herds had at least 1 animal for which results of mycobacterial culture were positive. Risk factors for seropositivity included water source, use of dairy-type nurse cows, previous clinical signs of paratuberculosis, species of cattle (*Bos taurus* vs. *Bos indicus*), and location.

Another study presented results for a random sample of Wisconsin dairy herds (158 herds and 4,990 cattle) analyzed by an absorbed ELISA procedure. Fifty percent of herds and 7.29% of cattle had positive test results. The only management factor found to be significantly associated with herd prevalence was housing of calves after weaning. Unexpectedly, herds with higher prevalence were associated with use of calf barns and hutches for calves after weaning rather than pens in the cow barn (Collins et al., 1994). In another work, Goodger et al. (1996) found that factors such as environmental conditions, newborn calf care, grower calf care, bred heifer care, and manure handling were significantly associated with paratuberculosis prevalence in Wisconsin dairy herds.

Cetinkaya et al. (1997) analyzed the relationships between the presence of clinical JD and farm and management factors in England. Two binary outcomes (case reported in 1993, case reported in 1994) and 27 predictor variables were considered. Farms on which Jersey and

Guernsey or their cross were predominant were associated with an increased risk of reporting disease (odds ratios from 10.9 to 12.9). The presence of farmed deer on the farm also increased the risk of reporting disease.

In a cross-sectional study (Jakobsen et al., 2000) using milk samples from 1,155 cows from 22 Danish dairy herds, several risk factors for paratuberculosis were identified. Eight point eight percent (8.8%) of the animals were ELISA positive, and 19 out of the 22 dairy herds had ≥1 test-positive cows. The significant risk factors were: Jersey versus large breeds, high parity, the first month after calving, and large herd size.

Nielsen and Toft (2007) studied management-related risk factors for within-herd transmission of MAP in 97 Danish dairy herds. Four significant risk factors were identified: housing of cows in bed stalls compared to housing in tie stalls; low level of hygiene in the feeding area of calving areas; low amounts of straw in the bedding of the calving area; high animal density among young stock >12 months of age.

In a study from Muskens et al. (2003), 370 randomly selected Dutch dairy farms with ≥20 dairy cows were surveyed. All cattle aged ≥3 years were serologically tested for paratuberculosis using an ELISA. Significant factors associated with seropositive animals were herd size, presence of cows with clinical signs of paratuberculosis, prompt selling of clinically diseased cattle and feeding milk replacer.

Transmission of infection

There is agreement on the role of the fecal-oral route as the main entry of MAP in the host through ingestion of fecal contaminants, milk or colostrum (Chiodini et al., 1984; Whitlock and Buergelt, 1996), and additional intrauterine transmission has been suggested. Seitz et al. (1989) obtained tissue specimens at a packing plant from pregnant dairy cows and their fetuses and from cows with clinical signs of paratuberculosis and from their fetuses. Of 407 lymph nodes from

cows, 8.4% were culture positive for MAP; 26.4% of these culture-positive cows had fetuses from which specimens were also culture positive. The results estimated the risk of fetal infection with MAP to be 26.4%.

Buergelt et al. (2006) reported that, in a group of 11 pregnant MAP infected Holstein cows, tissues or fluid from fetuses were positive on 36% of the pregnancies and 2 of 4 placentomes tested and 9% of allantoic fluids resulted on positive PCR reaction products.

In another study, Aly et al. (2005) estimated the extent to which MAP infection in a large herd was attributable to the infection status of the respective dams. Serologic test results were compared between cows and their dams. Cows with seropositive dams were 6.6 times as likely to be seropositive, compared with cows of seronegative dams. For seropositive cows born to seropositive dams, 84.6% of seropositivity was attributable to being born to a seropositive dam. For the herd as a whole, the seropositive status in 34% of seropositive cows was attributable to being born to a seropositive dam. The explanation is based on the subsequent transmission of MAP from infected dams to their daughters, either congenitally or via exposure to feces and colostrum of the dam shortly after birth.

A meta-analysis (Whittington and Windsor, 2007) suggested that about 9% (95% CI 6-14%) of fetuses from subclinically infected cows and 39% (20-60%) from clinically affected cows were reported infected with MAP ($p < 0.001$). The estimated incidence of calf infection derived via the in utero route depends on within-herd prevalence and the ratio of sub-clinical to clinical cases among infected cows. Assuming a rate of 80:20 for this ratio, estimates of incidence were in the range 0.44-1.2 infected calves per 100 cows per year in herds with within-herd prevalence of 5%, and 3.5-9.3 calves in herds with 40% prevalence. Contrarily, Kruip et al. (2003) investigated whether cows shedding MAP possessed oocytes and early embryos that were

carriers of the bacterium. The results suggested that neither in vivo embryos nor oocytes are carriers of the bacteria and do not form an extra risk at embryo transfer.

Immune Response to MAP

The immune response to mycobacteria is a complex sequence of coordinated events, leading to clearance of the pathogen but more likely to adequate control of infection. The loss of control observed in some hosts may be due to genetic factors or may be caused by exogenous stressors such as parturition, malnutrition, or secondary viral or bacterial infections (Tiwari et al., 2006).

The precise mechanism of acquired resistance to disease is unknown but may involve maturation of the immune system, including the balance between various T-cell subsets and the specific tissue distribution of immune cells (Coussens, 2001).

The events that determinate whether cattle eliminate the infection or become permanently infected remain unclear. In vitro studies have shown that MAP organisms proliferate within bovine macrophages. This may be, in part, due to inhibition of phagosome acidification and phagosome-lysosome fusion. Cytokines appear to regulate killing of the organisms in macrophages, and pretreatment of monocytes with IFN-g, granulocyte macrophage colony-stimulating factor, or high doses of tumor necrosis factor-α (TNF-α) restricted MAP growth in vitro (Weiss et al., 2004; Woo and Czuprynski, 2008).

The first line of defense against invading MAP in the ruminant gut involves M cells and phagocytic macrophages. M cells most likely pass MAP on to naïve immune cells located beneath the surface of the intestinal epithelial cells. MAP readily infects unactivated intestinal macrophages, and non-opsonized MAP is readily phagocytosed by bovine macrophages in vitro (Coussens, 2001).

MAP, as an intracellular parasite, remains within the phagocytic vacuole, and therefore, immunology to paratuberculosis is dependent on cell mediated immune responses; humoral immune factors have little or no protective value (Chiodini and Davis, 1993). Major histocompatibility complex (MHC) class II antigen presentation occurs predominantly when bacteria reside within the phagosome. Here, the cellular immune response to the pathogen depends on MHC II antigen presentation to lymphoid cells which produce IFN-g in response to antigen recognition (Kaufmann and Kaplan, 1996).

Development of immunity to facultative intracellular organisms in general involves the co-operative action of T lymphocytes as specific inducers and macrophages as non-specific effector cells. Cellular and humoral responses work in a reciprocal fashion through T helper type-1 and type-2 cells, with several immune cells falling into a Th0 classification characterized by production of both Th1 and Th2 cytokines. The clinical stages of bovine paratuberculosis, characterized by large numbers of bacilli, high antibody levels, and diminished cellular responses to specific and non specific antigens suggest a shift in the immune response from a primarily pro inflammatory and cytotoxic response (Th1-like) to an antibody-based response (Th2-like) (Chiodini and Davis, 1993; Coussens et al., 2004a).

It is, however, unlikely that this is a sharp transition; rather, there is a slow progression along the classical Th1-Th2 line. The same shift in immune responses is associated with development of clinical disease in human tuberculosis and other mycobacterial diseases (Coussens et al., 2004a).

The main cytokines implicated in the protective response are IFN-g and TNF-α. IL-2 plays a central role in priming T cells and NK to secrete higher levels of IFN-g, increasing the Th1

response. Il-10 is the cytokine which down regulates the protective response in these atypical mycobacterial infections (Kaufmann and Kaplan, 1996).

The mechanism responsible for loss or reduction of type 1-like responses to MAP are not well understood but may be related to undefined host genetic factors, constant exposure of immune cells to antigen released from infected macrophages, the type of antigen presenting cell, the cytokine environment, the antigen dose and affinity for the cell receptor, the timing and the level of co-stimulatory signals delivered by both the antigen presenting cells to the T cell, and by the T cell to the antigen presenting cells during priming and secondary responses, or to the development of antigen-specific or general regulatory cell populations (Brown et al., 1998; Coussens, 2004).

Intermittent destruction of infected macrophages within granulomas could account for the sporadic bacterial shedding observed in fecal cultures from subclinically infected animals, and infrequent shedding of MAP also leads to continuous low level stimulation of the humoral immune response. As a result of this stimulation, detectable levels of antibody return in the mid to late stages of subclinical infections (Coussens, 2001).

In a study from Stabel and Ackermann (2002), the role of αβ and γδ T cells in resistance to MAP infection was investigated. Results suggested that αβ T cells play a major role in resistance to infection with MAP and that γδ T cells may play a lesser role and potentially confound protective immune responses.

However, human γδ T cells are activated directly by various mycobacterial super antigens as a critical component of the host's early defense against mycobacterial infections, including MAP. Given the large proportion of T cells in calves that bear γδ receptors and their propensity to localize to the intestinal epithelium, these cells could be an important source of IFN-g, TNF-α

and perhaps other cytokines during early stages of MAP infection. Early release of significant amounts of IFN-g, TNF-α by antigen activated γδ T cells could protect many bovine gut macrophages by activating them before actual infection (Coussens, 2001).

Weiss et al. (2005) evaluated the role of interleukin (IL)-10 in the inability of monocyte-derived bovine macrophages to kill MAP organisms in vitro. Neutralization of IL-10 enabled macrophages to kill 57% of MAP organisms within 96 hours. It also resulted in an increase in expression of TNF-α, IL-12, IL-8, MHC class II, and vacuolar H+ ATPase; increase in acidification of phagosomes; apoptosis of macrophages; and production of nitric oxide.

Diagnostics

Diagnostic tests for paratuberculosis can be divided into two categories: those detecting the organism and those that evaluate the host response to infection. The first category includes fecal smear and acid-fast stain, bacteriologic culture (from fecal or tissue specimens), and polymerase chain reaction test (PCR). The second category, detection of host response, includes clinical signs in combination with gross and microscopic pathology and detection of immune response to infection, which comprise cellular immune response (increased IFN-g production, delayed type hypersensitivity reaction, lymphocyte proliferation), and humoral (antibody) response to MAP (National Research Council, 2003).

However, despite the availability of different tests, ante mortem diagnosis of paratuberculosis has been characterized by inaccuracy due to the lack of sensitivity (in most of the cases) or specificity of the current diagnostic tools. This is of special importance during the earlier stages of infection where a single effective diagnostic tool has not yet been identified (Stabel and Bannantine, 2005).

With the idea of comparing among available tests, four stages of the disease presented by Whitlock and Buergelt (1996) will be used. Briefly, stage I corresponds to silent infection in young cattle; stage II is the subclinical disease with carrier adults; stage III is clinical disease and, finally, stage IV is advanced clinical disease. As an attribute upon which to compare different tests, sensitivity of a test is defined as its ability to detect diseased (infected) animals, i.e. the proportion of the diseased (infected) animals that test positive. Specificity of the test is its ability to detect non-diseased (non-infected) animals and is defined as the proportion of non-diseased (non-infected) animals that test negative (Martin et al., 1987).

Tests Based on Agent Detection

Fecal smear and acid-fast stain

Fecal smear is used to screen feces for acid-fast staining microorganisms. The main disadvantage of this procedure is its low sensitivity due to the irregular shedding pattern and the variability in MAP concentration in feces.

The acid-fast stain procedure utilizes the physical property of some mycobacteria to resist decolorization by acids during staining procedures. The most common staining technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain, in which the bacteria are stained bright red and stand out clearly against a blue background (National Animal Monitoring System, 1997; Palomino et al., 2007). Acid-fast staining is also used to detect the bacteria in tissue samples through an impression smear made from ileum, mesenteric lymph nodes or other specimens.

Bacteriologic culture in feces

Detection of MAP by cultivation from fecal or tissue specimens has been the basis of paratuberculosis diagnosis for a century and remains one of the most widely used diagnostic test for the infection (Collins, 1996).

However, MAP culture is insidious and requires long incubation (8–16 weeks), decontamination of the specimen to selectively kill faster growing non-mycobacterial organisms, and concentration of organisms before inoculation of the medium (National Research Council, 2003). Conventional fecal culture involves monitoring for at least 16 weeks and PCR can be used to confirm positive results. Radiometric systems (BACTEC), based on detection of C^{14} labeled CO_2, can reduce the monitoring time to half, and non-radiometric automated culture systems, recording differences in oxygen and CO_2 pressure, are now available with similar time requirements (National Research Council, 2003).

Specificity of fecal culture has widely been reported as nearly 100%, but the possibility of pass-though of orally ingested organisms by uninfected cattle does exist. Research has shown that cattle may ingest fecal matter loaded with MAP and become transiently fecal culture positive (Whitlock et al., 2000). This condition was reported by Sweeney et al. (1992) who recovered MAP from feces in heifers orally infected with contaminated feces. MAP was detected 18 hours after entry up to day seven. All heifers remained seronegative and had negative results to the intradermal Johnin test. After necropsy, MAP was not isolated from mesenteric lymph nodes, but was recovered from ileal mucosal samples from each heifer.

One of the main limitations of fecal culture is its sensitivity that has been reported ranging from 23% to 74% depending on the method and stage of infection (Nielsen and Toft, 2008). The test has no ability to detect animals in stage I of disease, but sensitivity increases going from disease stage II to IV (Whitlock et al., 2000; National Research Council, 2003). Shedding of MAP organisms in feces can be intermittent and detection by culture is complicated by contamination with other microorganisms, and especially when few MAP specimens are shed in the feces.

As an example, one study by Whitlock et al. (2000) examined the sensitivity and specificity of fecal culture for MAP from seven dairy herds. A cohort of 954 cattle, cultured every 6 months and followed over 4 years was the basis to determine the test sensitivity. For all animals, the sensitivity of fecal culture to detect infected cattle on the first sampling was 38%, while sensitivity was 42% for a cohort of parturient cattle.

Because of the moderate sensitivity of conventional fecal culture, and because MAP grows very slowly on artificial media, different procedures have been successively proposed (National Research Council, 2003). The double-incubation method (Cornell method) is commonly used for decontamination and includes a pre-incubation step with brain-heart infusion medium that initiates germination of bacterial and fungal spores, followed by centrifugation, and then a second step with the addition of antibiotics (amphotercin B, vancomycin, and nalidixic acid) to kill the spores that subsequently germinate (Whitlock et al., 1991). Subsequent centrifugation leads to an increase in sensitivity of the process. Solid media supplemented with mycobactin J are most commonly used for inoculation (Herrold's egg yolk medium, modified Lowenstein-Jensen medium). Some modifications to this method have been introduced subsequently to improve the test performance in the Cornell modified decontamination and inoculation method (Whitlock and Rosenberger, 1990).

Stabel (1997) proposed a modified method for MAP fecal culture based on centrifugation of the total fecal sample supernatant and the use of a 2-step decontamination protocol. The growth rate of MAP and contamination rate of cultures when using this method were compared to 3 other published methods: sedimentation, centrifugation, and Cornell. Sensitivity was lowest for the Cornell method; however, contamination was not observed. Contamination was the most severe in the centrifugation and the sedimentation method. The authors stated that the proposed

method was 10-fold more sensitive for detection of MAP colonies and contamination was significantly reduced compared to other 3 methods.

The association between fecal culture performance and other diagnostic tests is not well established. Muskens et al. (2003a), working with fecal samples of 422 ELISA-positive cattle cultured for the presence of MAP, found that the percentage of samples with positive culture results was 17.3%. Of the positive cultures, the number of colonies varied from 1–10 (22% of cultures), 11–100 (22%), to more than 100 (55%).

In a study by Nielsen and Toft (2006) during a period of 3 years, repeated sampling of milk and feces was performed in a total of 1,985 Danish dairy cows. Milk samples were analyzed using an ELISA, and fecal samples were analyzed by culture. The results of the study indicated that the ability of both tests to detect infection increased almost linearly from 2 to 5 yr of age.

Nielsen et al. (2002b) evaluated two ELISA and a fecal culture using maximum-likelihood estimation of sensitivity and specificity. The authors concluded that sensitivity of the fecal culture was 20-25% when used for screening in a population with an intermediate level of infection. However, sensitivity increased to the range of 60-70% if fecal culture was used as a confirmatory test on cows with a high ELISA reading.

Recently, pooled fecal culture from several animals within a herd has been suggested as a screening tool (Kalis et al., 2004). Strategic pooling of fecal samples would increase diagnostic sensitivity, would decrease the costs, and would be valuable when a herd is suspected to be negative.

Wells et al. (2003) determined the sensitivity of bacteriologic culture of pooled fecal samples in detecting MAP, compared with bacteriologic culture of individual fecal samples in 24 dairy cattle herds. Ninety four and 88% of pooled fecal samples that contained feces from at least

1 animal with high (≥ 50 colonies/tube) and moderate (10 to 49 colonies/tube) concentrations of MAP, respectively, were identified by use of bacteriologic culture of pooled fecal samples. Prevalence of paratuberculosis determined by bacteriologic culture of pooled and individual fecal samples were highly correlated (r=0.96).

In another study (Wells et al., 2002), the sensitivity of different methods of bacteriologic culture of pooled bovine fecal samples for MAP detection was compared. The homogeneity in number of MAP in pooled fecal samples was also evaluated. The authors reported that, compared with concurrent bacterial culture of individual infected samples, 37 to 44% of pooled samples with low bacterial concentrations (mean <2.5 CFU/tube) yielded positive culture results and 94% of pooled samples with high bacterial concentrations (mean >10 CFU/tube), yielded positive results.

Automated systems

BACTEC system (Becton Dickinson Laboratories, Sparks, Maryland, USA) is a modified mycobacterial radiometric culture devise designed for human clinical laboratories. It is an automated, faster, and more sensitive method, and requires the use of radioisotopes (C^{14}-labeled palmitic acid). The instrumentation detects the C^{14}-labeled CO_2 that is produced by metabolism of the labeled palmitic acid, and IS*900* PCR is required to confirm positive results (National Research Council, 2003).

Non-radiometric automated systems are currently available and offer a reduction in the detection time for positive specimens (BACTEC MGIT series). The systems require special, defined media and incorporate a detector system that reacts to alterations in oxygen, CO_2, or pressure within a sealed tube (Hanna et al., 1999; Nielsen et al., 2001).

Five methods for whole herd fecal culture were compared in three herds by Eamens et al. (2000). These included two methods based on primary culture on Herrold's egg yolk medium

with mycobactin J (HEYM): conventional (1) decontamination with sedimentation and primary culture on HEYM; Whitlock decontamination and culture on HEYM (2). The remaining three methods were based on radiometric (BACTEC) culture: decontamination and filtration to BACTEC medium (3); modified Whitlock decontamination to BACTEC medium (4) and Whitlock decontamination to BACTEC medium (5). For BACTEC cultures, two methods were compared as confirmatory tests for MAP: mycobactin dependence on conventional subculture to HEYM and IS900 PCR analysis of radiometric media. In identifying shedder cattle, method 5 was the most sensitive, followed by methods 2, 4, 1, and 3. The number of BACTEC cultures confirmed by mycobactin dependence or PCR was similar.

Polymerase chain reaction (PCR)

The introduction of diagnostic probes based on specific bacterial DNA sequences has allowed fastidious microorganisms, such as MAP, to be rapidly identified. In 1989, a novel DNA insertion sequence (IS*900*) in MAP was reported (Collins et al., 1989; Green et al., 1989; Eglund, 2002). IS*900* is present in multiple copies (14-18) in MAP genome and consists of 1,451 base pairs (bp) of which 66% is G + C showing a degree of target sequence specificity (Green et al., 1989).

Polymerase chain reaction tests based on this insertion element have been the most widely used for MAP identification (Harris and Barletta, 2001). However, the detection of the etiologic agent is limited by the frequency and number of the organism that are present in the body fluid or tissue being tested. In the case of fecal detection, contamination, which inhibits PCR, has been a strong limitation to date. Many efforts are focused at the present on an effective method for DNA purification from feces that allows the use of PCR.

The isolation of MAP from sites distant to the intestinal tract, such as udder, fetus, kidney, liver, male reproductive tract and blood, have suggested active dissemination of MAP. This

opens the possibility for detection of the agent by PCR in fluids such as milk and blood of suspicious animals.

The PCR test is considered a useful test in detection of animals in stages II to IV of disease. Of the currently available methods for detection of MAP, PCR-based assays have the highest potential analytic sensitivity. Equally important as a test's analytic sensitivity is the sample that is to be tested. Especially important is the ability of the sample to have a high likelihood of containing MAP or leucocytes infected with MAP in early-stage animals, and to be devoid of factors that inhibit PCR, such as those found in feces (Buergelt and Williams, 2004).

Sensitivity of PCR is difficult to determine because PCR is almost certainly more sensitive than the great majority of existing diagnostic tests, making a gold standard impractical to establish (Kelly et al., 2005). One study has reported PCR sensitivity for fecal, blood, milk, and liver samples in advanced subclinically MAP infected cows as 87%, 40%, 96% and 93% respectively (Barrington et al., 2003).

Although specificity of IS*900* based PCR is considered nearly 100%, recent studies suggest that insertion sequences similar to IS*900* would be present in other mycobacterial species and such sequences would also be positive in most of the current IS*900* PCR systems (Cousins et al. 1999; Tasara and Stephan, 2005). Another concern, because of the high analytical sensitivity of PCR, is the possibility of false positive results arising from cross contamination of samples.

Several molecular targets, other than IS*900*, (HspX, L1/L9 integration sequences, IS*Mav2*, and F57) have been evaluated for MAP detection (Rajeev et al., 2005). The F57 and HspX sequences occur as single copies, while at least three copies of IS*Mav2* are present in the MAP genome (Tasara and Stephan, 2005) and has no similarity to known mycobacterial IS elements

although it shows more than 50% identity to a non-composite transposon of *Streptomyces coelicolor* at the DNA and protein level (Strommenger et al., 2001).

Tasara et al. (2005) developed a multiplex PCR system designed to enhance specificity for MAP detection in a single PCR reaction. Multiplex PCR is a variant of PCR which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. This PCR assay co-amplifies the *Mycobacterium* species 16S rRNA gene, MAP IS*900* and F57 sequences. The multiplex PCR assay was highly specific, but the nested PCR system was also positive for several other *Mycobacterium* species.

Li et al. (2005), working with a common clone of MAP, strain K-10, identified 17 copies of the previously described insertion sequence IS*900*, seven copies of IS*1311*, and three copies of IS*Mav2* in the K-10 genome. A total of 16 additional MAP insertion sequence elements were identified in the analysis, totaling 19 different insertion sequences with 58 total copies in the K-10 genome.

Bhide et al. (2006) presented a PCR-based detection of IS*900*, from the buffy coat of cattle (n=262) and sheep (n=78), and direct genotyping by single strand conformational polymorphism (SSCP). A total of 30 cattle and one sheep were positive for MAP-IS*900*. SSCP analysis grouped the MAP-IS*900* into four distinct clusters based on different band patterns. Nucleotide sequence variability between MAP detected from sheep and cattle was noticed in the study.

Real-time sequence detection methods based on two different chemistries were presented by Ravva and Stanker (2005). One was based on the detection of SYBR Green bound to PCR products and the second more specific method, detected the cleavage of a fluorogenic (TaqMan) probe bound to a target sequence during primer extension phase. Novel primers and probes that amplify small fragments (<80 bp) of the MAP specific insertion sequence, IS*900*, were designed.

Both the SYBR green and TaqMan assays are able to detect 3 to 4 fg of DNA extracted from MAP strain ATCC19698 (0.6 to 0.8 cells per assay). Both SYBR Green and TaqMan assays were highly specific for the detection of MAP.

PCR on milk

Pillai and Jayarao (2002) evaluated the application of IS*900* PCR for the detection of MAP from raw milk. This assay was based on IS*900* PCR detection including DNA extraction and PCR assay using commercially available kits. Detection of MAP by IS*900* PCR was consistent when about 100 CFU/ml were present, whereas detection was variable at concentrations as low as 10 CFU/ml. IS*900* PCR was also evaluated with pooled quarter milk samples from 211 cows from five herds with known history of JD. Out of 211 animals examined, 4% and 33% were positive for MAP by milk culture and IS*900* PCR from milk, respectively. A total of 20 bulk tank milk sample aliquots were also examined, of which 50% were positive for MAP by IS*900* PCR. By contrast, only 5% bulk tank milk sample aliquots were positive by culture.

Rodriguez-Lazaro et al. (2005) presented a real-time PCR assay for quantitative detection of MAP amplifying IS*900* insertion. The assay detected <3 genomic DNA copies with a 99% probability. Using prior centrifugation, the assay was able to detect 10^2 MAP cells in 20 ml artificially contaminated drinking water. With a detergent and enzymatic sample pretreatment before centrifugation and nucleic acid extraction, the assay was able to consistently detect 10^2 MAP in 20 ml artificially contaminated semi-skimmed milk.

Tasara and Stephan (2005) developed a light cycler-based real-time PCR assay amplifying the F57 sequence of MAP, including an internal amplification control template. The assay had a reproducible detection limit of about 10 MAP cells per ml, starting with a sample volume of 10 ml of MAP-spiked milk.

Jayarao et al. (2004), evaluated sensitivity, specificity, and predictive value of IS*900*-PCR for MAP detection in pooled quarter milk and bulk tank milk. Culture analysis resulted in 10.9%, 2.8%, and 20.6% of fecal, pooled quarter milk and bulk tank samples positive for MAP, respectively. While 13.5% and 27.5% of pooled quarter milk samples and bulk tanks were positive by IS*900* PCR, respectively. The IS*900* PCR assay using pooled quarter milk samples had a sensitivity, specificity and positive predictive value of 87%, 95% and 71%, respectively. The IS*900* PCR assay using bulk tank milk had poor sensitivity (21%), specificity (50%) and predictive value (60%).

In another study (Giese and Ahrens, 2000), milk and fecal samples from cows with clinical signs of paratuberculosis were tested by culture and PCR to determine the presence of MAP. The bacteria were cultivated from feces or intestinal mucosa in eight of 11 animals. A few colonies were cultivated (<100 CFU per ml) in milk from five fecal culture positive cows. Milk samples from two cows were PCR positive (both animals positive for fecal culture, and one positive for culture in milk). One cow was culture negative on intestinal mucosa, but culture positive in milk, and two cows were negative in culture and PCR from both feces and milk.

PCR on feces

Different methods have been proposed for PCR detection of MAP in feces, but the high analytical sensitivity of this test is seldom achieved on fecal specimens. Possible causes of this problem include nonspecific DNA derived from the host or other microbes, presence of inhibitory substances, and quality of the genomic DNA preparation (Khare et al., 2004).

Vary et al. (1990) presented the results obtained by DNA probes that hybridize IS*900*. Tests were found to be highly specific for MAP. The authors report that direct detection of MAP DNA in feces from infected cattle was highly specific, with a sensitivity equal to or greater than

that obtained by standard culture techniques with an important reduction in time to results when compared to culture.

Van der Giessen et al. (1992) presented three assays for MAP detection on fecal samples of dairy cattle, using dot spot hybridization of PCR products. The first two tests used PCR primers and a DNA probe derived from MAP-specific sequences of the 16S rRNA gene and insertion element IS*900*, respectively. The 16S rRNA test was able to detect 107 bacteria per g of feces, and the IS*900* test detected 104 to 105 per g of feces. These two tests and a commercially available test (IDEXX Corp.) were used twice with an interval of 3 months on fecal samples of 87 cows from two dairy herds with a history of JD. Results were compared with those of culturing. The tests showed a high specificity (89 – 100%) but the sensitivity ranged from 3 to 23%.

Khare et al. (2004) proposed a method based on immuno-magnetic bead separation coupled with bead beating and real-time PCR for the isolation, separation, and detection of MAP from milk and/or fecal samples from cattle. The authors report that by conventional and real-time IS*900*-based PCR, 10 or fewer MAP organisms were consistently detected in milk (2-ml) and fecal (200-mg) samples.

In another work (Tadei et al., 2004), three commercially available assays for IS*900*-PCR on fecal samples were compared with a conventional culture method. Sixty seven percent of 80 culture-positive samples were positive for an assay that detects MAP DNA by dot spot hybridization of PCR products (IDEXX Laboratories, ME), 60% were positive by an assay using ethidium bromide staining for agar gel visualization of amplification products (Adiavet paratub PCR, France), and 61.3% were positive by an assay with a colorimetric detection system (Institut

Pourquier, France). Specificity was 100% based on results from 20 culture-negative samples from a MAP-free herd.

Fang et al. (2002) reported an automated fluorescent PCR for detection of MAP in bovine feces. When the PCR was compared with culture of fecal samples, kappa scores of 0.94 to 0.96, a sensitivity of 93 to 96%, and a specificity of 92% were obtained. Results were quantified by use of a standard curve derived from a plasmid containing IS*900* and a minimum quantity of 1.7 x 10-4 pg of DNA, correlating to 1 to 8 CFU, was detected.

Another study reported a comparison between a real-time PCR and fecal culture (Bogli-Stuber et al., 2005). In fecal samples derived from 13 dairy herds in Switzerland real-time PCR identified 31 of 310 animals as positive within this population whereas culture identified 20 positive animals. The observed agreement of the two tests used in the study was 91.3%, whereas the kappa-value was 42%.

A high-throughput TaqMan PCR assay for MAP detection, targeted to *Mav2* insertion sequence was evaluated by use of fecal samples from naturally infected herds and herds considered free of paratuberculosis (Wells et al., 2006). Fecal, blood, and milk samples were subjected to the PCR-based assay, three different fecal culture procedures for MAP, two ELISAs, and one milk ELISA. Results showed that specificity of the PCR assay was 99.7%. Twenty-three percent of the dairy cows that were fecal culture positive by at least one of the three methods were positive by the PCR assay.

In another study, using Bayesian non-"gold standard" analysis methods, the TaqMan PCR assay had a higher specificity than serum ELISAs (99.3%) and sensitivity similar to that of the serum ELISAs (29%). By classical methods, the estimated relative sensitivity of the fecal PCR

assay was 4% for light and moderate fecal shedders (compared to 12 to 13% for the ELISAs) and 76% for heavy fecal shedders (compared to 67% for the milk ELISA).

Detection of Host Response to Infection

Clinical signs, gross and microscopic pathology

Clinical signs appear in the stage III of paratuberculosis and include gradual weight loss in spite of a normal appetite. With the progress of disease, manure consistency becomes more fluid and diarrhea may be intermittent. Serum and biochemical changes include low concentration of total protein, albumin, triglycerides and cholesterol. Muscle enzymes levels increase as a result of muscle wasting. However these changes are not specific enough to be useful as diagnostic tests. During stage IV animals become increasingly lethargic, weak, and emaciated. Most animals are culled before this stage because of reduced milk production or severe weight loss. Intermandibular edema, cachexia and persistent diarrhea characterize the terminal stage (Whitlock and Buergelt, 1996).

Gross lesions are confined to the terminal portion of the small intestine and associated lymph nodes. Lymph nodes are enlarged and edematous and sub-serosal lymphatics appear tortuous, dilated and thickened, and intestinal mucosa becomes thickened and corrugated.

Pathognomonic cellular changes include clustered epithelioid macrophages and /or inflammatory giant cells of Langhans' type and subtle histopathological alterations can be found even during stage I of the disease. Ziehl-Neelsen staining technique is the traditional method to demonstrate the presence of MAP in the tissues (Whitlock and Buergelt, 1996). At present, the gold standard for paratuberculosis diagnosis is necropsy followed by extensive culture and histological examination of multiple sections of lower small intestine and associated lymph nodes (National Research Council, 2003).

Cellular immune response

Because cell-mediated immune (CMI) responses are the first and strongest host response to mycobacterial infections, CMI tests may be useful for early detection of MAP infection (Robbe-Austerman et al., 2007).

Interferon gamma assay (IFN-g)

A method of measuring CMI response is the gamma interferon (IFN-g) assay, a laboratory test initially developed for the diagnosis of tuberculosis, but also available for the diagnosis of paratuberculosis (Kalis et al., 2003). The test is based on production and release of IFN-g by sensitized bovine lymphocytes in response to in vitro stimulation with a series of mycobacterial antigens. Because cellular immunity is developed soon after infection, this test is considered the most sensitive during early infection, and is deemed useful in detection of animals in stages II and III (Billman-Jacobe et al., 1992; Collins, 1996, 2003; Stabel, 2001). To improve diagnostic specificity, IFN-g levels released in response to bovis purified protein derivative (PPD) are compared with IFN-g levels released in response to avium-PPD and IFN-g levels in non-stimulated samples (Kalis et al., 2003). Reported sensitivities range from 72% to 99% for subclinical cases, without and with fecal shedding, respectively (National Research Council, 2003).

However, the disadvantage of IFN-g assay is its low specificity. The test is subject to cross reactivity with other mycobacteria (Huda et al., 2003), and specificity values are controversial, ranging from 26 to 97.6% (Kalis et al., 2003). These authors re-examined CMI specificity. The IFN-g assay specificity was estimated in 35 uninfected dairy herds by use of a newly developed algorithm, resulting in 93.6%. When interpreted according to two alternative algorithms the assay had specificities of 66.1 and 67.0% (Kalis et al., 2003).

In another work (Stabel, 2001), blood samples were obtained from infected dairy herds and tested by a modified IFN-g analysis. Blood samples were incubated alone (non-stimulated), with concanavalin A, and with *M. avium* PPD, *M. bovis* PPD, or a whole cell sonicate of MAP for 18 h to elicit antigen-specific IFN-g production. After incubation, plasma was analyzed for IFN-g by ELISA. Values for IFN-g for non-stimulated blood samples were consistently low. In contrast, concanavalin A stimulation of blood samples evoked a significant secretion of IFN-g regardless of infection status. Antigen-specific IFN-g results were positively correlated with MAP infection status. Accuracy of the IFN-g assay for correctly predicting infection status of individual cows in the herds with low levels of infection ranged from 50 to 75% when used as a single test.

Huda et al. (2004) presented a study based on repeated blood and fecal sampling for culture of feces, assessment of IFN-g secreted by MAP antigen stimulated whole-blood lymphocytes, and measurement of antibody responses against MAP in serum and milk by ELISA. The IFN-g test diagnosed higher proportions of infected and exposed animals than the antibody ELISAs. The highest sensitivity of IFN-g test was in infected cattle 2 or more years of age. IFN-g test had a better performance than antibody tests of animals of 1 and 2 years of age, with a similar performance for animals of 3 or more years old.

Hypersensitivity reaction (Skin test)

A well-known CMI test for mycobacterial infections is the intradermal skin test which measures delayed type of hypersensitivity to mycobacterial antigens. The swelling formed three days after injection of a mycobacterial PPD (Johnin PPD) is measured using callipers to determine the increase of skin thickness (Colins, 2003; Kalis et al., 2003).

In spite of successful application of the skin test in the control of bovine tuberculosis, it is only occasionally used in the control of paratuberculosis because its specificity has been reported

to be low. Sensitivity of this test has been reported to be close to IFN-g assay; however, specificity is lower because MAP shares antigens with environmental mycobacteria resulting in numerous cross reactions (Collins, 1996). However, a study (Kalis et al. 2003) reported a specificity of 93.5% for the skin test and a fair agreement ($\kappa = 0.41$) between skin test and IFN-g assay based on the analysis of 1631 animals.

In another study, Jungersen et al. (2002) estimated specificities from 95 to 99% by Johnin PPD stimulation, irrespective of interpretation relative to bovine PPD or no-antigen stimulation alone. For a limited number of test-positive animals, no change in the test results could be observed with increasing antigen concentrations but IFN-g responses were significantly reduced. In both MAP-free and MAP-infected herds, false positives were observed when the test was applied to calves less than 15 months of age.

Lymphocyte proliferation

The lymphocyte transformation test is an *in vitro* test based on the fact that lymphocytes, previously sensitized by an antigen, transform into blasts and proliferate when they are again exposed to this antigen. This proliferation is determined by measurement of the incorporation of H^3-thymidine or bromodeoxyuridine into replicating DNA. The assay for paratuberculosis detection uses antigen Johnin PPD to stimulate lymphocytes co-incubated with radio-labeled deoxiuridine to measure the rate of DNA synthesis (Buergelt et al., 1977, National Research Council, 2003). Although sensitivity is acceptable, like the previous test, it suffers from specificity problems related to exposure to other mycobacteria. Another concern is the use and disposal of radioisotopes, the expensive instrumentation and the large volume of blood required.

De Lisle and Duncan (1981) reported a whole blood lymphocyte transformation test to examine cattle infected with MAP. Minimally infected animals responded to Johnin PPD in the lymphocyte transformation test but did not routinely react on serological and/or skin testing.

Heavily infected animals showed considerable variation in their lymphocyte transformation responses to antigen and some of them were consistently unresponsive. Antigen induced lymphocyte transformation reactions were recorded in 7.6 to 41.5% of animals whose negative infection status was determined by bacteriology and/or histopathology.

Humoral immune response

Given that antibody response occurs late in the course of infection, pathobiology of paratuberculosis limits the ability of tests for serum antibodies to detect animals in the early stages of infection (Collins, 1996).

Complement fixation and agar gel immunodiffusion (AGID).

Complement fixation was one of the earliest serologic tests for paratuberculosis but in the present is not widely used because of its moderate sensitivity and low specificity. Agar gel immunodiffusion test was developed as a quick test for animals showing clinical signs. Positive results correlate well with clinical signs, but failure to detect subclinical infection is the main limitation. Sensitivity and specificity have been reported as 18.9 and 99.4% for the detection of subclinically infected animals (Sherman et al., 1984). AGID test is considered useful in detection of animals in stages III and IV.

Enzyme-linked immunosorbent assay (ELISA)

Most ELISA tests in current use are modifications of the method developed by Yokomizo et al. (1983) who developed an ELISA for MAP detection in cattle sera. The aim was to minimize the nonspecific reactions caused by IgM by measuring only IgG1 against a bacterial protoplasmic antigen. The sensitivity reported for this assay was 58% of cattle positive to fecal culture. The authors reported 4% of the sera from fecal culture negative animals giving a false positive result.

Three years later the same group reported that pre-absorption treatment of sera with *Mycobacterium phlei* increased the specificity of the ELISA test by removal of cross-reacting antibodies (Yokomizo, 1986). At present, ELISA test kits or services are commercially available from a number of sources (IDEXX, Portland, Maine, USA; Allied Monitor, Fayette, Missouri, USA; Synbiotics, San Diego, California, USA; Biocor Animal Health, Inc., Omaha, Nebraska, USA; CSL, Parkville, Victoria, Australia; Pourquier, Institut Pourquier, Montpellier, France), and sensitivity and specificity of ELISA for MAP detection have been described in numerous published reports (National Research Council, 2003). ELISA sensitivity is usually reported in reference to fecal culture. In the decade since the absorbed ELISA was introduced, the reported sensitivity has gradually decreased from 57% (Milner et al., 1990) to a more current estimate of 45% (Sweeney et al., 1995). Despite the fact that commercial kits are marketed as herd-level diagnostic tools, they are commonly used as cow-level tests. Because of its moderate sensitivity, the ELISA test rarely gives a positive result in animals under 2 years of age and frequently fails to detect individuals in the early phases of infection (Juste et al., 2005). Regardless of these disadvantages, ELISA testing of sera is still the method of choice for epidemiological studies and herd-based diagnosis (Bottcher and Gangl, 2004).

There are multiple estimations for ELISA sensitivity and specificity. Bech-Nielsen et al. (1992) reported an increase in pre-absorbed ELISA response for animals with heavy MAP fecal shedding when compared with the response in low shedders or culture negative animals. The specificity reported for this pre-absorbed ELISA in two fecal culture negative herds was 100% compared with 62.9% when the sera was not pre-absorbed.

In another study, two commercial ELISAs (Allied Laboratories, [Glenwood Springs, Colorado, USA] and the CSL, Limited, [Parkville, Victoria, Australia]) were evaluated (Sockett

et al., 1992). A subclinical case of bovine paratuberculosis was defined as the isolation of MAP from fecal samples or internal organs of cattle without diarrhea or weight loss. The Allied ELISA, and the CSL ELISA had sensitivities of 58.8, and 43.4%, respectively, and specificities of 95.4, and 99.0%, respectively. The Allied ELISA, and the CSL ELISA detected 65.7%, and 56.5% of the MAP fecal shedders, respectively.

In a review from Collins and Sockett (1993) the limitations of ELISA were presented. The authors stated that sensitivity is a direct function of the infection stages in the tested population, with a better ability for detection in the advanced stages of the infection. In this review, the estimates of sensitivity range from 24.6% to 88.2%, for stages 1 and 3 of infection, respectively, with a combined estimation of 45.5%. The specificity reported is 99.7% for pre-absorbed ELISA.

Whitlock et al. (2000) examined the sensitivity and specificity of the ELISA and fecal culture tests for paratuberculosis in dairy cattle. Infected dairy herds tested concurrently with both fecal culture and ELISA resulted in more than double positive animals by culture compared to ELISA. ELISA had a higher sensitivity in animals with a heavier bacterial load (75%) compared to low shedders (15%).

Another study reported sensitivity for ELISA of 45% for a group of 1146 cows, with values ranging from 15% to 87% as the disease progressed to clinical stages (Thorne and Hardin, 1997). On the other hand, sensitivities between 15.4 to 88.1% were presented by Dargatz et al. (2001) for serum ELISA, depending on the clinical stage and bacterial shedding status of the cattle.

It has been suggested that the measurable humoral immune response to MAP in subclinical cows can even vary widely from day to day (Barrington et al., 2003). It is suspected that this

variation in ELISA results is due to fluctuation in antibody production, variable losses by way of the gastrointestinal tract, or a combination of both (Buergelt and Williams, 2004).

Gasteiner et al. (2000) tested two ELISA-methods (A-ELISA, Allied Monitors; H-ELISA, Veterinary University Hannover) with serum samples from healthy, infected and diseased cattle as well as positive and negative reference sera. In both ELISA-methods total agreement between antibody detection and shedding of MAP was found for diseased animals. Reference serum samples of culturally negative cattle were negative in 98% by H-ELISA and in 82% by A-ELISA, and those of positive animals were positive in 59% by H-ELISA and in 82% by A-ELISA.

Jubb et al. (2004) estimated the sensitivity of a serum ELISA (Parachek, CSL, Parkville) in dairy herds participating in a control program in Australia. Values reported are 16.1%, 14.9% and 13.5% for herds with 5, 6 and 7 annual tests, respectively.

In another work, Nielsen et al. (2002) studied the ELISA response to MAP by cow characteristics and stage of lactation. The results showed that the probability of being ELISA-positive was 2 to 3 times lower for cows in first parity relative to cows in other parities (milk and serum). The probability of a positive result was higher at the beginning of the lactation for milk ELISA, but for serum ELISA the odds of being positive was higher at the end of the lactation.

Van Schaik et al. (2005) presented a kinetic ELISA with multiple cutoff values to detect fecal shedding of MAP. The sensitivity and specificity relative to culture reported were 67% and 95%, 31% and 99.7%, and 11% and 99.9% for three different cutoff values respectively. The authors suggested that cutoff values for this kinetics ELISA should be determined based on the apparent within herd prevalence of infection.

In a recent review, Nielsen and Toft (2008) examined multiple studies reporting sensitivity values for serum multiple ELISA with values ranging from 7% to 94%, depending on the particular test under analysis and the group reporting the results.

However, the high specificity of ELISA has been questioned by some recent works. Osterstock et al. (2007) evaluated the effect of exposure to environmental mycobacteria on results of 2 commercial ELISAs (A: HerdCheck, IDEXX laboratories Inc and B: ParaCheck, CSL Biocor). Weaned crossbred beef calves were inoculated with 1 of 5 mycobacterial isolates derived from herds with high proportions of false-positive serologic reactions for paratuberculosis, MAP, or mineral oil. By use of ELISA-A, ≥1 false-positive reaction over time was detected in 2, 3, 3, and 1 of the 3 calves injected with *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, or *Mycobacterium terrae*, respectively. By use of ELISA-B, only *M. scrofulaceum* induced false-positive reactions.

In a subsequent study, Roussel et al. (2007) evaluated the seroprevalence of paratuberculosis by use of the 2 previously cited commercial ELISAs (A,B) in association with prevalence of fecal shedding of mycobacteria within beef cattle herds in 6 affected beef herds and 3 geographically matched herds without high seroprevalence of paratuberculosis. Cattle from affected herds were 9.4 times as likely to have environmental mycobacteria isolated from feces. The authors suggested that beef herds with persistently high rates of false positive ELISA results may be associated with recovery of environmental mycobacteria from feces.

Agreement among serum ELISA kits

Inter-laboratory reproducibility of an absorbed ELISA kit for detection MAP serum antibodies (Johne's Absorbed EIA®, CSL Limited, Parkville, Australia) was evaluated by Collins et al. (1993). A panel of bovine sera was tested in triplicate microtiter wells at 8 different laboratories. Between-well CVs averaged 6.7% ± 2.8% (mean ± standard deviation), and

between-day CVs averaged 14.5% ± 9.8% among laboratories. Among 1392 assays in 7 laboratories, 98.6% were in agreement indicating that the absorbed ELISA kit provided reproducible results within and between laboratories.

However, strong discrepancies between different commercial ELISAs when performed concomitantly on the same animal have been reported. McKenna et al. (2006) presented a range of kappa coefficients for combinations of three different commercial ELISA tests from 0.18 to 0.33, which is slight and fair agreement, respectively.

Five diagnostic ELISA tests were evaluated by using individual serum or milk samples from cattle in paratuberculosis-free and infected dairy herds (Collins et al., 2005). The specificity of three ELISAs (two on serum, one on milk) was >99.8%. The specificity of the remaining two ELISAs, (serum), was 94.9 and 84.7%. Four of the five ELISAs evaluated produced similar sensitivity in detecting fecal culture-positive cattle (27.8 to 28.9%). One serum ELISA had the lowest specificity (84.7%) and the highest sensitivity (44.5%). Assay agreement (kappa coefficient) ranged from 0.47 to 0.85 for categorical assay interpretations (positive or negative), but linear regression of quantitative results showed low correlation coefficients (r=0.40 to 0.68).

In a recent study, three different commercially available serum-ELISA (Svanovir-ELISA, Svanova, Uppsala, Sweden; IDEXX-ELISA, IDEXX Laboratories, Maine, USA; Pourquier-ELISA, Institut Pourquier, Montpellier, France) and two milk ELISA (Svanovirm-ELISA Svanova, Uppsala, Sweden; Pourquier-ELISA, Institut Pourquier, Montpellier, France) were compared. Blood-, milk- and faecal samples were monthly taken from 63 selected animals. The highest number of blood- and milk samples with a detectable antibody-level was found by the Svanovir-ELISA. There was a significant correlation between serum- and milk- Svanovir-ELISA results, whereas the agreement between ELISA and faecal culture/PCR was low.

Significant correlations between Svanovir-serum-ELISA results and milk somatic cell counts were estimated (Geisbauer et al., 2007)

Milk/serum ELISA

Sensitivity and specificity for milk ELISA has been reported in a range from 21%-61% and 83%-100%, respectively (Nielsen and Toft, 2008). Hardin and Thorne (1996) compared milk and serum ELISA for MAP detection, using concurrent samples, and estimating the correlation between milk and serum tests. McNemar's chi square were significant (p = 0.05), but analysis of correlation and regression analysis were low (r^2=0.02), indicating a low association between both tests results.

Milk and serum samples from 35 dairy herds in the US were evaluated for cow- and herd-level MAP antibody test agreement (Lombard et al., 2006). Evaluation of 6,349 samples suggested moderate agreement between milk and serum ELISA results, with a kappa value of 0.50. Cow-level sensitivity for 18 dairy operations with 1.921 animals was evaluated relative to fecal culture results, with values of 21.2 and 23.5% for the milk and the serum ELISA, respectively.

Stabel et al. (2002) reported that from a population of 651 cows, only 25% of animals with fecal-culture positive results tested positive for milk ELISA and over 6% of cows that were fecal-culture negative tested ELISA-positive. ELISA for bulk-tank milk also has been developed for estimating the level of paratuberculosis infection in dairy cattle (Nielsen et al., 2001). Those authors describe a sensitivity of 97% and specificity of 83%. However, the number of infected animals that must be present in the herd to result in a positive bulk-tank sample is apparently unknown.

Disease Control

Epidemiological Factors in Control

Host factors

Transmission of paratuberculosis is mainly via ingestion of MAP in colostrum or milk, exposure of young to infected feces, or through *in utero* infection of calves (Seitz et al., 1989; Valentin-Weigand and Goethe, 1999; Buergelt et al., 2006; Mitchell et al., 2008; Wittington and Winsor, 2007). Level of exposure (dose of organisms) and age at the time of exposure are major factors in determining whether an animal eventually becomes infected with MAP (McKenna et al., 2006). Infection is believed to occur mainly in young individuals, with age-resistance occurring later (Nielsen and Toft, 2007). It is suspected that, on rare occasions, certain animals that are exposed to MAP can generate a protective immune response resulting in full clearance of the bacteria (Buergelt et al., 2004a; McKenna et al., 2006).

On the other hand, genetic factors have been associated with differences in host susceptibility to infection with MAP and subsequent disease. Estimations indicate a range of moderate heritability values for susceptibility to infection (Koets et al., 2000; National Research Council, 2003; Mortensen et al 2004, Gonda et al., 2006). Research has recently been aimed at detecting associations between susceptibility differences and polymorphisms of candidate genes, with no definitive results (Hinger et al., 2007; Taylor et al., 2006; Gonda et al., 2005, 2007).

A breed effect has been proposed in some studies with Jersey and Shorthorn cows having a higher susceptibility for paratuberculosis infection (Cetinkaya et al., 1999; Jakobsen et al., 2000). However, confounding variables such as different husbandry practices could have played a role in the reported differences.

Natural reservoirs and environmental factors

One important threat to paratuberculosis control programs is infection in feral maintenance hosts that cannot be controlled and could potentially reintroduce infection in livestock (Biet et al., 2005). There are many studies reporting the presence of MAP infection in non-domestic animal populations. Among the wild species in which paratuberculosis has been reported are other ruminants, such as deer (Stehman, 1996), bison (Buergelt et al., 2000, Ellingson et al., 2005a), and elk, as well as non-ruminants, such as wild rabbit (Greig et el., 1997), rat, wood mouse, hare, their predators, including fox and stoat, weasel, and primates, such as mandrill and macaque (Beard et al., 2001; Zwick et al., 2002), indicating a wide host range (Alifiya et al., 2004).

Analysis of the molecular diversity and comparative molecular pathology of MAP would help to establish the degree of heterogeneity in strains isolated from a variety of host species. The extent of strain sharing across a variety of hosts would reflect the degree of interspecies transmission (Motiwala et al., 2003, 2004).

MAP is primarily transmitted by the fecal-oral route with the bacteria shed in the feces of infected individuals and then ingested by susceptible animals. The level of transmission of MAP by indirect contact depends on the number of organisms shed in the feces and the organism's survival characteristics in the environment (National Research Council, 2003).

While MAP is unable to replicate in the environment, some characteristics, such as a peculiar lipid-rich cell wall, enable the organism to persist in the environment and contribute to its resistance to low pH, high temperature, and chemical agents (Manning, 2001). The relationship between MAP and the environment is complex, involving factors such as the physical characteristics of the substrate material (feces, water, milk, manure slurry, dust,

environmental surface, dirt), temperature, pH, water activity or content, and competing microorganisms (National Research Council, 2003).

Raizman et al. (2004) characterized the distribution of MAP in the environment of infected and uninfected Minnesota dairy farms. Eighty infected and 28 uninfected herds were sampled. Two environmental samples were obtained from each farm from various locations, and samples were tested using bacterial culture for MAP. Environmental samples were cultured positive in 78% of the infected herds, and one negative herd had one positive environmental sample. The study results indicated that targeted sampling of cow alleyways and manure storage areas appears to be an alternative strategy for herd screening and Johne's infection status assessment.

Lombard et al. (2006a) studied the distribution of MAP in the environment and assessed the relationship between the culture status of the bacteria in the farm environment and herd infection status. A total of 483 environmental samples were collected, and 218 (45.1%) were culture-positive for MAP. Positive environmental cultures resulted from parlor exits (52.3%), floors of holding pens (49.1%), common alleyways (48.8%), lagoons (47.4%), manure spreaders (42.3%), and manure pits (41.5%). Sixty-nine of the 98 operations (70.4%) had at least one environmental sample that was culture-positive. Of the 50 herds classified as infected by fecal culture, 76.0% were identified by environmental culture. Of the 80 operations classified as infected based on serum ELISA-positive results, 76.3% were identified as environmental-positive, whereas 20 of the 28 operations identified as infected based on milk ELISA were detected by environmental sampling.

Due to the particular characteristics of its cell wall, along with the clumping behavior of this bacteria, MAP appears to be more thermal resistant than other mycobacteria, making

pasteurization of milk and milk products somewhat problematic (Chiodini et al., 1984; Lund et al., 2000; Grant et al., 2001).

MAP DNA and viable bacteria has been reported on commercially pasteurized retail milk (Millar, 1996; Ayele et al., 2005; Ellingson et al., 2005), calling into question the validity of feeding pasteurized milk products to calves as a possible means of lowering the risk of MAP infection (McKenna et al., 2006).

Population factors

Different models have been proposed to explain the transmission and persistence of MAP within dairy herds, considering variables such as prevalence in different categories of animals, and taking into account that susceptibility decreases with age. In general terms, three adult-shedding categories (low, high and clinical) are usually considered, and risk factors are established according to the level of exposure as a result of environmental contamination. In these models the probability of a successful infectious contact increases as animals advance in subsequent stages of disease. In a study by Mitchell et al. (2008), it was concluded that in all models high-shedding animals have an important impact on prevalence of MAP infection in a herd. However, these animals were not the only ones responsible for disease persistence and infection in a low-prevalence herd, actively managing shedding animals. Infection could be maintained by other factors, such as dam-to-daughter and calf-to-calf transmission. A recent simulation model proposed by Nielsen et al. (2007) indicated the need of adjusting by covariates such as mean prevalence in the herd, the age adjusted prevalence of the herd, the rank of the age adjusted prevalence, and a threshold-based prevalence.

From the diagnosis point of view, a study presented by Kudahl et al. (2007) predicted that an increase in milk-ELISA sensitivity, used in a “test-and-cull” strategy would result in a more effective reduction of MAP prevalence, with a milk production level comparable to a non-

infected herd if initial prevalence was moderate (25%) or an increased milk production, if initial prevalence was high (80%). However, it was predicted that after 10 years, a persistent high replacement rate would limit progress because of a restricted replacement base.

Control Programs

In general, disease control programs have 3 main objectives: decrease the number of new infections; decrease the number of clinically diseased or shedding animals; and decrease the duration of disease or its infective period (McKenna et al., 2006a).

In the past, almost all of the JD control programs worldwide have been based on early identification and rapid elimination of clinically infected animals, and the implementation of preventive measures (Benedictus and Khalis, 2003). However, many of them have been ineffective in the reduction of the disease prevalence (Groenendaal et al., 2003), and numerous programs based only on test and cull have been terminated because of their high costs and because success or failure can only be demonstrated over a long period of time (Whitlock et al., 1994).

Groenendaal and Galligan (2003) presented a simulation model for paratuberculosis control on midsize dairy herds in the US. The results suggested that test-and-cull strategies alone do not reduce the prevalence of paratuberculosis in cattle and are costly for producers to pursue. Vaccination did not reduce the prevalence but was economically attractive. Finally, improved calf-hygiene strategies were found to be critically important and economically attractive in every paratuberculosis control program.

To quote some examples, The Netherlands started a national control program in 1998 based on repeated herd fecal cultures combined with sanitary and zootechnical management methods. France started a control program combining culture of feces and management strategies and Australia and some states of USA have started voluntary control programs, based on various

diagnostic methods (bacteriological and serological) supplemented with management recommendations to prevent the further spread of MAP within and between herds (Benedictus and Khalis, 2003). The voluntary program in Australia, modified in 2003, protects the status of non-infected herds and regions, reducing the social, economic and trade impact of JD at herd, regional and national levels. This program has been based on the introduction of a herd scoring system related to the risk of JD in a herd, thus facilitating trade of dairy cattle in a less regulated environment and providing a pathway for herds to progress with JD control.

In general, control programs have relied on management techniques to identify infected herds and then clear those herds of infection, and results of vaccination strategies have been controversial (Khalis et al., 2001; Muskens et al., 2002). In addition, because of the long subclinical phase and the limited sensitivity of diagnostics, eradication programs require a long term commitment (National Research Council, 2003). In New Zealand voluntary vaccination is practiced in infected herds. However, although vaccination reduces the incidence of clinical disease in cattle, it does not prevent infection and transmission (Benedictus and Khalis, 2003). Israel started a voluntary control program in 2003 aimed at detecting infected herds and providing management approaches for the reduction or prevention of herd infection. The program is based on ELISA testing and fecal culture of positive cows together with management practices focused on maternity hygiene, colostrum use, culling of fecal shedders, and categorization of herds according to their infectious status (Koren et al., 2005).

Many attempts have been made in the past decade to establish a nationwide management program in the US. At present, there is a national program designed as a model for improving the equivalency of state control and herd certification programs. The program is voluntary, so

producer incentive for participation relies on the potential for the added market value associated with products from known-status herds.

In April 2002, the US Department of Agriculture, Veterinary Services section, published the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program. This program recommended an advisory committee in each state to assist the state veterinarian in establishing and operating a JD program.

The structure of the program has 3 parts. Part 1 is education of the producers, part 2 is an assessment of on-farm risk and herd management plans, and part 3 involves herd testing and classification into 4 levels. Testing in the initial stage is done by ELISA on 30 randomly selected animals 36 month of age or older. Program levels are reached by successive testing of statistical subsets of second- or higher- lactation animals within a specific time frame (National Research Council, 2003)

Justification

Bovine paratuberculosis causes serious economic losses to the cattle industry worldwide and could become an important threat to international commerce.

From the point of view of public health, numerous studies have suggested an association between MAP and Crohn's disease in humans (Chiodini, 1989; Sechi et al., 2005; Shanahan and O'Mahony, 2005). However, presently, there is insufficient evidence to prove or disprove that MAP is the cause of even some of the cases of Crohn's disease in humans (National Research Council, 2003; Sartor, 2005). An additional concern is the fact that MAP is becoming more widespread in the environment and in the food chain.

Finally, the fact that MAP host range includes ruminant and non ruminant wildlife (Greig et al., 1999) raises the concern that the spread of the infection could alter wildlife populations,

and, if wildlife reservoirs become established, it could limit the ability to control or eradicate JD in domesticated livestock.

Management practices

Control principles are divided into three categories. First, management practices preventing or reducing the likelihood of highly susceptible newborn and young animals from ingesting manure from infected animals. Second, reducing infections by colostrum and milk management, and third, reducing farm contamination with MAP by management of infected animals. Test-and-cull strategies are not likely, by themselves, to be effective in herd MAP control, and hygiene and management practices should be included.

According to the previous statements, most control measures fall into one of three referred categories of MAP control (National Research Council, 2003); protection of calves, management of milk and colostrum, and reduction of MAP load in the farm.

The protection of young stock from older animals and from feces-contaminated feed and water considers the following measures:

- Cleaning and disinfection of maternity and calf pens after each use.
- Maintaining dedicated, clean, and dry maternity pens.
- Removal of calves immediately after birth to clean, dry calf pens, stalls, or hutches.
- Raising calves separate from the adult herd for at least the first year of life.
- Use of separate equipment for handling feed and manure.
- Design and maintenance of feed-bunks and waterers to minimize risk of contamination with manure.
- Applying manure from the adult herd only to cropland or to pasture grazed by adult stock.
- Not allowing shared feed or water between adults and young stock; nor offering feed refusals from adult cattle to young stock.
- Avoiding vehicular and human traffic from adult animal areas to young stock areas.

- Testing and managing test-positive cows at dry-off, before introduction to the maternity pen.

The reduction of infections by colostrum and milk management includes the following points:

- Feeding colostrum only from test-negative cows.
- Not colostrum pooling.
- After colostrum feeding, use of pasteurized milk or milk replacer.

Finally, the reduction of total farm exposure to the organism is based on:

- Immediately culling of all animals with clinical signs of JD.
- Cull culture-positive animals as soon as possible; for cows with low or moderate fecal culture colony counts, removal at the end of lactation may be acceptable.
- Test adult cattle at least annually by serum or fecal tests; positive serum test results should be confirmed by fecal culture.
- Consider calves from test-positive cows to be high risk individuals for later developing the disease, and consider culling recent offspring of test-positive cows.
- Purchase replacement animals from test-negative herds.

Testing and diagnostics in control programs

The control and eradication of JD is severely impaired by imperfect diagnostic tests, prolonged incubation time, the presence of undetected subclinical cases, and the lack of knowledge of strain diversity (Kudahl et al., 2007; Motiwala et al., 2003).

Tests for detection of antibodies to MAP, such as ELISA present the major disadvantage of moderate to low sensitivity, and the usefulness of serologic tests is compromised by the variability of the immune response, depending on the stage of disease (Collins et al., 2006). The ELISA test infrequently detects infected animals less than 2 years of age and frequently fails to detect individuals in the early phases of infection (Juste et al., 2005). Low agreement between results from different commercially available ELISA kits is another drawback of this test

(McKenna et al., 2006). However, ELISA testing of sera is still the most common method used in epidemiological studies and herd-based diagnosis (Bottcher and Gangl, 2004).

Tests based on the detection of the agent, likewise, present the problem of a low sensitivity, and it has been estimated that fecal culture detects only about 50% of cattle infected with MAP (Stabel, 1997). New methods detecting specific bacterial DNA sequences have allowed a more rapid identification of MAP. Polymerase chain reaction (PCR) tests based on the insertion element IS*900* have been widely used for MAP identification (Harris and Barleta, 2001).

A combination of independent tests is a common method to improve reliability of laboratory diagnostic tools. As a result of the limitations of MAP diagnosis, such strategies have already been implemented by using a combination of bacterial fecal culture and PCR or serological screening and bacterial fecal culture (Collins et al., 2006). Moreover, a combination of tests with different sensitivities and specificities allows a better classification of animals and herds relative to the probability of MAP infection (Bottcher and Gangl, 2004).

Delayed detection of infected cows was investigated by Nielsen and Ersbøll (2006). They analyzed the age at which cows tested positive by ELISA and fecal culture (FC) by use of time-to-event analyses. Repeated ELISA testing detected 98 and 95% of cows classified as high and low shedders, respectively, suggesting that most infected cows develop antibodies. Among the high shedders, 50% were positive before 4.3 yr of age. Repeated FC detected only 72% of the cows that were ELISA-positive, and 50% of the ELISA-positive cows were detected by FC at 7.6 years of age. The highest probability of testing positive by ELISA was from 2.5 to 4.5 yr of age, and the highest probability of testing positive by FC was from 2.5 to 5.5 yr of age.

For control programs, and from and epidemiological stand point, it is important to make the distinction between test performance at the individual animal level and test performance at the herd level. The ELISA is a valuable screening test for control programs. Although the test has relatively low sensitivity at the individual animal level, it exhibits fairly good sensitivity at the herd level. It also has significant advantages over fecal culture for screening, which is important in large-scale control programs. These advantages include relatively low cost, simplicity, and rapid results (National Research Council, 2003).

On the other hand, recently, pooled fecal culture from several animals within a herd has been suggested as a cost effective screening tool (Kalis et al., 2004). Strategic pooling of fecal samples would increase diagnostic sensitivity and would be valuable when a herd is suspected to be negative.

Vaccination

Vaccination against paratuberculosis was first described in 1926, at which time live vaccines were used. Conventional veterinary vaccines against MAP have generally comprised killed organisms in oil injected subcutaneously in young animals. Field vaccination is effective in decreasing the incidence of clinical disease and attenuating pre-existing infection, irrespective of whether live or killed vaccines are used (Khalis et al., 2001).

The currently available vaccines consist of a range of variations of whole bacterins with adjuvants and have shown a variable efficacy in field studies. Current vaccine prevents the occurrence of the clinical stage of the disease to a high degree, thereby limiting a substantial amount of the direct economical damage (Koets et al. 2006). In cattle, however, the vaccine does not prevent infection and subclinically infected animals shed bacteria in their feces intermittently. Another major drawback of the whole bacterin vaccines is the interference with tuberculosis and paratuberculosis diagnostics; about half of the animals receiving whole killed

MAP vaccines become false positive using the conventional tuberculin skin test diagnostic for bovine tuberculosis (Khalis et al., 2001; Muskens et al., 2002). A third weakness is that some vaccines cause substantial local tissue reaction, resulting in prolonged swelling and granuloma formation at the site of injection (Huntley et al., 2005; Koets et al. 2006).

Koets et al. (2006) reported the use of a recombinant MAP Hsp70 as a subunit vaccine in cattle experimentally infected with MAP. In previous studies, this subunit vaccine has been shown to produce a cell mediated immune response. The results of the study showed that recombinant MAP Hsp70 significantly reduced shedding of bacteria in feces during the first 2 years following experimental infection.

Vaccination is available on a limited basis in the US. The vaccine that has been in use in US is an oil suspension of killed Strain-18 organisms, a closely related strain of *M. avium*. The efficacy of vaccination has been questioned, and the current consensus is that vaccination may reduce the incidence of clinical disease, and to a lesser extent the prevalence of infection, but vaccinates are not fully protected from infection (Uzonna et al., 2003).

In a study by Uzonna et al. (2003), vaccination induced a persistent serologic, non-protective humoral response in 90% of animals within 6 months. The poor success of vaccination might be related to the inability of the vaccine to induce a protective Th1 response, mediating resistance against the disease, instead of an apparent induction of cell-mediated immunity as measured by intradermal testing, lymphocyte proliferation and cytokine assays.

A study from Spangler et al. (1991) documented the effect of calf vaccination for MAP on a serologic ELISA. Fifteen calves vaccinated with a killed paratuberculosis vaccine and 5 unvaccinated control calves were tested by serum ELISA from the first through the fifteenth month of life. All calves were ELISA-negative prior to vaccination. Thirteen of 15 vaccinated

calves became ELISA-positive between 2 and 6 months after vaccination indicating that the use of vaccine may interfere with diagnosis of paratuberculosis and with control programs based on serologic tests.

Kalis et al. (2001) analyzed whether vaccination with a killed vaccine prevented fecal shedding of MAP, and compared effectiveness of a culture and cull program in vaccinated and non-vaccinated herds, on 58 commercial Dutch dairy herds. Differences were not detected among the 25 herds that were vaccinated; culture results were positive for MAP in 4.4% of herds. In 29 herds that had not been vaccinated, culture results were positive in 6.7%. The authors concluded that vaccination of calves with a killed vaccine did not prevent transmission of MAP.

In another study (Kohler et al., 2001), after immunization of four calves with a live modified MAP vaccine (Neoparasec, Rhone-Merieux, Lyon, France), the humoral and cell-mediated immune reactions were studied during 2-years. The possibility of shedding of the vaccine strain and the influence of the vaccination on the tuberculin skin test were determined. A cell-mediated immune reaction developed much earlier than humoral immunity, with a transient increase in antibody titers. Cell-mediated immunity remained detectable until the end of the study period. Fecal shedding of the vaccine strain was not detected. Positive or inconclusive skin reactions against a *M. bovis* PPD reflected the possible interference with diagnosis of bovine tuberculosis.

In the past decades, vaccination against paratuberculosis in cattle was performed in The Netherlands only on a limited scale (Muskens et al., 2002); vaccination was restricted to herds with a high prevalence of clinical cases of paratuberculosis. This author reported a study designed to evaluate the immune response resulting from vaccination with a heat-killed

paratuberculosis vaccine. Over a period of 12–14 years, data showed a marked and prolonged effect of the vaccination on both cellular and humoral immune responses. It is concluded that a long lasting interference is to be expected with the available immunodiagnostic methods for both bovine tuberculosis and paratuberculosis.

DNA vaccines can offer an alternative approach that may be safer and elicit more protective responses. A genomic DNA expression library was generated and subdivided into pools of clones to determine DNA vaccine efficacy by immunizing mice via gene gun delivery and challenging them with live, virulent MAP. Four clone pools resulted in a significant reduction in the amount of MAP recovered from mouse tissues compared to mice immunized with other clone pools and non-vaccinated, infected control mice. Comparison of the protective clone array sequences implicated 26 antigens that may be responsible for protection in mice (Huntley et al., 2005).

Treatment

Treatment for paratuberculosis is rarely indicated; however, it may be considered for animals of genetic value or companion animals. St-Jean and Jernigan (1991) presented in a review some antibiotics for the treatment of paratuberculosis including isoniazid, rifampin, streptomycin, amikacin, clofazimine, and dapsone. They reported that treatment of paratuberculosis requires daily medication for extended periods and results in palliation of the disease rather than a definitive cure. The recommended treatment for paratuberculosis is based on isoniazid, rifampin, and an aminoglycoside.

Monensin sodium is a polyether ionophore with a broad spectrum of antimicrobial activity that includes several gram-positive bacteria. Hendrick et al. (2006) studied the role of monensin sodium in protecting cows from being milk-ELISA positive for paratuberculosis in Ontario, Canada dairy herds. In total, 4,933 dairy cows from 94 herds were enrolled in a cross-sectional

study. Composite milk samples were collected from all lactating cows and tested with a milk-ELISA for antibodies to MAP. In 48 herds in which paratuberculosis had not been diagnosed previously, the use of calf hutches and monensin in milking cows were both associated with reduced odds of a cow testing positive (OR=0.19 and 0.21, respectively). In 46 herds with a prior history of paratuberculosis, feeding monensin to the breeding-age heifers was associated with decreased odds of a cow testing positive (OR=0.54).

In another report, Hendrick et al. (2006a) enrolled 228 cows from 13 herds into a randomized clinical trial. Fecal culture and PCR were used to identify 114 cows as potential fecal shedders, while 114 cows were enrolled as ELISA negative, herd and parity matched controls. Cows received either a monensin controlled release capsule or a placebo capsule. Serial fecal culture and serum ELISA was performed over a 98-day period. On day 98 of the study, treatments were switched and cows were followed for another 98 days with a similar sampling protocol. During the first 98 days of the study, cows treated with a monensin were found to shed 3.4 CFU per tube less than placebo treated cows ($p = 0.05$). Treatment with monensin did not reduce the odds of testing positive on serology, and only cows shedding MAP on day 0 were found to have a reduced odds of testing positive on fecal culture when treated with monensin (OR=0.27; $p = 0.03$).

In another study, Brumbaugh et al. (2000) analyzed histopathological findings on 19 adult cows naturally infected with paratuberculosis. Thirteen cows were treated with monensin sodium and six remained untreated. Monensin had a beneficial effect in the ileum ($p = 0.07$), liver ($p = 0.03$) and rectal mucosa ($p = 0.05$), but not in mesenteric lymph nodes ($p = 0.35$).

Productive and Economic Impact of Johne's Disease

Paratuberculosis contributes both to direct and indirect losses in the cattle industry due to reduced milk production, premature culling, additional losses from higher cow replacement costs

and lower cull cow revenues (Bennett et al., 1999, National Research Council, 2003). The disease also involves losses due to potential limitations in domestic and international trade (National Research Council, 2003). In an early work, Buergelt and Duncan (1978) analyzed age and milk production data from Holstein cows reporting a significantly shorter life expectancy and reduced milk production of MAP infected cows when compared with non-infected herd mates. Benedictus et al., in 1987, reported a decrease in milk production of 19.5% compared with the lactation two years before culling of animals showing clinical signs of paratuberculosis. The reduction in production was 5% when compared to the previous lactation.

In another study, Collins and Nordlund (1991) compared the milk production (mature equivalent at 305 days, ME305) for ELISA positive cows with their test negative herdmates, with 5.36% less production for infected cows. This effect was significant in lactation number three or greater. Test positive cows produced less protein ME305 and fat ME305. The net economic effect on productivity of cows increased with each lactation reaching over $200/test positive cow by lactation number three.

Nordlund et al. (1996), in a cross-sectional epidemiologic survey in 23 dairy herds in Wisconsin found that ELISA-positive cows had a ME milk production of 376 kg/lactation less than that for ELISA-negative herdmates. However, significant difference was not found in lactation average percentages of fat and protein, or somatic cell count (SCC) linear score. Subclinical paratuberculosis infections were associated with a 4% reduction in milk yield.

In a study by Baptista et al. (2008), the association between the presence of antibodies to MAP and SCC was analyzed. A causal relationship between high SCC and antibodies to MAP was not found, but the results suggested a strong association and a potentially increased risk of MAP transmission when milk with high SCC is fed to calves.

Vanleeuwen et al. (2001) estimated the impact of subclinical infection in dairy cattle in 90 randomly selected herds in Canada. Milk production for ELISA-seropositive cows was lower than that for seronegative cows; in their 1st and 5th lactations, ELISA-seropositive animals produced 573 and 1273 kg less than seronegative cows, respectively.

Gonda et al. (2007) estimated the effect of MAP infection on milk, fat, and protein yield deviations, pregnancy rate, lactation somatic cell score, and projected total months in milk (productive life). A serum ELISA and fecal culture for MAP were performed on 4,375 Holsteins in 232 DHIA herds throughout the US. Infected cows (ELISA or fecal culture positive) produced 303.9 kg less milk/lactation, 11.46 kg less fat/lactation, and 9.49 kg less protein/lactation ($p \leq 0.003$) and had higher pregnancy rates (1.39% greater, $p = 0.03$) and lower productive life (2.85 months less, $p \leq 0.0001$). Somatic cell score was not affected. The fecal culture-positive population of cows had larger effects on all traits than ELISA-positive population of cows.

In a longitudinal study Lombard et al. (2005) determined the effects on production and risk of removal related to MAP infection at the individual animal level (serum ELISA) in dairy cattle. A total of 7,879 dairy cows from 38 herds in 16 states were analyzed. Cows with strong positive results had ME305 milk production, ME305 maximum milk production, and total lifetime milk production that were significantly lower than cows in other categories. No differences were observed for ME305-day fat and protein percentages, age, lactation, and lactation mean linear somatic cell count score between cows with strong positive results and those with negative results. After accounting for lactation number and relative herd-level milk production, cows with strong positive results were significantly more likely to have been removed by 1 year after testing.

Alternatively, some studies (Johnson-Ifearulundu et al., 2001) also reported non significant differences in production between infected and non-infected cows. These could be reflected in a difference of diagnostic tests used, average lactation number for the herds under study, or an effect of average lactation of the herds under analysis.

Johnson-Ifearulundu et al. (2001) measured the effect of subclinical infection on ME milk, protein, and fat production in a sample of Michigan dairy herds. Subclinical paratuberculosis test-positive status (fecal culture) had no statistically significant effect on ME milk, fat, or protein production. This is in agreement with results from Hendrick et al. (2005) where no difference in 305-day milk or fat production was detected in cows with positive results of serum ELISA, compared with seronegative cows.

Tiwari et al. (2005) reported that for cows culled for all reasons in four Canada provinces, MAP-seropositive cows had a 1.38 (1.05-1.81, 95% CI) times increased hazard of being culled compared to MAP-seronegative cows. Among cows that were culled because of either decreased reproductive efficiency or decreased milk production or mastitis, MAP-seropositive cows were associated with 1.55 (1.12-2.15, 95% CI) times increased hazard compared to MAP-seronegative cows.

In a review by McKenna et al. (2006a) it was stated that there was a 2.4 times increase in the risk of their being culled for cattle positive by ELISA, with a decrease in ME305 milk production by at least 370 kg. Host level factors included age, level of exposure and source of exposure, such as manure, colostrum, or milk. Agent factors involved the dose of infectious agent and strains of bacteria.

An epidemiological study in Ontario, Canada, based on 304 dairy herds analyzed the association between production and MAP serological status (lipoarabinomannan

enzyme-immunoassay). MAP positive status was associated with higher somatic cell counts at herd and individual levels, but no association was found with calving interval and milk production (McNab et al., 1991).

In another study, the effect of MAP infection on the shape of lactation curves was reported (Kudahl et al., 2004). Milk samples from 6,955 cows in 108 Danish dairy herds were tested with ELISA. The lactation curves after peak yield were significantly less persistent in young infected cows, where an increase of one standardized optical density (OD) unit was associated with a depression of the milk yield per day of 3.7 kg of fat corrected milk in first parity and 2.7 kg in second parity. In third and older parities, the model indicated exponentially increased losses with increased ODs. This study showed significant correlations between antibody response to MAP in milk and milk production, and it links infection to poor persistency and considerable milk loss.

Johnson-Ifearulundu et al. (2000), based on a prospective cohort study design, evaluated the impact of subclinical MAP infection on days open in dairies in Michigan. ELISA-positive cows had a 28-day increase in days open when compared to ELISA-negative cows. The authors concluded that reduced estrus expression or an increased post-partum anestrous period would occur in the subclinically infected ELISA-positive animals, probably due to a negative energy balance associated with MAP infection.

Hendrick et al. (2005) determined the effect of paratuberculosis on culling, milk production, and milk quality in infected dairy herds using a cross-sectional design. Results showed that cows positive for bacteriologic culture of feces and milk ELISA produced less milk, fat, and protein, compared with herd mates with negative results. The survival analyses indicated that cows with positive results of each test were at higher risk of being culled than cows with

negative results. Paratuberculosis status was not associated with milk somatic cell count linear score.

A study by Raizman et al. (2007) evaluated the lactation performance of cows shedding MAP in feces before calving and of cows culled with clinical signs consistent with JD during the subsequent lactation. Fecal culture was performed in 1,052 cows before calving. Signs of clinical disease (milk fever, retained placenta, metritis, ketosis, displaced abomasum, lameness, mastitis, pneumonia, and JD), and production and reproduction data were recorded for each cow. In 8% of cows fecal samples were positive for MAP. In multivariable analysis, light, moderate, and heavy fecal shedding cows produced on average 537, 1,403, and 1,534kg, respectively, less milk per lactation than fecal negative cows. Fecal culture positive cows were less likely to be bred and conceive. In the multivariable analysis the 56 cows culled with presumed JD produced approximately 1,500kg/lactation or 5kg/day less than all other cows.

Diverse estimations of the economic losses due to paratuberculosis have been presented by different authors.

Chiodini et al. (1984) estimated that JD produced an annual loss in New England of $15.4 million and cost the Wisconsin dairy industry $54 million per year. The cost suffered by chronically infected herds would reach an annual economic loss of $75-100 per adult animal. Braun et al. (1990), based on the prevalence of infection and extrapolating data from previous studies, calculated a net loss due to JD of $9 million annually in Florida. In another study, Stabel (1998) estimated that the economic impact of paratuberculosis on the US national cattle industry was over $1.5 billion per year.

In a study by Losinger (2005) the analysis of the economic impacts of JD indicated that reduced milk production, associated with the determination of dairy operations as JD-positive,

reduced consumer surplus by $770 million ± $690 million, and resulted in a total loss of $200 million ±$160 million to the US economy in 1996.

The USDA National Animal Health Monitoring System's (NAHMS) 1996 national dairy study analyzed the impact of paratuberculosis on herd productivity and economy in US dairy herds. Positive herds experienced a loss of almost $100 per cow when compared to JD-negative herds due to reduced milk production and increased cow-replacement costs (Ott et al., 1999). Herds reporting at least 10% of their cull cows as having clinical signs consistent with JD, had losses over $200 per cow. These herds experienced reduced milk production of 700 kg per cow, culled more cows with lower cull-cow revenues, and had greater cow mortality than JD-negative herds. Averaged across all herds, JD costs the US dairy industry, in reduced productivity, $22 to $27 per cow or $200 to $250 million annually.

Economic losses attributed to paratuberculosis in herds with a disease control program were estimated by Groenendaal and Galligan (2003) by use of the simulation model. Mean loss increased considerably from $35/cow/y in year 1 to > $72/cow/y in year 20. Lower milk production accounted for 11% of the total loss attributable to paratuberculosis, and 12% of the loss resulted from a lower slaughter value of culled infected cattle and treatment costs of clinically affected cows. Finally, most of the loss (77%) attributable to paratuberculosis was categorized as loss of future income as a result of suboptimal culling.

One study in the Maritime Provinces of Canada (Chi et al., 2002) estimated an annual cost due to paratuberculosis for an average, infected, 50 cow herd of $2,472. This estimation considers direct production losses and treatment costs.

Elzo et al. (2006) evaluated cow and calf genetic and environmental factors for their association with ELISA scores for paratuberculosis in a multi-breed population of beef cattle.

Regressions indicated that poorer maintenance of cow weights was associated with higher ELISA scores. The data also indicated that cows with greater ELISA scores tended to produce lighter calves at birth and/or calves with slower pre-weaning growth. These results suggest that subclinical paratuberculosis may be negatively affecting cows and their offspring.

Crohn's Disease

The Disease

Inflammatory bowel disease (IBD) comprises a group of chronic, relapsing, idiopathic, inflammatory illnesses of the gastrointestinal tract usually presenting as Crohn's disease (CD) or ulcerative colitis (UC), which predominantly affect the colon (CD and UC) and /or the distal small intestine (CD) in either a superficial (UC) or transmural (CD) manner (Blumberg et al., 1999). Both disease entities primarily affect young adults and are often accompanied by extra-intestinal manifestations such as arthritis, uveitis or primary sclerosing cholangitis, as well as associated illnesses (e.g. osteoporosis or secondary colon carcinoma) (Hoffmann et al., 2002-2003).

CD was recognized as a distinct entity 75 years ago (Economou and Pappas, 2007) and, although CD and UC share many clinical and pathological characteristics, they also have some different features suggesting that the main pathological processes in these two diseases are distinct (Bouma and Stober, 2003). Epidemiological and clinical observations point toward a multi-factorial model, where clinical disease is trigged by the association of multiple elements involving genetic, immune-related, environmental, and infectious factors (Bouma et al., 1997; Selby, 2000; Gazouli et al., 2005; Trinh and Rioux, 2005; Economou and Pappas, 2007). However, one of the hallmarks of the disease is the activation of nuclear factor kappa B (NF-kB) that drives the increased expression of pro-inflammatory cytokines (Schreiber, 2005).

Crohn's disease is a chronic, relapsing inflammatory condition affecting any part of the human gastrointestinal tract, with the distal ileum most commonly involved. It is characterized by transmural inflammation with deep ulceration, thickening of the bowel wall and fistula formation, and non-caseating granuloma. Clinical presentation depends upon the site of the inflammation, and it includes general malaise, chronic weight loss, abdominal pain, and diarrhea. Extraintestinal manifestations develop in up to 25% of patients and perianal disease is also frequent (Selbi, 2000; Sechi et al., 2005).

Uzoigwe et al. (2007) reviewed some aspects of CD epidemiology. The disease occurs throughout the world, it is most prevalent in Europe and North America. It exhibits a prevalence of 161–319 cases/100,000 people in Canada and affects between 400,000 and 600,000 people in North America alone. Prevalence estimates for Northern Europe have ranged from 27-48/100,000, with around 13 people per 100,000 reported for the population in the UK (Sechi et al., 2005, Uzoigwe et al., 2007).

The incidence of CD in North America has been estimated at 6/100,000 per year, and is thought to be similar in Europe, but lower in Asia and Africa. The incidence of CD in industrialized parts of the world has been reported to be increasing, and the disorder occurs most frequently among people of European origin, and has been reported to be 2-4 times more common among those of Jewish descent than among non-Jews. The disease appears in individuals of any age, but commencement between 15 and 30 years of age is more common, and it can also occur in early childhood or later in life. (Sugimura et al., 2003; Sechi et al., 2005; Uzoigwe et al., 2007).

The course of clinical disease is chronic and intermittent and treatment includes anti-diarrheal and anti-inflammatory agents to treat symptoms, immunosuppressive drugs aimed at disease remission, and surgery (Sechi et al., 2005).

Etiology

The precise causes of CD remain unknown. Hypotheses include an aberrant or autoimmune host inflammatory response to undefined antigens, infectious etiology, including MAP, and aberrant immune response to a specific infectious agent, but consensus has not been achieved (National Research Council, 2003).

Other causes of CD have been proposed including chronic ischemia and micro-infarction, persistent measles infection, chronic viral infection, infection with pathogenic *E. coli*, abnormal response to a dietary component, and abnormal inflammatory response to normal intestinal micro flora, or components of the flora, in genetically predisposed individuals (National Research Council, 2003). Various polymorphisms of a human gene, caspase recruitment domain 15 gene (*CARD15*, former *NOD2*), that confers increased susceptibility to CD, have been reported, and the role of this gene, which may function as an apoptosis regulator, is currently unclear.

Crohn's Disease and *CARD15/NOD2* Gene

It has long been suspected that certain individuals may be genetically predisposed to developing CD (Newman and Siminovitch, 2005; Grant, 2005; King et al., 2006). As early as 1934, CD was recognized as a familial disorder. This observation was further confirmed by many groups (Hugot, 2006), with a proportion of familial aggregations of 8% to 10% on average.

In a review by Tysk (1998), population based studies were presented showing that the relative risk of IBD is increased 10–20 times in first-degree relatives of the proband with CD, with the highest risk in siblings.

Research has confirmed that as many as 50% of monozygotic twins are affected by CD whereas the dizygotic-twin concordance is not significantly different from that for all siblings. Reported concordance rates for ulcerative colitis are seen in approximately 6-17% and 0-5% for monozygotic and dizygotic twins, respectively (Tysk, 1998; Bouma and Strober, 2003; Lakatos et al., 2006). A high heritability index close to 1.0 was presented for CD, and this estimation remained high after correction for shared environmental factors (Tysk, 1998).

Linkage studies have revealed a number of putative IBD-susceptibility loci, suggesting that several genes are involved in predisposition to IBD.

In 2001, three research groups independently reported an association between mutations in a gene on chromosome 16, (*CARD15/NOD2* gene) and CD (Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001). Mutations increasing susceptibility to CD up to 40 times were mapped to this locus (Maeda et al., 2005). A recent meta-analysis analyzed the disease risk associated to *CARD15/NOD2* mutations providing odds ratios for CD in mutation carriers equal to 2.2 (95% CI: 1.84-2.62), 2.99 (95% CI: 2.38-3.74), and 4.09 (95% CI: 3.23-5.18) for the three main mutations R702W, G908R, and 1007fs. In addition, the odds ratio for double mutants was estimated to be 17.1 (95% CI: 10.7-27.2, Economou et al., 2004).

In European populations, having one copy of the risk alleles confers a 2-4-fold risk for developing CD, whereas double-dose carriage increases the risk 20-40-fold. Carriage of *CARD15/NOD2* risk alleles is associated with ileal location, earlier disease onset, and structuring phenotype (Bonen and Cho, 2003).

The product of *CARD15/NOD2* gene is an intracellular element responsible for the indirect recognition of bacterial peptidoglycan through the binding of muramyl dipeptide, a component of both Gram negative and positive bacterial cell walls in monocytes, macrophages and dendritic

cells, where it is mainly expressed (Ogura et al., 2001a; Maeda et al., 2005). The protein is a member of the Ced4-APAF1 protein super family and is expressed in cells such as monocytes, dendritic cells, Paneth cells and intestinal epithelial cells. Structurally, CARD15/NOD2 is composed of three segments: the first being composed of two NH2-terminal caspase recruitment domains (CARD units), the central portion consisting of nucleotide-binding domain and finally, a leucine-rich repeat (LRR) region as is found in toll like receptors (Hugot, 2006; Lakatos et al., 2006).

The binding of CARD15/NOD2 to this bacterial motif causes its binding to a second CARD15/NOD2 molecule, thus forming a dimmer. Further interaction with other cytosolic proteins leads to the ultimate activation of NF-kB, eliciting pro-inflammatory reactions (Ogura et al., 2001a). NF-kB is nuclear transcription factor that regulates expression of a large number of genes that are critical in ruling apoptosis, viral replication, tumor genesis, inflammation, and autoimmune diseases. It is still unclear whether NF-kB expression is elevated or depressed in CD due to conflicting observations and studies. *In vitro* experiments demonstrated that the declining activity of this protein indicates a loss-of-function effect (Lakatos et al., 2006).

The LRRs are involved in the interaction with infecting bacterial lipopolysaccharides (LPS) and peptidoglycan, whereas the CARDs enable the protein to induce apoptosis and the NF-kB signaling pathways (Lesage et al., 2002). *NOD2* variations identified so far are evenly distributed along the entire coding sequence except in its 5' portion encoding the first CARD domain.

The three main mutations of *NOD2* (R702W, G908R and L1007fsinsC), including a frame shift mutation encoding a truncated protein, occur in the LRR domain or in its vicinity, suggesting that they alter the recognition of the bacterial LPS. This hypothesis has been

supported by functional experiments that have demonstrated that the 1007fs mutation decreased the NF-kB activation by the LPS (Lesage et al., 2002; Bonen et al., 2003). Two other members of the NOD-LRR family have been experimentally demonstrated to serve a role in resistance to bacterial pathogens: NOD1 for *Helicobacter pylori* and Naip5/Birc1e for *Legionella pneumophila* (Behr and Schurr, 2006). This theory, also, would support the controversial role of MAP infection in patients with CD.

Evidence suggests that the CD-associated mutations result in a loss of functional phenotype (Behrn and Schurr, 2006). However, variant CARD15/NOD2 proteins apparently present inflammation-promoting functions. Two main hypotheses provide an explanation for this apparently contradictory point. The first advocates that mutant CARD15/NOD2 is defective in performing critical functions required for limiting inflammation (loss-of-function). The second proposes that the variant proteins directly activate pro-inflammatory signaling pathways (gain-of-function). The hypotheses are not contradictory and may be a valid combination (Zelinkova et al., 2005).

Cells of healthy persons and CD patients harboring mutant *CARD15/NOD2* alleles do not respond to muramyl dipeptide ex vivo, pointing to a loss-of-function phenotype even in people without disease. Therefore, an important etiological consideration is whether only a subset of persons with susceptible alleles experience specific microbial exposures or whether additional compensatory mechanisms are ineffective in these patients (Behrn and Schurr, 2006).

An estimation of the proportion of cases of CD that could be attributed to *CARD15/NOD2* mutations has been proposed at 15–30%, leaving space for a number of other factors in the pathogenesis of CD. The association of gene polymorphisms to an increased susceptibility to develop disease does not preclude the possibility that the disease may be infectious in etiology,

and CD could result from bacterial insult in genetically susceptible individuals (Van Heel et al., 2001; Newman and Siminvitch, 2003).

In three works (Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001) it was determined that the *CARD15/NOD2* mutant (L1007fsinsC) found in CD patients was inefficient in killing bacteria, when compared with wild-type *CARD15/NOD2*, supporting the proposed link between bacterial detection and bacterial killing. Taken together studies on *CARD15/NOD2* provide a conceptual link between CD and bacterial sensing.

Some works provide more evidence of the link between bacterial infection, gene polymorphisms, and CD. Sechi et al. (2005a) analyzed the proportion of people in Sardinia with or without CD that were infected with MAP and had allelic variants of *CARD15/NOD2*. The results showed that more than 70% of CD affected individuals carried at least one of the *CARD15/NOD2* alleles associated with susceptibility and were also infected with MAP.

In one study (Heresbach et al., 2004) the association of *CARD15/NOD2* mutations with CD in different subsets of CD phenotypes was studied. Carriers of at least one *CARD15/NOD2* variant were significantly more frequent in CD than in controls, and were significantly associated with ileal involvement, and structuring evolution. Granuloma formation was found to be associated with the mutant R702W allele.

Mycobacterium Paratuberculosis and Crohn's Disease

In the early part of the 20th century, the similarities between this human intestinal disease and JD in cattle were identified. JD in cattle shares some similarities with human CD as diarrhea, wasting, and a predilection for the ileum (Chiodini, 1989; Bernstein et al., 2004).

CD and JD have been compared clinically and pathologically, but the similarity of the two diseases has been exaggerated in some cases. Some differences include an extra-intestinal manifestation in CD, but not in JD, and macroscopic features such as fistulas and pseudo polyps

in CD. Similarities and differences have been interpreted by experts both in favor of and in opposition to the view that MAP is a cause of CD (Selby, 2000; National Research Council, 2003).

Chiodini et al. (1984a) reported a previously unrecognized *Mycobacterium* species isolated from two patients with CD. The organism was an acid-fast, mycobactin-dependent *Mycobacterium* with unique particularities. The bacterium was pathogenic for mice, and a goat inoculated orally developed both humoral and cell-mediated immunologic responses and granulomatous disease of the distal small intestine, with noncaseating, tuberculoid granulomas. These findings raised the possibility that a *Mycobacterium* could play an etiologic role in at least some cases of CD. Few years later MAP was isolated for the first time from a CD patient (Chiodini, 1989).

Shanahan and O'Mahony (2005) suggested supporting observations of a causal link between MAP and CD. They presented *Helicobacter pylori,* as an example of an infectious agent contributing to peptic ulcer and gastric cancer. Also, genetic and patho-physiologic indication of heterogeneity of CD, suggest distinct deficiencies leading to a similar clinical manifestation. This would imply that a subset of disease might have an infectious basis. Monozygotic twin studies, with concordance rate of only about 50%, indicate an environmental contribution to the pathogenesis of CD, and the increasing incidence, particularly in developed nations is consistent with an environmental influence.

In general terms, evidence supporting a link between MAP and CD includes: clinical and pathological similarities between JD and CDs; higher detection rates of MAP by PCR and culture in gut samples from Crohn's patients compared with controls; demonstration of a serological response to MAP antigens in Crohn's patients; and anti-MAP antibiotic therapy

resulting in remission, or improvement in disease condition; presence of MAP in food chain (milk, meat) and water supplies, and detection of MAP in human breast milk by culture and PCR (Naser et al., 2000; Grant, 2005; Sartor, 2005; Bernstein et al., 2007).

In recent years, the idea of a link between both diseases has been supported by reports of MAP detected in tissues of patients with CD by culture and by molecular methods (Sechi et al., 2005). In addition, detection of MAP DNA in milk has been stated as a plausible transmission via cattle to human and raises concerns about public health safety (Shanahan and O'Mahony et al., 2005).

Detection of MAP by molecular techniques in human intestine tissue has produced variable results; the majority of studies have detected MAP DNA or cultured the bacteria in higher frequency from tissues of CD affected individuals than from controls, although the reported frequency of recovery of MAP in CD and ulcerative colitis have ranged from 0% to 100%. However, this opens the possibility that this organism may selectively colonize the ulcerated mucosa of CD patients but not initiate or perpetuate intestinal inflammation (Sartor, 2005).

Naser et al. (2004) tested for MAP by PCR and culture in buffy coat preparations from individuals with CD, with UC, and without IBD. MAP DNA in uncultured buffy coats was identified by PCR in 13 (46%) individuals with CD, four (45%) with ulcerative colitis, and three (20%) without inflammatory bowel disease. Viable MAP was cultured from the blood of 14 (50%) patients with CD, two (22%) with ulcerative colitis, and none of the individuals without inflammatory bowel disease.

In another study, Sechi et al. (2005) found that twenty-five patients (83.3%) with CD and 3 control patients (10.3%) were IS*900* PCR positive in intestinal mucosal biopsies. MAP was

cultured from 19 Crohn's patients (63.3%) and from 3 control patients (10.3%). The finding of the organism colonizing a proportion of people without CD would be consistent with what occurs in other conditions caused by a primary bacterial pathogen in susceptible hosts.

Ghadiali et al. (2004), reported two alleles found by analysis of short sequence repeats of MAP isolated from CD patients. Both of these alleles clustered with strains derived from animals with JD. Identification of a limited number of genotypes among human strains could imply the existence of human disease-associated genotypes and strain sharing with animals.

Autschbach et al. (2005) examined IS*900* in a large number of gut samples from patients with CD and UC, and in non-inflamed control tissues. IS*900* PCR detection rate was significantly higher in CD tissue samples (52%) than in UC (2%) or control (5%) specimens ($p < 0.0001$). In CD patients, IS*900* DNA was detected in samples from both diseased small bowels (47%) as well as from the colon (61%). No association between MAP specific IS*900* detection rates and clinical phenotypic characteristics in CD was established.

Collins et al. (2000) analyzed results of multiple diagnostic tests (PCR, ELISA, and IFN-g tests) for MAP in IBD patients and controls. Most assays were adaptations of diagnostic tests for this infection performed routinely on animals. The authors concluded that MAP, or other mycobacterial species, infect at least a subset of IBD patients.

Abubakar et al. (2008) presented a meta-analysis of studies using nucleic acid-based techniques to detect MAP in patients with CD compared with controls. Based on 47 studies, the pooled estimate of risk difference in the detection of MAP in CD patients compared with non-IBD controls from all studies was 0.23 using a random effects model. Similarly, MAP was detected more frequently from patients with CD compared with those with ulcerative colitis (risk difference 0.19). The data confirms that MAP is detected more frequently among CD patients

compared with controls, but the pathogenic role of this bacterium in the gut remains uncertain. The analysis suggested an association between MAP and CD, but this association remained inconclusive, and its strength and consistent detection of MAP DNA does not prove causation.

Bernstein et al. (2004) had as an objective to determine whether CD subjects were more likely to be MAP seropositive than controls in a sample from Manitoba population (Canada). Using an ELISA for serum antibodies to MAP, initially developed for cattle but adapted for human use, the rate of positive ELISA results there was no different in MAP seropositivity rate among CD patients (37.8%), UC patients (34.7%), healthy controls (33.6%), and non-affected siblings (34.1%). In the same population, another study could not find an interaction between the NOD-2 genotype and MAP serology in relationship to CD or ulcerative colitis (Bernstein et al., 2007).

Effectiveness of anti-mycobacterial drugs to control CD as a factor of association with MAP is controversial. However, disease remission following antibiotic therapy has been reported. Chamberlin et al. (2007) presented a case of a 63 years old patient tested previously positive for MAP in blood, that after antibiotic treatment showed a complete remission with accompanying negative results for MAP detection by PCR on blood. Another case of a man who had persistently active CD that was not responding to medical therapy was reported (Behr et al., 2004). Tissue from mesenteric lymph nodes was examined by IS*900* PCR and MAP DNA was detected. After anti-MAP antibiotic therapy the patient's condition markedly improved within a few months. The man was found to possess the susceptibility alleles of the *CARD15/NOD2* gene. Recently, an altered T cell function associated with the presence of MAP in CD patients was reported. Higher levels of IL-4 and IL-2 were found in these patients when compared to MAP

negative CD cases, indicating a skewed Th2 immune response and providing a new antecedent for the link between MAP detection and CD (Ren et al., 2008).

Some of the evidence against the causal association between MAP and CD is based on the information presented by different authors. CD is less common in rural areas and it is not known to be an occupational hazard of farming; Johnes et al. (2006) found no association between CD prevalence in dairy farmers and exposure to clinical cases of bovine paratuberculosis.

Sartor (2005) enumerated some other points such as: lack of epidemiological support of transmissible infection; lack of epidemiological evidence of transmission from water or milk products; no evidence of transmission to humans in contact with animals infected with MAP; genotypes of CD and bovine origin MAP isolates are not similar, and variability in detection of MAP by PCR and serological testing. Environmental conditions such as poor sanitization and overcrowding which should favor transmission of infection appears to protect against CD.

On the other hand, there is no evidence for vertical or horizontal transmission of CD, and sustained clinical responses to immunosuppressive drugs and to antitumor necrosis factor-alpha seem to be at variance with a chronic infection. Disseminated MAP in CD has not been reported associated with immunosuppression due to drugs used in therapy (Shanahan and O'Mahony, 2005).

Another point relates to the fact that detection of bacterial DNA in the granuloma of intestinal CD is not specific to MAP; other forms of bacterial DNA are also present, which could reflect disturbed host-flora interactions in patients with CD and is consistent with other observations of increased mucosal bacteria in CD (National Research Council, 2003).

A systematic review (Feller et al., 2007) presented 28 case-control studies comparing MAP in patients with CD with individuals free of inflammatory bowel disease or patients with

ulcerative colitis. The authors concluded that the analysis assessed the evidence for an association between MAP and CD. Some possible confounding and bias on some of the studies considered in the review were; different source populations for controls and cases; higher propensity of inflamed tissue to become infected with MAP, no MAP-specific test validated for human beings is available. In this meta-analysis, the pooled odds ratio of MAP presence for individuals suffering IBD from studies using PCR in tissue samples was 7.01 (95% CI 3.95-12.4) and was 1.72 (1.02–2.90) in studies using ELISA in serum. The association of MAP with CD appeared to be specific, but its role in the etiology of CD remains unclear.

Although there is indication of an association between MAP and CD, to date the evidence appears to be insufficient to either establish or refute a causal connection between JD and CD. The available scientific evidence has been reviewed by a number of expert groups in recent years. The consensus opinion, at present, is that the available information is insufficient to prove or disprove that MAP is a cause of CD, but the hypothesis is still plausible (National Research Council, 2003; Vinh and Bersrtein, 2005). The discovery of a susceptibility gene in Crohn's patients, *CARD15/NOD2*, does not preclude a role for MAP in the pathogenesis of at least some cases of CD, as the function of this gene is bacterial sensing in the gut. If MAP does contribute to the causation of CD then it may not be acting as a conventional infectious agent (Grant, 2005).

Genetics in Animal Production

Genetic Basis of Disease Resistance and Susceptibility

Host-pathogen relationships are shaped by co-evolutionary mechanisms between host defense mechanisms and pathogen genetic diversity (Detilleux, 2001). The animal genome influences susceptibility to disease, however because of the vast variety of pathogens and complex host defense mechanisms involved, the understanding of this interplay is very complex (Adams and Templeton, 1998).

Cattle show considerable variability in their response to a wide range of disease challenges, and much of the variability is genetic (Morris, 2007). The improvement and utilization of host genetic resistance to disease is an attractive option as a component of livestock disease control in a wide range of situations. The main requisites for a successful intervention are: sufficient genetic variation for disease resistance, economic and social benefits, and the option of using other complementary methods of disease control (Gibson and Bishop, 2005).

A distinction is important in the defense against pathogenic organisms. Resistance refers to the ability to limit infection and tolerance to the ability to limit the disease severity induced by a given agent. Therefore, selecting the host for resistance would reduce disease transmission but possibly impose selective pressure upon the pathogen (Råberg et al., 2007).

Resistance to pathogens based on innate immunity includes the following elements: impenetrable barriers, absence of appropriate receptors in cellular membranes, failure to survive after entrance, inability to replicate in the host, and elimination by host defense mechanisms (phagocytes). On the other hand, the resistance by adaptive immunity involves lymphocyte-mediated host responses, (cytotoxic T cells, helper T cells, and B cells), natural killer cells and macrophage-mediated phagocytosis, humoral-mediated responses (including antibodies and complement), and production and regulation by cytokines (Adams and Templeton, 1998).

Justifications for including disease resistance in a breeding program include the constraints on productivity from monetary losses, unfavorable genetic correlation between productivity and disease, increased demand by consumers for animal products of high quality from healthy animals, increased resistance to antimicrobial drugs, loss of biodiversity in naïve populations, and positive epidemiological response due to a decreased disease transmission when the

proportion of resistant animals increases in the population (Stear et al., 2001; Detilleux, 2001; Detilleux, 2002).

As presented by Morris (2007), multiple cases of a genetic effect on disease susceptibility have been previously reported in livestock including mastitis, nematode parasites, external parasites, eye diseases such as keratoconjunctivitis and squamous cell carcinoma, respiratory disorders, tuberculosis and brucellosis.

Under current animal production systems and consumer demand for healthy foods, genetic selection for better resistance to infectious disease may become an alternative or an accompanying measure to already existing prophylactic measures (Detilleux, 2001).

Genetic Component in Mycobacterial Infection

At the present, there is considerable evidence that host genome is important in determining the outcome of infection (Veazeyet al., 1995; Mallard, 1999; Bellamy, 2003; Vergne et al., 2004; Morris, 2007). Genetic factors have long been suspected of determining susceptibility and resistance to mycobacterial infection. As a comparison with a close relative of MAP, a high proportion of the world human population has been exposed to *Mycobacterium tuberculosis*, but not all individuals in contact with the bacteria become infected, and only a fraction of infected individuals develop clinical disease. Both infection and clinical tuberculosis result from interactions between the infectious agent, environmental factors, and the host. Recent population-based studies have reported associations between some candidate genes and clinical tuberculosis, but the molecular basis of the genetic control of disease progression remains unclear (Bellamy, 2003; Vergne et al., 2004).

Studies on animal models for mycobacterial infection have also found evidence that genetic factors influence disease susceptibility. In the 1940s, it was established that inbred strains

of rabbits, designated resistant and susceptible, exhibited two patterns of disease following infection with virulent *M. bovis* (Bellamy and Hill, 1998; Bellamy, 2003).

In a study by Mackintosh et al. (2000), testing the genetic resistance to experimental infection with *M. bovis* in red deer (*Cervus elaphus*), strong evidence was found for a genetic basis to resistance to tuberculosis (heritability of 0.48).

In successive studies, some candidate genes have been proposed for mycobacterial resistance. Solute Carrier 11A1 gene (*SLC11A1*, formerly *NRAMP1*) has been associated with innate resistance to *Salmonella typhimurium*, *Leishmania donovani* or *Mycobacterium bovis* BCG infection. Also a mutation was identified in the gene encoding interferon-gamma receptor type 1 (IFNGR1) as the cause for a homozygous recessive genetic disorder causing increased susceptibility to atypical mycobacterial infection (Blackwell, 2001).

The identification of families with increased susceptibility to mycobacterial infection, and the association of gene mutations (IFN-g and interleukin-12 receptor) with individuals having this condition indicate that these alterations would generate partial dysfunction of macrophage pathways (Levin and Newport, 1999).

Genetics and Paratuberculosis

Bovine paratuberculosis has largely been suspected to have a genetic component. Estimations indicate a range of moderate values for heritability to infection (Koets et al., 2000; Elzo et al., 2006; Gonda et al., 2006a). Roussel et al. (2005), working with pure breed beef cattle from 115 beef ranches in Texas, found an increased risk to be seropositive to MAP associated with the Brahman breed or *Bos indicus* cattle. Cattle from *Bos indicus*-based herds were more than 17 times as likely to be seropositive as were cattle *Bos taurus*-based herds, and cattle from interspecies-based herds were 3.6 times as likely to be seropositive as were cattle from *Bos taurus*-based herds.

In another study, Centinkaya et al. (1997) evaluating the relationship between the presence of JD and farm management factors in dairy cattle in England reported that farms on which Channel Island breeds (Jersey and Guernsey) were predominant were associated with an increased risk of reporting disease; odd ratios ranged from 10.9 to 12.9 relative to Friesian or its crosses and other breeds. It has been suggested that this susceptibility may be related to increased exposure rather than to increased susceptibility or may be confounded by some factors that play an important role in the development of clinical disease such as lower culling rate in Channel Island breeds.

Koets et al. (2000), working with Dutch dairy cattle, report an estimated heritability of susceptibility to MAP infection of 0.06 for a population composed of vaccinated and non vaccinated animals. In the subpopulation of vaccinated animals the estimated heritability was 0.09. This provides evidence for the presence of genetic variation in the susceptibility of cattle to paratuberculosis (diagnoses based on postmortem examinations at slaughter house). The estimated heritabilities for susceptibility of cattle to MAP are comparable to many other diseases traits.

In general, the genetic control of disease and resistance is polygenic, and several quantitative trait loci (QTL) will be responsible for the genetic component of variation in individual resistance to infectious disease. Candidate genes involved in QTL may be the bovine major histocompatibility antigens and genes involved in innate resistance, such as natural resistance associated with macrophage growth (*SLC11A1* gene). The *SLC11A1* gene has been shown to be linked to resistance to mycobacterial infection, including murine models of paratuberculosis (Koets et al., 2000).

Mortensen et al. (2004) estimated the genetic variation and the heritability of antibody production against MAP in a population of 11,535 Danish Holstein cows in 99 herds. The model based on antibody (IgG) levels in milk determined using an ELISA and measuring optical density (OD) values, showed a significant heritability of 0.102 and a genetic variance of 0.054. When a sire model was used, considering only the pedigree of sires of the cows rather than the entire pedigree, the estimated heritability was 0.091. This suggests that the genes influence the shift to the undesirable humoral immune response where the infection is out of control.

A study from Nielsen et al. (2002a) aimed at determining the proportion of transmission of paratuberculosis in dairy cattle attributable to the dam with emphasis on vertical transmission, including *in utero* transmission, direct contact of dam with the newborn, and through milk and colostrum consumed. This study found for 1,056 pairs of dam-daughter Danish dairy cows, using the level of antibodies to MAP in milk, an effect explained by the sire of 6.35% and an effect from dam-daughter pairs of 7.7% ($p < 0.05$). These results suggest that the parental contribution was significant and both heritability of susceptibility and vertical transmission should be considered in any control program of paratuberculosis in cattle.

A longitudinal study (Aly and Thurmond, 2005) based on pairs of dam-daughter dairy cows found that daughters born to MAP seropositive dams were 3.6 to 6.6 times more likely to be seropositive than those born to seronegative dams. Excluding involvement of vertical and horizontal transmission, a possible explanation for some of these results could be a genetic predisposition to postnatal infection with MAP in which the higher risk of infection for daughters of seropositive cows would be related to an inherited susceptibility to infection.

Gonda et al. (2007a) analyzed twelve paternal half-sib families with the aim of identifying QTL affecting susceptibility to MAP infection in US Holsteins. Serum and fecal samples from

4,350 daughters of these 12 sires were obtained for disease testing. Case definition for an infected cow was a positive ELISA, a positive fecal culture or both. Infected cows were matched with two of their non-infected herd-mates in the same lactation to control for herd and age effects. Eight chromosomal regions putatively linked with susceptibility to MAP infection were identified, using a Z-test ($p < 0.01$).

Probability of infection based on both diagnostic tests was estimated for each individual and used as the dependent variable for interval mapping. Based on this analysis, evidence for the presence of a QTL segregating within families on *Bos taurus* (BTA) chromosome 20 was found.

Recently, familial aggregation of paratuberculosis was described in beef cattle by use of pedigree information and microsatellite markers. In one study significant associations between ancestors and offspring ELISA status were reported. The results in a second study reported increased odds of having at least one positive paratuberculosis test result for two out of nine clusters compared to the cluster with the lowest proportion of positive paratuberculosis test results after conditioning on herd (Osterstock et al., 2007a, 2008).

Candidate Genes

The development of mycobacterial diseases is the result of a complex interaction between the host and pathogen influenced by environmental factors. Numerous host genes are likely to be involved in this process. However, only a small part of the total familial clustering observed in tuberculosis can be explained by the host genes identified to date (Bellamy, 2003). Susceptibility to infectious disease is influenced by multiple host genes, most of which are low penetrance QTLs that are difficult to map (Lipoldová and Demant, 2007).

At present, some studies have explored the association between paratuberculosis susceptibility and candidate host genes. Those few have not succeeded in finding conclusive

associations (Taylor et al., 2006; Hinger et al., 2007), likely due to limitations in sample size and sensitivity of the diagnostic test used. However, nine chromosomal regions putatively associated with MAP infection have been documented based on quantitative trait loci mapping (Gonda et al., 2005, Gonda et al., 2006b). As for susceptibility to many infectious diseases that are probably not controlled at the genetic level by a single gene, variation in susceptibility to bovine paratuberculosis is likely controlled by a group of genes, or many genes (multifactorial inheritance).

Coussens et al. (2001) reported the identification of a collection of over 40 genes whose expression in bovine peripheral blood mononuclear cells (PBMC) from a Johne's afflicted animal appears to be specifically repressed by MAP. Tentative gene identities have been assigned to many of these transcription units, based on basic local alignment search tool (BLAST) analysis against the Genbank database. The activity is focused on identifying these genes and their protein products.

Candidate Genes in Study

Caspase Recruitment Domain 15 gene (*CARD15*, formerly *NOD2*)

Details on this gene are presented in Chapter 2 section "Crohn's Disease and *CARD15/NOD2* Gene".

Solute Carrier 11A1 (*SLC11A1*), Formerly Natural Resistance Associated Macrophage Protein 1 (*NRAMP1*)

Solute Carrier family 11 (proton-coupled divalent metal ion transporters) member A1 gene (*SLC11A1*) codes for an integral membrane protein, which is expressed exclusively in macrophage/monocytes and polymorphonuclear leukocytes. The protein is localized to the endosomal/lysosomal compartment of the macrophage and is rapidly recruited to the membrane of the particle-containing phagosome upon phagocytosis (Govoni and Gross, 1998).

This protein/divalent cation transporter regulates iron homeostasis in macrophages, and plays a crucial role in macrophage activation altering the microenvironment of the phagosome to affect microbial killing.

SLC11A1 gene was originally mapped and positionally cloned on the basis of its ability to regulate resistance and susceptibility to a range of intramacrophage pathogens, including *Salmonella*, *Leishmania*, and *Mycobacterium bovis* (Feng et al., 1996; Li et al., 2006; Stober et al., 2007).

*SLC11A1*gene is highly conserved in many mammalian species and it shows considerable correspondence in structure between mice and humans. In human beings, the *SLC11A1* gene is located on chromosome region 2q35, and it encodes an integral membrane protein of 550 amino acids. Several polymorphisms have been described in the human *SLC11A1* gene facilitating studies on the relevance of this gene to mycobacteria susceptibility in human populations (Bellamy, 2003; Sechi et al., 2006).

The bovine homolog to this gene was mapped to BTA 2 (2q43-q44). It is expressed primarily in macrophages in liver, spleen, and lung, and is presumed to encode a protein with 12 trans-membrane segments, with one hydrophilic amino-terminal region containing a SH3-binding motif located at the cytoplasmic surface (Feng et al., 1996). Bovine *SLC11A1* intron sizes show a considerable degree of conservation when compared to murine and human *SLC11A1* introns (Coussens et al., 2004).

Role in immunity

It has been proposed in numerous reports that *SLC11A1* polymorphisms play a role in susceptibility to infection by intracellular bacteria, including mycobacteria (Sechi et al., 2006). It has been reported that in the mouse, resistance or susceptibility to infection with pathogens such as *Salmonella*, *Mycobacterium* and *Leishmania* is controlled by this gene located on

chromosome 1, influencing the rate of intracellular replication of these organisms in macrophages (Govoni and Gros, 1998; Gruenheid and Gros, 2000). A glycine and aspartic acid substitution at position 169 of the mouse SLC11A1 protein is invariably associated with the resistant and susceptible phenotypes, respectively (Ables et al., 2002). In the mouse, the progression of the *Mycobacterium avium* infection has also been reported to be highly dependent on the *SLC11A1*gene (Gomes and Appelberg, 1998). A recent study reported that different inbred mouse strains infected with MAP exhibited differences in bacterial replication associated to *SLC11A1* polymorphisms. This was also associated to differences in time and magnitude in IFN-g production (Roupie et al., 2008)

Case-control studies in human have confirmed the importance of this gene in susceptibility to mycobacteria (Govoni and Gross, 1998). Genetic studies have found that allelic variants at the human *SLC11A1* gene are associated with susceptibility to leprosy (*Mycobacterium leprae*) and tuberculosis (*M. tuberculosis*) and possibly with the onset of rheumatoid arthritis (Govoni and Gross, 1998; Skamene et al., 1998; Stokkers et al., 1999; Ables et al., 2002; Awomoyi et al., 2002).

Some research indicates a role of the *SLC11A1* product as an iron pump that depletes the phagosomal compartments of this nutrient and leads to starvation of the pathogen of this essential cation as a way to control mycobacterial proliferation (Gomes and Appelberg, 1998; Govoni and Gross, 1998; Coussens et al., 2001).

SLC11 defines a novel family of functionally related membrane proteins including *SLC11A2*, which was recently shown to be the major transferrin-independent uptake system of the intestine in mammals. This observation supports the hypothesis that the phagocyte-specific

SLC11A1 protein may regulate the intraphagosomal replication of antigenically unrelated bacteria by controlling divalent cation concentrations at that site (Govoni and Gross, 1998).

SLC11A1 expression has an effect on phagosomes during *M. bovis* (BCG) infection. Phagosomes containing mycobacteria retain the ability to fuse with early endosomes but are unable to fuse with lysosomes. The mycobacterial capacity to inhibit phagosome–lysosome fusion is reduced if not abrogated in the presence of a functional host cell *SLC11A1* (Gruenhei and Gros, 2000).

Several approaches have been used to analyze how expression of *SLC11A1* at the phagosomal membrane may influence survival of *Mycobacterium avium* and affect its ability to modulate the fusogenic properties of the phagosome in which it resides. *SLC11A1* expression appears to have a bacteriostatic effect and abrogation of *SLC11A1* restores the bacteria's capacity to replicate within macrophages. (Frehel et al., 2002).

Role in inflammatory bowel disease

Recently, Sechi et al. (2006) reported a strong association between Crohn's disease and polymorphisms at the 823C/T and 1729 + 55del4 loci in the *SLC11A1* gene in the Sardinian population. Although previous studies have suggested that *SLC11A1* mutations may favor microbial survival, the study failed to find any association between *SLC11A1* polymorphisms and MAP infection. Kojima et al. (2001) investigated the association of IBD with three different *SLC11A1* alleles found in a Japanese population. The allele frequency of one allele was significantly higher in patients with Crohn's disease (11.1%) and ulcerative colitis (11.2%) than those in the healthy control group (4.5%). The results suggest that the novel promoter polymorphism of the *SLC11A1* gene may influence susceptibility to IBD in this population.

However, in another study no difference was found in allele frequencies of *SLC11A1* promoter alleles between healthy donors and CD patients in a population of Ashkenazi Jews,

indicating that differences in *SLC11A1* promoter polymorphism play no role in CD in that population (Chermesh et al., 2007).

Association with disease susceptibility in cattle

Nucleotide sequence polymorphism due to a variation in the number of GT dinucleotide repeats has been reported in the 3' untranslated region (nucleotide positions 1781–1804) of the bovine *SLC11A1* gene (Feng et al., 1996; Horin et al., 1999). The association of *SLC11A1* gene polymorphisms with disease in cattle has been mainly focused on this single microsatellite. Coussens et al. (2004) report the nearly complete structure of the bovine *SLC11A1* gene, including sizes and positions of 13 introns relative to the bovine *SLC11A1* gene coding sequence and the DNA sequence of intron-exon junctions. Comparison of the bovine, murine and human *SLC11A1* gene structures revealed a high degree of conservation in intron placement (Coussens et al., 2004).

Ables et al. (2002) aimed at detecting polymorphisms in the *SLC11A1* gene from different cattle and buffalo breeds. Five breeds of cattle and four breeds of buffalo were used in the study. Sequencing showed two nucleotide substitutions and one amino acid substitution that was observed at nucleotide position 1202 in exon V of the Japanese black Angus, Philippine and Bangladesh swamp-type buffaloes which coded for threonine. On the other hand, the Korean cattle, Holstein, African N'dama, and buffalo had isoleucine, at this position.

Research analyzing the role of *SLC11A1* gene in resistance to disease in cattle has been contradictory. In recent works, an association between bovine *SLC11A1* gene polymorphism and susceptibility to diseases such as brucellosis and mastitis in cattle has been reported (Adams and Templeton, 1998; Joo et al., 2003; Ganguly et al., 2007). Mastitis-resistant cows were reported producing more *SLC11A1*-mRNA than the susceptible cattle, and ratios of *NRAMP1*:β-actin expression were higher in resistant cows (Joo et al., 2003). Ganguly et al. (2007) screened a

population of Murrah breed of buffalo (*Bubalus bubalis*) to identify polymorphism at 3'UTR of *SLC11A1* gene and evaluate their association with the macrophage function. Four allelic variants (GT13, GT14, GT15 and GT16) were identified. Macrophages, after maturation, were challenged with *Brucella* LPS to assay the macrophage function in terms of H_2O_2 and NO production. The $(GT)_{13}$ allele was significantly ($p < 0.01$) associated with increased production of H_2O_2 and NO, indicating a significant association with the improved macrophage function in buffalo. These results are in agreement with previous results from Qureshi et al. (1996) who found that the macrophages from cattle resistant to *in vivo* challenge with *Brucella abortus* were significantly superior ($p < 0.05$) in controlling intracellular growth of *B. abortus*, *M. bovis* BCG and *Salmonella dublin* than macrophages from susceptible animals.

In another study, Reddacliff et al. (2005) reported possible associations of particular *SLC11A1* protein and MHC alleles with susceptibility or resistance to JD in sheep. Adult sheep were phenotypically classified as having severe, mild or no disease on the basis of clinical, pathological and cultural tests for paratuberculosis, and as positive or negative in tests for humoral or cell mediated immunity. Correlations with phenotype were found for particular *SLC11A1* and MHC alleles.

A case-control study in a naturally infected herd analyzed the association of 3 polymorphic markers for the *SLC11A1* gene with susceptibility to JD. Only one polymorphism at the 3'-UTR microsatellite showed a different frequency between cases and control groups. These results suggest that *SLC11A1* is involved in lesion progression (Estonba, 2006).

On the other hand, there are some reports that are unable to determine an association between the gene and particular diseases in cattle. One report did not find associations between resistance and susceptibility to infection with *M. bovis* and polymorphism in the *SLC11A1* gene,

or between the magnitude of the lesions and various RFLP types of *M. bovis* isolates. The study concluded that the *SLC11A1* gene does not determine resistance and susceptibility to infection with *M. bovis* in cattle (Barthel et al., 2000). In another study, Estrada-Chavez et al. (2001) demonstrated, by Western blotting, a high-level expression of SLC11A11 proteins in peripheral blood cells and granulomas of *Mycobacterium bovis*-infected bovines. Immunohistochemistry of granulomatous lesions showed heavily labeled epithelioid macrophages and Langhans cells, suggesting that *M. bovis* infection enhances *NRAMP1* expression and that active tuberculosis can occur despite this response.

In another work (Kumar et al., 2005), the presence of $(GT)_{13}$ allele in *SLC11A11* gene even in a homozygous condition could not provide enough resistance to brucellosis in a naturally infected herd. Kumar et al. (1999), testing the association between polymorphisms and variation in susceptibility to tuberculosis in cattle, did not find differences in the frequency of the alleles between the infected and random groups questioning the role of a specific *SLC11A11* sequence on tuberculosis resistance/susceptibility in bovines.

In another study, no association was found between the *SLC11A1*-resistant alleles and the resistant phenotype in either experimental or naturally occurring brucellosis. Bacterial intracellular survival was assessed in bovine monocyte-derived macrophages from cattle with either the resistant or susceptible genotype, but no difference was observed in the rates of intracellular survival of *B. abortus* (Paixão et al., 2007).

Finally, Hinger et al. (2007) tested the association between a set of microsatellites in different candidate genes and MAP antibody response in Holstein cows. The authors reported an inability of the test to demonstrate an effect of polymorphisms in *SLC11A11,* given that the study

population showed only 3 alleles, 2 of which displayed very low frequency and were therefore not informative.

Interferon Gamma

Host defense against intracellular pathogens such as mycobacteria depends on effective cell-mediated immunity (CMI), in which interactions between T cells and macrophages are crucial (Frucht and Holland, 1996, Shtrichman and Samuel, 2001). A major effector mechanism of CMI is the activation of infected macrophages by type 1 cytokines, particularly interferon gamma (IFN-g). The IFN-g protein is produced by antigen-specific type 1 helper T cells (Th1 cells) and natural killer (NK) cells, and binds to IFN-g-receptor (R) R1/R2 complexes at the macrophage surface. Interferon-g, in conjunction with tumor necrosis factor- alpha (TNF-α), has been also demonstrated as an element activating anti-mycobacterial microbicidal mechanisms in mouse macrophages (Ottenhoff et al., 2002), and cytotoxic T cells as well as B cell differentiation (Schmidt et al., 2002). Concurrently, suppression of Th2 effector cell functions induced by IFN-g stimulates cellular Th1 responses and Th1-mediated autoimmunity. These diverse effects place IFN-g in a key position in the regulation of the immune system (Schmidt et al., 2002).

Interferon gamma alone, or in conjunction with lipopolysaccharide or TNF-α, can activate murine macrophages to kill or inhibit mycobacteria by the induction of nitric oxide, however, according to the study presented by Zhao et al. (1997), the amount of nitric oxide (NO) produced by activated bovine monocytes *in vitro* may be insufficient to kill or inhibit intracellular MAP. This suggested that production of nitric oxide by activated bovine mononuclear phagocytes might not be a major anti-mycobacterial mechanism against MAP infection. On the other hand, Cooper et al. (2002) proposed that the acquired cellular response plays a double role in mycobacterial disease. On the one side, it is required to limit bacterial growth, yet on the other it

must itself be limited to reduce damaging inflammatory responses. This dichotomy could contribute to the chronicity of mycobacterial infections. The authors suggest that for chronic mycobacterial infection, IFN-g and NO exert a negative-feedback regulatory effect on the cellular and inflammatory response.

This regulatory role is supported by the results of studies, such as those with IFN-g knockout mice, where invasion by different pathogens caused dramatic increases in mortality (Schmidt et al., 2002). Reports of familial clusters of disseminated *Mycobacterium avium* complex infections in humans have suggested that the activation pathways leading to the generation of IFN-g are critical to effective protection against this intracellular pathogen; in these instances susceptible patients had a defect in IFN-g production (Frucht and Holland, 1996).

In humans INF-g genetic deficiency is associated to mutations in IFN-g receptor. This is a heterogeneous syndrome with different clinical, genetic, immunological and histopathological types. Complete deficiency in IFN-g receptor is associated with severe or fatal outcomes after infection with non-tuberculous mycobacteria or *M. bovis* BCG, and is accompanied by poor granuloma formation, multibacillary lesions and progressive infection, often despite intensive antibiotic treatment. In contrast, individuals with partial deficiency in IFN-g receptor often develop milder, though still severe, infections (Huang et al., 1998, Ottenhoff et al., 2002). However, a 40-year-old woman with disseminating *M. avium* infection has been reported with an acquired deficiency in IFN-g resulting from the presence of serum auto-antibodies that specifically neutralized IFN-g (Ottenhoff et al., 2002).

Denis et al. (2005) analyzed the impact of INF-g on *Mycobacterium bovis* replication, cytokine release and macrophage apoptosis. The authors concluded that virulent *M. bovis* is a major determinant of release of pro-inflammatory cytokines by macrophages, and IFN-g

amplifies the macrophage cytokine release in response to *M. bovis*. Induction of apoptosis is closely linked to the emergence of macrophage resistance to *M. bovis* replication, which is dependent on endogenous TNF-α release.

Moreover, an association study on susceptibility to nematode parasites in sheep suggested that a polymorphic gene conferring increased resistance to gastrointestinal nematode parasites is located at or near the interferon gamma gene, supporting previous reports which have mapped a quantitative trait locus (QTL) for resistance to this region in domestic sheep (Coltman et al., 2001).

IFN-g gene maps to a single locus located on the long arm of chromosome 12 in the human, chromosome 10 in the mouse, and chromosome 5 in cattle. Transcripts of the *IFN-g* gene possess four exons and three introns. Mature *IFN-g* mRNA is ~1.2 kb and encodes a protein of ~17 kDa. IFN-g functions as an *N*-glycosylated homodimer (Shtrichman and Samuel, 2001).

The organization of the *IFN-g* gene into four exons is evolutionarily highly conserved, as has been proven for humans, for experimental model animals, and for domestic animals, such as the sheep, horse, pig, and chicken. The most prominent sequence variations within the *IFN-g* genes described to date are polymorphic intronic microsatellites, as demonstrated for humans, cattle, sheep, and pigs (Shtrichman and Samuel, 2001). Schmidt et al. (2002) analyzed polymorphisms in the bovine *IFN-g* gene reporting four distinct series of single nucleotide polymorphisms found in functionally important regions of Bo*IFNG*. The region between the two intron 1 microsatellites contained the highest density of SNPs in *Bos taurus* breeds.

Toll-Like Receptors

Innate immune responses to pathogens are mainly coordinated by monocytes/macrophages, granulocytes, and dendritic cells, which act as a first line of defense against invading microorganisms. Discrimination of non-self from self is achieved by numerous

host proteins equipped with the ability to recognize structures or molecular patterns, present on foreign organisms. One major group of proteins is the Toll-like receptor family (TLR), also referred to as pattern recognition receptors (PRRs) (Underhill et al., 1999; Schröder and Schumann, 2005). These recognition receptors of the innate immune system have been conserved in both the invertebrate and vertebrate lineages, and recognize a variety of endogenous and exogenous ligands; many of the latter are conserved molecules essential for pathogen survival (Roach et al., 2005).

Ligation of TLRs by pathogen-specific receptors initiates a signal transduction pathway in the host cell that culminates in the activation of NF-kB and the induction of cytokines and chemokines that are crucial to eliciting the adaptive immune response against the pathogen (Wang et al., 2002; Werling et al., 2006). Consequently, activation of TLR is an important link between innate cellular response and the subsequent activation of adaptive immune defense against microbial pathogens (Bhatt and Salgame, 2007).

The involvement of the Toll receptors in innate immunity was first described in *Drosophila*. *Drosophila* Toll was originally identified as a type I trans-membrane receptor required for the establishment of dorso-ventral polarity in the developing embryo and in the induction of an antifungal response in adult flies (Wang et al., 2002; Takeda et al., 2003; Schröder and Schumann, 2005). Soon after the discovery of the role of the *Drosophila* Toll in the host defense against fungal infection, a mammalian homologue of the *Drosophila* Toll was identified. Subsequently, a family of proteins structurally related to *Drosophila* Toll was identified, collectively referred to as the Toll-like receptors. The TLR family is known to consist of 13 members characterized structurally by the presence of a leucine rich repeat (LRR) domain

in their extracellular domain and a Toll/Interleukin 1 receptor homology (TIR) domain in their intracellular region (Wang et al., 2002; Takeda et al., 2003; Quesniaux et al., 2004).

Role of TLRs

Ectopic over-expression of TLR4, the first mammalian TLR identified, was shown to cause induction of the genes for several inflammatory cytokines and co-stimulatory molecules. Therefore, it was anticipated that the TLRs might be involved in immune responses, especially in the activation of innate immunity (Takeda et al., 2003). The TLRs are capable of recognizing several classes of pathogens and coordinating appropriate immune responses, involving both innate and adaptive immunity (Takeda et al., 2003).

Lipoproteins in which the N-terminal cysteine is triacylated are recognized by TLR2 in combination with TLR1. Diacylated lipoproteins are recognized by TLR2 in combination with TLR6. Double-stranded RNA is recognized by TLR3. Lipopolysaccharide is recognized by TLR4. Flagellin is recognized by TLR5. Cyclic compounds such as nucleic acids and heme are recognized by the family consisting of TLR7–9 (Wang et al., 2000).

TLR2 has been implicated in the activation of NF-kB following interaction of macrophages with lipoproteins from pathogens such as Gram-negative bacteria, *Mycoplasma* and spirochetes, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycoinositolphospholipids from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermidis*, zymosan from fungi, glycolipids from *Treponema maltophilum*, and porins that constitute the outer membrane of *Neisseria*. Furthermore, TLR2 recognizes several atypical types of lipopolysaccharides (LPS) from *Leptospira interrogans* and *Porphyromonas gingivalis*, in contrast to TLR4, which recognizes LPS from enterobacteria such as *Escherichia coli* and *Salmonella* spp. TLR2 and TLR4 may

differentially recognize these structural variations in LPS (Wang et al., 2002; Takeda et al., 2003, Arko-Mensah et al., 2007).

In addition to controlling the development of adaptive immunity, activation of TLRs appears to be directly involved in induction of antimicrobial activity, and TLR2 activation leads to nitric oxide–dependent and –independent killing of intracellular *Mycobacterium tuberculosis* in mouse and human macrophages, respectively (Takeda et al., 2003).

A study from Swiderek et al. (2006) that analyzed the association between TLR polymorphisms and natural bacterial infections in the mammary gland in sheep reported 6 different alleles for TLR2 and a protective association for one allele in relation to bacterial infection. Numerous studies *in vitro* and *in vivo* have shown that whole mycobacteria or mycobacterial components act as agonists for TLRs. Recent studies show that certain TLR knockout mice are more susceptible than wild-type mice at an early stage of respiratory tract infection; in particular, TLR2–/– mice are more susceptible than wild type mice. In addition, defective capability of intracellular killing was correlated with impaired production of TNF-α, which is vital for containment of mycobacterial infections (Jo et al., 2007).

Underhill et al. (1999) showed that TLR are required for the induction of TNF-α in macrophages by *Mycobacterium tuberculosis*. Expression of a dominant negative form of myeloid differentiation primary-response protein 88 (MyD88) in a mouse macrophage cell line blocks TNF-α production induced by *M. tuberculosis*. TLR2 was identified as the specific TLR required for this induction.

According to Quesniaux et al. (2004), the present *in vivo* evidence suggests that TLR signaling has only a modest effect on acute mycobacterial infection and the generation of adaptive immunity, but may be more relevant for the long-term control of infection.

In a recent work, Sweet and Schorey (2006) found that glycopeptidolipids, which are highly expressed surface molecules on *M. avium*, can stimulate the nuclear factor-kB pathway and production of pro-inflammatory cytokines when added to murine bone marrow-derived macrophages. This stimulation was dependent on TLR2 and MyD88, indicating that glycopeptidolipids can function as TLR2 agonists and promote macrophage activation in a MyD88-dependent pathway.

Toll-like receptor 4 gene

In 1998, TLR4 was shown to be involved in the recognition of LPS, a major cell wall component of Gram-negative bacteria. Subsequently, other members of the TLRs family were shown to be essential for the recognition of a range of microbial components. The structural similarity of TLRs seems to reflect their common function in the recognition of microbial components (Takeda et al., 2003).

The Toll-like receptor 4 gene codifies for a type I transmembrane protein with an extracellular domain consisting of a leucine-rich repeat region (LRR) and an intracellular domain homologous to that of the human interleukin-1 receptor. It mainly recognizes conserved pathogenic motifs of Gram-negative bacteria, activating the nuclear factor NF-kB (Browning et al., 2007). Other ligands for TLR4 are structures such as respiratory syncytial virus, human heat shock protein 60 (hsp60), and fibrinogen. Expression of TLRs is modulated by a variety of factors such as microbial invasion, microbial components, and cytokines. Infection by *Mycobacterium avium* induces variation in TLR4 mRNA expression in macrophages and leads to chromatin remodeling (Takeda et al. 2003).

In recent years TLR2, TLR4 and latterly, TLR1/TLR6 that heterodimerise with TLR2, have been implicated in the recognition of mycobacterial antigens (Quesniaux et al., 2004; Yadav et al., 2006, Weiss et al., 2008). In a work by Ferwerda et al. (2005), it was reported that

CARD15 and TLRs are two non-redundant recognition mechanisms of *M. tuberculosis*. Chinese hamster ovary fibroblast cell lines transfected with human TLR2 or TLR4 were responsive to *M. tuberculosis*. TLR4-defective mice released 30% less cytokines after stimulation with mycobacteria, compared to controls. These results indicated that *CARD15* and TLR pathways are non-redundant recognition mechanisms of *M. tuberculosis* that synergize for the induction of pro-inflammatory cytokines.

In a second study, Ferwerda et al. (2007) showed that TLR2, TLR4, and CARD15 are pattern recognition receptors for *M. paratuberculosis*, mediating cytokine production and stimulation of host defense. In the study, it was demonstrated that murine and human TLR2 recognize sonicated MAP. However, the role of TLR4 becomes apparent if human mononuclear cells are infected with live *M. paratuberculosis*, as under these conditions, cytokine responses were reduced by inhibition of TLR4. In addition, in this study it was demonstrated that TLRs, such as TLR2 and TLR4, as well as CARD15 are independent recognition systems of *M. paratuberculosis*.

In a recent work by Mendez-Samperio et al. (2008) it was proposed that CXCL8 chemokine, which has an important role in mediating the inflammatory conditions following invasion of *M. tuberculosis*, is activated through the ERK1/2 MAPK pathway induced by TLR2 and TLR4. In another study, Fremond et al. (2003) infected with BCG TLR4 mutant C3H/HeJ and control C3H/HeOUJ. TLR4 mutant mice experienced an arrest of body weight gain and showed signs of increased inflammation, with persistent splenomegaly, increase in granuloma number and augmented neutrophil infiltration. *TLR4* mutant mice show normal macrophage recruitment and activation, granuloma formation and control of the BCG infection, but this is associated with persistent inflammation. The authors suggested that TLR4 signaling is not

essential for early control of BCG infection, but it may have a critical function in fine tuning of inflammation during chronic mycobacterial infection.

Branger et al. (2004) intranasally infected *TLR4* mutant (C3H/HeJ) and wild-type (C3H/HeN) mice with live *Mycobacterium tuberculosis*. *TLR4* mutant mice were more susceptible to pulmonary tuberculosis, as indicated by a reduced survival and an enhanced mycobacterial outgrowth, suggesting that TLR4 may be involved in the generation of acquired T cell-mediated immunity. These results would indicate that TLR4 plays a protective role in host defense against lung infection by *M. tuberculosis*.

A study by Abel et al. (2002) reported that *TLR4* mutant mice had a reduced capacity to eliminate mycobacteria from the lungs, spread the infection to spleen and liver, at higher levels than the wild-type mice and succumbed within 5–7 months, whereas most of the wild-type mice controlled infection. The lungs of *TLR4* mutant mice showed chronic pneumonia with increased neutrophil infiltration, reduced macrophages recruitment, and abundant acid-fast bacilli. The purified mycobacterial glycolipid, phosphatidylinositol mannosides, induced signaling in both a TLR2- and TLR4-dependent manner. Macrophage recruitment and the proinflammatory response to *M. tuberculosis* would be weakening in *TLR4* mutant mice, resulting in chronic infection with impaired elimination of mycobacteria. This indicates that TLR4 signaling is required to mount a protective response during chronic *M. tuberculosis* infection.

In a study by Reiling et al. (2002), mice defective in CD14, TLR2, or TLR4 were infected with *M. tuberculosis* by aerosol. Following infection with mycobacteria, either mutant strain was as resistant as congenic control mice. Granuloma formation, macrophage activation, and secretion of pro-inflammatory cytokines in response to low-dose aerosol infection were identical in mutant and control mice. However, high-dose aerosol challenge with 2000 CFU *M.*

tuberculosis revealed TLR2-, but not TLR4-defective mice to be more susceptible than control mice. In conclusion, while TLR2 signaling contributes to innate resistance against *M. tuberculosis* in borderline situations, its function, and that of CD14 and TLR4, in initiating protective responses against naturally low-dose airborne infection is redundant (Reiling et al. 2002).

However, there is also conflicting information about the role of TLR4 in susceptibility of mice to *M. tuberculosis* infection, as presented by Shim et al. (2003). Their results indicated that infection of TRL4-competent and TLR4-deficient mice on the C3 H inbred mouse strain background had similar outcomes, measured in terms of the course of the disease, cell accumulation patterns in the lungs, and lung histopathology.

Toll-like receptor 4 maps to human chromosome 9q32-33, while it has been mapped to the distal end of bovine chromosome 8 (McGuire et al., 2006). White et al. (2003b) analyzed the structure of *TLR4* in bovine, and comparative analyses show gene order conservation between the bovine chromosome 8 region and human chromosome 9. The coding sequence of bovine *TLR4* is divided into three exons, with intron-exon boundaries and intron sizes similar to those of human TLR4 transcript variant 1. White et al. (2003a) amplified each exon in 40 individuals from 11 breeds and screened the sequence for single nucleotide polymorphisms (SNPs). Thirty two SNPs were identified, 28 of which are in the coding sequence, for an average of one SNP per 90 bp of coding sequence. Eight SNPs were non-synonymous and potentially alter specificity of pathogen recognition or efficiency of signaling.

The association between *TLR4* and disease in bovine has been scarcely studied. Sharma et al. (2006) analyzed the association between polymorphisms in the *TLR4* gene and somatic cell score and lactation persistency in the Canadian Holstein bull population. A total of 3 single

nucleotide polymorphisms (SNP) of *TLR4* were detected, and one was found to be associated with higher lactation persistency and lower somatic cell scores.

Goldammer et al. (2004) demonstrated that mastitis strongly increased (4- to 13-fold) the mRNA abundances of all of TLR2 and TLR4 genes. The number of TLR2 copies correlated well with those of TLR4, indicating coordinated regulation of these two PRRs during infection of the udder.

In another study, Yang et al. (2008) demonstrated that bovine TLR2 and TLR4 receptors recognize this *S. aureus* strain resulting in enhanced activation of NF-kB factors in these cells, similar in strength to that in response to *E. coli* and LPS.

Case Control Genetic Association Studies

Genetic Epidemiology

Genetic epidemiology investigates the role of genetic determinants in the causation of complex diseases. This is a discipline related to traditional epidemiology that focuses on the familial, and in particular genetic, determinants of disease and the joint effects of genes and non-genetic determinants (Burton et al., 2005).

Until the last decade, association between genetics and disease had been mainly restricted to the study of relatively rare familial diseases controlled by a single major gene. However, studies of animal models and epidemiological studies in humans have shown that many apparently non-hereditary diseases, including infectious diseases, develop predominantly in genetically predisposed individuals, and that this predisposition is caused by multiple genes (Lipoldova and Demant, 2006).

The principal distinction between simple monogenic diseases and complex genetic diseases is that the latter do not exhibit classical Mendelian patterns of inheritance and are likely influenced by multiple genetic and environmental factors (Silverman and Palmer, 2000).

Complex diseases are most likely influenced by genetic heterogeneity (multiple genetic causes leading to the same disease), environmental phenocopies (purely environmental forms of the disease), incomplete penetrance (subjects inheriting a disease gene but not developing the disease), genotype-by-environment interactions (non-additivity of genetic and environmental influences on disease development), and multi-locus effects (more than one gene influences disease development) (Silverman and Palmer, 2000).

Identification of low-penetrance genes may be useful in the identification of individuals at high risk of disease, it would increase the understanding of the molecular mechanisms that underlie disease, and it would help to identify therapeutic targets. In the case of infectious disease, the mapping of low-penetrance disease-susceptibility genes is difficult, not only by the heterogeneity of populations, but also by differences in environment and lifetime exposure to infections, which obscure the already relatively weak individual effects of these genes (Lipoldova and Demant, 2006).

Genetic variants—or polymorphisms—arise from new mutations. The simplest type of polymorphism is a single base mutation, which substitutes one nucleotide for another, referred to as a single nucleotide polymorphism (SNP). Insertions of additional sequences or deletions can also occur and these range in size from one to several thousand base pairs (Daly and Day, 2001). SNPs do not necessarily have any relevance to disease or outcome; they can be anonymous variants within or between genes, or could be functional, causal mutations. In general, functionally significant effects associated with genetic polymorphisms are most likely when they are associated with an amino acid substitution in the gene product, when a deletion or insertion results in a frame shift in the coding region, when a gene is completely deleted or when the polymorphism directly affects gene transcription, RNA splicing, mRNA stability or mRNA

translation. More SNPs are thought to exist in the human genome than any other type of polymorphism (Daly and Day, 2001; Cardon and Palmer, 2003).

The arrangement of genetic polymorphisms within a single chromosome in an individual is known as a haplotype. Frequently certain haplotypes are more common as a result of linkage than would be expected if each polymorphism was inherited randomly. Two genetic loci are linked if they are transmitted together from parent to offspring more often than expected under independent inheritance. They are in linkage disequilibrium if, across the population as a whole, they are found together on the same haplotype more often than expected (Daly and Day, 2001; Teare and Barrett, 2005).

Population Candidate Gene Association Studies

The alternative to family studies and to population approaches that rely on linkage disequilibrium is to perform population candidate gene association studies. Genetic association studies aim at detecting association between one or more genetic polymorphisms and a trait, which might be some quantitative characteristic or a discrete attribute or disease (Cordell and Calayton, 2005). In other terms, their aim is to determine if there is a statistical relation between genomic variation at one or more sites and phenotypic variation, usually represented by the presence or absence of a disease or by levels of a disease related trait (Hattersley and McCarthy, 2005).

Some advantages to this approach include the fact that such studies may provide adequate power to detect relative risks as low as 1.5 which is usually not possible in familial studies. In addition, as most candidate gene studies are focusing directly on a single gene and frequently look directly at functionally significant polymorphisms, concerns about the extent of linkage disequilibrium and the adequacy of SNP markers to detect associations are not primordial. It has

also been discovered that the issue of population stratification and spurious association can be manageable through design and data analysis (Li, 2008).

In population candidate gene association studies, DNA samples from cases and population controls are genotyped for polymorphisms situated in or close to a gene which prior knowledge suggests might play a role in the pathogenesis of the disease of interest, based on a comparison of unrelated affected and unaffected individuals from a population (Lander and Schork, 1994; Blackwell, 2001; Daly and Day, 2001; Li, 2008).

Two types of association are explored in these studies. The first of these forms of association is termed direct association, and target polymorphisms, which are themselves putative causal variants. This type of study is the easiest to analyze and the most powerful, but the difficulty is the identification of candidate polymorphisms. In the second type of association, the polymorphism is a surrogate for the causal locus and this type of association allows searching for causal genes in indirect association studies. However, indirect associations are even weaker than the direct associations they reflect, and it will usually be necessary to type several surrounding markers to have a high chance of picking up the indirect association (Cordell and Clayton, 2005).

If a mutation increases disease susceptibility, then it can be expected to be more frequent among affected individuals (cases) than among unaffected individuals (controls) (Pritchard and Donnelly, 2001).

There are a number of important issues to consider in the design of case-control studies of this type. These include: choice of candidate gene and polymorphism for study, sample size requirements, genotyping quality, recruitment methods, matching of cases and controls, number

of subjects to be studied, and data analysis and interpretation (Daly and Day, 2001; Hattersley and McCarthy, 2005).

Even if case-control association studies have some weaknesses with regard to potential confounding factors such as population stratification, they remain an important tool in genetic epidemiology and are often preferred to family-based studies (Burton et al., 2005; Guedj et al., 2007).

Tests of association are used as a first step in the analysis process. Various tests are proposed based on either genotypes, such as the genotypic, Hardy-Weinberg equilibrium or Cochran-Armitage tests, or alleles, such as the allelic test (Zheng and Tian, 2005). The basic aim of the association-study design is to correlate genotypes and disease phenotypes that are obtained from a sample of individuals (Zondervan and Cardon, 2004). In a case–control study, the objective is to compare exposure to risk factors (environmental or genetic) between affected individuals and unaffected controls, which have been selected from the same population as cases, to find associations between risk factors and disease. In these association studies the susceptibility or causal alleles are themselves evaluated (Zondervan and Cardon, 2004). The standard measure of effect in the case–control study is the odds ratio (OR), defined as the odds of exposure among cases divided by the odds of exposure among controls (Clayton and McKeingue, 2001; Zondervan and Cardon, 2004).

A key issue in case-control association studies is how the case subject met the criteria for the affectation phenotype, but in practice, diagnostic error may be present when an imperfect gold standard or reference test is used instead of a definitive diagnosis. Also, control subjects must clearly represent the opposite end of the phenotypic expression of disease (Weiss et al., 2001).

Positive results in a case-control association study may be due to a direct effect of the polymorphism in question, linkage disequilibrium, or population stratification (artifact of population admixture), a spurious association due to differences in allele frequency between poorly matched cases and controls, resulting from differences in ethnic origins (Lander and Schork, 1994; Weiss et al., 2001). Confounding, in al aspects of epidemiology, raises the possibility both of generating false findings (positive confounding) or obscuring true causal associations (negative confounding) (Cordell and Clayton, 2005).

Procedures that account for population stratification consist in matching cases and controls for ethnicity or use of multiple unlinked markers. Another way of tackling this problem is to collect data about ethnicity from the members of the sampled group and then stratify the analysis according to reported ethnicity (Pritchard and Donnelly, 2001).

An additional criterion for evaluation of the quality of the case-control study is assessment of Hardy-Weinberg equilibrium in the markers studied within the control group. This provides a check to ensure that genotyping errors, mutation, or population stratification do not explain the observed results (Weiss et al., 2001).

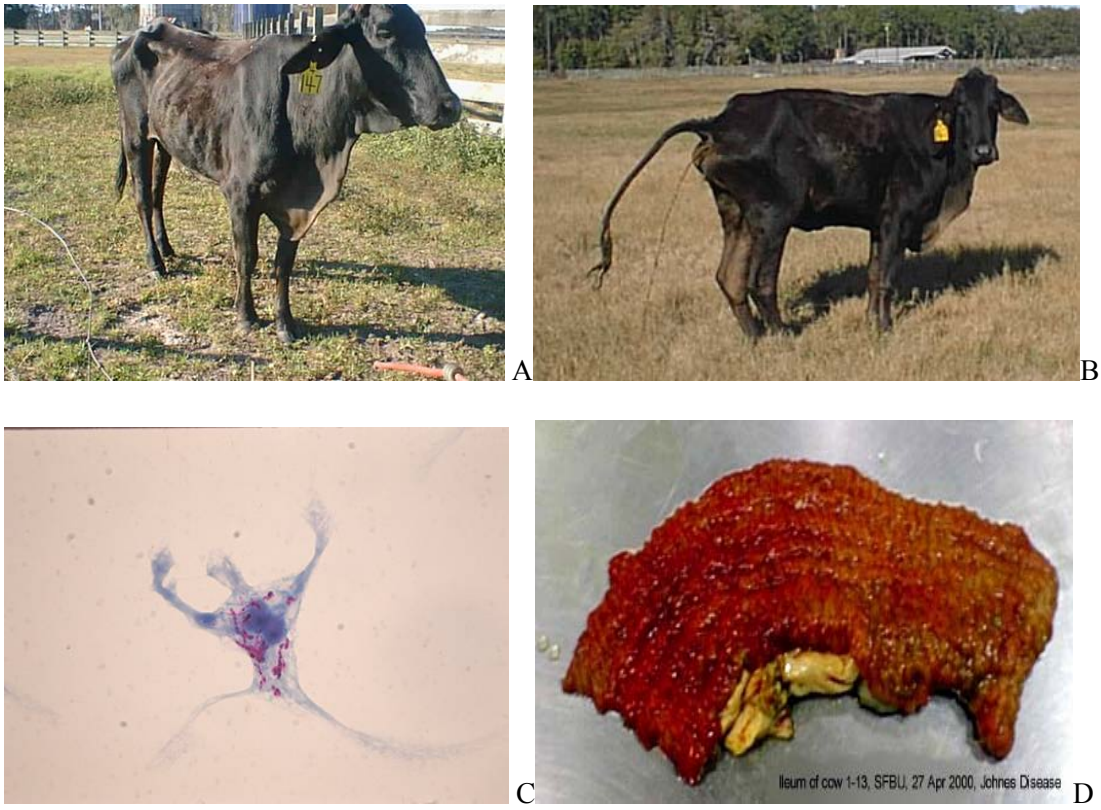

Figure 2-1. Clinical cases. A & B) Two adult females affected by paratuberculosis showing a deteriorated body condition (courtesy of Dr. Owen Rae),C) monocyte containing MAP bacilli (courtesy of Dr. Claus Buergelt), D) section of an affected ileum exhibiting a thickened and corrugated mucosa (courtesy of Dr. Owen Rae).

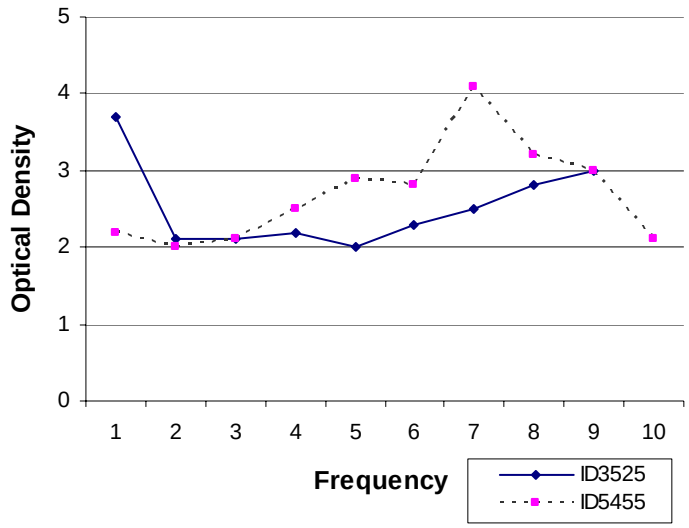

Figure 2-2. Variation in ELISA optical density in two Holstein cows (Data provided by Dr. C.D. Buergelt).

CHAPTER 3
ASSOCIATION AMONG RESULTS OF SERUM ELISA, FECAL CULTURE, AND NESTED PCR ON MILK, BLOOD, AND FECES FOR THE DETECTION OF PARATUBERCULOSIS IN DAIRY COWS

Summary

Paratuberculosis is a chronic, infectious disease of ruminants that entails a serious concern for the cattle industry. One of the main issues relates to the inefficiency of diagnosis of subclinically infected animals. The objective of this field study was to analyze the association among results of a serum ELISA, fecal culture, and nested PCR tests on milk, blood, and feces for *Mycobacterium avium* subsp. *paratuberculosis* detection in dairy cows. Feces, blood and milk samples were collected from 328 lactating dairy cows in four known infected herds. Results were analyzed to determine associations and levels of agreement between pairs of tests. A total of 61 animals (18.6%) tested positive when all the tests were interpreted in parallel. The agreement between results in different pairs of tests was poor, slight and fair in two, five and three of the ten possible combinations, respectively. Fecal culture and fecal PCR resulted in the highest kappa coefficient (0.39; fair agreement), with the lowest agreement being for ELISA and blood PCR (-0.036; poor agreement). Fisher's Exact Test resulted in statistically significant associations ($P \leq 0.05$) between the following test pairs; ELISA:fecal culture; ELISA:fecal PCR; milk PCR:fecal PCR, blood PCR:fecal PCR and fecal culture:fecal PCR. ELISA showed the highest complementary sensitivity (CS) values for all the possible two-test combinations, followed by fecal PCR. The combined use of ELISA and fecal PCR displayed the highest potential to increase the overall sensitivity for the diagnosis of paratuberculosis infection.

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Introduction

Paratuberculosis is a chronic, infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis*, and characterized by progressive weight loss and profuse diarrhea (Chiodini et al., 1984). The disease has a worldwide distribution and is categorized by the Office International Des Epizooties as a list B disease, which is a serious economic or public health concern (OIE, 2004).

Most cattle with JD are infected as calves by fecal-oral transmission, and *in utero* transmission has also been reported (Seitz et al., 1989; Whitlock and Buergelt, 1996). However, young animals manifest no clinical signs and the incubation period is variable, ranging from 2 to 10 years (Bassey and Collins, 1997; Whitlock et al., 2000; Stabel and Ackerman, 2002).

The diagnosis of paratuberculosis is hampered by a lack of accurate tests. Available methods fail to identify all infected animals (false negative results), and some produce substantial numbers of false positives (Chiodini et al., 1984). Tests for detection of antibodies to MAP, such as enzyme-linked immunosorbent assays (ELISA) present the major disadvantage of moderate to low sensitivity. The usefulness of serological tests is compromised by the variability of the immune response depending on the stage of disease. For this reason, it is generally accepted that their sensitivity in detecting infected animals is only about 30% (Collins et al., 2006), and the ELISA test rarely gives a positive result in animals under 2 years of age. It frequently fails to detect individuals in the early phases of infection (Juste et al., 2005). Low agreement between results from different commercially available ELISA kits is another drawback of this test (McKenna et al., 2006). Despite these disadvantages, ELISA testing of sera is still the method of choice for epidemiological studies and herd-based diagnosis (Bottcher and Gangl, 2004).

Tests based on the detection of the agent likewise present the problem of low sensitivity. The shedding of MAP organisms in feces can be intermittent and detection by culture is imperfect, especially because of contamination, and when few organisms are shed in feces. It has been estimated that fecal culture detects only about 50% of cattle infected with MAP (Stabel, 1997). The advent of diagnostic methods based on specific bacterial DNA sequences has allowed fastidious microorganisms, such as MAP, to be rapidly identified. Polymerase chain reaction (PCR) tests based on the insertion element IS*900* have been the most widely used for MAP identification (Harris and Barleta, 2001). However, the detection of the etiologic agent is limited by the presence of inhibitory substances, and the frequency and number of organisms that are present in the body fluid or tissue being tested. The isolation of MAP from sites other than the intestinal tract, such as udder, kidney, liver, male reproductive tract and blood, have suggested active dissemination of the bacteria and opens the possibility for detection of the agent by PCR in fluids such as milk and blood of suspicious animals (Buergelt and Williams, 2004).

A combination of independent tests is a common method to improve reliability of laboratory diagnostic tools. As a result of the setbacks of MAP diagnosis, such strategies have already been implemented by using a combination of bacterial fecal culture and PCR or serological screening and bacterial fecal culture (Collins et al., 2006). Moreover, a combination of tests with different sensitivities and specificities allows a classification of animals and herds relative to the probability of MAP infection (Bottcher and Gangl, 2004).

A broader knowledge of the behavior and association between different diagnostic tests is desirable for the implementation of strategies based on the combination of different tests, which could be a useful approach to improve the sensitivity of MAP detection. The hypothesis of this study was that different degrees of association exist among tests detecting MAP infection. Our

objective was to analyze the association among results of a serum ELISA, fecal culture, and nested PCR on milk, blood, and feces for MAP detection in dairy cows.

Materials and Methods

Study Population

Blood, milk and fecal samples were collected from 328 lactating dairy cows in four herds near Gainesville, Florida, USA. Dairies A, B and C were composed by 70, 500 and 600 Holstein cows, respectively, while dairy D was a 100 cow Jersey herd. The four herds had a past history of clinical and ELISA positive paratuberculosis cases, with some individuals confirmed by necropsy in herds B and C (submitted to the College of Veterinary Medicine, University of Florida). Fifty six (56), 122, 100 and 50 cows were sampled from herds A, B, C, and D, respectively. No formal randomization in the selection of animals was attempted, and the inclusion criteria for the animals was that they be lactating, without any clinical sign of paratuberculosis at the time of collection (diarrhea and/or weight loss). Each animal was sampled only once during the study to avoid the effect of correlation among repeated measures within the same individual in the statistical analysis. A maximum of 10 animals was sampled in each farm visit and all samples were collected between December 2004 and December 2006.

Sample Handling

Milk samples

Before collection, the teats were cleansed with alcohol to avoid sample contamination from skin. Milk (30-40 ml) was collected in a sterile 50 ml centrifuge tube from the four quarters by hand milking. The first 10-15 ml of milk were discarded. The milk samples were centrifuged at 1,000 *g* for 15 min and the supernatant discarded. The resultant pellet was washed thrice in phosphate buffered saline (PBS, pH 7.3) and centrifuged at 500 *g* for 15 min. The pellet was resuspended in 1 ml of PBS, centrifuged and resuspended in 100 µl of 0.2 N NaOH.

Extraction of DNA on milk

Extraction was performed by heating the resuspended pellet at 110°C for 20 min, followed by centrifugation at 500 *g* for 3 min. The final product was stored at -20°C for subsequent PCR.

Blood samples

After cleansing with alcohol, 10 ml of blood per cow were collected from the coccygeal vein into Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) with and without EDTA. For the blood PCR procedure, 3 ml of EDTA blood was added to 4 ml of Ficoll-Isopaque® Plus Gradient (Amersham Pharmacia, Piscataway, NJ, USA, density 1.078 g/ml) and centrifuged for 40 min at 500 *g* at 18°C. The buffy coat was collected, then washed twice with PBS, and centrifuged at 500 *g* for 15 min. Cells from the pellet were counted with a hemocytometer, resuspended in 100 µl of 0.2 N NaOH.

Extraction of DNA on blood

Extraction was performed by heating the resuspended pellet at 110°C for 20 min, followed by centrifugation at 500 *g* for 3 min. Neutralization was not attempted. The final product was stored at -20°C for subsequent PCR.

Enzyme-Linked Immunosorbent Assay

Serotesting of samples was done by use of the ELISA developed by Allied laboratories Inc. (Ames, Iowa, USA) with crude, soluble *M. paratuberculosis* strain 18 protoplasmic antigen (Allied Monitor Missouri, MO, USA), based on the protocol of Braun et al. (1990). Antigen was diluted to a concentration of 0.1 mg/ml in 0.05M sodium carbonate buffer at pH 9.6. This dilution (100 µl per well) was incubated over night at 4ºC. A suspension of *Mycobacterium phlei* was prepared by adding 5 g of dry, heat-killed *M. phlei* to 1 L of phosphate buffered saline solution containing 1% gelatin and 0.05% Tween 80 (PBS-TG). Three ml of this base suspension were added to 97 ml of 0.85% NaCl solution for use. Test sera (200 µl) including positive and

negative controls were pre-absorbed over night with this suspension (200 µl) to reduce nonspecific reactions. Samples were centrifuged at 600 *g* for 10 min and 20 µl of supernatant were added to 1 ml of PBS-TG. The sensitized plates were washed 3 times with a 0.85% saline solution containing 0.05% Tween 80, allowing 3 minutes/wash. Diluted samples (100 µl) were added to three wells followed by incubation at room temperature (2 h). The wells were then washed 3 times with PBSS-TG as before. Horseradish peroxidase conjugated with antibovine IgG was diluted to 1:2,000 in PBS-TG. Diluted conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) (100 µl) was added to each well followed by incubation at room temperature (2 h). The wells were washed 3 times with PBS-TG and 100 µl substrate added to each well. The latter was prepared by adding 125 µl of a 40 mM solution of 2,2'-azino-bis(3-ethilbenzthiazolinesulfonic acid) and 100 µl of a 1M solution of hydrogen peroxidase to 25 ml of citrate buffer (10.5 g of citric acid monohydrate/l, adjusted to pH 4.0 with NaOH). ELISA results were calculated as ELISA ratios (ER) from wavelength readings (optical density, OD, at 405 nm) in triplicates as sample OD divided by a value equivalent to ¼ of the OD of the positive control. This value was typically in the range of 0.13 to 0.14. Results were recorded as negative (<1.5), suspicious (1.5 to 1.9), low positive (2.0 to 2.5), and high positive (>2.5) as reported previously (Buergelt and Williams, 2003, 2004).

Fecal Culture

Culture of MAP was accomplished using Herrold's egg-yolk method with sedimentation (modified from Whitlock and Rosenberger, 1990). Briefly, 2 grams of fecal matter were suspended in 35 mL of sterile distilled water and shaken for 30 minutes. After allowing the sample to precipitate for 30 minutes, the 5 top mL of supernatant were transferred into 25 ml of HPC/BHI broth and allowed to stand undisturbed overnight at 35-37^{o}C. Samples were

centrifuged for 30 min at 900 *g* at 10°C. The pellet was suspended in 1 mL antibiotic brew and incubated 12 hours at 35-37°C. An aliquot (0.25 ml) of sediment was distributed over each of four commercial medium slants (BD BBL™, Becton, Dickinson and Company, USA). Three of the slants contained mycobactin, and one was prepared without mycobactin. Results were evaluated weekly up to 16 weeks of incubation. Positive cultures were confirmed by PCR analysis (IS900) of the growing colonies, following the same protocol described for blood, milk and feces.

Extraction of DNA on Feces

A sub sample consisting of 0.25 g of feces was prepared for PCR analysis using a commercial kit (PowerSoil™ DNA Isolation Kit, MO BIO Laboratories Inc, USA) following the manufacturer's instructions.

Nested Polymerase Chain Reaction

After DNA extraction, 1 µl (milk, blood) and 5 µl (feces) of the previously described product was submitted for PCR. A commercial reaction mix (Hot Master Mix®, Eppendorf North America, Westbury, NY, USA) was used according to the manufacturer's specifications.

Samples were tested in a nested PCR. The first reaction was based on primers P90 and P91 which target a 413 bp sequence of IS*900* in MAP. The second reaction used 1 µl of amplified product and primers J1 and J2 which overlap and span a 333 bp region within the insertion sequence (Vary et al., 1990; Gwozdz et al., 1997; Buergelt and Williams, 2003). The protocol for the first stage PCR was 35 cycles at 94°C for 30 sec, at 58°C for 15 sec and at 72°C for 60 sec. The protocol for the second stage (nested reaction) consisted of 36 cycles of 30 sec at 94°C, 15 sec at 63°C and 60 sec at 72°C.

A volume of 10 μl of the PCR product was run on 1.5% agarose gel by electrophoresis in TAE running buffer (Continental Lab Products, CA, USA). Extracted DNA from an isolate previously obtained from a clinically affected cow confirmed at necropsy by histopathology and culture was used as positive control and sterile water was used as negative control for the PCR assay and included in each of the reactions.

Gel inspection was done using ultraviolet light and recorded with a computerized digital camera (UVP Transilluminator System).

Statistical Analysis

Results for the five diagnostic tests were analyzed to establish associations between all the possible combinations of test pairs. Maximum possible agreement beyond chance level, and Cohen's kappa coefficient were used as a measure of agreement between each pair of tests.

The following ranges were considered for interpretation of the kappa coefficient (Landis and Koch, 1977); poor agreement: less than 0.00; slight agreement: 0.00-0.20; fair agreement: 0.21 to 0.40; moderate agreement: 0.41 to 0.60; substantial agreement: 0.61 to 0.80; very good: 0.81 to 1.00.

Fisher's Exact Test was used to test whether there was any non-random association between both variables (tests results). This test was chosen because in some cases the 2x2 contingency tables were highly imbalanced (low values in the "+" cell for both tests). The right-sided probability value was used considering the alternative hypothesis of a positive association between both tests results (observations tending to lie in upper left and lower right cells of the 2x2 contingency table).

McNemar's Test was used to test significant differences in the proportion of positive results between each pair of tests, and crude odds ratios were estimated between pairs of tests to

determine the effect of a positive result in one test to the odds of a positive result in a different test.

Complementary sensitivity (CS) was estimated for each pair of tests as presented by Juste et al. (2005). For tests A and B, CS for test A corresponds to the ratio of the number of positive results only in test A (negative for B) to the total number of positive results in test B, expressed as a percentage. This variable represents the additional detection efficacy of one method over the other, assuming that both are highly specific (Sockett et al., 1992).

Data were analyzed using the SAS statistical package for Windows (SAS Systems for Windows Version 9.00, SAS Institute Inc. Cary, NC, USA) using the PROC FREQ, PROC GENMOD and the CHISQ EXACT procedure. Values of $P \leq 0.05$ were considered significant for all tests.

Results

A total of 61 animals (18.6%) in the overall population were positive in at least one of the five tests being analyzed. By herd, 26.8%, 18%, 19%, and 10% positive animals were detected in herds A, B, C and D, respectively. The ELISA produced the highest number of positive results (33 animals), followed by fecal PCR (27 animals), while the lowest number of positive results was for blood PCR (7 animals) (Table 3-1). A cross-classification of the number of positive animals for the five tests is presented in Table 3-2. The maximum possible agreement beyond chance level for all tests combinations is shown in Table 3-3, with values ranging from 5.3% (blood PCR:fecal culture and blood PCR:fecal PCR) to 16.6% (ELISA:fecal PCR).

Kappa coefficients for the agreement of results in test pairs ranged from -0.036 ±0.01 (poor level of agreement) to 0.39 ±0.10 (fair level of agreement), for ELISA:blood PCR and fecal culture:fecal PCR, respectively (Table 3-4). Fisher's Exact Test, applied to test the null

hypothesis of no association between outcomes in each pair of tests (10 possible combinations), resulted in no significant values for 5 of the possible combinations. On the other hand, significant association was found for the test pairs ELISA:fecal culture; ELISA:fecal PCR; milk PCR:fecal PCR, blood PCR:fecal PCR and fecal culture:fecal PCR (Table 3-5). Results for McNemar's Test for differences in proportion of positive results are presented in Table 3-5.

Estimations of the odds of a positive result in a test given a positive result in a different test are provided in Table 3-6. The odds ratios were greater than one for most of the combinations (9 out of 10 pairs), indicating that a positive result in one test increased the odds of a positive result in a different test. Estimated complementary sensitivity for each test, when combined with the results of a different test (20 combinations) is provided in Table 3-7. Estimated values indicate diverse magnitudes in the additional percentage of infected individuals detected when the complementary test is added. The highest value corresponded to ELISA when combined with blood PCR (471%).

Discussion

The total of cows that tested positive for MAP infection by herd ranged from 10 to 26.8%, when the five tests were interpreted in parallel. Since the cows were not selected by a formal random process, the proportion of reactors may not represent the true prevalence of infection in the herds studied. These values are probably affected by factors such as involuntary biases in the selection of tested animals, the sensitivity of the tests when used in different stages of the disease, and the application of different tests in parallel as was done in this work.

Considering the proportion of reactive animals by test, the greatest number of positive results corresponded to the only test in the study that is based on the host response to the infection (33 cows for ELISA), followed by fecal PCR (27 cows). It was not the purpose of this study to determine differences in sensitivity and specificity for the five tests under analysis, but

to establish the association in their respective outcomes. The lack of an *in vivo* gold standard makes the estimation of a test's accuracy beyond the scope of this study. However, the limitation in sensitivity of available diagnostic tests for the detection of subclinical stages of MAP infection was one of the challenges motivating this work.

Estimated agreements between ELISA and three other tests (milk and blood PCR and fecal culture) were low, including a negative value for the kappa coefficient for blood PCR. In rare situations, kappa can be negative demonstrating an agreement between results less than that expected by chance. In this case, because the value is very close to zero, it would simply indicate very low agreement. On the other hand, the kappa coefficient for agreement between ELISA and fecal PCR was fair (0.303±0.08), which could be an indication of higher sensitivity of fecal PCR when compared to fecal culture.

The ability of IS*900* PCR to detect MAP in milk has been analyzed in raw bulk tank milk and in individual cows. Pillai and Jayarao (2002) reported a detection limit for bulk tank milk of 10 to 100 CFU/ml of MAP, which is in agreement with values reported by Giese and Ahrens (2000) for cows exhibiting clinical signs. In the same study (Pillai and Jayarao, 2002), MAP was detected in 4% of pooled quarter milk samples by culture and 33% by IS*900* PCR, from individuals in infected herds. According to these authors, the variation in the detection ability of these tests for low MAP concentrations could be due to loss of some organisms in the cream fraction after centrifugation of milk. This could also be the explanation for some of our PCR negative results in cows testing positive by other tests. Our results, indicating poor association between ELISA and blood PCR, are in agreement with a previous study (Juste et al., 2005) that reported kappa values for the association between these two tests of -0.36, 0.44 and -0.166 for cows, heifers and both, respectively. One possible interpretation is that each method detects

different populations or stages of MAP infection because their respective targets (bacteria and antibodies) do not have parallel dynamics. Two tests with relatively high specificities might have a low kappa value because they detect different populations of infected animals and this could be the explanation for low kappa values reported in the present study.

The low percentage of positive blood and milk PCR results compared to ELISA positives in the present study differs from previous results (Buergelt and Williams, 2004). In the referred study, working with clinically and subclinically infected dairy cows, the combination of blood and milk PCR was able to detect animals at least to the same level of detection as the ELISA. In that study, while the number of clinically infected animals detected was similar with PCR on blood and milk or ELISA methodologies, subclinically infected animals gave positive PCR signals in subgroups when the ELISA reading was negative or suspicious. This finding was interpreted to be a likely indicator of early infection. If the association between PCR and ELISA results shows that each method is detecting different stages of infection, the complementary use of both tests could result in an increase of the overall sensitivity.

Considering the fact that the immune response to MAP infection is characterized by an initial cell-mediated response followed by humoral immunity, with the former fading during the course of the disease, the efficacy of ELISA in a population would depend on the prevalence of individual animals among each stage of disease (Whitlock and Buergelt, 1996). After initial MAP infection, most animals begin to develop a type 1-like T-cell response (Th1), characterized by release of pro-inflammatory cytokines such as gamma interferon, as a key factor in controlling mycobacterial infections (Coussens, 2004; Coussens et al., 2004a). Following a variable period post infection, the host immune system shifts to a Th2-like response, that elicits

non-protective immunoglobulin G_1 antibody production from B cells (Coussens et al., 2002; Whittington and Sergeant, 2001).

The later (clinical) stages of bovine paratuberculosis are characterized by a predominance of Th2 activity, with high antibody levels, large numbers of bacilli and diminished cellular responses to specific and non-specific antigens (Chiodini and Davis, 1993; Bassey and Collins, 1997). According to this immunologic dynamics, sensitivity of tests measuring MAP-specific antibodies in the first subclinical stages would be relatively low. ELISA sensitivity has been estimated at 9 to 17% in subclinically infected animals shedding low numbers of MAP detectable by fecal culture (Jubb et al., 2004; Whitlock et al., 2000). Values ranging from 15% to 88% are reported as the disease progresses to clinical stages with increasing levels of fecal shedding (Dargatz et al., 2001; Sweeney et al., 1995).

Fecal culture, which is considered as the most specific test for paratuberculosis diagnosis, showed strong discrepancies with some of the other tests in this study (kappa coefficient: -0.027 to 0.39, Table 3-4). One of the main limitations of this type of test is its moderate sensitivity that in general has been reported ranging from 30% to 55%, again depending on the stage of infection (Whitlock and Buergelt, 1996; Buergelt and Williams, 2004). Whitlock et al. (2000) followed a cohort of 954 cattle cultured every 6 months over a period of 4 years, and reported a sensitivity of 38% for fecal culture. Eamens et al. (2000) using five different fecal culture methods reported sensitivities from 26% to 89%. Research on subclinically infected cows showed the existence of daily variation in fecal shedding determined by conventional fecal culture techniques (Barrington et al., 2003). These results suggest caution when interpreting negative results from a single fecal culture. Cows in the first stages of infection would intermittently shed low numbers of the

bacteria in feces, and only 15-25% of these animals could be detected by fecal culture on a single test (Buergelt and Williams, 2004).

In the present study some variation in levels of fecal shedding is suspected given the fact that only 11 fecal cultures were positive. This could also be explained by low sensitivity of the method used for culture or a low rate of animals in advanced stages of the infection. Our values for the association between ELISA and fecal culture are in agreement with data presented by other authors, where results from cows concomitantly tested by both tests showed low agreement (Sweeney et al., 1995; Nielsen et al., 2002b; Muskens et al., 2003a).

In our study, fecal culture also exhibited poor agreement and lack of association with PCR on milk and blood. MAP has been reported in different organs and tissues such as blood, milk, semen, lymph nodes and fetuses (Seitz et al., 1989; Buergelt and Williams, 2003; Buergelt et al., 2004; Juste et al., 2005). This suggests that intermittent bacteremia occurs and readily obtainable samples as blood and milk could be used to examine for the presence of the bacteria. However, at the present, there is insufficient information regarding the occurrence and duration of these events in the course of the infection. Further analysis should determine the likelihood of intermittent haematogenous spread of MAP and the variation in presentation of the agent in milk.

Our data is not sufficient to clarify if the lack of agreement between fecal culture and the other tests was due to low sensitivity of this method or to low specificity (false positives) of the other four tests in the study. In the overall population, only 3.4% of animals tested positive for fecal culture. As could be expected, the agreement between the results of fecal culture and fecal PCR was the highest (kappa coefficient=0.39) among all tests combinations. Our estimation is in agreement with results of an earlier study yielding kappa values ranging from 0.38–0.45 for three different fecal PCR methods when compared to fecal culture (Taddei et al., 2004). A plausible

explanation would be that both tests have a similar target, and the detection differences only depend on each test's sensitivity in detecting the presence of MAP. In this regard, the higher proportion of reactors in fecal PCR would suggest a higher sensitivity when compared to fecal culture, which requires viable bacteria in numbers of 10^2 CFU/g of faeces (Halldórsdóttir et al., 2002). Of the currently available methods for detection of MAP, PCR-based assays have the highest potential analytic sensitivity. Equally important to a test's analytic sensitivity is the sample that is to be tested. Especially crucial is the ability of the sample to have a high likelihood of containing MAP (or leucocytes infected with the agent) in early-stage animals, and to be devoid of factors that inhibit PCR, such as those found in feces (Barrington et al., 2003). The sensitivity of PCR is difficult to determine because PCR has higher analytical sensitivity than most existing tests (Kelly et al., 2005). The diagnostic sensitivity of a nested PCR for fecal, blood, milk, and liver samples in cows with advanced subclinical paratuberculosis was found to be 87%, 40%, 96% and 93%, respectively (Barrington et al., 2003) but sensitivity in early stage disease is unknown.

The concept of complementary sensitivity (CS) applied in this study, as previously reported (Juste et al., 2005), seems a useful tool when a gold standard is not available in practical terms. Complementary sensitivity provides a measure of the efficiency of combining two methods with high specificity to increase sensitivity in MAP detection. In this case, the requisite for specificity is accomplished with high values for ELISA and PCR reported by several studies (Sockett et al., 1992; Sweeney et al., 1995; Whitlock et al., 2000; Buergelt and Williams, 2004), although some caution is suggested by those studies reporting the presence of IS*900* in mycobacteria other than MAP (Cousins et al., 1999). The CS values for the tests ranged from 4 to 471% (Table 3-7), indicating, in some cases, a significant improvement in the percentage of

infected cows detected when two different tests were combined. These results, combined with considerations such as sensitivity, specificity and cost of particular tests, provide antecedents for the application of test combinations on field. In our study ELISA showed the highest CS values for all the possible two tests combinations, followed by fecal PCR. Considering that both tests detected the highest number of infected animals, this appears to be the best combination for diagnostic applications. The reported high specificity of all the tests analyzed in this study was an assumption in the estimation of CS. However, there is no absolute certainty that extra positive animals detected for one method over the other are not actually false positives. Recently some studies have presented evidence of the contribution of environmental mycobacteria to false-positive results in a commercially available serum ELISA kit (Osterstock et al., 2007). Accordingly, the main limitation for our study was the lack of a practical gold standard to determine the real status and stage of infection of the animals in the study.

In conclusion, the low agreement and the lack of association between results in most of the tests presented, together with the CS values estimated in this study, provide information for the possible use of different test combinations in the detection of different stages of infection. According to our results, the combined use of ELISA and fecal PCR has the potential to increase the overall sensitivity for the diagnosis of paratuberculosis infection.

Table 3-1. Number and proportion of positive results for ELISA, nested PCR on milk, blood and feces, and for fecal culture among 328 dairy cattle in four herds.

	Herd A (%)	Herd B (%)	Herd C (%)	Herd D (%)	Total (%)
ELISA	7 (12.5)	13 (10.6)	12 (12.0)	1 (2.0)	33 (10.0)
Milk PCR	4 (7.1)	5 (4.0)	4 (4.0)	0 (0.0)	13 (3.9)
Blood PCR	2 (3.5)	1 (0.8)	3 (3.0)	1 (2.0)	7 (2.1)
Fecal culture	0 (0.0)	7 (5.7)	1 (1.0)	3 (6.0)	11 (3.4)
Fecal PCR	4 (7.1)	12 (9.8)	7 (7.0)	4 (8.0)	27 (8.2)
All tests in parallel	15 (26.8)	22 (18.0)	19 (19.0)	5 (10.0)	61 (18.6)

Table 3-2. Cross classification of number of positive results for the five tests (above the diagonal). The diagonal shows the number of positive animals for each test (n=328)

	ELISA	Milk PCR	Blood PCR	Fecal culture	Fecal PCR
ELISA	33	2	0	3	11
Milk PCR		13	1	1	4
Blood PCR			7	0	6
Fecal culture				11	8
Fecal PCR					27

Table 3-3. Maximum possible agreement beyond chance for each combination of test pairs (%).

	ELISA	Milk PCR	Blood PCR	Fecal culture	Fecal PCR
ELISA		13.2	11.8	12.7	16.6
Milk PCR			5.9	7.1	11.5
Blood PCR				5.3	5.3
Fecal culture					11.0
Fecal PCR					

Table 3-4. Kappa coefficient ± asymmetric standard error (above diagonal) and agreement interpretation (below diagonal) for each combination of test pairs.

	ELISA	Milk PCR	Blood PCR	Fecal culture	Fecal PCR
ELISA		0.032 ± 0.05	-0.036 ± 0.01	0.138 ± 0.07	0.303 ± 0.08
Milk PCR	slight		0.074 ± 0.09	0.048 ± 0.08	0.154 ± 0.08
Blood PCR	poor	slight		-0.027 ± 0.01	0.330 ± 0.10
Fecal culture	slight	slight	poor		0.390 ± 0.10
Fecal PCR	fair	slight	fair	fair	

Table 3-5. Right-sided P ≥ F for Fisher's Exact Test for each combination of test pairs (above the diagonal). P ≥ S for McNemar's Test for each combination of test pairs (bellow the diagonal).

	ELISA	Milk PCR	Blood PCR	Fecal culture	Fecal PCR
ELISA		0.38	1.00	0.017	< 0.001
Milk PCR	0.002		0.24	0.36	0.016
Blood PCR	<0.0001	0.15		1.00	< 0.001
Fecal culture	0.0002	0.67	0.34		< 0.001
Fecal PCR	0.33	0.01	<0.0001	0.0006	

For Fisher Exact Test values equal to or less than 0.05 indicate significant association between test results. For McNemar's Test values equal to or less than 0.05 indicate significant difference in the proportion of positive results between test pairs.

Table 3-6. Odds ratios (95% CI) for positive results in pairs of tests.

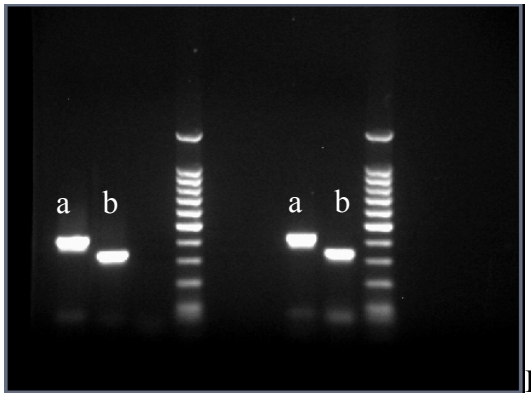
ELISA	Milk PCR	Blood PCR	Fecal culture	Fecal PCR
ELISA	1.66 (0.35-7.8)	0.57 (0.03-10.2)	5.67 (1.56-20.54) †	8.65 (3.5 – 20.9) †
Milk PCR		4.29 (0.47-38.5)	2.54 (0.3-21.49)	5.60 (1.6-19.5) †
Blood PCR			1.80 (0.09-33.46)	85.14 (9.8-740) †
Fecal culture				41.54 (10.1-169) †
Fecal PCR				

† Significant at 95% confidence level.

Table 3-7. Complementary sensitivity (CS) for each test when combined with a different test (%).

	ELISA	Milk PCR	Blood PCR	Fecal culture	Fecal PCR
ELISA		33	21	21	48
Milk PCR	238		46	77	177
Blood PCR	471	171		157	300
Fecal culture	264	109	64		173
Fecal PCR	81	33	4	11	

Presented values correspond to CS of the test indicated at the top of each column when combined with the corresponding test indicated at the left of each row.


A


B

Figure 3-1. Diagnostic tests. A) MAP colonies in positive samples for fecal culture. B) Positive PCR results for standard (a) and nested (b) PCR.

CHAPTER 4
Mycobacterium paratuberculosis SHEDDING INTO MILK: ASSOCIATION OF ELISA SEROREACTIVITY WITH DNA DETECTION IN MILK

Summary

The objective of this study was to analyze the association between ELISA seroreactivity and *Mycobacterium avium* subspecies *paratuberculosis* DNA presence in bovine milk as detected by nested PCR. An irregular pattern of detection was observed for milk PCR outcomes along with fluctuations in serial ELISA results. Cows testing positive by milk PCR had negative and inconclusive ELISA results in 23.5% and 11.8% of the cases, respectively. A kappa coefficient of 0.012 indicated a slight agreement between both tests; Fisher's Exact Test did not indicate a significant association between test outcomes ($p = 0.55$). Ability of serum ELISA as indicator of the likelihood of milk shedding of *Mycobacterium paratuberculosis* in dairy cows is questionable.

Introduction

Mycobacterium avium subspecies *paratuberculosis* is the cause of a chronic granulomatous intestinal disease (Johne's disease) in ruminants, characterized by progressive weight loss and profuse diarrhea (Whitlock and Buergelt, 1996). Paratuberculosis represents a significant problem for the dairy industry, and one of the main issues relates to the efficiency of subclinical diagnosis.

MAP isolation from milk was first reported in 1935, in association with advanced clinical paratuberculosis (Taylor et al., 1981). More recent studies have found MAP isolation rates in milk of up to 45% in clinically affected animals (Giese and Ahrens, 2000) and of up to 22% in colostrum or 8% in milk in subclinical cases (Streeter et al., 1995).

Reprinted with permission from Pinedo, P.J., Buergelt, C.D., Monif, R.G., Williams, J.E., Rae, O. *Mycobacterium avium* subspecies *paratuberculosis* shedding into milk: Correlation of Serum ELISA Titers with DNA Detection in Milk. J Appl Res Vet Med. In press.

The concern about MAP in milk and milk products centers on its apparent heat resistance and in the controversial role that the bacteria could play in Crohn's disease in human (Naser et al., 2004; Grant et al., 2005). In testing units of whole pasteurized milk from retail outlets throughout central and southern England, it was found that, over three month periods, up to 25% of commercial units sampled were affected by the presence of MAP DNA (Millar et al., 1996). In a study in the Czech Republic, MAP was cultured from 1.6% of commercially pasteurized retail milk (Ayele et al., 2005). A United States' study found viable MAP in 2.8% of milk samples taken from grocery stores in three states (Ellingson et al., 2005).

ELISA testing for MAP has been used as a herd tool from which producers could make management decisions (Whitlock et al., 2000). Using nested PCR, Buergelt and Williams (2004), showed a positive correlation between high MAP ELISA readings in blood and increased probability of detection of MAP DNA in milk of clinical cows. However, a clear association was not found when comparing a sub group of subclinically infected animals. These latter findings bring into question whether detection of subclinical infection by ELISA is an effective tool for identification of cows shedding MAP into milk as a first step of protecting the food chain.

The purpose of this report was to analyze the association between ELISA seroreactivity and the presence of MAP DNA in milk based upon a nested PCR. The information reported here is retrospective and based on data from dairy cows tested concurrently by serum ELISA and milk PCR, or with prolonged serial observations to detect MAP DNA in milk.

Materials and Methods

Study Population

Blood and milk samples were derived from cows belonging to the University of Florida's Dairy Research herd (USA), composed of 500 Holstein cows, and known to be infected with MAP. As a routine, cows were tested for MAP by serum ELISA annually and, in some cases,

milk samples were obtained for PCR analysis. Research data on 98 adult cows, tested during 2003 and 2004, were selected on the sole basis of their having had a PCR analysis for MAP in milk concurrent with the routine ELISA test. No formal randomization in the selection of animals was attempted and the samples analyzed do not represent a particular status of paratuberculosis infection. Thirteen animals were considered for the serial analysis. One particular animal (cow Id#3900) was successively tested for ELISA and milk and blood PCR for about nine months and finally submitted for necropsy. All the procedures involving animal handling were in agreement with the animal care protocols of the University of Florida.

Sample Handling, Milk Samples

Before collection, the teats were thoughtfully cleansed with alcohol to avoid sample contamination from skin. Milk (30-40 ml) was collected in a sterile 50 ml centrifuge tube from the four quarters by hand milking, discarding the first 10-15 ml. The milk samples were centrifuged at 1,000 *g* for 15 min and the supernatant discarded. The resultant pellet was washed thrice in phosphate buffered saline (PBS, pH 7.3) and centrifuged at 500 *g* for 15 min. The pellet was resuspended in 1 ml of PBS, centrifuged and resuspended in 100 µl of 0.2 N NaOH. After boiling at 110^{o}C for 20 min to extract DNA, the material was centrifuged at 500 *g* for 3 min. The final product was stored at -20^{o}C for subsequent PCR.

Blood Samples

After cleansing with alcohol, 10 ml of blood per cow were collected from the coccygeal vein into Vacutainer® tubes (Becton Dickinson, NJ, USA) with and without EDTA. For the blood PCR procedure (cow Id#3900), 3 ml of EDTA blood was added to 4 ml of Ficoll-Isopaque® Plus Gradient (Amersham Pharmacia, NJ, USA) and centrifuged for 40 min at 500 *g* at 18^{o}C. The buffy coat was collected, then washed twice with PBS, and centrifuged at 500 *g* for 15 min. Cells from the pellet were resuspended in 100 µl of 0.2 N NaOH, boiled at 110^{o}C

for 20 min to extract DNA, and centrifuged at 500 *g* for 3 min. The final product was stored at -20^{o}C for subsequent PCR.

Nested Polymerase Chain Reaction (PCR)

After DNA extraction, 1 µl of the previously described product (milk and blood) was submitted for PCR. A commercial reaction mix (Hot Master Mix®, Eppendorf North America, NY, USA) was used according to the company's specification. Samples were tested with primers P90, P91 for IS*900*, which specifically recognize a 413 bp sequence of MAP. The reaction was followed by the nested PCR, where 1ul of previously amplified product was tested with second set of primers J1, J2 overlapping and spanning a 333 base pair region within the insertion sequence (Vary et al., 1990; Gwozdz et al., 1997; Buergelt and Williams, 2004).

A volume of 10 µl of the PCR product was run on 1.5% Agarose gel by electrophoresis in TAE running buffer (Continental Lab Products, CA, USA). Extracted DNA from the laboratory strain #295 was used as positive control and sterile water was used as negative control for the PCR assay. Gel inspection was done using ultraviolet light and recorded with a computerized digital camera (UVP Transilluminator System). Positive and negative controls were used in each of the reactions.

Enzyme-Linked Immunosorbent Assay

Serotesting of samples was done by use of the ELISA developed by Allied laboratories Inc. (Ames, Iowa, USA) with crude, soluble *M. paratuberculosis* 18 protoplasmic antigen (Allied Monitor Missouri, MO, USA), based on a previously documented protocol (Braun et al., 1990). Antigen was diluted to a concentration of 0.1 mg/ml in 0.05M sodium carbonate buffer at pH 9.6. This dilution (100 µl per well) was incubated over night at 4ºC. A suspension of *Mycobacterium phlei* was prepared by adding 5 g of dry, heat-killed *M. phlei* to 1 L of phosphate

buffered saline solution containing 1% gelatin and 0.05% Tween 80 (PBS-TG). Three ml of this base solution were added to 97 ml of 0.85% NaCl solution for use. Test sera (200 µl) including positive and negative controls were pre-absorbed over night with this suspension (200 µl) to reduce nonspecific reactions. Samples were centrifuged at 2,000 rpm for 10 min and 20 µl of supernatant were added to 1 ml of PBS-TG. The sensitized plates were washed 3 times with a 0.85% saline solution containing 0.05% Tween 80, allowing 3 minutes/wash. Diluted samples (100 µl) were added to three wells followed by incubation at room temperature (2 h). The wells were then emptied and washed 3 times with PBSS-TG as before. Horseradish peroxidase conjugated with antibovine IgG was diluted to 1:2,000 in PBS-TG. Diluted conjugate (100 µl) was added to each well followed by incubation at room temperature (2 h). The wells were then emptied and washed 3 times with PBS-TG as before. Substrate was prepared by adding 125 µl of a 40 mM solution of 2,2'-azino-bis(3-ethilbenzthiazolinesulfonic acid) and 100 ml of a 1M solution of hydrogen peroxidase to 25 ml of citrate buffer (10.5 g of citric acid monohydrate/l). Substrate (100 µl) was added to each well. ELISA results were calculated as ELISA ratios (ER) from wavelength readings (optical density, OD, at 405 nm) in triplicates as sample OD divided by a value equivalent to ¼ of the OD of the positive control (Bech-Nielsen et al., 1992). This value was typically in the range of 0.13 to 0.14. This value was typically in the range of 0.13 to 0.14. Results were recorded as negative (<1.5), suspicious (1.5 to 1.9), low positive (2.0 to 2.5), and high positive (>2.5) as reported previously (Buergelt and Williams, 2004).

Analysis

Results are presented in tables to demonstrate the association between the different tests. For the statistical analysis kappa coefficient was used as a measure of agreement between the two tests. The following ranges were considered for interpretation of the kappa coefficient; poor

agreement: less than 0.00; slight agreement: 0.00-0.20; fair agreement: 0.21-0.40; moderate agreement: 0.41-0.60; substantial agreement: 0.61-0.80; almost perfect: 0.80-1.00 (Landis and Koch, 1977).

Fisher's Exact Test was used to test whether there was a non-random association between either variable (respective tests results). For both methods of analysis, inconclusive results (suspicious category) for the ELISA test were not considered and ELISA categories "strong" and "low positive" were deemed as one group (positive).

Data were analyzed using the PROC FREQ procedure of the SAS statistical package for Windows (SAS Systems for Windows® Version 9.00, SAS Institute Inc., NC, USA).

Results

Cows tested by serum ELISA and the nested PCR in milk were grouped by ELISA categories in Table 4-1. For ELISA categories negative and high positive 16.6% and 77.5% of the results were in disagreement with PCR test respectively. A total of 23.5%, and 11.8% of the individuals that had evidence of MAP DNA in milk were negative or inconclusive for ELISA outcomes respectively (6 animals).

Results for ELISA and milk PCR from 83 cows were the basis for the statistical analysis and are presented in a two by two contingency table (Table 4-2).

There was agreement between ELISA and milk PCR in 31 of the 83 animals (37.3%) included in the analysis. Four cows were positive for PCR, but negative for ELISA and 48 were positive for ELISA but PCR negative. The kappa coefficient (±1.96 asymmetric standard error) for the association of both tests was 0.012 (±0.059) which is a slight level of agreement.

Fisher's Exact Test did not resulted in significant values ($p = 0.55$), indicating that there was not sufficient evidence to reject the null hypothesis of no association between test outcomes.

Serial samples taken for individual cows evidenced a variable pattern of MAP shedding into milk, measured as the presence of bacterial DNA by nested PCR. Table 4-3 presents serial results for a particular cow tested during 9 months (21 times), and confirmed as a clinical case by necropsy. An irregular pattern of detection can be observed for milk and blood PCR results, along with fluctuations in ELISA readings.

Serial results for serum ELISA and milk PCR in a different group of 5 cows with fluctuations in the milk shedding status are presented in Table 4-4. For this group, as shown for cow Id#3900, data suggest a poor association between detection of the bacteria in milk and serum ELISA results.

Cows that tested positive for milk DNA had a variable pattern for ELISA ODs over time. ELISA test results from 29 cows that tested positive for milk PCR are summarized in Table 4-5. The data suggests that ELISA seroreactivity may have a negative status despite the fact that the cow is shedding the bacteria in milk, as shown by PCR detection.

Cow Id#6142 was tested by milk PCR using samples taken from separate quarters on six different occasions. While milk from all four quarters was demonstrated to be positive on two instances, milk from three of the four quarters in a given test was negative on two occasions, and from two or one quarter in one sampling each (Table 4-6), indicating that not pooling milk from all four quarters increases the risk of obtaining a false-negative result for the animal.

Discussion

Serologic tests for MAP are most useful in establishing the herd prevalence infection, for presumptive identification of infected animals, and for confirming the diagnosis of JD in animals presenting compatible clinical signs (Nielsen et al., 2001).

ELISA testing has been advocated as a herd tool from which individual producers could make management decisions, though, the ELISA for MAP has the disadvantage of moderate to

low sensitivity in cows shedding low numbers of bacteria (Whitlock et al., 2000). There are multiple commercial MAP ELISA tests available and, despite the fact they are marketed as herd-level diagnostic tools, they are commonly used as cow level (McKenna et al., 2006). Considering that one of the aims of diagnostic tests in animal production is to help to control the introduction of potential pathogens into the human food chain, the ability of serologic tests as ELISA to detect individuals that are more likely to shed MAP into milk is crucial. However, from the data presented, it can be stated that a given ELISA outcome is not conclusive as to whether or not a given cow is shedding MAP into its milk (Table 4-1).

It has been suggested that the measurable humoral immune response to MAP in subclinical cows can vary widely over time, even from day to day (Barrington et al., 2003). This information is in agreement with our findings (Figure 2-2, Tables 4-3 and 4-4). It is suspected that this variation in ELISA results is due to fluctuation in antibody production, protein enteropathy, variable losses by way of the gastrointestinal tract, or a combination of these.

Further, strong discrepancies between different commercial ELISAs when performed concomitantly on the same animal were found by McKenna et al. (2006). In their study, the highest and lowest kappa coefficients for combinations of three different commercial ELISA tests were 0.33 and 0.18, which is fair and slight agreement, respectively.

MAP has been reported in different tissues and fluids such as blood, milk, semen, lymph nodes and fetuses suggesting that intermittent bacteremia occurs accompanied by dissemination of MAP to body fluids like milk (Taylor et al., 1981; Juste et al., 2005).

The ability of IS*900* PCR to detect MAP in milk has been analyzed in raw bulk tank milk and in individual cows. Pillai and Jayarao (2002) reported a detection limit for bulk tank milk of 10 to 100 CFU/ml of MAP, which is in agreement with values reported by Giese and Ahrens

(2000) for cows exhibiting clinical signs. In the same study, MAP was detected in 4% and 33% of pooled quarter milk samples, in individuals from infected herds, by culture and IS*900* PCR, respectively (Pillai and Jayarao, 2002). According to these authors, the variation in the detection ability for low MAP concentration could be due to loss of some organisms in the cream fraction after centrifugation of milk. This could also be the explanation for some of our PCR negative results in cows previously positive or exhibiting high ELISA values.

Because of the apparent intermittent pattern of MAP dissemination, shedding into milk may not be ascertained from a single milk sample. As Tables 4-3 and 4-4 evidence, MAP shedding appears to be irregular over an extended period of time and herd management decisions based upon a single analysis of milk can not rule out MAP shedding into milk at another point in time.

Poor agreement between ELISA results and bacterial DNA detection in blood was previously reported, with kappa values for serum ELISA vs. blood PCR results of -0.36, 0.44 and -0.166 for cows, heifers and the two combined, respectively, suggesting a poor to moderate agreement between tests (Juste et al., 2005). The interpretation offered is that each method detects different populations, or stages of MAP infection, because their respective targets might not have parallel dynamics. This explanation may apply to our results as well (Table 4-3), based on the possibility of different temporal patterns for the humoral immune response to MAP and the presence of MAP in milk. A higher number of individuals positive for MAP PCR detection in milk would be desirable in our study to determine, more accurately, the likelihood of having an accompanying negative result for the serum ELISA. One limitation of this study, because of the retrospective nature of the analysis, is that this condition was restricted to only 17 individuals.

An additional factor governing the presence or absence of MAP in milk is the means by which a sample is obtained. As demonstrated in Table 4-6, in order for a milk sample to be deemed adequate for analysis, the milk should be obtained from all four quarters (pooled sample).

Based on the results of this study, it is concluded that MAP shedding in milk, as detected by PCR, has a slight association with the concurrent ELISA seroreactivity; ability of serum ELISA as indicator of the likelihood of milk shedding of MAP in dairy cows is questionable.

Table 4-1. DNA detection of MAP in milk by nested PCR grouped by ELISA result categories (number and % of animals, n: 98 cows).

	ELISA ratio and interpretation				
	High positive	Low positive	Suspicious	Negative	Total
Milk PCR + (%)	9 (52.9)	2 (11.8)	2 (11.8)	4 (23.5)	17
Milk PCR -	31	17	13	20	81
Total	40	19	15	24	98

Table 4-2. ELISA results and DNA detection of MAP in milk by nested PCR.

	ELISA ratio and interpretation		
	Positive (≥ 2.0)	Negative (< 1.5)	Total
Milk PCR + (%)	11 (73.3)	4 (26.7)	15
Milk PCR -	48	20	68
Total	59	24	83

Inconclusive ELISA results were removed, and low and strong positive categories are presented as positive (number and % of animals).

Table 4-3. Results for 21 serial testing (9 months) in cow Id#3900. Milk and blood PCR results are given relevant to ELISA categories in concurrent testing.

	ELISA ratio and interpretation			
	Negative	Suspicious	Low positive	High positive
Milk PCR +	0	1	0	5
Milk PCR -	0	3	2	10
Blood PCR +	0	3	0	3
Blood PCR -	0	1	2	12

Table 4-4. Serial results for milk PCR, and serum ELISA in a group of five individuals.

Cow Id	Days from previous tests	Milk nested PCR	ELISA ratio[†]
3475	-	+	1.7
	14	-	4.6
3763	-	+	1.8
	2	+	1.5
	3	+	<1.5
	1	+	<1.5
	1	-	<1.5
	1	+	1.6
3838	-	+	5.6
	1	-	5.8
	1	+	3.6
	4	-	4.9
	1	+	4.9
	1	-	4.9
	1	-	4.9
3976	-	+	2.6
	24	+	<1.5
	13	+	<1.5
6044	-	+	<1.5
	27	-	1.7
	35	+	<1.5

[†]ELISA categories: negative (<1.5); suspicious (1.5 to 1.9); low positive (2.0 to 2.5); high positive (>2.5).

Table 4-5. Cows that were positive to MAP DNA by PCR detection in milk are grouped by their corresponding serum ELISA status.

ELISA Category	Number of test results[#]	ELISA ratio range
negative	7	0.5-1.4
suspicious	8	1.5-1.9
low positive	2	2.2-2.5
high positive	18	2.2-3.9

[#] Six animals with multiple testings.

Table 4-6. Serial results for cow Id#6142 tested by MAP PCR on individual quarter milk samples and concurrent serum ELISA.

	PCR on milk by quarter				
Sample date	RF	LF	RR	LR‡	ELISA ratio
9/24/2002	-	+	+	-	2.9
12/10/2002	-	+	-	-	1.5
12/30/2002	+	+	+	-	2.0
1/21/2003	+	+	+	+	2.6
1/28/2003	+	+	+	+	2.5
2/4/2003	n.a.*	-	-	-	2.3

* No milk was obtained from RF quarter. ‡RF= right front, LF= left front, RR= right rear, LR= left rear.

CHAPTER 5
ASSOCIATION BETWEEN *CARD15* GENE POLYMORPHISMS AND PARATUBERCULOSIS INFECTION IN FLORIDA CATTLE

Summary

Paratuberculosis represents a major problem in farmed ruminants and at the present is considered a potential zoonosis. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis*, and susceptibility to infection is suspected to have a genetic component. Caspase recruitment domain 15 *(CARD15)* gene encodes for a cytosolic protein implicated in bacterial recognition during innate immunity. Crohn's disease is an idiopathic inflammatory bowel disease in humans comparable in many features to bovine paratuberculosis involving an abnormal mucosal immune response. The association between mutations in the *CARD15* gene and increased risk of Crohn's disease has been described. The objective of this candidate gene case-control study was to characterize the distribution of three polymorphisms in the bovine *CARD15* gene and test their association with paratuberculosis infection in Florida cattle. Three previously reported single nucleotide polymorphisms (E2[-32] intron 1; 2197/C733R and 320/Q1007L) were screened for the study population (431 adult cows). The statistical analysis resulted in significant differences in allelic frequencies between cases and controls for SNP2197/C733R ($p < 0.001$), indicating a significant association between infection and variant allele. In the analysis of genotypes, a significant association was also found between SNP2197/C733R and infection status ($p < 0.0001$); cows with the heterozygous genotype were 3.35 times more likely to be infected than cows with the reference homozygous genotype ($p = 0.01$). Results suggest a role for CARD15 gene in the susceptibility of cattle to paratuberculosis infection. These data contribute to the understanding of paratuberculosis, suggest new similarities with Crohn's disease and provide new information for the control of bovine paratuberculosis.

Introduction

Paratuberculosis (Johne's disease) is a chronic, infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and characterized by progressive weight loss and profuse watery diarrhea (Chiodini et al., 1984). The disease has a worldwide distribution and is categorized by the Office International Des Epizooties as a list B disease, which is a serious economic or public health concern (OIE, 2004).

Crohn's disease (CD) is a chronic idiopathic inflammatory bowel disease in humans similar in many features to bovine paratuberculosis and involves an aberrant mucosal immune response in genetically susceptible individuals (National Research Council, 2003; Scanu et al., 2007). The association between mutations in the caspase recruitment domain 15 gene (*CARD15*, formerly *NOD2*) and increased risk of CD has been described in different geographical populations (Hugot et al., 1996, 2001; Ogura et al., 2001a; Hugot 2006).

CARD15 is an intracellular element responsible for the indirect recognition of bacterial peptidoglycan through the binding of muramyl dipeptide, a component of both Gram negative and positive bacterial cell walls in monocytes, macrophages, dendritic cells, and intestinal epithelial cells, where it is mainly expressed (Ogura et al., 2001). Structurally, CARD15 is composed of three segments: NH2-terminal caspase recruitment domains (two CARD units), a nucleotide-binding domain (central portion), and finally, a leucine-rich repeat (LRR) region as is found in toll-like receptors (Hugot 2006; Lakatos et al., 2006).

Three main mutations of *CARD15* have been found to be associated with an increased risk of CD. These mutations occur in the LRR domain or in its vicinity, suggesting an alteration in the recognition of the bacterial components (Lesage et al., 2002). Research suggests that the CD-associated mutations result in a "loss of function" phenotype, however, variant CARD15 proteins apparently present inflammation-promoting functions. A possible explanation suggested that

CARD15 is defective in performing critical functions required for limiting inflammation (loss-of-function) and the variant proteins directly activate pro-inflammatory signaling pathways (gain-of-function) (Zelinkova et al., 2005).

Johne's disease is considered a potential zoonosis. A role for MAP, the causal agent of JD, in the etiology of CD has been repeatedly suggested and, while an association between MAP and CD has been documented in diverse studies (Sechi et al., 2005, 2005a; Scanu et al., 2007), a causal link has not been proven or shown (Feller et al., 2007).

Genetic factors have also been associated with differences in susceptibility to bovine paratuberculosis, and estimations indicate a range of moderate values for heritability of infection (Koets et al. 2000; Elzo et al., 2006; Gonda et al., 2006a). The function of *CARD15* in the coordination of immunity against bacteria, the similarities shared by JD in cattle and CD in human, and the controversial role of MAP in this disease make this gene a good candidate to test the role of genetics in the susceptibility to bovine paratuberculosis.

The central hypothesis of this study was that a combination of particular alleles in the candidate gene would be present in higher frequency in case individuals compared to controls, suggesting a role in susceptibility to infection. The objective of this candidate gene case-control study was to characterize the distribution of three polymorphisms in the bovine *CARD15* gene and test their association with paratuberculosis infection in Florida dairy and beef cattle.

Materials and Methods

Study Population

The study sample size was estimated based on an exposure rate for the controls of 15% for the allele related to higher susceptibility. An odds ratio value of 2.0 was established as a threshold for significance, with a ratio for cases and controls of 1:2 (power=80%,

confidence=95%). Based on these parameters, the required number of cases and controls was 119 and 239 cows respectively for a one-tailed analysis (Win Episcope 2.0).

Consequently, 431 adult cows, consisting of 299 Holstein, 50 Jersey and 82 Brahman-Angus crosses were selected for this study. The population was recruited from three Holstein and one Jersey dairy herds, and one Brahman-Angus cow-calf herd near Gainesville, Florida, USA. The five herds had a past history of clinical paratuberculosis cases, with some individuals confirmed by necropsy examination (submitted to the College of Veterinary Medicine, University of Florida).

A case-control design was used based on the infection status of the animals following multiple tests. MAP infection was determined by parallel interpretation of five diagnostic tests (serum ELISA, milk PCR, blood PCR, fecal PCR and fecal culture) or by necropsy examination (gross pathology, histopathology and PCR on tissues). The infection status of 402 cows was established by using the *in vivo* tests, whereas 29 cows were evaluated by necropsy.

In determining the case-control sub-populations, a case was defined as an animal being positive for any of the tests, considered in parallel, and a control was defined as an individual negative to all the tests that it was subjected to. As a result, the final population consisted of 126 cases and 305 controls (1:2.4 ratio).

Diagnosis

Refer to chapter 3 for details in diagnostic procedures.

Genotyping

Extraction of DNA

DNA from the study population was extracted from whole blood or tissues using the QIAamp DNA Blood Mini Kit (Qiagen Inc., CA, USA), according to the manufacturer's directions and stored at -20^{o}C before use.

Allele determination

Three previously reported single nucleotide polymorphisms (SNPs) within the bovine *CARD15* gene were tested (designated as SNP_0, SNP_1, and SNP_2) (Taylor et al., 2006). SNP_0 [SNP E2 (-32) G>A] is located in the intron 1, positioned 32 bp upstream of the first base of the second coding exon, and is considered a putative regulatory region identified by homology across seven animal species (Taylor et al., 2006). SNP_1 [2197, T>C (C733R)] is situated in the leucine-rich repeat domain and is responsible for an amino acid substitution (cysteine to arginine). SNP_2 [3020 A>T (Q1007L)] is located outside the last domain and is also responsible for an amino acid substitution (glutamine to leucine) (Taylor et al., 2006).

The bovine *CARD15* gene was genotyped by using the fluorescence-based TaqMan® allelic discrimination genotyping method (Applied Biosystems, Foster City, USA), via the Applied Biosystems 7900 HT SNP genotyping platform. Five µl reactions in 384-well plate were prepared using Eppendorf epMotion 5070 (Eppendorf North America, Inc., Westbury, NY, USA), and Packard Multi Probe II HT Systems (PerkinElmer Las, Inc., Shelton, CT, USA), liquid handling/sample processing robotics and the assays were performed and analyzed according to the manufacture's recommendations. All the genotyping was performed in the laboratories of the University of Florida Center for Pharmacogenomics.

Statistical Analysis

Genotype frequencies were tested for departure from Hardy-Weinberg equilibrium (HWE) by using Fisher Exact Test and Chi-square test with one degree of freedom. Both tests were also used to check whether there was any non-random association between allele or genotype frequencies versus infection status of the cows. The analysis was performed for each SNP and combination of polymorphisms.

The univariable analysis for logistic regression considered the infection status as response variable and included 5 possible explanatory variables; SNPs, breed, herd and age. Multivariable logistic regression models were proposed to estimate the odds of infection for "susceptibility allele" carriers compared to non allele carriers, controlling for possible confounding variables. The models considered the infection status as response variable (yes/no), and included genotypes (based on alleles provided by different SNP's), breed, herd and age as explanatory variables. Genotypes were analyzed as ordinal variables [major homozygous (0); heterozygous (1); minor homozygous (2)] and as class variables with the major homozygous genotype deemed as baseline. Breed and herd were considered as class variables, and age (months) was considered as an ordinal variable with three levels demarcated by the mean ± one standard deviation.

The complete model was tested by backward elimination procedure to determine the variables to be included in the final model based on the deviance value.

Data were analyzed using the SAS statistical package for Windows (SAS Systems for Windows Version 9.00, SAS Institute Inc. Cary, NC, USA) using the PROC FREQ, PROC GENMOD and the CHISQ EXACT procedure. Values of $P \leq 0.05$ were considered significant for all tests.

Results

The resultant ratio of cases to controls was 1:2.4 (126 cases vs. 305 controls) with an average age for cases and controls of 64 and 65 months, respectively ($p = 0.7$). No variation was found in the population for SNP_0, with all the population containing the G allele, and consequently, this SNP was removed from the analysis. From a total of 431 individuals submitted for the genotyping analysis, 5 and 4 samples failed to provide a result for SNP_1 and SNP_2 respectively.

Frequencies for the major allele in SNP_1 (A_1) and SNP_2 (B_1) were 0.94 and 0.54, respectively (variant alleles for SNP_1 and SNP_2 were denominated A_2 and B_2, respectively). Values for the coefficient of linkage disequilibrium (LD), normalized LD, and correlation between the two SNPs were -0.027, 1.0, and -0.23, respectively, and Chi-square test indicated that SNP_1 and SNP_2 were in linkage disequilibrium ($p < 0.001$). The same analysis, performed for each herd separately, indicated also that both polymorphisms were in linkage disequilibrium. The genotype frequencies of SNP_2 did not deviate from HWE; however the distributions of SNP_1 did not fit HWE in our control population.

The statistical analysis resulted in significant differences in allelic frequencies between cases and controls for SNP_1 ($p < 0.0001$) indicating an association between infection and variant allele. The frequency for the variant allele was 11.4% and 3.8% in cases and controls, respectively ($p = 0.002$). However, no association was found between SNP2 and infection status ($p = 0.10$) (Table 5-1). In the analysis of genotypes, a significant association was only found between SNP1 and infection status ($p < 0.0001$) (Table 5-2). A significant association between allele combinations and infection status was found ($p < 0.0001$) when both SNPs were considered simultaneously in the genotype (Table 5-3).

This connection was also tested within the Brahman-Angus subpopulation, where the highest proportion of the variant allele was found, resulting in a significant value for Fisher's Exact Test ($p = 0.02$) and, consequently, supporting the role of this polymorphism in susceptibility to infection.

Estimated odd ratios (OR) for the univariable logistic regression models are presented in Table 5-4. The models tested the effect of each of the explanatory variables on the individual animal odds of paratuberculosis infection (dichotomous variable; yes/no). The analysis indicated

significant ORs for SNP1 when considered as ordinal ($p = 0.0009$), and class variable [genotype A_1A_2 referred to the major genotype A_1A_1 ($p < 0.0001$)]. Genotype A_2A_2, however, resulted in a not significant OR ($p = 0.37$) when the variable was considered as class. Breed effect was significant for all the classes. No statistical significance was found for variables SNP_2 and age.

Different models for multivariable logistic regression were tested in the aim of controlling for potential confounding variables such as breed, herd, and age. The intension was, consequently, to isolate the effect of our variables of interest (SNP_1 and SNP_2). The final model for multivariable logistic regression is presented in Table 5-5. The model includes SNP_1 genotype (class) as an explanatory variable of interest, controlling for breed and age. The analysis indicated that, after controlling for breed and age, the effect of SNP_1 was significant, and the estimated odds of infection for cows with the A_1A_2 genotype were 3.35 times the estimated odds of cows with the major (A_1A_1) genotype ($p = 0.01$).

Discussion

Genetic factors have long been suspected in association with susceptibility and resistance to mycobacterial infection, including bovine paratuberculosis (Abel and Casanova, 2000; Koets et al., 2000). Several studies report a breed effect in the variation in susceptibility to paratuberculosis (Cetinkaya et al., 1997; Roussel et al., 2005; Elzo et al., 2006) and estimations of heritability to MAP infection ranging from 0.06 to 0.159 have been reported (Koets et al., 2000; Mortensen et al., 2004; Gonda et al., 2006a).

At present, few studies have explored the association between paratuberculosis susceptibility and candidate host genes. Some of them have not succeeded in finding strong associations (Taylor et al., 2006; Hinger et al., 2007), likely due to limitations in sample size and sensitivity of the diagnostic test used. However, nine chromosomal regions putatively associated with MAP infection have been documented based on quantitative trait loci mapping (Gonda et

al., 2005, 2006b). As for susceptibility to many infectious diseases that are probably not controlled at the genetic level by a single gene, variation in susceptibility to bovine paratuberculosis is likely controlled by a group of genes, or many genes (multifactorial inheritance).

The present results indicate a significant association between a polymorphism in the *CARD15* gene and paratuberculosis infection in the cattle population under study.

CARD15 has been mapped to the bovine chromosome 18 (BTA18) and its transcript is 5105 bp long and the protein comprises 1013 amino acids. This compares to human and murine *CARD15* with transcript lengths of 4485 bp and 4585 bp, respectively (Taylor et al., 2006).

The role of *CARD15* in innate immunity as an intracellular bacterial receptor could provide a biological explanation for the observed association. The function of this gene was first appreciated in human by its identification as a locus linked to Crohn's disease. Defects in the *CARD15* gene have been shown to disturb the expression of the cytokines necessary to coordinate the balance between immune suppression and activation. Disturbances in this equilibrium, required for healthy functioning of the adaptive immune system in the intestine, lead to a dysfunctional inflammatory reaction accompanied by epithelial injury (Maeda et al., 2005; Strober et al., 2006; Fishbein et al., 2008).

In the present study, the mutation associated with a higher susceptibility to paratuberculosis infection (C733R) is found in the leucine rich repeats (LRR) domain, responsible for the interaction with the bacterial cell wall component peptidoglycan. Coincidently, one of the three most common genetic variants in *CARD15* is the insertion frameshift mutation at nucleotide 3020 (3020insC), leading to truncation of the C terminal 33 amino acids in the LRR region (Ogura et al., 2001a). This mutation is associated with

hyporesponsiveness to peptidoglycan and attenuated NF-kB activation, and leads to a overexpression of the *CARD15* protein accompanied by a diminished antibacterial defense function in epithelial cells (Strober et al., 2006).

To the authors knowledge, this is the first large scale study taking into account the serious limitations affecting the performance of paratuberculosis diagnosis, and showing an association between *CARD15* and paratuberculosis infection in cattle. One of the potential limitations in a case control study is that the diagnosis of paratuberculosis may be hampered by a lack of test accuracy. Available methods fail to identify all infected animals (Chiodini et al., 1984). These potential false negative results raise the concern of misclassification of cases and controls. To address this limitation, parallel interpretation of multiple diagnostic tests was used as a means to improve overall sensitivity. The procedure considered five highly specific diagnostic tests to avoid a potential increase of non-specific results (false positives), producing clearly determined subpopulations of cases and controls. Another important aspect of candidate gene case control studies is the need to achieve a study population large enough to allow multiple statistical tests to establish significant findings. According to our sample size estimations, this requisite was achieved in this study.

The failure in fitting the assumption of HWE for SNP1 could be explained by the fact that domestic populations are likely to be under inbreeding conditions and suffer assortative mating. This constitutes a limitation for this study, however, the possibility of population stratification is low as indicated by the absence of association with paratuberculosis infection in other unlinked markers analyzed (data not presented). This concern is also reduced, given that cases and controls were matched from the same subpopulations.

The present study was not designed to determine the effect of breed in susceptibility, but this variable was by necessity considered in the analysis. Furthermore, the fact that the variant allele for SNP1 (2197, T>C) was found mainly in the Brahman-Angus subpopulation raised the concern of breed as a potential confounding factor. Two tests were performed to clarify this point; logistic regression analysis with a model controlling for breed (Table 5-5) and Fisher's Exact Test within the Brahman-Angus subpopulation. Both analyses resulted in significant associations between this polymorphism and the infection status of the cows (OR=3.35, p = 0.01; p = 0.02, respectively).

Variables included in the final logistic regression model were SNP1, breed and age. Although this last variable was not significant in the univariable model, because of its biological relevance, it was maintained in the final model. According to the analysis, cows with the A_1A_2 genotype were 3.35 times more likely to be infected than cows with the A_1A_1 genotype (p = 0.01). However, the resulting odds of infection for cows with the A_2A_2 genotype relative to the reference were not significant (p = 0.97). A possible explanation for this lack of significance could reside in the small number of animals carrying this variant genotype (11 cows).

It is concluded that amino acid substitution C733R (SNP1) appears to be associated with susceptibility to paratuberculosis infection in this population of Florida cattle. The information presented in this study adds new evidence relative to the potential association of bovine paratuberculosis and Crohn's disease. According to the conclusions of the Committee on Diagnosis and Control of Johne's Disease (National Research Council, 2003), the identification of a common susceptibility gene for paratuberculosis in animals and CD in humans would offer a moderate evidence of causation for the role of MAP in CD. These data also contribute to the

understanding of this disease and present new information for the control of bovine paratuberculosis.

Table 5-1 Alleles: Cross classification of number (%) of cases (+) and controls (-) and alleles for SNP1 and SNP2.

	Infection status		
Alleles	+	-	P value
SNP1			
A_1	209 (89%)	592 (96%)	<0.001†
A_2	27 (11%)	24 (4%)	
SNP2			
B_1	140 (59%)	324 (53%)	0.1
B_2	98 (41%)	292 (47%)	

† Significant at $p \leq 0.05$.

Table 5-2. Genotypes: Cross classification of number (%) of cases (+) and controls (-) and genotypes for SNP1 and SNP2.

	Infection status		
Genotype	+	-	P value
SNP1			
A_1A_1	95 (81%)	291 (95%)	<0.0001†
A_1A_2	19 (16%)	10 (3%)	
A_2A_2	4 (3%)	7 (2%)	
SNP2			
B_1B_1	38 (32%)	83 (27%)	0.19
B_1B_2	64 (54%)	158 (51%)	
B_2B_2	17 (14%)	67 (22%)	

† Significant at $p \leq 0.05$.

Table 5-3. Complete genotypes: Cross classification of number (%) of cases (+) and controls (-) and genotypes combining SNP1 and SNP2.

	Infection status		
Genotype	+	-	P value
A_1A_1 B_1B_1	24 (20%)	66 (22%)	<0.0001†
A_1A_1 B_1B_2	53 (45%)	156 (51%)	
A_1A_1 B_2B_2	17 (15%)	67 (22%)	
A_1A_2 B_1B_1	10 (9%)	9 (3%)	
A_1A_2 B_1B_2	9 (8%)	1 (0.3%)	
A_2A_2 B_1B_1	4 (3%)	7 (2%)	

† Significant at $p \leq 0.05$.

Table 5-4. Univariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida.

Variable	Type		Odds ratio	95% Confidence interval	P value
SNP1	Ordinal[#]		2.32[†]	1.41 – 3.83	0.0009
SNP2	Ordinal[##]		1.30	0.56 – 1.05	0.10
SNP1	Class	A_2A_2 vs A_1A_1	1.76	0.51 – 6.17	0.37
		A_1A_2 vs. A_1A_1	5.87[†]	2.63 – 13.0	<0.0001
SNP2	Class	B_2B_2 vs B_1B_1	0.55	0.28 – 1.07	0.08
		B_1B_2 vs. B_1B_1	0.83	0.51 – 1.36	0.47
Breed	Class[###]	BA vs H	2.67[†]	1.59 – 4.47	0.0002
		J vs H	0.32[†]	0.12 – 0.98	0.02
Herd	Class	1 vs 5	0.90	0.44 – 1.84	0.77
		2 vs 5	2.32[†]	1.30 – 4.13	0.004
		3 vs 5	0.70	0.38 – 1.27	0.24
		4 vs 5	0.28[†]	0.10 – 0.77	0.01
Age	Ordinal[####]		1.28	0.88 – 1.86	0.18

[#] $A_1A_1(0)$; $A_1A_2(1)$; $A_2A_2(2)$. [##] $B_1B_1(0)$; $B_1B_2(1)$; $B_2B_2(2)$. [###] BA: Brahman x Angus (Herd 2); H: Holstein (Herds 1,2,5); J: Jersey (Herd 4). [####] Age ranges: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$

Table 5-5. Multivariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida.

Variable	Type		Odds ratio	95% Confidence interval	P value
SNP1	Class[#]	A2A2 vs A1A1	0.97	0.24 – 3.90	0.97
		A1A2 vs. A1A1	3.35[†]	1.25 – 9.01	0.01
Breed	Class[##]	BA vs H	1.65	0.81 – 3.38	0.17
		J vs H	0.32[†]	0.12 – 0.84	0.02
Age	Ordinal[###]		1.14	0.76 – 1.71	0.51

[#] $A_1A_1(0)$; $A_1A_2(1)$; $A_2A_2(2)$. [##] BA: Brahman x Angus; H: Holstein; J: Jersey. [###] Age ranges: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$.


A

B

C

D

Figure 5-1. Beef cattle population. A-D) Series of individuals from the Brahman-Angus cow-calf herd under analysis (courtesy of Dr. Owen Rae).


A

B

Figure 5-2. Dairy cattle population A-B) Series of individuals from the UF dairy research unit under analysis.

CHAPTER 6
ANALYSIS OF THE ASSOCIATION BETWEEN THREE CANDIDATE GENES *(BOIFNG, TLR4, SLC11A1*) AND PARATUBERCULOSIS INFECTION IN CATTLE

Summary

Paratuberculosis represents a major problem in the world dairy and beef industry and at the present is considered a potential zoonosis. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis*, and susceptibility to infection is suspected to have a genetic component. The objective of this candidate gene case-control study was to characterize the distribution of polymorphisms in three candidate genes related to the immune function; *BoIFNG, TLR4*, and *SLC11A1* genes and test their association with paratuberculosis infection in a cattle population. The statistical analysis demonstrated significant differences in allelic frequencies between cases and controls for *BoIFNG* $SNP_1$2781 ($p = 0.006$) and *SLC11A1* microsatellites, indicating a significant association between infection and variant alleles. In the analysis of genotypes, a significant association was also found between infection status and *BoIFNG* $SNP_1$2781 ($p = 0.03$) and *SLC11A1-275-279-281* microsatellites ($p = 0.04$, $p = 0.018$, and $p = 0.001$, respectively). However, when variables breed and age were included in the multivariate logistic regression analysis, the statistical significance of polymorphisms in the odds of infection disappeared, leaving in question the role of these candidate genes in host susceptibility to paratuberculosis.

Introduction

Paratuberculosis is an economically significant, chronic, infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis,* and characterized by progressive weight loss and a nonresponsive, persistent or intermittent diarrhea (Chiodini et al., 1984).

Genetic factors have been associated with differences in host susceptibility to bovine paratuberculosis, and estimations indicate a range of moderate values for heritability of infection

(Koets et al., 2000; National Research Council, 2003; Mortensen et al 2004, Gonda et al 2006). Research has also been aimed at detecting associations between susceptibility differences and polymorphisms at candidate genes with no definite results (Hinger et al., 2007; Taylor et al., 2006; Gonda et al., 2005, 2007).

Interferons constitute a multi-gene family of inducible cytokines. A member of this group, interferon gamma (IFN-g), plays a crucial role in the innate host response to intracellular bacteria, including mycobacteria (Huang et al., 1993; Shtrichman et al., 2001). Release of IFN-g after the initial MAP entry into the host, as part of a protective type 1-like T-cell response, has been claimed as a key factor in the control of infection and disease manifestation (Coussens, 2004; Coussens et al., 2004a).

Toll-like receptors (TLR) are a family of trans-membrane structures capable of recognizing several classes of pathogens and responsible for coordination of appropriate innate and adaptive immune responses (Wang et al., 2002; Kiyoshi et al., 2003, Quesniaux et al., 2004). One member of this family, TLR4 has been implicated in the recognition of mycobacterial antigens (Quesniaux et al., 2004; Yadav and Schorey; 2006, Weiss et al., 2008) mediating cytokine production and stimulation of host defense (Ferwerda et al., 2007).

The *SLC11A1* (solute carrier family 11 member 1) gene (coding for natural resistance-associated macrophage protein 1, NRAMP1) is associated with natural resistance against intracellular pathogens as *Mycobacterium* sp., *Salmonella* sp., and *Leishmania* in the mouse, and plays an important role in innate immunity, preventing bacterial growth in macrophages during the initial stages of infection (Paixao et al., 2007). The role of polymorphisms within the (GT)n microsatellite of the *SLC11A1* gene in natural resistance against *Brucella abortus* in cattle is controversial (Feng et al., 1996; Paixao et al., 2007) and two studies reported possible

associations of particular *SLC11A1* alleles with susceptibility or resistance to Johne's disease in sheep (Reddacliff et al., 2005) and with lesion progression in affected cattle (Juste et al., 2005).

The critical function of *BoIFNG, TLR4* and *SLC11A1* genes in the coordination of immunity against bacteria suggest a potential involvement of these candidate genes in the variation in bovine paratuberculosis susceptibility.

The central hypothesis of this study was that different arrangements of particular alleles in three candidate genes would predominate in case compared to control populations, suggesting a role in susceptibility to infection. The objective of this candidate gene case-control study was to characterize the distribution of polymorphisms in three candidate genes; *BoIFNG, TLR4*, and *SLC11A1* genes and test their association with paratuberculosis infection in cattle.

Material and Methods

Study Population

The same population of cattle presented in Chapter 5 was considered in this study. Briefly, 431 adult cows, consisting of 299 Holstein, 50 Jersey and 82 Brahman-Angus crosses were included in the analysis. Animals were recruited from three Holstein and one Jersey dairy herds, and one Brahman-Angus cow-calf herd near Gainesville, Florida, USA.

A case-control design was used based on the infection status of the animals following multiple tests to reduce misclassification of individuals. MAP infection was determined by parallel interpretation of five diagnostic tests (serum ELISA, milk PCR, blood PCR, fecal PCR and fecal culture) or by necropsy examination (gross pathology, histopathology and PCR on tissues). In determining the case-control sub-populations, a case was defined as an animal being positive for any of the tests, considered in parallel, and a control was defined as an individual negative to all the tests that it was subjected to. As a result, the final population consisted of 126 cases and 305 controls.

Diagnosis

Diagnostic procedures were performed as reported in chapter 5.

Genotyping

DNA extraction: DNA from the study population was extracted from whole blood or tissues using the QIAamp DNA Blood Mini Kit (Qiagen Inc., CA, USA), according to the manufacturer's directions and stored at -20^{o}C before use.

Allele determination

***Bovine IFNG* and *TLR4* genes**

Two previously reported single nucleotide polymorphisms (designated here as SNP_1 and SNP_2) within the bovine *IFNG* gene were tested (Schmidt et al., 2002). The SNP_1 [2781, G/T (G134V)] is situated in the coding region of exon 1, position 134 of *BoIFNG* cDNA, and causes an amino acid change from glycine to valine in the signal peptide of the BoIFN pre-protein. The SNP_2 [6811 G/A] is located in the coding region of exon 4 (Schmidt et al., 2002).

Three previously reported single nucleotide polymorphisms (named here as SNP_3, SNP_4, and SNP_5) within the bovine *TLR4* gene were tested (White et al., 2003). The SNP_3 [1040, C/A (A347E)] is responsible for an amino acid substitution (alanine to glutamic acid) located in the extracellular domain of the protein (White et al., 2003). The SNP_4 [1142 A/G (K381R)] is responsible for an amino acid substitution (lycine to arginine) situated in the extracellular domain. The SNP_5 [2021 C/T (T674I)] is also responsible for an amino acid substitution (threonine to isoleucine) situated in the transmembrane/cytoplasmic domain of the receptor (White et al., 2003).

The PCR and sequencing primers used for the *BoIFNG* exon1 $SNP_1$2781G/T were; Forward: 5'- CGATTTCAACTACTCCGGCCTAAC -3'; Reverse: 5'-Biotin-GGCCATAAGAACCAGAAAAACCC -3'; Forward sequencing primers:

5’-TCTTAGCTTTACTGCTCTGT -3’. The sequence to analyze for *BoIFNG* exon1 $SNP_1$2781G/T was: GG/TGCTTTT GGGTTTTTCT.

The PCR and sequencing primers used for the *BoIFNG* exon 4 $SNP_2$6811 G/A were; Forward: 5’- Biotin-GATCCAGCGCAAAGCCATA -3’; Reverse: 5’-TCTCTTCCGCTTTCTGAGGTTAG -3’; Reverse sequencing primers: 5’-GCTTTCTGAGGTTAGATTTT -3’. The sequence to analyze for *BoIFNG* exon 4 $SNP_2$6811 G/A was: GGC/TGACAG GTCATTCATC.

The PCR and sequencing primers used for the bovine *TLR4* cSNPs 1040C/A, and 1142 A/G were; Forward: 5’-GGATAGCGTACTTGGACAAA-3’; Reverse: 5’-Biotin-CCCAAAATCAGTGTGAGAACAGC-3’; Forward sequencing primers for $SNP_3$1040C/A: 5’-TGACTTTGACAAGTTTCC-3’, and Forward sequencing primers for $SNP_4$1142 A/G: 5’-AAGCCTTCAGTATCTAGATC-3’. The sequence to analyze for $SNP_3$1040C/A, and $SNP_4$1142 A/G were TGC/AATTGA AGCTCAGTTC , and TCAA/GAAGA AATCACT, respectively.

The PCR conditions for pyrosequencing were; 95 ºC for 2 min, 45 cycles consisting of, denaturation at 94 ºC for 30 s, annealing at 56 ºC for 30 s (for *BoIFNG* exon 4 $SNP_2$6811 G/A and bovine TLR4 cSNPs 1040 C/A and 1142 A/G, and at 58ºC for 30 s (for *BoIFNG* exon1 $SNP_1$2781G/T), and extension at 72 ºC for 1 min, followed by final extension at 72 ºC for 7 min. The PCR products were processed and prepared for genotyping.

Genotyping was performed in the laboratories of the University of Florida Center for Pharmacogenomics. Genotypes were determined by either pyrosequencing (Biotage, Uppsala, Sweden) or by use of the fluorescence-based TaqMan® platform (Applied Biosystems, Foster City, USA).

Genotyping for, *BoIFNG* exon1 $SNP_1$2781G/T; *BoIFNG* exon4 $SNP_2$6811 G/A; bovine *TLR4* cSNPs 1040C/A; and 1142 A/G was performed by pyrosequencing and according to the published protocol (Langaee and Ronaghi, 2005).

The *TLR4* gene $SNP_5$2021 C/T was genotyped using TaqMan® allelic discrimination genotyping method (Applied Biosystems, Foster City, USA), sing the Applied Biosystems 7900 HT SNP genotyping platform. Five μL reactions in 384-well plate were prepared using Eppendorf epMotion 5070 (Eppendorf North America, Inc., Westbury, NY, USA), and Packard Multi Probe II HT Systems (PerkinElmer Las, Inc., Shelton, CT, USA), liquid handling/sample processing robotics and the assays were performed and analyzed according to the manufacture's recommendations.

Microsatellite analysis for *SLC11A1* gene

A region corresponding to 275 bp, targeting the 3′UTR of bovine *SLC11A1* gene (GenBank Acc. No. U12862) was PCR amplified using primer pairs ($N_1$5'-GCCACGGGTGGAATGAGT-3', $N_2$5'- TGAGCTAGGAAATAGCAGG-3'). This region contains different numbers of the microsatellite repeat (GT). Polymerase chain reaction (PCR) was carried out in a final volume of 2 μl reaction mixture, PCR assay buffer [10 mM Tris–HCl (pH 8.8 at 25°C), 50 mM KCl, 0.8% Nonidet P40], 2.0 mM of $Mg2^+$, 200 mM dNTPs except dATP, 160 mM of dATP, 0.25 ml of a35SdATP, 10 pM of each primer and 2.0 U of Taq DNA polymerase.

PCR conditions were: initial denaturation consisting in three cycles of 94°C for 3 m, 55°C for 20 s, and 72°C for 10 s; followed by 31 cycles of 94°C for 1m, 50°C for 40s, and 72°C for 20s, and a final extension of 72°C for 5 m.

In order to detect the length variations of (GT)n repeats, amplicons were subjected to analysis in the University of Florida Interdisciplinary Center for Biotechnology Research, using

a DNA sequence analyzer from Amersham (MegaBACE1000 and 4500), combining 1 µl of PCR product sample with 5.86 µl of 0.1% Tween and 0.14ul of ET Rox550 size standard. Data were analyzed using GeneMarker v 1.4 (SoftGenetics, LLC, State college, PA 16803)

Statistical Analysis

Genotype frequencies were tested for departure from Hardy-Weinberg equilibrium (HWE) by using Fisher Exact Test and Chi-square test. Both tests were also used to check whether there was any non-random association between allele or genotype frequencies versus infection status of the cows. The analysis was performed for all the polymorphisms in each of the study genes.

Three genotypes per polymorphism were considered in the analysis for *BoIFNG* and *TLR4* [major homozygous (0); heterozygous (1); minor homozygous (2)]. Given that *SLC11A1* gene presented more than two alleles, three categories for each of the alleles were set for the genotype analysis [non allele carrier homozygous (0); heterozygous (1); homozygous for the particular allele (2)]

The univariable analysis for logistic regression considered the infection status as categorical response variable (yes/no), and polymorphism (SNPs, microsatellite), breed, herd and age were included as possible explanatory variables. Multivariable logistic regression models were proposed to estimate the odds of infection for "susceptibility allele" carriers compared to non allele carriers, controlling for possible confounding variables. The models considered the infection status as response variable, and included genotypes (based on alleles provided by the analyzed polymorphisms), breed, herd and age as explanatory variables. Genotypes were considered as ordinal variables (as previously described; 0, 1, and 2) and as class variables with the major homozygous genotype deemed as baseline. Breed and herd were considered as class variables, and age (months) was considered as an ordinal variable with three levels demarcated by the mean ± one standard deviation.

The complete model was tested by backward elimination procedure to determine the variables to be included in the final model based on the deviance value.

Data were analyzed using the SAS statistical package for Windows (SAS Systems for Windows Version 9.00, SAS Institute Inc. Cary, NC, USA) using the PROC FREQ, PROC GENMOD and the CHISQ EXACT procedure. Values of $P \leq 0.05$ were considered significant for all tests.

Results

The ratio of cases to controls was 1:2.4 (126 cases vs. 305 controls) with an average age for cases and controls of 64 and 65 months, respectively ($p = 0.7$). From a total of 431 individuals submitted for the genotyping analysis, 2, 7, 1, 0, 15 and 19 failed to provide a result for SNP_1, SNP_2, SNP_3, SNP_4, SNP_5, and *SLC11A1* microsatellite, respectively.

Bovine IFNG Gene

Frequencies for the major allele in SNP_1 and SNP_2 were 0.95 and 0.66, respectively. Values for the coefficient of linkage disequilibrium (LD), normalized LD, and correlation between the two SNPs were -0.015, 0.02, and -0.15, respectively, and Chi-square test indicated that SNP_1 and SNP_2 were in linkage disequilibrium ($p < 0.001$). The genotype frequencies of SNP_2 did not deviate from HWE; however the distributions of SNP_1 did not fit HWE in our control population.

The statistical analysis resulted in significant differences in allelic frequencies between cases and controls for SNP_1 ($p = 0.006$) indicating an association between infection and variant allele. The frequency for the variant allele was 7.98% and 3.54% in cases and controls, respectively. However, no association was found between SNP_2 and infection status ($p = 0.63$).

Significant differences in genotype frequencies between the 2 groups of individuals testing positive or negative for paratuberculosis infection were found only for *BoIFNG*2781G/T polymorphism (SNP_1) ($p = 0.036$, Table 6-1).

Estimated ORs for the univariable logistic regression models are presented in Table 6-2. The analysis indicated significant ORs for SNP_1 when considered as ordinal ($p = 0.0193$), and class variable [genotype A_1A_2 referred to the major genotype A_1A_1 ($p < 0.03$)]. However, when SNP_1 was included in the final model for multivariable logistic regression, controlling for breed and age (Table 6-4) the significance for this effect was lost ($p = 0.97$ and $p = 0.85$ for SNP_1 when considered as ordinal or class variable, respectively).

***Toll-Like Receptor 4* Gene**

Frequencies for the major allele in SNP_3, SNP_4 and SNP_5 were 0.98, 0.98, and 0.86, respectively. Values for the coefficient of linkage disequilibrium (LD), normalized LD, and correlation between the SNP_3 and SNP_5 were -0.002, 0.003, and -0.849, respectively, and Chi-square test indicated that SNP_3 and SNP_5 were in linkage disequilibrium ($p = 0.03$). The genotype frequencies of SNP_4 and SNP_5 did not deviate from HWE; however the distributions of SNP_3 did not fit HWE in our control population.

The statistical analysis resulted in no significant differences in allelic frequencies between cases and controls for SNP_3, SNP_4, and SNP_5 ($p = 0.07$, $p = 0.06$, and $p = 0.7$, respectively).

No significant differences in genotype frequencies between the cases and controls were found in the three *TLR4* polymorphisms under analysis (Table 6-1). The univariable logistic regression analysis did not result in significant values for any of the polymorphisms analyzed (Table 6-2).

Solute Carrier Family 11 Member 1 Gene

The microsatellite analysis resulted in five different alleles (sizes 273bp, 275bp, 277bp, 279bp, and 281bp) with frequencies 0.074, 0.567, 0.288, 0.044, and 0.027 respectively. None of the genotype frequencies fit HWE in our control population.

The statistical analysis resulted in significant differences in allelic frequencies between cases and controls for *SLC11A1* microsatellite ($p = 0.002$) indicating an association between infection and *SLC11A1* alleles.

Significant differences between genotype frequencies between the case and control groups were found for SLC11A1-275, 279 and 281 microsatellite alleles ($p = 0.04$, $p = 0.018$, and $p = 0.0017$, respectively, Table 6-1).

Estimated ORs for the univariable logistic regression models are presented in Table 6-3. The analysis indicated significant ORs for SLC11A1-275, 279, and 281 when considered as class variables [genotype $\text{Allele}_{275,279,281}$/* referred to the major genotype */* ($p = 0.027$, $p = 0.007$, and $p = 0.004$, respectively)]. However, when SLC11A1-275, 279, and 281 microsatellite alleles were included in the final model for multivariable logistic regression the significance for these effects was lost (Tables 6-5 to 6-7). The final models included genotype (class) as an explanatory variable of interest, controlling for breed and age.

Discussion

Numerous studies have reported a role for genetics in determining differences on susceptibility and resistance to mycobacterial infection including bovine paratuberculosis (Cetinkaya et al., 1997; Abel and Casanova, 2000; Koets et al., 2000; Mortensen et al., 2004; Roussel et al., 2005; Elzo et al., 2006; Gonda et al., 2006). Some authors have focused on the participation of candidate host genes with discordant results (Gonda et al., 2005, 2007; Taylor et al., 2006; Hinger et al., 2007).

In our study allele frequencies for SNP_1 (*IFNG* gene) and for (GT)n microsatellite of the *SLC11A1* gene were significantly different for our population of cases and controls ($p = 0.006$ and $p = 0.002$, respectively), indicating a potential association between infection status and the referred polymorphisms. This finding is in agreement with the results presented by Estonba et al. (2005), where one (GT)n microsatellite allele of the *SLC11A1* gene had a frequency significantly higher in the ELISA seronegative (0.22) than in the seropositive (0.07) group. However, converse results were presented by Hinger et al. (2007) who did not find a significant association between paratuberculosis infection status (based on serum ELISA) and both *SLC11A1,* and *INFG* gene polymorphisms.

Our results in the univariate logistic regression indicated a significant effect on the individual animal odds of paratuberculosis infection for *BoIFNG* SNP_1 (expressed as ordinal and class variable) and for *SLC11A1* 275-allele (class variable), 279-allele (class variable), and 281-allele (ordinal and class variable).

In the analysis, variables herd and breed were also significant, indicating differences in prevalence in the herds and, consequently, in the breeds included in this study. However, due to the case-control nature of the design, conclusions can not be derived relative to differences in breed susceptibility and, consequently, the analysis for the variables of interest (polymorphisms) must control for these possible confounders.

The effect of the previously significant polymorphisms appears to be clarified in the multivariate analysis. After the backward elimination procedure, when variables breed and age are incorporated in the final model, the significant effect of *BoIFNG* gene and *SLC11A1* gene polymorphisms (as genotypes) was lost ($P > 0.05$). Only one genotype (the heterozygous for *SLC11A1*-275) indicated a tendency to significance with a P value of 0.09.

One of the potential limitations in a case control study is the misclassification of individuals in these categories. This is especially important for paratuberculosis given the lack of accurate *in vivo* diagnostic tests (Chiodini et al., 1984). In this study, parallel interpretation of multiple diagnostic tests was used as a mean to improve overall sensitivity. The procedure considered five highly specific diagnostic tests to avoid a potential increase of non-specific results (false positives), producing a more clearly determined subpopulations of cases and controls.

Some of the polymorphisms under study failed in fitting the assumption of HWE, and this could be explained by the fact that domestic populations are likely to be under inbreeding conditions and suffer assortative mating. This constitutes a limitation for this study; however, the possibility of population stratification is low as indicated by the absence of association with paratuberculosis infection in other unlinked markers analyzed concurrently. This concern is also reduced, given that cases and controls were matched from the same subpopulations.

Although some of the analyses in this study suggest a potential connection between polymorphisms in *BoIFNG and SLC11A1* genes and paratuberculosis, subsequent examination controlling for potential confounding dismissed this association. Further analysis including a larger population and different polymorphisms in these two genes is warranted.

It is concluded that the present study could not demonstrate a definitive association between paratuberculosis infection in cattle and the proposed candidate genes (*IFNG, TLR4,* and *SLC11A1* genes).

Table 6-1. Frequency of cases per genotype and significance for the association between genotype and infection status (*BoIFNG, TLR4* and *SLC11A1* genes).

Polymorphism	Genotype	Infected (%)	Total (n)	P value[#]
*IFNG*2781G/T (SNP_1)	GG	26.1	394	0.036[†]
	GT	44.8	29	
	TT	50.0	6	
*IFNG*6811G/A (SNP_2)	GG	27.6	192	0.87
	GA	27.0	178	
	AA	24.1	54	
TLR4 1040 C/A (SNP_3)	CC	27.0	415	0.18
	CA	46.2	13	
	AA	50.0	2	
TLR4 1142 A/G (SNP_4)	AA	27.3	422	0.07
	AG	55.6	9	
	GG	0.0	0	
TLR4 2021 C/T (SNP_5)	CC	29.5	315	0.43
	CT	22.8	92	
	TT	22.2	9	
SLC11A1-273	*/*	28.7	359	0.19
	273/*	31.1	45	
	273/273	0.0	8	
SLC11A1-275	*/*	27.7	141	0.04[†]
	275/*	40.0	75	
	275/275	24.5	196	
SLC11A1-277	*/*	28.5	284	0.86
	277/*	33.3	18	
	277/277	27.3	110	
SLC11A1-279	*/*	27.3	388	0.018[†]
	279/*	66.7	12	
	279/279	25.0	12	
SLC11A1-281	*/*	26.7	393	0.0017[†]
	281/*	62.5	16	
	281/281	66.7	3	

[#]Chi square and Fisher's Exact Test. [†] Significant at $p \leq 0.05$.

Table 6-2. Bovine *IFNG* and *TLR4* genes: Univariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida.

Variable	Type		Odds ratio	95% Confidence interval	P value
BoIFNG					
SNP1	Ordinal[#]		1.98	1.11 - 3.51	0.0193[†]
SNP2	Ordinal[#]		0.93	0.68 - 1.28	0.645
SNP1	Class	A_2A_2 vs A_1A_1	2.85	0.56 - 14.1	0.207
		A_1A_2 vs. A_1A_1	2.29	1.06 - 4.90	0.03[†]
SNP2	Class	B_2B_2 vs B_1B_1	0.83	0.41 - 1.67	0.6
		B_1B_2 vs. B_1B_1	0.96	0.61 - 1.53	0.89
TLR4					
SNP3	Ordinal[#]		2.03	0.83 - 4.92	0.11
SNP4	Ordinal[#]		3.33	0.88 - 12.6	0.07
SNP5	Ordinal[#]		0.73	0.46 – 1.17	0.2
SNP3	Class	A_2A_2 vs A_1A_1	2.69	0.16 - 43.5	0.48
		A_1A_2 vs. A_1A_1	2.31	0.76 - 7.02	0.13
SNP4	Class	B_2B_2 vs B_1B_1	n.a.	n.a.	n.a.
		B_1B_2 vs. B_1B_1	3.33	0.88 - 12.6	0.07
SNP5	Class	C_2C_2 vs C_1C_1	0.68	0.13 - 3.34	0.63
		C_1C_2 vs. C_1C_1	0.7	0.41 - 1.21	0.2
Other					
Breed	Class[##]	BA vs H	2.49	1.49 - 4.12	0.0004[†]
		J vs H	0.32	0.12 - 0.84	0.02[†]
Herd	Class	1 vs 5	3.28	1.09 - 9.85	0.03[†]
		2 vs 5	7.76	2.79 - 21.5	<0.001[†]
		3 vs 5	2.44	0.86 - 6.88	0.09
		4 vs 5	3.59	1.33 - 9.67	0.01[†]
Age	Ordinal[###]		1.26	0.87 - 1.84	0.2

[#]non allele carrier homozygous (0); heterozygous (1); homozygous (2). [##] BA: Brahman x Angus; H: Holstein; J: Jersey. [###] Age range: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$. n.a.: Not available.

Table 6-3. Univariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida (*SLC11A1* gene).

Variable	Type		Odds ratio	95% Confidence interval	P value
SLC11A1-273	Ordinal[#]		0.77	0.44 - 1.36	0.37
SLC11A1- 275	Ordinal[#]		0.89	0.71 - 1.13	0.37
SLC11A1-277	Ordinal[#]		0.97	0.76 - 1.24	0.84
SLC11A1-279	Ordinal[#]		1.35	0.79 - 2.3	0.27
SLC11A1-281	Ordinal[#]		3.56	1.52 - 8.24	0.0032[†]
SLC11A1-273	Class	273/273 vs */*	n.a.	n.a.	n.a.
		273/* vs. */*	1.12	0.57 - 2.18	0.73
SLC11A1- 275	Class	275/275 vs */*	0.85	0.52 - 1.36	0.49
		275/* vs. */*	2.01	1.08 - 3.76	0.027[†]
SLC11A1-277	Class	277/277 vs */*	0.93	0.57 - 1.53	0.8
		277/* vs. */*	1.25	0.45 - 3.44	0.66
SLC11A1-279	Class	279/279 vs */*	0.88	0.23 - 3.32	0.85
		279/* vs. */*	5.31	1.56 - 17.9	0.007[†]
SLC11A1-281	Class	281/281 vs */*	5.48	0.49 - 60.9	0.16
		281/* vs. */*	4.54	1.62 - 12.8	0.004[†]

[#]non allele carrier homozygous (0); heterozygous (1); homozygous (2). [†] Significant at $p \leq 0.05$.
n.a.: Not available.

Table 6-4. Bovine *IFNG*: Multivariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida.

Variable	Type		Odds ratio	95% Confidence interval	P value
SNP1	Ordinal[#]		0.98	0.49 - 1.97	0.97
SNP1	Class	A2A2 vs A1A1	1.11	0.20 - 6.04	0.9
		A1A2 vs. A1A1	0.91	0.36 - 2.31	0.85
Breed	Class[##]	BA vs H	2.45	1.31 - 4.75	0.005[†]
		J vs H	0.32	0.12 - 0.83	0.02[†]
Age	Ordinal[###]		1.09	0.73 - 1.62	0.66

[#]non allele carrier homozygous (0); heterozygous (1); homozygous (2). [##] BA: Brahman x Angus; H: Holstein; J: Jersey. [###] Age range: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$.

Table 6-5. Allele *SLC11A1*-275: Multivariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida.

Variable	Type		Odds ratio	95% Confidence interval	P value
SLC11A1-275	Class	275/275 vs */*	0.90	0.54 – 1.50	0.7
		275/* vs. */*	1.73	0.91 – 3.32	0.09
Breed	Class[##]	BA vs H	2.72	1.54 – 4.78	0.0005[†]
		J vs H	0.34	0.13 – 0.91	0.03[†]
Age	Ordinal[###]		1.08	0.72 – 1.63	0.68

[##] BA: Brahman x Angus; H: Holstein; J: Jersey. [###] Age range: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$.

Table 6-6. Allele *SLC11A1*-279: Multivariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida.

Variable	Type		Odds ratio	95% Confidence interval	P value
SLC11A1-279	Class	279/279 vs */*	0.38	0.09 - 1.57	0.18
		279/* vs. */*	2.63	0.73 - 9.48	0.14
Breed	Class[##]	BA vs H	2.84	1.57 - 5.10	<0.001[†]
		J vs H	0.31	0.12 - 0.82	0.01[†]
Age	Ordinal[###]		1.12	0.74 - 1.69	0.58

[##] BA: Brahman x Angus; H: Holstein; J: Jersey. [###] Age range: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$.

Table 6-7. Allele *SLC11A1*-281: Multivariate analysis of the individual animal odds of paratuberculosis infection among dairy and beef cattle in Florida ().

Variable	Type		Odds ratio	95% Confidence interval	P value
SLC11A1-281	Ordinal[#]		1.85	0.75 - 4.4	0.18
Breed	Class[##]	BA vs H	2.43	1.32 - 4.43	0.004[†]
		J vs H	0.31	0.12 - 0.83	0.01[†]
Age	Ordinal[###]		1.08	0.72 - 1.62	0.69
SLC11A1-281	Class[##]	281/281 vs */*	2.32	0.19 - 27.1	0.5
		281/* vs. */*	2.07	0.67 - 6.42	0.2
Breed	Class[##]	BA vs H	2.39	1.29 - 4.39	0.005[†]
		J vs H	0.31	0.12 - 0.82	0.01[†]
Age	Ordinal[###]		1.08	0.72 - 1.63	0.68

[#]non allele carrier homozygous (0); heterozygous (1); homozygous (2). [##] BA: Brahman x Angus; H: Holstein; J: Jersey. [###] Age range: Low (0); Medium (1); High (2). [†] Significant at $p \leq 0.05$.

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BIOGRAPHICAL SKETCH

Pablo J. Pinedo was born in 1967 in Santiago de Chile, where he was raised, enjoying a childhood surrounded by family and friends. In 1987 he enrolled in the College of Veterinary Medicine in the Universidad de Chile where he graduated with honors. He moved to an agricultural region in the south of Chile, and started working at a dairy cooperative, focusing on cattle genetics and dairy record management. During this time he met Pilar and they were married three years later. After some years, two of the most important events in his life occurred: the arrival of his two sons, Santiago and Pablo (today 5 and 7 years old). After 10 years practicing veterinary medicine, the opportunity of continuing his education by pursuing a PhD presented itself when he was awarded the UF College of Veterinary Medicine Alumni Fellowship. Leaving behind a structured life, Pilar and Pablo decided to face this new adventure and the family flew to Gainesville, Florida. Since fall 2004, he has been in the Doctor of Philosophy program in the Large Animal Clinical Sciences Department of the College of Veterinary Medicine at the University of Florida under the supervision of Dr. Owen Rae, working on diagnostics and genetics of bovine paratuberculosis.