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DISSERTATION

SCREENING AND DIAGNOSIS OF PARATUBERCULOSIS IN YOUNG AND ADULT DAIRYCATTLE

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2004

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COLORADO STATE UNIVERSITY

OCTOBER 30TH, 2003

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARIA CELIA ANTOGNOLI "SCREENING AND DIAGNOSIS OF PARATUBERCULOSIS IN YOUNG AND ADULT DAIRY CATTLE" BE ACCEPTED AS FULLFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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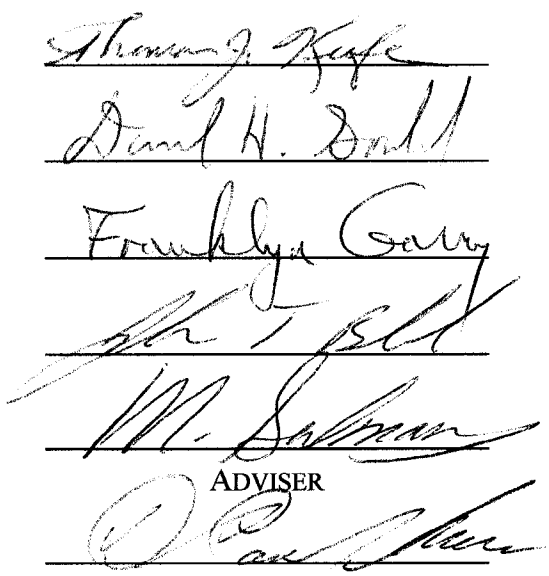

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ABSTRACT OF DISSERTATION

SCREENING AND DIAGNOSIS OF PARATUBERCULOSIS IN YOUNG AND ADULT DAIRY CATTLE

A gamma interferon (γ-IFN) test, a skin test, and a commercial ELISA were evaluated for the detection of Paratuberculosis (PTB) exposure in cohort of calves at 2, 4, 6, and 8 months of age. The cohort was selected from two dairy farms with 11% (farm A) and 2% (farm B) sero-prevalence of PTB based on the serological status of the dams. The association between the PTB status of the dams and the cell-mediated immune response in the calves was explored.

Results from this study indicated that: [a] the humoral response is not relevant in calves younger than 8 months; [b] the γ-IFN levels in plasma are high in 2-month old calves but decrease steadily with age; [c] positive skin test reactions are observed more frequentely in calves older than 4 months of age in the cervical site; [d] poor agreement was observed between the γ-IFN and the skin test; and [e] no agreement was observed between CMI-based tests and fecal shedding in 8-month old heifers. Although Map was detected in 1.5% of the calves from herd A (1.5%) by culture and PCR, these two tests did not agree in their positive results. Due to the low number of potentially infected calves, the proposed tests could not be fully characterized. Reference tests with improved accuracy would be required to further evaluate these tests in young cattle.

The association between a positive γ-IFN or skin test and risk factors related to the dams and to the calves was explored via logist regression analysis. This study showed that heifers born from dams shedding ***Mycobacterium avium*** subsp ***paratuberculosis***

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(Map) in feces at 60 days in milk are 2.7 times more likely to have a positive γ-IFN test at 8 months of age than those born from non-shedders.

Methods for detection of Map in feces were evaluated in detail. Conclusions from these studies indicate that the accuracy for detection low shedders increases with the parallel use of either a One-Tube Semi Nested PCR and conventional culture, or the non-radiometric and the conventional fecal culture.

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I would like to express my gratitude to the dairy owners who made this study possible. They allowed me to visit their facilites and sample their cattle once a month for approxiamtely three years without questioning my work. Thanks are also due to Dr Greg Goodell for his assistance in the identification of the animals and intellectual support during the progression of the study.

The role of our field team was central since this study demanded a great load of physical work separating and restraining heifers. Numerous students and visitors collaborated with this task and I am thankful to all of them; however, I would like to express special gratitude to Jim Barker and Heather Hirst, who were a permanent component of the field support team. Without them this study would not have been completed in a reasonable time frame.

The evaluation of tests for detection of PTB has been the central component of this study and, as such, support form the laboratory was critical. Special thanks go to Sarah Jensen, and Stacy Spykeman, who participated directly in the boring task of processing fecal samples one day after the other to obtain PCR results. The participation of Joni Triantis in the initial stages of the PCR optimization, and Katja Boegli-Stuber in the development of the DNA extraction protocol is gratefully recognized.

My adviser, Dr Mo Salman, has been a great mentor. He has provided support and various opportunities for learning including travelling. He believed in this study at

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moments when even I couldn't see the benefits from it. Without his encouragement I would not have completed this project.

I am thankful for the scientific input provided by the members of my graduate committee; Dr Keefe, Dr Belisle, Dr Gould and Dr Garry. Their help in improving the quality of my research is much appreciated.

Special thanks go to my husband Glenn Maxey for his understanding and his company during the last years of this study. My gratitue also goes to my mom, Noemi Fasolo, for her encouragement form the very beginning of my academic life. She taught me to be self-confident and this has helped me throughout my studies in the US.

Lastly, I would like to acknowledge the College Resarch Council, and the Program of Economically Important Infectious Animal Disease at CSU for funding this reaserch project.

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DEDICATION

To Glenn and Noemi

for their love and support

To Isabella, Tuxedo, Petunia, and Jonesy

for their company during the progression of this study

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CHAPTER 1 INTRODUCTION AND DESCRIPTION OF THE STUDY

1.1 Paratuberculosis: General Aspects and its Potential Link to Crohn's Disease

Paratuberculosis or Johne's disease is chronic granulomatous enteritis, which affects mainly domestic ruminants, but also wild ruminants and other wild animals, such as rabbits and macaques.[21] It was first described by Johne and Frothingham in Germany, in 1895, as an atypical form of bovine Tuberculosis (TB), characterized by the presence of acid fast bacilli in the intestinal mucosa. In 1910, with the identification of the causal agent by Twort, Johne's disease was reported as a separate entity from bovine TB. Between 1902 and 1910 the disease was reported in several other European countries as well as in the United States. Today, Paratuberculosis (PTB) is found in almost all countries around the world, with a few known exceptions, which include Sweden and Western Australia.[2;44] In the United States, a national study based on conventional culture from ileocecal lymph nodes collected during slaughter performed in 1987, showed a 2.9% prevalence of infection in culled dairy cows.[27] Several other regional studies based on either serology or fecal culture attempted to estimate herd prevalence.[3;9;41] However, it was in 1996 with National Dairy study performed by the National Animal Health Monitoring Systems (NAHMS) that more accurate estimates of herd and cow level prevalence of PTB were obtained. This study indicated that at least 22% of the dairy herds in the United States are infected with PTB based on serology.[2]

The disease caused by ***Mycobacterium avium*** subsp ***paratuberculosis*** (Map), is characterized by the thickening of the intestinal mucosa due to granuloma enteritis with consequent malabsoption and emaciation. Clinical signs of PTB in cattle include

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intermittent non-treatable diarrhea, progressive weight loss, emaciation, mandibular edema due to hypoproteinemia, significant reduction in milk production in dairy cows, and eventually death. PTB, however, is mainly a subclinical infection. The complete spectrum of disease in an infected herd or flock could be characterized as follows:[7;21]

- Infected animals with clinical manifestations of disease, heavy shedding, high antibody levels and low cell mediated immune responses.
- Animals in a late subclinical phase, with moderated fecal shedding, moderate levels of cell mediated and humoral immunity, and reduced milk production.
- Animals in an early subclinical phase, with light intermittent shedding, no evidence of reduced milk production, potentially high cell mediated immunity and a weak humoral immune response.
- Animals in a carrier status, with limited inmmunological evidence of infection and absence of bacteria in feces. Young calves are a typical example of this stage. In this latent stage, diagnosis can be achieved by biopsy of mesenteric lymph nodes.
- Uninfected animals, which have generated a protective immunity and have succeeded in claring the bacteria from their systems.

After a long incubation period only a few animals develop clinical signs, which are indicators of terminal disease. Among the factors associated with the onset of clinical signs are the infective dose, the age of the animals at the time of infection and other stress-related factors, such as parturition, immunosupression, intensive farming systems, poor nutrition and transportation. Dietary iron and vitamin D intake have been reported as factors that modulate the course of the disease.[21] Clinically infected animals are

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considered the "tip of the iceberg"; it has been estimated that, for each symptomatic animal in a farm with an establish infection and transmission cycle, another 25 infected animals would be expected with no visible signs of disease.[42] The detection and culling of subclinically infected animals shedding Map into the environment is central to any effort of controlling the disease. This is a major challenge, since the historically used screening and/or diagnostic methods (conventional culture and ELISA) are not sensitive enough to detect most subclinical shedders.

PTB is not only responsible for causing significant financial losses to the livestock industry, but the causative agent is also suspected to share a common etiology with Crohn's disease in humans. Similar to PTB, Crohn's is a chronic debilitating intestinal disease. Clinical signs of Crohn's disease are often first observed in adolescents until their mid-20s, and also in later years between the ages of 50 to 60 and older. Crohn's disease can be present in three forms: predominantly inflammatory, stricturing and stenosing, or fistulizing. The disease is incurable, and surgery is the treatment of choice in 74% of the patients.[18] The true cause of Crohn's disease is still a topic for debate. Even though environmental, genetic, and immunological factors are believed to play a role in the etiology of the disease, there are currently two leading theories explaining the etiology of Crohn's disease: (1) the autoimmune theory, which suggests that Crohn's is a product of immunological imbalances; and (2) the infectious theory, which suggests that Map is a major participant in the etiology of the disease.

The following is a list of evidence in support of the possible association of Map and Crohn's disease is the following:

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- Map causes PTB in livestock, which is chronic granulomatous enteritis similar to Crohn's disease. Because Map could be present in dairy products and might resist pasteurization, humans could be expose to it by consumption of dairy products containing viable bacteria.[28]
- Map has been detected in approximately 30% of the samples from Crohn's patients by PCR targeting IS900. The agent has also been cultured from feces in a low percentage of the patients. [14;19]
- Crohn's patients have an increased humoral response to several intestinal microorganisms including Map.[16]
- The use of anti-mycobacterial drugs for treatment of Crohn's disease was reported to be effective in a significant number of patients, although relapse still occurs.[19]

Contrasting with the evidence presented in support of the association between Map and Crohn's disease, the following statements support the lack of association between them:

- Contrary to what it would be expected if Map was associated with Crohn's disease, the disease is more prevalent in urban than in rural populations.[31;34]
- Techniques for direct detection of Map in clinical specimens, such as culture, polymerase chain reaction and in situ hybridization failed to detect the bacteria in a significant percentage of Crohn's patients. Moreover, the IS 900 sequence, which is the specific target DNA for PCR, has been detected in intestinal tissues form healthy individuals. In the last 5 years, studies on PTB in livestock have questioned the specificity if the IS900.[12;15]

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- Not all patients responded succesfully to antimycobacterial drug treatment, which suggests that the etiology of Crohn's disease could be multifactorial.[18]

In my opinion, there is not enough evidence to prove or disprove that Map participates in the etiology of Crohn's disease. It seems reasonable to believe that Crohn's is a disease of multifactorial etiology. From an epidemiological point of view, a long debate on the association between Map and Crohn's disease might be a waste of our time. Instead, the control of PTB in cattle, based on measures aimed at reducing the spread of infection via manure, could have multiple advantages: (a) if there is in fact a participation of Map in the etiology of Crohn's disase, the control of PTB in cattle will reduce human exposure to the agent, thereby reducing the risk of contracting Crohn's disease; (b) it will reduce the prevalence of PTB in the herds, reducing their financial losses due to the disease; and (c) it will improve the overall herd health by reducing the exposure of cattle other agents transmitted via manure, such as Salmonella and pathogenic E. Coli species.

1.2 Epidemiology

The epidemiology of Johne's disease is complex, and the study of its components is critical for a better implementation of prevention and control measures.

1.2.1 The Agent

Map is genetically and phenotypically similar to other Mycobacterium avium subspp, which are opportunistic or environmental microorganisms. The ability to differentiate Map from other mycobacterial species is central to the surveillance and control of Johne's disease in cattle. Molecular techniques have allowed for the differentiation of

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subspecies by either identification of specific insertion sequences in the genome or by species-specific fingerprinting patterns detected by restriction endonuclease analysis (REA) or restriction fragment length polymorphism (RFLP). Differences among mycobacteria causing PTB have been reported in the literature. Cattle and sheep strains (C and S strains) differ, not only in their genotype, but also in their growth rate, nutritional requirements, and host specificity (see chapter 2). Whittington et al.[43] found differences among strains from dairy and beef cattle, as well as from cattle in different regions of Australia (C1 and C3 strains). There seems to be a host preference in Map strains, which is important to know for managing the disease in mixed grazing situations. Nevertheless, it is possible that different types of Map strains have evolved by adapting to more than one host. In the environment, Map can survive for extended periods of time, which presents a significant challenge to the successful control of PTB.

1.2.2 Host Factors

Neonates in particular are at a higher risk of infection than other age categories since they are exposed not only to a contaminated environment (contaminated manure from other adults in the herd), but also to the ingestion of milk and colostrum from the mother or from other cows (pooled colostrum). The naive immune system characteristic of the neonate might also contribute to bacterial colonization of the intestine. Natural resistance to PTB infection seems to develop with age; however, older cattle may also acquire infection given a significant bacterial dose.[17] Studies involving experimental infection and monitoring of cattle with PTB suggested that animals infected as adults are less likely to develop clinical signs of disease than those animals infected within the first months of life.[24]

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1.2.3 Environmental Factors

Map remains viable for 163 days in river water, 270 days in pond water, 11 months in bovine feces, and 7 days in urine.[5] Although laboratory studies have reported that temperatures over 35° C appear to reduce the viability of the microorganisms , the relevance of moisture, UV light, and temperature has not yet been determined.[21] Evidently, Map has good survival skills under a wide variety of environmental conditions, which poses a challenge to the successful control of the disease.

1.2.4 Transmission

The main sources of PTB infection are the infected animals, which are shedding Map into the environment. Thus, pastures, soil, water, feedstuff, colostrum, and milk from teats splashed with contaminated feces could be a source of infection. Map can infect a wide variety of animal species, but it is more commonly found in domestic ruminants. The epidemiological significance of PTB infection in deer and other wild animals, such as rabbits and predators, has not been determined. It is assumed that most cases of PTB infection in wildlife are the consequence of contact between wildlife and infected farm animals. Nevertheless, the existence of an animal reservoir to which Map has adapted and developed a sylvatic cycle is a possibility that should not be overlooked.

It is believed that Map enters the host mainly by the oral route via fecal-oral contact. However, a recent editorial letter from Corner et al.[11] suggested that experimental studies did not provide convincing evidence that the major route of transmission is fecal-oral. Instead, these researchers suggested that it would be valuable to look at inhalation of Map as a major route of transmission of PTB in cattle. In support to this theory, a personal

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communication with Dr.Barbara Martin[a] indicated that Map has been isolated from lung tissue in experimentally infected calves.

Intrauterine transmission has also been described in cattle, sheep and goats; however, the magnitude of this mode of transmission is unclear. On the one hand it has been reported that Map has been found in low numbers in uterine fluids and semen only in animals with severe PTB infection.[13] On the other hand, studies by Sweeney and Stehman indicated that 26 % of the infected cattle and 10% of the goats with subclinical infection produce infected fetuses.[36;38] The isolation of Map from parasites of the gastro-intestinal tract in ruminants generated speculation about a potential indirect route of transmission by means of intestinal parasites. Indirect transmission via parasites, as well as venereal transmission, seems less likely to occur than the fecal oral form.

1.2.5 Risk Factors Associated with PTB

The following risk factors have been associated with PTB infection in the literature:[9;39]

- Herd Size: The size of a herd is strongly related to herd prevalence. It has been reported that herds with more than 300 cows are 4.6 times more likely to have PTB than those with less than 300 cows.[39]
- Introduction of Replacement Animals: The incorporation in the herd of stock with unknown status increases the risk of infection transmission
- Calf Management: Increased time of separation between calf and dam allowing calf to suckle, and lack of adequate cleaning of the udder before collecting colostrum

[a] Dr Barbara Martin, National Veterinary Service Laboratories, US Department of Agriculture

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increase the risk of ingestion of contaminated milk or colostrum form an infected dam, thereby increasing the risk of infection in the calf.

- Group Housing for Peri-Parturient Cows: This practice increases the risk of PTB infection transmission to the susceptible neonates by allowing the access of multiple cows to the calving areas.
- Group Housing for Pre-Weaned Calves: Group housing of pre-weaned calves increases the probability of calf-calf infection transmission of any disease compared to indiviadual housing.
- Soil Acidity: It has been reported that acidic soils may enhance the viability of Map by making environmental iron available to the bacteria. This is, however, a controversial finding, since PTB diagnosed in small ruminants in South Africa was associated with alkaline soils.[22]

1.3 Economic Impact

The sources of economic losses attributable to PTB are the following:

- Reduced Feed Efficiency: Infected cows with clinical signs of disease have a significant reduced feed efficiency due to malabsorption compared compared to healthy cows.[30]
- Reduced Milk Production: Abbas et al. reported a 15% reduction in annual milk production in infected cows compared to non infected cows within the same herd.[1] Whitlock et al.[40] demonstrated that sublinically infected cows produce 12 pounds less per day than culture negative cows. In addition, ELISA positive cows produce 4% less milk during their productive life than those ELISA negative.[29] Not only the

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quantity of milk is affected in infected cows, but there is alos a decline in the total amounts of milk fat and protein. It has been estimated that the reduction in milk fat and protein in subclinically infected cows costs $205 per cow per lactation.[29]

- Reduced Fertility: It has been reported that asymptomatic Johne's cows have a higher rate of culling due to infertility than Johne's negative cows.[4;27] In addition, the calving interval in infected cows was reported to be 1.7 months longer than in negative cows.[1]
- Premature Culling: PTB infected animals are slaughtered before reaching their production potential.[4]
- Increased Incidence of Mastitis: PTB infection was found to be associated with a 22.6% risk of mastitis in asymptomatic Johne's cows compared to 3.6% in non infected cows. In addition, MacNab demonstrated that PTB infected cows have a higher somatic cell count than non-infected.[26] The 1996 dairy NAHMS study, estimated the annual losses due to PTB in US infected herds with more than 10% of clinical cases to be approximately U$ 200 per cow.[2] This estimate comprised direct costs due to premature culling, reduced milk production, and reduced body weight at slaughter.

Indirect costs due to Johne's disease result from restrictions to animal trading, reduced accessibility to the markets, laboratory and veterinary fees, PTB research funding, and preventive management practices. Other less obvious costs are the so-called "inapparent costs", which include the loss of genetic potential due to early culling and trading restrictions. Both indirect and inapparent costs are difficult to estimate because they depend on the market prices and the production system.

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1.4 Prevention and Control

A successful control plan for PTB should include risk assessment and the implementation of management strategies aimed at preventing the spread of infection at the individual, herd, and regional levels.[21] Control of PTB at the regional level is based on surveillance methods, such as notification of suspicion, investigation of suspected clinical cases, investigation of high-risk herds, movement testing and accreditation testing aimed at preventing the spread of infection. The limitations of these procedures are mainly related to the poor sensitivity of the currently available tests for detection of infected herds and animals, as well as the lack of notification of suspect cases. The least biased method to achieve a reliable estimate of PTB prevalence at the regional level includes abattoir surveillance for gross lesions or serology combined with fecal culture, and purposive surveys. Zoning is another tool that could be used to prevent the dissemination of infectious animal diseases from infected areas to free zones. This procedure has been used in Australia to maintain free zone areas (such as Western Australia). At the herd level, close management is central to avoid the introduction of infection in PTB free herds. In herds with open management, breeding animals should be purchased from low-risk or negative PTB herds. Within herd transmission of PTB can be avoided by the implementation of the following management practices: [21]

- Secure a clean environment for delivery of calves
- Ensure that neonates receive colostrum from their mother or from a negative dam
- Minimize the time of contact between the neonates and their mothers (no more than 12 to 24 hours after birth)

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- Rear calves separated from adults and avoid fecal cross-contamination of water, pastures, milk/colostrum and utensils between adult and young stock
- Cull of clinical and subclinically infected animals or have them managed separately.

The increase of herd immunity by vaccination is another way of reducing the contact rate in infected herds. The available vaccines (killed or attenuated) for PTB, however, do not produce a significant increase of immunity at the individual level and do not prevent infection in all animals either. The benefit of vaccination against PTB is to reduce the incidence of clinical disease and consequent environmental contamination with Map. The downsides of vaccination are that vaccinated animals continue to shed Mycobacteria, they also react to serological and intradermal skin tests, and they may develop a granuloma at the injection site.[23;35;37]

1.5 Description of the Study and Dissertation Structure

1.5.1 Rationale

Natural PTB infection seems to occur in young animals (less than 30 days old) mainly due to ingestion of infectious fecal matter via milk or colostrum. Clinical manifestations and fecal shedding of the bacteria, however, do not typically start until the adulthood. As with most mycobacterioses, the first immune response elicited by the host is a cell-mediated type.[6] A humoral immune response is often found in adult animals (older than three years of age) shedding the agent in their feces with or without manifesting clinical signs of disease. It has been described that more than 90% of the infected cattle in an adult herd may be subclinically affected.[32] These animals, characterized by the absence of clinical manifestations, shed the bacteria mainly in feces, milk, or colostrum and

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constitute a permanent source of infection for the rest of the herd. Map has also been found in fecal samples from calves experimentally and naturally infected with Map.[10;25] The confirmation of PTB status is performed by using direct diagnostic methods based on the detection of the agent, or indirect diagnostic techniques based on either the detection of specific antibodies or cell-mediated immunity. Bacterial isolation by conventional fecal culture on solid media is the most commonly used direct method for the confirmation of the disease in suspected individuals. This procedure, for years considered the "gold standard", is time and labor consuming (approximately 16 weeks) and has low sensitivity. For these reasons, the detection of PTB infected animals has moved towards the use of serological methods, such as enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID) or complement fixation (CF). The ELISA has been reported to have the best sensitivity of the three.[33] The complex immune response towards the presence of the agent, the poor understanding of the disease pathogenesis, and the mentioned limited sensitivity of the diagnostic tests have hampered the efforts to control Johne's disease.

The accurate detection and culling of fecal shedders is central for the control of PTB in cattle populations. The identification of a strategy for early diagnosis of PTB infection in heifers and young bulls would be a significant contribution to the livestock industry by avoiding the costs of raising PTB infected animals. Early detection would also minimize the risk of dissemination by culling animals prior to the stage of heavy shedding. Improved reference tests and diagnostic strategies are needed in order to improve the accuracy of detecting subclinically infected adult and young cattle.

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This study was aimed at exploring new diagnostic strategies for detection of PTB infection and fecal shedding in young and adult animals. It was also a comprehensive attempt to describe the evolution of the cellular and humoral immune response in calves from herds with management practices and levels of PTB infection typical for the state of Colorado. The association between the PTB status of the dams and the immune response of their heifer calves was also explored in order to identify potential indicators of infection in young animals. Overall, this study was intended to better understand the immunopathogenesis dynamics in young heifers exposed to moderate to low levels of PTB infection and to identify a diagnostic strategy that would improve the accuracy of detection of sublinically infected young and adult dairy cattle,

1.5.2 Hypotheses

a. Tests based on the detection of CMI could be used for the detection of young dairy cattle exposed to PTB infection

b. The PTB status of the dam (measured by ELISA and fecal shedding of Map) is associated with the CMI responses in calves younger than one year old and could be used as a predictor of the calves' CMI response.

c. OTSN-PCR used in parallel with fecal culture will increase the accuracy of detection of fecal shedders more than using fecal culture alone.

d. The use of conventional fecal culture (HEYM) in combination with another rapid culture system, such as the liquid non-radiometric ESP culture system II, will improve the detection of fecal shedders compared with the use of only one of the two fecal culture systems.

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1.5.4 Methodology

1.5.4.1 Selection of Study Animals

Animals for this study were selected from two well-characterized dairy herds with high (11% positives, 22% positives plus suspects) and low (2% positives, 5% positives plus suspects) sero-prevalence of PTB infection determined by IDEXX ELISA.[20] Both herds had participated in a serological monitoring program for PTB together with 18 other dairy herds of the Northern Colorado area. The high seroprevalence herd (hereafter referred as herd A) had approximately 2500 milking cows and a history of PTB infection confirmed by fecal culture and presence of clinical cases. The low seroprevalence herd (hereafter referred as B) had approximately 1000 milking cows and no history of clinical cases. One decisive aspect for the selection of these herds was the willingness of the owners to participate in a long-term cohort study.

1.5.4.2 Enrollment

Three hundred dam/heifer pairs from herd A and 64 from herd B were enrolled in the study based on the dams' serological status. The enrollment was performed monthly when the female calves were approximately 2 months old (+/- 15 days). A total of 100 seropositive, 100 suspect and 100 negative dams (and their heifers) from herd A, and 64 sero-negative dams (and their female calves) from herd B were enrolled in the study. The classification of the serologic status from the IDEXX ELISA was proposed by Dr. M. Collins.[8]

1.5.4.3 Collection of Samples

1.5.4.3.1 Dams

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Serum, feces and milk samples were collected only once, at the time of enrollment. Serum samples were collected and stored at –70°C until tested by IDEXX ELISA. Forty ml of composite milk samples from the four quarters and approximately 10 grams of feces were collected from every dam enrolled in the study. Feces and milk were kept frozen at –70°C until processed by culture and/or PCR.

1.5.4.3.2 Female Calf Cohort

Females calves were tested at 2, 4, 6, and 8 months of age. Testing procedures included intradermal skin testing, γ-IFN test, and ELISA. Fecal samples were collected from the rectum at 8 months of age for detection of Map in feces by culture and PCR. Heparinized and non heparinized blood samples were collected in vacutainer tubes for ELISA and G-IFN testing.

1.5.4.4 Tests under Evaluation

γ-IFN Test: Heparinized blood samples for γ-IFN were transferred into a cell culture plate and stimulated with Map and *M. avium* PPDs in separate wells of the same plate, both at a concentration of 0.3 mg/ml. A negative control sample was stimulated with PBS. Samples were incubated overnight at 37°C and 5% CO_2, according to manufacturer's instructions (BOVIGAM ™ CSL, Ltd. Parkville, Australia). After overnight incubation, stimulated plasma was collected and stored at –70°C until tested.

Intradermal Skin Test: The intradermal skin test was performed simultaneously to the collection of blood in order to avoid any interference with the immune response detected by the other methods. Cervical (left/right) and caudal fold (left/right) injection sites were randomized a priori for every calf, and 0.1 cc of Map PPD (NVSL, 1 mg/ml)

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was injected intradermally. Delayed Type Hypersensitivity (DTH) reactions were measured with a standard caliper 72 hours post injection. Any reaction or sign of inflammation was recorded in mm of skin thickness.

ELISA: Non-heparinized blood samples were centrifugated at 3000 rpm for 20 minutes and serum was transferred into 1 ml aliquots and stored at –70°C until tested. The tests were performed according to the manufacturer's instructions. Results were classified according to the sample-positive (S/P) ratio (S/P ratio= Sample OD at 650 - Neg. control mean /Pos. control mean - Neg. control mean).

Positive = S/P > 0.25

Suspect = 0.1 < S/P < 0.24

Negative = S/P < 0.1

Fecal Culture: Feces collected from the dams and heifer calves were tested by conventional culture on solid media (HEYM) for isolation of Map. A subset of paired fecal samples were tested both by conventional culture and the ESP II system, which is a non-radiometric culture in liquid media.

PCR in Feces: A one tube semi-nested PCR (OTSN-PCR) for detection of Map in spiked feces was optimized at the Animal Population Health Institute (APHI) laboratory. The target DNA was the species-specific Insertion Sequence (IS) 900, and the selected primers, as well as the DNA extraction procedures and amplification conditions, are detailed in Chapter 5.

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1.5.4.5 Confirmation of Disease Status

Confirmation of disease status in the dams was achieved mainly by fecal culture. However, results from PCR in fecal samples were also considered as reference tests for evaluation of the indirect diagnostic tests.

1.6 Dissertation Structure

Chapter 1 provides a general introduction to the disease, a description of the study design, and the methodology. The Methodology section in Chapter 1 overlaps with the Materials and Methods described in each of the other chapters. Each specific study, however, provides a more detailed description of the research approach used for those specific objectives.

Chapter 1 is a literature review specifically intended to provide information about methods for diagnosis and screening of Paratuberculosis, their advantages and limitations.

Four chapters comprise the main body of this dissertation. Each chapter addresses one of the four objectives described in section 1.5.3

- Chapter 3 "Evaluation of three tests for the detection of Paratuberculosis in a calf cohort at 2, 4, 6, and 8 months of age"
- Chapter 4 "Risk factors associated with positive gamma and Johnin skin test results in 8-month-old dairy heifers"
- Chapter 5 "Evaluation of a One tube semi-nested PCR for detection of Paratuberculosis in young and adult dairy cattle: Comparison to other diagnostic and screening tests"

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- Chapter 6 " Comparison of two fecal culture systems for the detection of ***Mycobacterium avium*** subsp ***paratuberculosis*** in adult and young dairy cattle".

Lastly, Chapter 7 , is a summary of the conclusions and recommendations based on the findings of this study.

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CHAPTER 2 LITERATURE REVIEW: DIAGNOSIS AND SCREENING OF PARATUBERCULOSIS

2.1 Paratuberculosis: Infection, Exposure, and Related Issues

There are two possible ways of detecting Paratuberculosis (PTB)-infected cattle, either by detecting the presence of the agent in clinical specimens, or by detecting the immune response elicited by the agent after colonization of the host. Hereafter, the former will be called the direct and the latter the indirect methods for detection of PTB.

It is relevant to note that the identification of the agent in clinical specimens is not always synonymous with infection detection. Infection has been defined as the "invasion and multiplication of pathogenic microorganisms in the body, with or without the manifestation of disease".[68] Exposure, on the other hand, does not imply colonization of the host. In the particular case of PTB, the detection of the causal agent in feces, is generally considered an indication of infection. There are cases, however, when the causal agent is isolated or detected in host's fecal matter even though it might be passing through the intestinal tract. The frequency of the occurrence of this phenomenon is unknown, and it is acknowledged as a topic that requires further research. The detection of a specific immune response towards the causal agent could be an indication of either infection or exposure. Validation of indirect methods for infection detection seems to require the use of a direct diagnostic method that would confirm PTB infection leaving no doubts that the agent being detected had colonized the host.

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2.2 Etiology

The causal agent for Johne's disease was first isolated by Twort and Ingram in 1910 [13;96] and it has received different names (***Mycobacterium enteriditis chronicae pseudotuberculosae bovis johne,*** and ***Mycobacterium johnei***) until its present classification as ***Mycobacterium avium*** subsp ***paratuberculosis*** (Map).[95] For several years, research on Johne's disease was performed by using strain 18, an ***M. avium*** serovar 2 strain, which was thought to be a laboratory adapted paratuberculosis strain characterized by rapid growth and mycobactin independence. With time, researchers suspected that strain 18 was more likely a laboratory contaminant, and the failure to cause Johne's disease in ruminants supported the suspicion. In 1968, based on the present evidence, the research community decided to withdraw strain 18 as a referent for Johne's disease. A new wild type was adopted as a reference strain and redesignated as American Type Culture Collection (ATCC) strain 19698. Decades later, the advances in DNA technology (absence of IS 900 in strain 18) confirmed that strain 18 was not the agent of Johne's disease but rather an ***M avium*** serovar 2, with similar phenotypic characteristics as Map.[12]

Map is an aerobic, non-spore forming, non-motile, Gram positive, and acid-fast bacillus.[83] It has a characteristic dependency on mycobactin, an iron-chelating agent, responsible for making iron available to Map cells. Iron plays an important role in basic biological redox reactions, and because of its scarce distribution in the environment, most microorganisms have developed mechanisms to transform insoluble environmental iron into a soluble state. Map depends on supplementation with mycobactin in the culture media for artificial growth. Even though it is known that under in vivo conditions Map

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obtains iron from the host, the mechanisms by which this process takes place are not well known.

Map requires between 6 to 8 weeks to produce visible colonies in solid media. Typical colonies look white, firm, glistening, rough or smooth, of approximately 1-5 mm diameter.[13] There are some phenotypic differences between strains, such as the presence of pigmentation in some of them, as well as differences of cultural requirements for ovine strains compared to cattle strains. For this reason, researchers differentiate between C strains (from cattle) and S strains (from sheep), which not only have different phenotype but also different fingerprinting pattern.[20] Although Map sheep strains have been notoriously difficult to cultivate in conventional solid media, they can grow in liquid Middlebrook media with the addition of egg yolk (BACTEC 12B).[107]

Typing of Map strains cannot be accomplished by biochemical testing due to the variability in reactions between strains. Current methods for strain identification are based on growth characteristics, morphology, mycobactin dependency, and presence of IS 900 at the genomic level, which is considered specific for Map. Antibiotic susceptibility is also variable among strains. However, in general terms, Map seems to be susceptible to neotetrazolium chloride, streptomycin, and rifampicin, and resistant to isoniazid and thiophen-2 carboxylic acid hydrazide (TCH).[13] Thin layer chromatography of cellular fatty acids has also been utilized for the differentiation of *M. avium* subspecies.[7;48] This technique is expensive and labor intensive, which makes it unsuitable for routine diagnosis.

In the environment, Map can survive for extended periods of time. Map remains viable for 163 days in river water, 270 in pond water, 11 months in bovine feces and 7

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day in urine.[13] Map is resistant to many disinfectants. After 10-minute exposure, formalin 5%, phenol (1:40), cresylic disinfectants (1:32), mercury bichloride (1:1000), and calcium hypocloride can effectively kill Map.[13]

2.3 Direct Detection of Mycobacterium Avium subsp. Paratuberculosis

As mentioned above, methods for direct detection of Map are those techniques involving the isolation and/or identification of the microorganism by specific staining properties, or the identification of species- specific portions of their genome by molecular techniques.

2.3.1 Culture

Isolation of Map from tissues or feces is the definitive test for confirmation of Johne's disease. Factors contributing to a successful isolation of Map could be grouped into those related to the cultivation process per se, and those related to the characteristics of the inoculum. Among factors related to the cultivation process are the type of media utilized, the process for concentration of bacteria to produce the inoculum, and the decontamination process.

2.3.1.1 Factors Associated with the Cultivation Process

2.3.1.1.1 Culture Media

There are currently three methods for culturing Map: conventional culture on solid media, radiometric culture and non-radiometric culture. Whipple et al.[101] published a comprehensive review on conventional methods for cultivation of Map on solid media. Herrold egg yolk with the addition of mycobactin J (HEYM) is the media most frequently used by most laboratories. Incubation time required for isolation of Map in HEYM is

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approximately 12 to 16 weeks or longer. The use of a modified Lowenstein Jensen media containing mycobactin, sodium pyruvate and antibiotics (chloramphenicol, cycloheximide, and penicillin) also called, Jorgensen media, has been recommended for cultivation of Map as well.[49] Development of colonies in Jorgensen media has been reported to be more abundant and faster than in HEYM media.[44] There is, however, a downside of using Jorgensen's media, which is the high proportion of contaminated samples. Radiometric culture methods were introduced in the early seventies to overcome the lengthy incubation time required for isolation of Map in solid media. The BACTEC system is a commercial radiometric liquid culture system for isolation of mycobacteria. This system contains radioisotope-labeled palmitic acid (with C_{14}), which is utilized by bacteria as a nutrient source. Mycobacterial growth is detected indirectly by measurement of the CO_2 released in the upper part of the culture bottles when bacteria metabolize palmitate. The BACTEC radiometric culture method (BACTEC-RCM) requires a minimum of 9 days for detection of growth after inoculation of the media with Map strains from cattle, and approximately 8 weeks to have a negative result available on clinical specimens (vs. 6 months required by traditional methods)[26;30] One limitation of the BACTEC-RCM is that it uses radioactive material. Laboratories utilizing this media should be accredited for handling radioactive C_{14}, which makes the use of this system less popular. An alternative to the BACTEC-RCM are the non-radiometric culture systems such as the Extra Sensing Power (ESP) system II (commercialized by TREK Diagnostics, Inc, Cleveland OH) and the Mycobacterium Growth Indicator Tube (MGIT) systems (commercialized by Beckton and Dickinson). They are based on the measurement of pressure changes within the headspace of a bottle or culture tube, which

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contains media specifically prepared to maximize the consumption of nutrients and the production of gas as by-product of bacterial metabolism. The ESP system coupled with acid fast stain of negative bottles and IS900-based PCR for typing of grown bacteria has become more widely used for detection of Map in feces in the last five years. This culture system was chosen as one of the reference tests for the Dairy National Animal Health Monitoring Systems (NAHMS) study 2002 (Dr Brian McCluskey, personal communication).

2.3.1.1.1.1Concentration of the Inoculum

The literature describes at least three different methodologies to obtain a pellet for decontamination of specimens, prior to inoculation in the media: Sedimentation, Centrifugation, Filtration.

- Sedimentation: In 1990, the National Veterinary Services Laboratories (NVSL) suggested the use of a 30-minute sedimentation as a standard procedure to obtain a pellet for decontamination.[101] Sedimentation prior to overnight decontamination, however was demonstrated to be less sensitive when compared with centrifugation of the samples.[88]

- Centrifugation: Centrifugation of samples after decontamination was reported to increase the amount of Map in the inoculum and the sensitivity of the system.[103 51] In 1989, Shin et al. described the so- called Cornell method, which incorporated a double decontamination and a centrifugation step at 1,700 xg for 20 minutes.[82] Contamination rates were reduced to less than 2% with this method. They also reported a 3-fold increase in the sensitivity (SE) using the double decontamination methods compared with the sedimentation method. Stable described what she called

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the National Animal Disease Center (NADC) method, which consisted of a double centrifugation of the total fecal sample supernatant combined with a 2-step decontamination.[88] According to her results the NADC method is more sensitive than the Cornell method due to the addition of one extra centrifugation and decontamination step to the whole procedure. In 1999, Kalis et al investigated the optimal speed at which the samples have to be centrifuged for a successful isolation of Map.[52] They evaluated the effect of two centrifugation speeds (high: 3,000 xg and low: 1,000 xg) on the isolation of Map using Jorgensen culture media. Their results showed that centrifugation at 3,000 xg yielded higher numbers of Map in the inoculum, as evidenced by the earlier detection of growth.

- Filter-Concentration: Polycarbonate filters (13 mm diameter) with a 3 um pore size were used by Collins et al. to enhance the detection of Map in BACTEC RCM in 1990.[22] The filter was used to separate Map from 10 ml of suspension, which contained 3 grams of feces in 30 ml of 1.0% hexadecilpiridinium chloride (HPC). Briefly, 10 ml of the fecal suspension were drawn into a 10-ml syringe with an 18 gauge needle. The needle was then substituted using the filter described above. The fecal suspension was slowly pressed through the filter. After rinsing with sterile water, filters were placed into the BACTEC RCM and the bottle of media was resealed with a rubber disk. Compared with conventional culture in HEYM media, radiometric culture of filter-concentrated fecal suspensions was capable of detecting 50 % more positive specimens.[22]

2.3.1.1.2 Decontamination of the Inoculum

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In the early 1960's, research was conducted to determine the efficacy of several decontamination agents to control fungal and bacterial contamination for isolation of Map from feces. Among the tested decontamination agents were 10% sulphuric acid, 0.2% NaOCl, 0.5% phenol, and 0.1% to 1% benzalkonium chloride .[63;64] These early studies recommended the use of 0.3% benzalkonium chloride for decontamination of the inoculum. In the eighties new studies on various methods to reduce bacterial and fungal contamination, including oxalic acid, sodium hydroxide, sodium hypoclorite, phenol, benzalkonium chloride and hexadecyl-cetylpyridinium chloride (HPC), concluded that the latter two methods were the most effective. At the present, most diagnostic laboratories rely on the use of either HPC (0.75% final concentration) or benzalkonium chloride. The use of 3 um pore size polycarbonate filters was reported to be helpful for the control of Bacillus spp., which has been identified as the most common contaminant when culturing bovine feces.[78] The addition of antimicrobials to the inoculum and/or the culture media is another effective way of controlling the growth of opportunistic flora. The Cornell method involves an overnight incubation of the fecal inoculum in a brain heart infusion broth (BHI) containing vancomycin, nalidixic acid and amphotericin B, and a posterior incubation in HEYM containing the same antimicrobials.[88] Current radiometric and non-radiometric culture methods (BACTEC and TREK ESP) use a cocktail of antibiotics commercially available named PANTA® plus (Becton Dickinson), Myco AS, or Myco PVNA. A comparison between the effect of PANTA, and a cocktail of vancomicin, amphotericin B and nalidixic acid (VAN), indicated that the latter was superior to the commercial product for the control of contaminant flora.

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2.3.1.2 Factors Related to the Characteristics of the Inoculum

A second group of factors, which affect the isolation of Map are more related to the characteristics of the microorganisms (viability and type of strain) and the quantity of Map in the inoculum. The mechanisms by which each of these factors could affect the development of Map in culture are explained as follows:

- Bacterial Viability: Bacterial viability is influenced by the condition, transportation, and storage of the specimen. If the specimen is feces, the success in the isolation of Map will depend on whether the sample is fresh (i.e., recently collected from the rectum and transported to the laboratory for isolation) or whether it has been subjected to the action of UV radiation. The latter is the case of fecal samples collected from the environment (i.e., pen samples), which could be affected by the bactericidal effect of direct sunlight, urine, and dehydration, thereby hampering the growth of Map in culture. Transportation and storage conditions may also affect the viability of Map. Dehydration of the specimen might cause damage to the mycobacterial cell wall, thereby reducing the chances of growth in culture media. Temperature and time of storage have proved to affect the success of isolation of Map by culture.[78] The viability of Map in fecal samples stored at room temperature (23° C) for approximately 10 days was found to be affected significantly when compared to storage at –70° C for 15 weeks.[78]

- Type of Strain: S strains from sheep are more demanding of certain nutrients in the culture media, such as egg yolks, than C strains from cattle. S strains also need longer time for development of visible colonies in culture.

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- Dose of Inoculum: Samples with low numbers of Map (low shedders) are less likely to yield a positive culture result than those with moderate or high numbers. For this reason, the sensitivity of culture is always higher for moderate and heavy shedders than for low shedders.

2.3.1.3 Sensitivity

A study comparing the sensitivity of the currently available culturing procedures on individual fecal samples has been published by Eamens et al. in 2000.[31] Five different culture techniques were evaluated in this study:

e. Sedimentation prior to solid culture media

f. Double incubation with antibiotics and inoculation into solid media (known as Whitlock's procedure)

g. Filtration to radiometric culture

h. Double incubation into radiometric media without initial saline mixing step (known as Whitlock's modification)

i. Whitlock's procedure into radiometric media

Conclusions of this study indicated that radiometric culture with a preparatory double incubation/centrifugation described by Whitlock and Rosenberger (1990) was the most sensitive (method e).[102] The second most sensitive was method b, which involved the same preparation procedure with inoculation into solid media. Methods based on sedimentation or filtration were significantly less sensitive than those based on double incubation with antibiotics and centrifugation.

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Pooled fecal culture (PFC) has been used as a flock-screening test for PTB in Australia where the testing of individual fecal samples is not an option due to large flock size. The main reason for using fecal culture instead of antibody tests for screening of PTB lays in the fact that the antibody response develops later than the beginning fecal shedding.[81;105] Therefore the early identification and culling of Map shedders is a helpful tool for controlling the spread of PTB in animal populations. Compared with serology, the screening of ovine PTB by PFC is more sensitive and cost efficient.[106;81] The sensitivity of PFC in sheep-flocks depends mainly on the number of multibacillary versus paucibacillary cases (the higher number of multibacillary cases, the more sensitive), and on the efficacy of mixing the pools of feces.[106] Whittington at al. reported a sensitivity between 83% to100% for detection of at least 1 multibacillary case in 49 uninfected pellets, and 23% to 73% for detection of at least 1 paucibacillary case in 49 uninfected pellets, using pools of 50 individual samples.[106] Seargent et al. reported a 95% sensitivity of PFC when applied to flocks with more than 2% prevalence of PTB, and 82% sensitivity when the prevalence was lower than 2 %.[81]

In cattle, a study from the Netherlands reported a 73% sensitivity of PFC versus 64% sensitivity of culture from individual samples.[51] This study recommends pooling the samples in age-dependent groups. This is called strategic pooled sampling and is aimed at accounting for the clustering of the disease by age group. A recent study performed in the US showed a 94% sensitivity when including at least 1 sample with high numbers of Map in a pool of 5, and 40% sensitivity when including at least 1 sample with low numbers of Map in a pool of the same size.[100]

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2.3.2 Histopathology and Acid Fast Stain

Gross and microscopic PTB lesions have different patterns in cattle and small ruminants such as sheep and goats. While cattle lesions are a diffuse granulomatous change without necrosis, inflammatory hyperemia, or fibrosis, sheep and goat lesions often resemble those of tuberculosis (TB) with caseation, calcification, and fibrosis. [13] Macroscopic lesions are mainly present in the intestine and in the draining mesenteric lymph nodes. Ileum and ileo-cecal valve are the most frequently affected areas in the intestine. Intestinal lesions could be diffuse or segmental. Macroscopically, the intestinal mucosa looks thickened, edematous, and its corrugated appearance does not disappear by manual stretching. Serosal and mesenteric lymphatic vessels are dilated, and mesenteric lymph nodes look pale, edematous and swollen.[8;104] Microscopically, the histological changes vary according to the severity of disease. In subclinical cases, lesions might be mild, characterized by scattered Langhans' giant cells with few macrophages in the villi and in the paracortical zone of the lymph nodes. As disease progresses, the number of Langhans' giant cells and macrophages infiltrating the intestinal wall and the lumen of the lymphatic vessels increases. The accumulation of giant cells obliterates the intestinal crypts and contributes to the thickening of the intestinal wall. Large amounts of acid fast bacilli could be demonstrated inside giant cells and macrophages. A consistent finding in clinical cases is the change of the lymphatic tissue characterized by the presence of plasma cells and lymphocytes surrounding the lymphatic vessels and clumps of epithelioid cells in the lumen. In severe stages of disease, epithelioid granulomas are formed in the wall of the lymphatic vessels and might protrude into the lumen. Microscopic focal granulomas could also be present in the liver, tonsils, kidneys, lungs and other systemic lymph-nodes.[74]

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In countries with an advanced Program for Control of Johne's disease, such as Australia and New Zealand, routine histopathology comprises the study of a standard set of tissues (ileocecal valve, ileocecal lymph nodes, pieces of ileum distal and proximal, one piece of proximal colon, and one piece of cecum). These tissues are stored in 10% buffered formalin and stained with the hematoxylin-eosine stain and the Ziehl-Nielsen method for acid fast bacilli. The criteria for interpretation of histological findings in Australia and New Zealand are the following (Dr D. Cousins personal communication based on the Australia and New Zealand Standard Procedures for PTB):

Indication of PTB infection: The presence of one or more single giant cells in any section, and/or one or more accumulation of epithelioid cells containing one or more acid fast bacilli (3 for New Zealand) in the lamina propria or in the paracortical zone of the lymph nodes.

Suggestion of PTB infection: If any section contains two single Langhans' giant cells and/or two accumulations of epithelioid macrophages in the intestinal lamina propria or in the cortical area or the lymph nodes, in the abscense of acid fast bacillus.

Pathologists in the United States use similar classification criteria. Lesions characterized by inflammatory giant cells and epithelioid macrophages in the absence of demonstrable acid fast bacilli are categorized as "paratuberculosis-suspicious", whereas a "paratuberculosis-positive" result requires the presence of non-caseating granulomas with Langhan's giant cells and epithelioid macrophages with at least one acid fast bacillus compatible with Map. The serial study of several sections of tissue is recommeded to identify inflammatory cells typical of PTB when individual inflammatory cells of doubtful morphology are present in the sample.[9;10]

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2.3.3 Molecular Genetic-Based Diagnostic Tests: Polymerase Chain Reaction (PCR)

PCR has been used for detection of Map in clinical samples such as feces, milk, and tissues. As a diagnostic tool, PCR offers the advantage of providing results in 24 hours compared with several weeks of culture. Target DNA sequences for molecular diagnosis by PCR could be divided in two groups: (a) insertion sequences, which are mobile, transposable elements within the genome, generally present in multiple copy numbers and, (b) single-copy genes. Within the first group, the most popular target DNA sequence utilized in PCR is the IS900 sequence, first reported in 1989 by Green et al.[43] IS 900 is present in 15 to 20 copies in Map's genome, which makes it particularly attractive for diagnostic purposes. The most commonly used sets of primers targeting IS900 were reported by Vary et al [98]and Millar et al.[65] There is only one commercial PCR kit for detection of Johne's disease developed by IDEXX laboratories, which is based on the IS900 sequence. Southern blot analysis using IS900 as a probe led to the identification of other insertion elements, IS901 and IS 902, present in some ***M. avium*** subsp ***avium*** (Maa) strains and in ***M avium*** subsp ***sylvaticum***.[55;69] These two insertion elements share 99% homology between them and belong to the IS900 family, but have not been detected in Map strains. In the last three years, the species-specificity of IS900 has been questioned by studies reporting the existence of IS900-like sequences in other mycobacterial species.[27;35] While Cousins et al. recommend the use of restriction enzyme analysis (REA) to confirm the specificity of the IS900 products, Englund et al., disagreed with this recommendation arguing that IS900-like sequences might have identical restriction sites as IS900. According to Englund et al., the definite method for identity confirmation of the IS900 product is sequence analysis.[35] Another important insertion element for differentiation of Map strains is the IS1311. This sequence present in both

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Map and Maa strains has five point mutations, which could be targeted by REA for differentiation between subspecies. In Australia, current management practices are based on the belief that cattle are only susceptible to PTB caused by the C strain of Map and not by the S strain. Because of this, it is considered safe for cattle to graze in pastures previously grazed by sheep. The need to confirm the safety of these management practices led to the development of a practical and rapid strategy for differentiation of sheep and cattle strains based on the use of REA targeting the 5 point mutations in IS1311.[60] In this way, IS1311 is useful for differentiation, not only between Maa and Map, but also between S and C strains within the subsp paratuberculosis.

Within the second group of target sequences for Map, there are other specific genes, such as a 620 base pair (bp) fragment named F57, and the *hspX* gene. The F57 fragment reported by Poupart et al.[76] in 1993, does not hybridize with DNA from other mycobacteria spp. and does not have common sequences with IS 900, the *hspX* gene, or any other known insertion sequence from Map. F57 encodes a 34-kDa antigenic protein, and has been used as a target sequence in a multiplex PCR aimed to differentiate, ***M. bovis***, Maa and Map.[19] On the other hand, the *hspX* gene first reported by Ellingson et al[32] in 1998, encodes a heat shock protein implicated in cell attachment and stimulation of phagocytosis, all of which are considered virulence factors.[46] A portion of the *hspX* gene, which is considered specific for Map, has been used as a target DNA sequence in a PCR panel for differentiation of Mycobacterium avium subspecies.[33] Not all Map strains tested in this study were positive for the *hspX* gene; 2 out of 12 strains failed to amplify the gene. Due to the lack of follow up on those two strains, it is unknown whether they lack the gene or whether the template did not have enough target DNA to be amplified.

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A review on DNA target sequences used for PCR diagnosis by Harris and Barletta[46] indicates that that this gene appears to have merit for diagnostic purposes. Different from insertion sequences, which are mobile transposable elements present in multiple copy numbers in the genome, *hspX* and F57 are single copy genes. This attribute makes single copy genes less suitable targets for DNA amplification since the sensitivity of the PCR is compromised due to the presence of only one copy of the target.

The analytical sensitivity of PCR on pure DNA could be as high as 1 microorganism, however, this is never achieved in practice due to limitations related to DNA extraction procedures and presence of inhibitors.[90] The sensitivity of PCR in clinical specimens is highly related to the following factors:

- Type of sample and presence of PCR inhibitors: Detection of Map has been reported in clinical specimens such as feces, milk,[25;41] and tissues.[36] Of these, fecal samples remain the most difficult specimen for DNA extraction and amplification due to the presence of inhibitors.[67] Acidic polysaccharides, heme and its metabolic products, and proteinases are known PCR inhibitors present in feces.[1;67] Map has also been detected by PCR in fresh and formalin-fixed tissues. According to Stevenson and Sharp, the sensitivity of PCR in tissues is higher than in fecal samples. With fresh tissues, the sensitivity of the system depends mainly on the method used for separating mycobacterial cells from tissue debris.[90] With formalin-fixed tissues, however, the sensitivity of the PCR could be affected due to DNA degradation by the formalin-paraffin processing.[39;66] Even though PCR inhibitors were described, milk is the least difficult sample to process for DNA extraction and amplification.[4] When PCR is utilized for identification of Map in primary radiometric culture, the egg yolk

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from the media, which inhibits PCR, could affect the sensitivity of the reaction. To solve this problem, it is necessary to either subculture the isolates in radiometric media without egg yolk , or perform an ethanol extraction followed by centrifugation in order to separate Map from inhibitors in primary culture.[108]

- Bacterial load present in the specimen: The number of Map cells present in the specimen is associated with the final amounts of DNA available for amplification. Most PCR systems can reliably detect cattle shedding more than 104 cells per gram of feces (moderate to heavy shedders). Animals shedding low numbers of bacteria are hardly ever detected by PCR.[90]

- DNA extraction procedure: The procedure utilized for DNA extraction is critical since it also associated with both the binding to PCR inhibitors and the yield of available DNA for amplification. A variety of procedures for DNA extraction from feces have been reported in the literature: centrifugation and heat treatment[98], freeze and boil coupled with TE-Triton100 lysis,[40] Xylol and proteinase K,[37]bead-beating with zirconium beads,[11;21;97], immunomagnetic separation,[42] solid-phase hybridization capture.[65] In a study comparing different physical and chemical methods for DNA extraction from fecal samples, a freeze and boil procedure combined with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mm EDTA) and Triton X-100 was found to be more sensitive, rapid, and simpler than any of the other tested methods (which included bead beating, microwaving, and heating in a dry bath combined with either TE Triton, guanidine isothiocyanate, or a commercial DNA extraction kit).[40]

- Type of PCR system used: The use of two sets of nested primers and two amplification cycles enhances not only the sensitivity but also the specificity of the

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PCR. This is the case of the nested PCR, which demonstrated to be more sensitive than the conventional PCR.[75] The real time or quantitative PCR based on the use of fluorescent molecular probes offers several advantages over the nested and conventional PCR systems such as increased sensitivity, quantification of target sequence in the sample, higher specificity (due to the inclusion of an internal hybridization probe), elimination of all post PCR processing (gel electrophoresis), and high reproducibility of results.[38;54]

2.4 Indirect detection of Johne's disease

Indirect diagnostic methods of PTB infection are those based on the detection of the host's immune response towards the presence of Map. These methods could be targeting the detection of CMI or specific antibodies.

2.4.1 Immune Response

PTB infection in cattle may be acquired either by in-utero transmission or by ingestion of contaminated milk or colostrum during the first months of life.[13] Although based on just one study, most researchers believe that after being ingested, Map penetrates the intestinal mucosa using the M cells as portal of entry. A particular feature of these cells is the lack of brush border micro-villi, digestive enzymes or surface mucus, which facilitates the attachment of microorganisms.[86] It is hypothesized that Map crosses the intestinal M cells by transcytosis and is expelled to the basolateral wall of the M cells, where they are phagocytosed by macrophages and dendritic cells. Live Map may remain intact within the phagocytic cells for a long time or they may be destroyed and presented to T lymphocytes thereby generating the immune response. Contact between antigen

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presenting cells and T lymphocytes induces the liberation of cytokines, which causes macrophage activation and granuloma formation. When activated, macrophages release interleukin-1(IL-1), which activates T cells. Activated T lymphocytes release interleukin-2 (IL-2), which results in the clonal expansion of CD4+ T helper cells and CD8+ cytolytic T cells. While the interaction between macrophages presenting antigen with CD4+ lymphocytes is in the context of MHC class II molecules, their interaction with CD8+ lymphocytes is associated with MHC class I molecules. TH_1 helper lymphocytes direct the cellular immunity processes by liberation of IL-2, gamma interferon (γ-IFN) and tumor necrosis factor-β (TNF-β).[14] TH_2 helper lymphocytes, on the other hand, are responsible for the development of the humoral response by releasing interleukins-4, 5, 6 and 10 (IL-4, IL-5, IL6, IL-10).[14;70] Cytokines released by TH_2 lymphocytes depress the mechanisms for development of CMI, while they enhance the production of specific antibodies.[14] The γ-IFN seems to be the most critical cytokine for the control of mycobacterial infections, as demonstrated by the occurrence of disseminated infection in young children with γ-IFN receptor deficiencies.[3] γ-IFN is secreted by CD4+, CD8+, γδ T cells, and natural killer cells (NK cells), and it is responsible for not only enhancing macrophages' phagocytic functions, but also releasing reactive oxygen and nitrogen intermediates, expression of Major Histocompatibility Complex (MHC) class II, and secretion of arachidonic acid metabolites.[86] In general, cattle in early stages of infection are characterized by the absence of clinical signs of disease and detectable antibodies.[91-93;104] Approximately four to eight weeks after the successful entry of Map in the host, a cellular immune response could be detected. A strong CMI response seems to be characteristic of the early subclinical stage.[18] Several

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studies focusing on tissue immunity have demonstrated the importance of CD4+ T cells in controlling the progression of PTB infection.[2;59] On the other hand, a study by Navarro et al.[71] showed that in advanced lepromatous stages of PTB in goats, the lamina propria of the small intestine has decreased CD8+ and increased CD4+. Local immunity to PTB seems to be represented mainly by CD4+ in the initial subclinical stages shifting to CD8+ as disease progresses into a clinical stage. In contrast to the immunologic events described for tissue, the immune response in peripheral blood during the first stages of disease is characterized by a proliferation of mononuclear cells mediated by CD8+ and γδ T cells with a gradual shift to a CD4+ expansion in later stages. [15;16] Mainly, the role of all CD8+, CD4+, and γδ T cells in PTB and other mycobacterial infections seems to be to provide cytokines for macrophage activation and T cell expansion. Differently from humans, for whom γδ T cells comprise between 1% to 5% of the circulating T cells, the percentage of circulating γδ T cells in calves can reach as high as 40% of the total lymphocyte population. An interesting feature of the γδT cells is that they do not require an interaction with MHC proteins for activation.[28] These cells are a major source of secreted γ-IFN, which is a potent activator of phagolysosome fusion in macrophages.[28] Finally, the role of B cells and specific antibodies in protecting the host against mycobacterial infections is unclear. It has been demonstrated that the T/B cell ratio in tissues is significantly high in experimentally infected lambs.[5] A notoriusly high percentage of B cells in peripheral blood and a decrease of antigen specific B-cell proloferative responses seems to characterize the clinical phase of the disease in cattle.[99] These findings suggest that antigen specific B cells miight migrate to other sites in the

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clinical phase of the disease in cattle with a possible simultaneous redistribution of T cells necessary for B-cell proliferation.[99]

In summary, due to the complex interactions between different cell populations and cytokines involved in the immune process, it seems that the successful detection of PTB infection via indirect diagnostic tests appears to rely on targeting the right immune response at the appropriate stage of infection.

2.4.2 Tests Based on CMI

2.4.2.1 Skin Test

Cellular immune responses can be evidenced by antigen-specific delayed type hypersensitivity reactions (DTH) measured by intradermal injection of the Johnin antigen, which is produced either in the form of Old Tuberculin (OT) or as a purified protein derivative (PPD). The OT Johnin consists of crude culture filtrate of ***Mycobacterium avium*** subsp. ***paratuberculosis*** grown in Dorset and Henley synthetic media. Johnin PPD is prepared similarly, but a precipitation step with trichloro-acetic acid is added. Both products contain crude antigens, which result in significant cross-reactivity with other Mycobacterium spp., Nocardia spp., Actinomyces spp., Dermatophilus spp., Streptomyces spp., Corynebacterium spp., and fungi.[13] Johnin PPD has, however, higher potency and specificity than OT, and can be stored as powder for long periods without loss of potency.[57]

The Johnin test can be performed by intradermal or intravenous injection. In infected animals, the intravenous administration of Johnin produces an increase in the body temperature due to cytokine release and change in the neutrophil:lymphocyte ratio. A

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rise of 1.5 °C or greater between at approximately 5 hours post injection is considered a positive reaction. The calculation of the neutrophil:lymphocyte ratio is more accurate than a measurement of a skin fold but also more laborious. A positive reaction is characterized by a shift in the ratio greater than 2:1. In addition to the recording of rectal temperature variations this procedure also involves preliminary basal blood counts, which makes it time consuming. The intradermal Johnin test is easier to perform and interpret than the intravenous test, and the results are read at the farm site. The intradermal Johnin could be injected either in the neck area or in the caudal fold. A study evaluating the effect of testing frequency and injection site of Johnin on the development of anergy suggested that a month interval between Johnin injections did not impact the immune response of artificially infected calves.[56] Additionally, the same study indicated that variation of injection sites prevents desensitization to the Johnin antigen.[56] Reactions to the intradermal Johnin injection are observed 48-72 hours later for a DTH reaction indicated by the presence of swelling, redness, heat, and edema. The sensitivity of the intravenous Johnin test has been estimated at 80% for heavy shedding cattle and 35% for light shedders.[58] These studies were based on the use of PPD from strain 18, which was thought to be a Map strain. Current scientific knowledge indicates that strain 18 is an ***M. avium*** strain unrelated to PTB in cattle.[12] Hence, the main weakness of the Johnin test is the lack of specificity. Because of this and the lack of new available antigens for testing, the Johnin skin test was abandoned as a routine diagnostic tool, limiting its use to fulfilling requirements for the importation of live livestock, such as llamas and sheep. Currently, there is no available information on the standardization of antigens used for diagnosis of PTB. Steadham et al.[89] designed a study with the purpose of producing and

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characterizing a reference PPD from Map strain 19698 in terms of protein concentration and potency. The evaluated PPD from strain ATCC 19689 was adjusted to a concentration of 1 mg/ml and to a potency capable of producing similar DTH reactions as the USDA Johnin OT 133-8707. Nevertheless, this reagent has yet to be validated under field conditions.

Because the evaluation of the Johnin test was conducted more than 50 years ago, and because reference tests for PTB have improved significantly in the last decade with the development of radiometric culture and PCR, it might be worth re-assessing the value of the skin test using the best battery of antigens available.

2.4.2.2 γ-IFN Test

It has been demonstrated that γ-IFN is an essential cytokine for the control of mycobacterial infections by activating the macrophages' phagocytosis capabilities.[111] A test to detect specific γ-IFN has been developed in Australia and was incorporated to their National Brucellosis and Tuberculosis Eradication Campaign.[80;87;109;110] This test is commercially available (Commonwealth Serum Laboratories, CSL) and measures the amount of γ-IFN produced by lymphocytes stimulated with mycobacterial antigens. Briefly, whole blood is stimulated with bovine and avium PPD overnight in presence of 5% CO_2. Plasma is collected by centrifugation of the stimulated blood and the interferon levels are detected by an ELISA system, which contains monoclonal antibodies against bovine γ-IFN. Compared to the Johnin skin test, the γ-IFN test has the advantage of being non-invasive, providing results that could be objectively measured, and requiring only one movement of cattle for collection of blood samples. The antigen specific stimulation of γ-IFN has been utilized as a measure of CMI responses for both TB and

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PTB. While the γ-IFN test for detection of early stages of TB has been proven useful, its use for detection of PTB is still under evaluation.[80;109;110]

In adult cattle, the γ-IFN ELISA demonstrated higher sensitivity than the absorbed ELISA for detection of subclinically PTB-infected animals.[6] On the other hand, the absorbed ELISA and the γ-IFN bioassay (evidenced by protection from cytopathic effect on a bovine kidney cell monolayer) demonstrated higher sensitivity for identification of animal in clinical stagesd of the disease.[6] Due to the need for maintenance of bovine kidney cell lines, the γ-IFN bioassay has not been further utilized. These findings were confirmed by Stable[85], who compared the γ-IFN secretion of subclinically vs.clinically infected cows. By showing a higher γ-IFN production in the subclinically infected group, the author concluded that the γ-IFN test could be valuable for the detection of cattle with subclinical PTB infection .[85] A more recent study reported the evaluation of a modified γ-IFN test in adult cattle from herds with high and low levels of PTB infection using both fecal culture and histopathology as reference tests.[87] Modifications to the γ-IFN test included the stimulation of blood with a mitogen (ConA) to detect false negative reactors and the use of a Map sonicate in addition to bovine and avium PPDs provided by the manufacturer of the γ-IFN kit. The sonicated Map was incorporated with the aim of evaluating a differential CMI response between Maa and Map infection. This study failed to demonstrate significant differences in the response to stimulation with the Map sonicate and the Maa PPD provided by CSL. The fact that Map sonicate is yet a crude antigen, which shares a high percentage of homology with Maa PPD could explain the lack of discrimination in the responses to these two antigens. In the same study, the sensitivity of the γ-IFN for detecting infecting cows from a herd with a low level of

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infection (mostly subclinical presentations) ranged between 50% to75%. The lack of γ-IFN response in the negative control herd suggested that the presence of false positive reactors to this test is unlikely. The γ-IFN test used in this study could not identify animals that were shedding the agent previously but only those that were shedding Map at the time when the test was performed. Because the combined used of γ-IFN and ELISA allowed for an accurate assessment of 73% of the infected animals in a highly infected herd, the authors considered recommending the use of both tests for screening of PTB.

The diagnosis of PTB infection as well as the development of the immune response in young cattle are issues that require further research. There are few studies focused on the evaluation of diagnostic tests for PTB in calves, however none of them have been performed on a large sample size of naturally exposed calves. After following up a cohort of PTB experimentally infected calves for 18 months, Collins and Zhao[24] reported that the detection of γ-IFN in calves under six months of age produced erratic results. Nevertheless, the γ-IFN test was able to accurately identify PTB infected and non infected-control animals after 17 months of age. The calves under study did not develop an antibody immune response, thereby confirming the concept that the humoral response develops towards adulthood. Confirmation of disease status and fecal shedding in the cohort was performed by radiometric culture of fecal samples (BACTEC). In a similar study, McDonald et al.[61] evaluated the performance of ELISA and γ-IFN test in naturally and experimentally PTB infected calves. Their study supported the idea that ELISA could be useful for the detection of specific antibodies in heifers older than 24 months and might contribute to the progress of a test-and-cull program for control of PTB.

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According to this study, the γ-IFN test produced frequent false positive results in calves between 1 and 25 months of age. Both previously mentioned studies agreed with the idea that the γ-IFN test yields a large proportion of non-specific reactions in calves younger than six months of age. Olsen et al.,[73] investigated the mechanisms for γ-IFN production in experimentally infected and non infected calves, and explored the potential causes responsible for the lack of specificity of the γ-IFN test in young animals. The study suggested that the poor specificity of the test in young calves (up to 5 months) could be due to both the lack of specificity of the available antigens and the production of innate γ-IFN by cell populations other than CD4+, such as natural killer (NK) and NK T cells. In view of these findings the concept that "The γ-IFN test has become an effective alternative measure of cell-mediated immunity in animals with mycobacterial diseases" [87] might need to be revised. The γ-IFN is a cytokine produced by certain cell populations, not only under stimulation of specific antigens, but also by innate mechanisms; hence, the detection of γ-IFN is not necessarily a reflection of CMI interactions.

One of the latest reports on the use of the γ-IFN test for detection of subclinical PTB in young cattle suggests that the value of this test in young animals relies on the finding of more specific antigens, as well as on the adjustment of the interpretation criteria in relation to the specificity and sensitivity of the test.[50]

2.4.3 Tests based on AB detection

The agar gel immunodifusion (AGID), complement fixation (CF), and the enzyme-linked immunosorbent assay (ELISA), are tests directed to detect the specific production of antibodies against Map. AGID and CF have been demonstrated less sensitive than ELISA.[53;62;77;84]

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AGID is simple and inexpensive, but requires large amounts of reagents and the interpretation of results is somewhat subjective. Although AGID has been demonstrated to have low sensitivity in cattle, it has shown a 70% to 93% sensitivity when used on sheep with multibacillary PTB infection.[17;47] The CF test has been required as the standard serologic test for detection of Johne's disease but, due to its low sensitivity and specificity, only a few countries still require its use for certification of herds and/or importation of live animals. The sensitivity of CF ranges between 10% in subclinically infected animals to 90% in animals showing clinical signs of disease.[53;77] The specificity of both AGID and CF depends upon the antigen used and it is limited by cross reactivity with other mycobacterial, nocardial and corynebacterial species.[13]

The serum ELISA is the most popular test used for screening of PTB in cattle. The sensitivity of the various ELISA protocols reported in the literature varies depending upon the antigens used, the age of the animals tested, the stages of disease, the type of sample tested (milk vs serum), and the gold standard used to estimate sensitivity and specificity values. The value of using milk for detection of specific antibodies against Map is still controversial. While some studies support the idea that the concentration of IgG_1 in milk is associated to the serum concentration,[94] Hardin and Thorne found a poor correlation and low kappa value between serum and milk ELISA results.[79] Milk is a more convenient sample to collect than serum since it can be collected simultaneously with those for the Diary Herd Improvement Association program (DHIA).[45]

It has been demonstrated that the ELISA is only valuable in adult cattle of at least two years of age, and the sensitivity is very limited (about 50 %) but improves in animals shedding the bacteria.[23] Reported sensitivity values for ELISA range from 15% in

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subclinically infected animals to 87% in clinically infected with heavy fecal shedding (more than 100 CFU/ gram of feces).[79;84] Most studies evaluating the use of ELISA for the detection of PTB agree on an overall sensitivity of approximately 45%.[91] The impact of the stage of disease on the sensitivity of the ELISA has been well described by Sweeney et al: lightly shedding cows yielded negative or weak results, and heavy shedders yielded sero-positive results.[91] Reported values for the ELISA's specificity oscillate between 95% to 99% depending on the pre-absorption of serum samples with an extract of ***Mycobacterium phlei***.[29;34;79] This procedure is aimed to minimize cross reactivity with other environmental mycobacteria. In addition, ELISA has demonstrated higher sensitivity and specificity than the CF and AGID.[88] Other variables influencing the sensitivity of ELISA are parity and stage of lactation. Nielsen et al. reported that the probability of being ELISA positive in both milk and serum samples was two to three times higher in the first parity than in other parities.[72] According to this study, the association between stage of lactation and ELISA varies depending upon the type of sample tested. Hence, the probability of being positive at the beginning of the lactation is higher when ELISA is performed in milk samples. On the other hand, serum ELISA has shown that the odds of a positive result are higher at the end of the lactation.[72] Despite these findings, the value of using ELISA in milk for screening of PTB is still under evaluation.

In addition to the variable degree of sensitivity, the repeatability of the ELISA in serum is somewhat questionable. It has been demonstrated that tested cattle had variable ELISA results when sampled twice, which hampered efforts to control the disease in infected herds based on results from this test. Hirst et al. indicated that the

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unpredictability of repeat test results is likely to be a consistent finding on dairies where repeat samples are routinely collected. This study also showed that highly test positive cows were more likely to maintain positive status than borderline test positive cows.

The use of antibody based tests in young calves is considered of low value since, in general, seroconversion takes place after 24 months of age. Sporadic positive ELISA and CF results have been observed in experimentally inoculated calves, but reactors to both tests are more frequently seen after 9 months of age.[61]

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CHAPTER 3 EVALUATION OF THREE TESTS FOR THE DETECTION OF PARATUBERCULOSIS IN A CALF COHORT AT 2, 4, 6, AND 8 MONTHS OF AGE

3.1 Preface

This chapter addresses the first objective/s of the study: To evaluate the use of the intradermal skin test using ***Mycobacterium avium*** subsp. ***paratuberculosis*** (Map) Purified Protein Derivative (PPD) produced by the National Veterinary Services Laboratories (NVSL), a modified gamma interferon (γ-IFN) test, and a commercial ELISA, as screening tests for exposure to Map in heifers at 2, 4, 6 and 8 months of age from two Colorado dairy farms with moderate and low seroprevalence of Paratuberculosis (PTB) infection. Another related objected was to determine agreement between the aforementioned tests applied to the calf cohort as well as among the tests applied to the dams (ELISA, fecal culture, fecal PCR).

A manuscript will be prepared for publiction with this material. Co-authors will be Salman MA, Hirst HL, and Garry F. This study was funded by the Program of Economically Important Infectious Animal Diseases (PEIIAD) at Colorado State University

3.2 Structured Abstract:

Objectives: To describe and evaluate the use of an intradermal skin test, a γ-IFN test, and a commercial ELISA as screening tests for exposure to Map in heifers at 2, 4, 6 and 8 months of age from two Colorado dairy farms with moderate and low seroprevalence of PTB infection. An additional objective was to determine agreement between the

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aforementioned tests applied to the calf cohort as well as among the tests applied to the dams (ELISA, culture of feces, Polymerase Chain Reaction-PCR- in feces).

Design: Prospective cohort study

Sample population: The heifer cohort was selected from two well-characterized dairy farms with 2% (farm B) and 11% (farm A) sero-prevalence of PTB determined by whole herd testing with a commercial ELISA. A total of 100 seropositive, 100 suspect, and 100 sero-negative dams (and their female calves) from farm A, and 64 sero-negative dams (and their female calves) from farm B were enrolled in the study.

Procedure: A modified γ-IFN test, a skin test, and a commercial ELISA were evaluated for the detection of PTB exposure in calves at 2, 4, 6, and 8 months of age. Fecal samples were collected from the calves when they reached 8 months of age to determine presence of Map in feces by culture and PCR. Feces and sera were collected from the dams at the time of enrollment (60 days in milk) to be tested by ELISA and fecal culture and PCR.

Results: Map was detected in feces from 1.5% of the calves by culture. The same percentage of calves was shedding Map in their feces according to PCR results. Atypical Mycobacteria were detected in 0.93% of the calves in the farm with 11% seroprevalence of infection. The majority of the skin test positive reactions were observed in calves after 4 months of age corresponding with the cervical injection site. Positive skin test results showed poor agreement with γ-IFN positive results or presence of Map in feces collected at 8 months of age. Calves shedding atypical Mycobacteria in feces did not show positive skin test results. The percentage of positive γ-IFN tests in the calves showed a

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steady decline dependent upon age. The proportion of ELISA positive results was significantly lower than the proportion of positives by γ-IFN and skin test.

Conclusions and clinical relevance: Calves exposed to PTB may shed low numbers of Map in their feces. Discrimination between active and passive shedding requires further research before screening tests for young cattle could be fully characterized. In addition, the characterization of CMI-based tests will require a more sensitive system for detection of shedders. This study showed that the parallel application of culture and PCR in feces enhances the sensitivity for detection of Map in young calves. The humoral response is not frequently found in calves naturally exposed to PTB. γ-IFN levels are initially high in 2- month old calves and decrease with age. The CMI measured by the skin test, however, does not seem to follow the same pattern. Culture and histopathology of tissues collected at post-mortem might help explain positive γ-IFN and skin test results in young calves.

3.3 Introduction

Present knowledge regarding the transmission of Paratuberculosis (PTB) and establishment of infection in young cattle, subsequent immunological events, as well as the progression of infection into adulthood, is incomplete. Although the cascade of events that characterize the immunopathogenesis of the disease in cattle has been the focus of various reviews, the majority of their scientific foundation originates from PTB murine models and in vitro studies, human studies of other mycobacterial diseases (such as human tuberculosis and leprosy), or gastrointestinal diseases caused by other microorganisms.[5;7] Several links in the chain of events of cattle PTB are speculative

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hypotheses and inferences from human mycobacterial diseases with the exception of a few studies performed in sheep and experimentally infected cattle.

It is believed that the most relevant type of immunity to PTB in early stages of the disease is cell-mediated.[5] In-vitro studies have shown that Map and its cell wall components are potent stimuli for the release of interleukin 1 (IL-1) by activated macrophages.[2] IL-1 would activate T cells, which are consequently expected to trigger the clonal expansion of both CD8+ cytolytic and CD4+ T helper cells (Th). Control of PTB by the host seems to be achieved mainly by the increased lytic capabilities of activated macrophages and the development of a cytotoxic response mediated by natural killer (NK) cells, CD8+ T lymphocytes, and γδ T cells. Th_1 lymphocytes produce interleukin 2 (IL-2), tumor necrosis factor β (TNF-β), and gamma interferon (γ-IFN), cytokines that are known to play a central role in the cell-mediated immune response by activating naïve macrophages.[5;7] As disease progresses, the immune response seems to shift from a cell mediated immunity regulated by Th_1 cells to a humoral immunity mediated by Th_2 cells.[4]

The test for detection of γ-IFN and the intradermal skin test (Johnin test) had been used for detection of cattle in early stages of PTB infection. The skin test measures the CMI response in vivo. It was evaluated in the 1950's using Protein Purified Derivative (PPD) or crude extracts (Old tuberculin) from strain 18, which is an ***M. avium*** strain.[15;22] The crude antigens used in these evaluations were responsible for a high proportion of false-positive results, which originated the idea that the skin test was not useful for detection of PTB in cattle. In addition to the non specific results caused by the use of crude antigens, past research involving the use of the Johnin test presents several

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limitations: (1) Most of the study subjects are experimentally-infected animals, which might not be representative of natural conditions (2) Sample sizes were small; and (3) Tests for in-vivo detection of CMI were evaluated between 20 to 50 years ago using reference tests of poor sensitivity. More recently, the United States Department of Agriculture National Veterinary Service Laboratories (USDA, NVSL) produced a PPD from Map American Type Culture Collection (ATTC) strain 19689, but there are no reports of the evaluation of this antigen in naturally-infected young cattle.

Alternatives to the in-vivo measure of CMI are the in vitro assays for cytokines, such as the γ-IFN test. This assay detects the release of γ-IFN produced by sensitized lymphocytes after stimulation with the appropriate antigen. The commercial γ-IFN assay uses ***M. avium*** subsp. ***avium*** (Maa) and ***M. bovis*** PPDs for lymphocyte stimulation and detects the release of γ-IFN by means of a monoclonal antibody. While this assay was successful in the detection of bovine tuberculosis (TB), its use for detection of PTB demonstrated low specificity and might require further standardization with more specific antigens.[28] Collins and Zhao[6] and McDonald et al.[17] concluded that the evaluation of γ-IFN concentration in both experimentally and naturally infected calves produced erratic results. In these studies, stimulation of blood was performed with both the Maa and ***M. bovis*** PPD's provided by the commercial kit. In addition to the conventional antigens, Stabel and Whitlock[27] evaluated the response to stimulation of lymphocytes with a whole cell sonicate from Map strain 19689. They failed to demonstrate a significant difference in the production of γ-IFN between samples stimulated with Maa PPD or the Map sonicate in infected adult cattle. Another evaluation of the γ-IFN test in Belgium concluded that more specific antigens from Map rather than Maa should be evaluated.[28]

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Since current knowledge about natural PTB infection in young stock is a combination of hypothetical inferences from experimental infection and in-vitro studies, further characterization of the CMI and humoral responses in naturally exposed young calves is warranted in order to find better strategies for early screening or diagnosis of PTB. The objective of this study was to describe and evaluate the use of the intradermal skin test using Map PPD produced by NVSL, a modified γ-IFN test, and a commercial ELISA, as screening tests for exposure to Map in heifers at 2, 4, 6 and 8 months of age from two Colorado dairy farms with moderate and low seroprevalence of PTB infection. An additional objective was to determine agreement between the aforementioned tests applied to the calf cohort as well as among the tests applied to the dams (ELISA, fecal culture, fecal PCR).

3.4 Materials and Methods

3.4.1 Selection of Herds and Cattle:

Animals for this study were selected from two well-characterized dairy herds with high (11%) and low (2%) sero-prevalence of PTB infection determined by a commercial ELISA.[13] Both herds had participated in a serological monitoring program for PTB together with 18 other dairy herds in Northern Colorado. The high seroprevalence herd (hereafter referred as herd A) had approximately 2500 cows and a history of PTB infection confirmed by fecal culture and presence of clinical cases. The low seroprevalence herd (hereafter referred as B) had approximately 1000 cows, with a few records of positive fecal culture in the past and no history of clinical disease. One decision criterion for the selection of these herds was the willingness of the owners to participate in a long-term cohort study. Three hundred dam/heifer pairs from herd A and

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64 from herd B were enrolled in the study on the basis of the dams' serological status. A total of 100 seropositive, 100 suspect, and 100 negative dams (and their female calves) from herd A, and 64 sero-negative dams (and their female calves) from herd B participated in the study. The enrollment was performed on a monthly basis, when the female calves were 2 months old (+/- 15 days).

3.4.2 Collection of samples

3.4.2.1 Dams:

Serum and fecal samples were collected only once, at the time of enrollment when most dams were approximately 60 days in milk (DIM). Approximately 10 grams of feces were collected from the rectum of every dam enrolled in the study. Feces and serum samples were sent to the Colorado Department of Agriculture -Rocky Mountain Regional Animal Health Laboratories (RMRAHL) to be tested by culture and ELISA, respectively.

3.4.2.2 Female calf cohort:

Females calves were tested at 2, 4, 6, and 8 months of age. Testing procedures included the intradermal skin test, γ-IFN, and ELISA. Fecal samples were collected from the rectum at 8 months of age for detection of Map in feces by culture and one tube nested PCR (OTSN-PCR). Heparinized and non-heparinized blood samples were collected in vacutainer tubes for ELISA and γ-IFN testing, respectively.

3.4.3 Tests:

3.4.3.1 Modified γ-IFN test:

Whole blood samples from each calf were collected in heparinized vacutainer tubes. Samples were transported in temperature controlled box to the Animal Population Health

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Institute (APHI) laboratory at Colorado State University within 6 to 8 hours post-collection. Upon arrival, a 1.5 ml aliquot of each sample was pippetted into each of 3 wells of a 24-well tissue culture plate.[a] Aliquots were stimulated with 100 ul of phosphate-buffered saline (PBS, negative control), Map PPD, and Maa PPD, respectively. This test was modified by substitution of ***M. bovis*** PPD provided by the manufacturer with Map PPD from NVSL. Map PPD from NVSL (1mg/ml) was diluted in PBS to a concentration of 0.3 mg/ml. Maa PPD was provided by the γ-IFN kit [b] and was used undiluted at a concentration of 0.3 mg/ml as well. After a mean incubation of 18 hours at 37° C, and 5% CO_2, the plates were centrifuged at 300 g for 10 min. The supernatant plasma was collected and shipped to the RMRAHL where they were tested with a commercial γ-IFN kit. Results were reported from the RMRAHL in crude absorbances for each antigen. Results were classified as positive or negative based on the following calculation, which was adapted from the clasification criterion recommended by the manufacturer:

POSITIVE = (OD of blood stimulated with Map PPD – OD of blood stimulated with PBS $\geq$ 0.100) *and* (OD of blood stimulated with Map PPD – OD of blood stimulated with Maa PPD $\geq$ 0.100), or NEGATIVE, if otherwise

3.4.3.2 Intradermal Skin test:

The intradermal skin test was performed at the time of the collection of blood to avoid any interference with the immune response detected by the other methods. Cervical (left/right) and caudal fold (left/right) injection sites were randomized a-priori

[a] Costar Corning Inc, Corning NY, 14831
b BOVIGAM , CSL Limited, Parkville

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for every calf. One tenth cc of Map PPD (NVSL, 1 mg/ml) was injected intradermally. Delayed Type Hypersensitivity (DTH) reactions were measured with a standard caliper 72 hours post injection and recorded in mm of skin thickness. The absence of reaction 72 hours post injection of Map PPD were categorized as negative, edematous reactions were recorded and categorized as suspects, and any well delimited and firm DTH reaction $\geq$1 mm of skin thickness was categorized as positive.

3.4.3.3 ELISA:

Non-heparinized blood samples were centrifuged at 1000 g for 20 min and serum was transferred into 1 ml aliquots and stored at –70°C until tested. The test was performed according to the manufacturer's instructions[c]. Results were classified according to the sample-positive (S/P) ratio (S/P ratio= Sample OD at 650 - Neg. control mean /Pos. control mean - Neg. control mean). Sera with S/P values $\geq$ 0.25 were classified as positives, whereas S/P values between 0.10 and 0.24 were classified as suspects, and S/P values < 0.10 were classified as negatives. This classification was used elsewhere.[13]

3.4.3.4 Fecal Culture:

Feces were cultured at RMRAHL on solid Herrold egg yolk media with the addition of mycobatin (HEYM). Results were reported as negative or positive, and for positive cultures the number of colony forming units (CFUs) present in the slant was noted. The reported scores were as follows:

4+ >70 CFU (heavy shedders)

3+ 31-70 CFU (moderate shedders)

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[c] IDEXX *Mycobacterium paratuberculosis* antibody test kit, IDEXX Laboratories Inc, Westbrook, ME

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2+ 8-30 CFU (low shedders)

1+ 1-7 CFU (low shedders)

3.4.3.5 PCR in feces:

OTSN-PCR for detection of Map in spiked fecal samples was optimized at the APHI laboratory. The target DNA was the species-specific Insertion Sequence (IS) 900, and the selected primers as well as the DNA extraction procedures and amplification conditions are detailed elsewhere.[d] Each sample was tested in duplicate. Results were categorized as positive (if all repeats positive) negative (if all repeats negative) or suspect (if at least one positive repeat). Each PCR run included a negative control to demonstrate the presence of cross-contamination and a positive control to verify the quality of the reagents and the performance of the PCR system. The identities of the amplicons obtained from positive samples were confirmed by sequencing.

3.4.4 Analysis:

3.4.4.1 Descriptive statistics and conventional epidemiological approach for determination of test accuracy:

Descriptive statistics were computed with the use of a software package[e] to tabulate percentage of positive, suspect and negative results to skin test, G-IFN and ELISA by herd and by age groups. The sensitivity (SE) and the specificity (SP) of the G-IFN, skin test, and ELISA applied to the heifer cohort at 8 months of age, as well as the apparent prevalence of disease, were calculated according to the following formulas:[23]

[d] Antognoli et al. (2003) Evaluation of a One Tube Nested PCR for detection of Paratuberculosis in young and adult cattle: Comparison to other diagnostic and screening tests. Manuscript to be submitted to AVJR.
[e] SAS version 8.02, SAS Institute, Cary, NC

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Se = (pT+/D+), where pT+ is the probability of a positive test results given disease is present (D+) and Sp = (pT-/D-), where pT- is the probability of a negative test result in disease free animals (D-). Diseased status was assigned to animals with a positive result to fecal culture or fecal PCR.

The prevalence of fecal shedders was calculated as the number of animals with presence of Map in feces detected by culture or PCR out of the total animals sampled.

3.4.4.2 Bayesian approach for evaluation of diagnostic tests in the absence of a gold standard:

Bayesian analysis was used to estimate herd prevalence, as well as the SE and SP of the skin test, γ-IFN test, and ELISA in 8-month old heifers from both study herds. The selected modeling strategy consisted of the use of three tests (two dependent and one independent) in one population. This model relies heavily on prior data, but it has been shown that as the third test becomes closer to a gold standard, it is possible to obtain accurate estimates of two dependent tests, in this case the γ-IFN and the skin test (Dr. Ian Gardner, personal communication). The modeling for test dependence was done using the Dendukuri and Joseph parameterization[9] that specified uniform priors for sensitivity and specificity covariances, as described in Gardner et al.[11] In addition, the same model was utilized to determine prevalence as well as Se and Sp of fecal PCR, fecal culture and ELISA in the adult cows from herd A, as the data in this age group had less sparse cells. The analysis was performed using WinBUGS software, which allows fitting various Bayesian models without the need to specify the full conditional distributions.[12] Information about prior distribution and modeling, cross classification of test results

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incorporated in the model, and a description of the code used in WinBUGS, are detailed in appendix A.

3.4.4.3 Agreement between tests:

Kappa scores were calculated to measure the agreement beyond chance among the γ-IFN test, the skin test, and the ELISA by age. In order to determine the agreement between tests, categorical responses were transformed into dichotomized based on biological criterion. All fecal culture positive results were considered positives and all negative and atypical results were considered negatives. For PCR in feces, the category suspect was merged with the negative because we were not able to achieve good repeatability with suspect results. Thus, a sample was positive to this test when it had at least two positive results after repeated testing. For the ELISA, the category suspect was merged with the negatives because it has been reported that the likelihood of PTB infection increases as the S/P ratio increases.[3] Hence, it was a more conservative approach to merge the suspects with the negatives. Lastly, for the skin test, a new variable was created, in which the suspect reactions were merged with the positives. This last classification was based on the Uniform Methods and Rules for Bovine TB eradication.[1] This was a more conservative but less specific approach.

3.5 Results

3.5.1 Descriptive Results of Tests Applied to the Dams:

Fecal Culture: A total of 250 dams from herd A and 59 dams from herd B were tested by fecal culture. The prevalence of fecal shedders was 10% (25/250) in herd A and 0% in herd B. Of 25 positive fecal samples from herd A, 44% were reported in the

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category 4+ (heavy shedders) and the remaining 56% were reported as 1+ or 2+, which represented light shedders.

PCR of Feces: A total of 268 fecal samples from herd A and 55 samples from Herd B were tested by PCR. Results for the presence of Map in fecal samples from Herd A were 87.7% negative, 5.8% suspect and 6.5% positive, whereas 96.4% of the tested animals in herd B were negative and 3.6% were suspects.

ELISA: Dams were enrolled in the study based on historical ELISA records. There were 33.8% ELISA negative dams, 33.1% suspects and 33.1% positives from herd A enrolled according to past records. Results from ELISA performed at the time of enrollment (a year later) on 297 dams indicated that 16.5% were positive, 20.9% were suspects, and 62.6% were negatives. During a one-year follow up, approximately 40% of the ELISA positive dams converted to negative, 25% converted to suspects, and 35% remained positives. The majority of the initially negative dams remained negative, with only a 5% of them seroconverting to positives. Within the suspects, 60% converted to negative, 9% converted to positives, and the rest remained as suspects in a year period. Historical ELISA records for the dams in herd B that were enrolled in the study indicated that 95.5% of the enrolled dams were negative and 4.5% were suspects. ELISA was performed on 61 dams from herd B at the time of enrollment. The majority of the dams in herd B remained negative after a year (78.5%), with only one suspect cow converting to positive. ELISA results obtained at the time of enrollment indicated that 80 % of the animals in this herd were negative, 4.9% were positives and 14.8% were suspects.

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3.5.2 Descriptive Results of Tests applied to Heifer Cohort

Fecal Culture: Map was isolated from 1.5% (4/263) of the fecal samples collected from heifers in herd A at 8 months of age. All positive cultures were reported as 1+ (low shedders). Atypical mycobacteria identified as Runyon group IV were detected in 2 of the 263 cultured fecal samples from herd A. Calves from herd B were negative.

PCR of Feces: A total of 262 fecal samples from 8-month old heifers in Herd A were tested by PCR. Positive results were detected in 1.5% of the samples and the percentage of suspects was 5.7% (15/262). Feces from all calves from Herd B were negative by PCR.

ELISA: A total of 231 heifers from herd A and 57 from herd B completed all four ELISA tests. Table 3-1 shows the results by herd and age group. Only one heifer (0.4%) from Herd A had two consecutive positive ELISA results at 4 and 6 months of age. None of the positive ELISA results were associated with the presence of Map in feces as detected by culture or PCR. Only one heifer from herd A, which had a suspect ELISA result at 6 months of age, was positive to fecal culture at 8 months of age.

Intradermal Skin Tests: A total of 254 heifers from herd A and 58 from herd B completed all four intradermal tests performed at 2, 4, 6, and 8 months of age. Table 3-2 shows the results of the skin test by herd and by age. The majority of the positive skin test reactions were observed in the cervical injection site at the ages of 4 and 6 months. Few heifers from herd A showed a positive skin test reaction during consecutive testing interventions. Two heifers were positive at 2 and 4 months of age, 6 heifers were positive at 4 and 6 months of age, and 4 heifers were positive at 6 and 8 months of age. None of the heifers from herd B reacted to consecutive skin tests.

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γ-IFN Test: A total of 261 heifers from herd A and 51 heifers from herd B were tested at 2, 4, 6 and 8 months of age. Table 3-3 shows the results by age and by herd. The percent of positive reactors to the γ-IFN test showed a steadily decrease with age in both farms. Two heifers (0.8%) from herd A reacted positively to all four tests but these results were not associated with the presence of Map in feces at 8 months of age.

3.5.3 Estimation of SE, SP and Apparent Prevalence of Fecal Shedding in 8 month old calves:

The apparent prevalence of Map infection relative to fecal shedding was 0 % in herd B and 2.7% in herd A, when OTSN-PCR and fecal culture are used in parallel. The apparent prevalence of shedders detected by either culture or OTSN-PCR when used separately was 1.5%. In farm A, the SE of the tests relative to the presence of Map in feces as detected by the parallel use of culture and PCR was 16% for the skin test and 0% for the γ-IFN test and the ELISA. The SP was 88.8%, 98.7% and 80.4% for the γ-IFN, ELISA and skin test, respectively. In farm B the sensitivity of all two tests was 0% and the SP was 96.8%, 96.9% and 97.3% for γ-IFN, ELISA and skin test, respectively.

3.5.4 Bayesian approach for evaluation of diagnostic tests in the absence of a gold standard:

Table 3-4 shows the posterior probabilities for the prevalence of infection in 8-months old heifers from herd A, as well as the SE and SP of the γ-IFN test, ELISA , and skin test. The broad intervals for the estimates indicated that the prior information on test accuracy and prevalence in the heifers was weakly informative. Similar results were obtained regarding the accuracy of tests and prevalence in the adult cows with the

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exception of fecal culture, which was assumed to have a very low false-positive rate (1 in 1000) with high probability (2.5% Posterior = 0.99 and 97.5% posterior =1.0).

3.5.5 Agreement between Tests

3.5.5.1 Adult Cows

The Kappa value for agreement between ELISA and fecal culture results at the time of enrollment was 35% (95% CI = 18% , 50%). The Kappa value for agreement between culture and PCR in fecal samples was 44% (95% CI= 26%, 62%).

3.5.5.2 Heifer Cohort

Fecal culture vs fecal PCR: Although McNemar's chi-square test indicated that there are no statistically significant differences in the proportions of positive results yielded by these two tests, there also was no agreement beyond chance between them, as indicated by a negative Kappa value (Kappa = -0.0117, 95% CI=-0.0207; -0.0027).

γ-IFN, ELISA, and Skin test by age group: Table 3-5 shows Kappa score for agreement between tests at 2, 4, 6, and 8 month of age. None or minimum agreement is expected beyond chance among these tests in the herd included in this study, as indicated by Kappa scores close to zero. Similarly, almost zero Kappa values (no agreement) were obtained between fecal culture or fecal PCR performed at 8 months and each of the three tests under evaluation (γ-IFN, ELISA and skin test) at any age.

3.6 Discussion

To our knowledge this study was different from any prior study in 3 ways: First, the study populations were typical of the naturally-occurring PTB infection and management conditions in Colorado, whereas most studies on the evaluation of diagnostic tests for

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detection of PTB in young cattle were performed on either experimentally infected calves or calves from herds with an extremely high prevalence of PTB infection. Second, the selection of the study animals (dam-heifer pairs) was based on ELISA results of the dam, the most commonly used screening test for PTB due to its low cost and rapidity. Third, tests under evaluation were intended to detect early infection by following up a cohort of heifers between the ages of 2 to 8 months. Consequently, this study is a comprehensive approach to characterize the humoral and CMI response in heifers at 2, 4, 6, and 8 month of age in a natural setting. Fourth and last, several tests for detection of PTB in young cattle were evaluated simultaneously and compared.

Culture results indicated that 10% of the dams enrolled in herd A were shedding Map in feces. Kappa values for agreement between ELISA and culture results in the adults suggested that the percentage of agreement beyond chance between these two tests was 34%. Kappa is strongly influenced by prevalence and, therefore, inferences regarding the agreement between ELISA and fecal culture to other populations should be avoided.[21] Previous studies have found that the agreement between serology and culture is more evident in heavy shedders.[8;22;25] In this study, only 44% of the culture positive dams were heavy shedders, and the remaining 56% were low shedders, which may have contributed to a low Kappa value. The absence of positive culture results in dams from herd B confirmed the assumption that the presentation of the disease was different in each of the study herds. Results from PCR indicated that 6.5% of the dams were shedding Map in feces, which was lower than the prevalence of shedders detected by culture (10%). Samples classified as suspects by PCR not only lacked repeatability but also did not show any association with either culture or ELISA results. For this reason, the

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category PCR-suspect was merged with the negatives. The agreement beyond chance between culture and PCR results in the adult cows was 44% (95% CI = 26%, 62%), and the agreement in positive results for the two tests was more evident in cows shedding high numbers of Map in feces (heavy shedders).

Fecal culture results from 8-month old heifers in herd A indicated that Map was present in 1.5% of the samples. A similar percentage of samples with presence of Map in feces was identified by PCR, however, culture and PCR positive results did not agree. In general, discordant results between fecal culture and PCR in paired samples could be explained by the following four reasons: (1) The distribution of bacteria in the sample is not homogeneous. Like other Mycobacterium spp, Map aggregates in clumps. If the number of Map cells in feces is small (especially in low shedders), and in addition, the few cells present in the sample are clumped together, then the probability of achieving the same result in paired samples is low (2) The DNA extraction, which is part of the procedure for PCR detection, is not 100% efficient. Therefore, if the sample contains low numbers of CFU and the efficiency is not 100%, it is possible that the template used for PCR has no Map DNA to be detected. (3) While PCR detects microorganisms that are either alive or dead, fecal culture depends on the viability of the bacteria for the development and detection of colonies. Thus, the lack of viability of Map in some subset of the samples could be one cause for discordant results between these two tests. (4) The non- homogeneous distribution of the DNA in the template can lead to PCR negative results. We have found that repeated testing of samples with high concentrations of DNA might produce inconsistent results if the template is not homogenized before its inclusion in the PCR reaction. Fecal culture results suggested that Map was present in low

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numbers (1-7 CFU/tube) in the 8-month old heifers, which might explain the discordant results between PCR and culture in these animals. Fecal shedding has been detected in experimentally-infected calves less than 8 months of age, although shedding was reported to be sporadic.[6;17] It is assumed that naturally-infected calves might shed Map in feces intermittently, and probably beyond the detection limits of the conventional methods.[29] To increase the accuracy in the detection of fecal shedders, both conventional fecal culture and PCR were included in this study as reference tests. The lack of discrimination between passive shedding and shedding due to active infection is a limitation of this approach. An alternative to overcome this limitation is by culture or histopathology of tissues, which was not feasible in this study. Map was not found in fecal samples from heifers in herd B, which confirms that this herd had a lower prevalence of PTB than herd A. Atypical Mycobacteria spp. found in feces from heifers in herd A indicated exposure to Mycobacteria other than Map in this farm. This exposure could be responsible for some false-positive results to antibody or to CMI-based tests evaluated in this study due to cross reactivity.

ELISA results in the heifers were similar in both herds. The highest percentage of positive ELISA results was 1.7 % in any of the four tests in both herds, which confirms that the humoral response is hardly ever detectable in young animals.[16;19] In addition, the percentage of positive results did not increase with age despite the repetitive stimulation with PPD antigens. Differences in percentage of positive observed ELISA results in both herds were not significant (P= 0.79) and could be attributed to assay variability.

Positive skin test results were most frequent in heifers older than 4 months, regardless the herd of origin. This finding could indicate that either the immune system was too

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immature to elicit a response when the first test was performed (2 months of age)[18;26]or that the onset of infection occurred later in life, when the calves were transferred to group pens post-weaning and exposed to more sources of environmental contamination (i.e. via manure from adult cows and from other calves). While the proportion of positive skin test reactions in herd A peaked at 6 months of age, the proportion of positive reactors in herd B increased steadily until the last test at 8 months of age. If these reactions were attributable to the onset of Map infection, differences in herd management and consequently in exposures, could have contributed to the differences in the percentage of reactors at 8 months. Another potential source of variability was due to operator bias, since two people were responsible for measuring and recording the skin test reactions.

The cervical injection site yielded a higher proportion of reactors than the caudal fold. This finding is consistent with previous studies on the evaluation of the intradermal skin test for screening of bovine TB, which demonstrated that the cervical injection site was more sensitive but less specific than the caudal fold.[10] In this study, skin test reactions in 8 month-old heifers from herd A agreed slightly with the detection of Map in feces by the reference tests. Early stages of infection could be characterized by the development of CMI in the absence of extensive intestinal granulomas and consequent fecal shedding.[14]

The γ-IFN test has been reported to be useful in the detection of adult cattle subclinically infected with PTB. [14;27] The γ-IFN test might be able to identify animals with possible subclinical PTB infection before they become positive by fecal culture.[27] Authors of studies that reached this conclusion did not use a reference test other than fecal culture, which is questionable given that cattle housed in contaminated environments might passively shed the organism in feces.[27] Some studies claimed that

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the γ-IFN test produced non-specific reactions in young calves and that its specificity improves with age. [6;17;20] Cell populations such as γδ and NK cells have been identified as the producers of innate G-IFN in young calves.[20] The steady decline of γ-IFN concentrations with age might indicate that the detected γ-IFN is the product of specific antigen stimulation rather than innate γ-IFN production. However, due to our inability to confirm infection status in these animals no conclusions could be drawn regarding the sensitivity or specificity of this assay.

The major limitation of this study was the low sensitivity of the reference tests to determine true disease status, particularly in the heifer cohort. The low percentage of positive reactors to some of the tests such as ELISA, fecal culture and PCR, limited the use of the conventional and the Bayesian analysis, which did not provide much information, as reflected by the wide probability intervals of the estimates. The only exception was the specificity of fecal culture in adult animals, which was estimated to be close to 100%.

Efficient control of PTB requires the detection of subclinically infected animals, which are silently contaminating the environment and contributing to the spread of the disease. Final stages of PTB control might have to face the challenge of detecting low shedders releasing Map to the environment in low but sustainable amounts. Unfortunately, the tests for detection of CMI evaluated in this study could not be fully characterized to be applied in such conditions. With fecal culture as the "gold standard" for PTB, calves may need to be followed for 1-2 years before Map can be detected in their feces. Improved detection of Map may be achieved by testing repeated fecal samples of the same calf and by the parallel use of several tests on the same sample.

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Further evaluation of CMI-based tests in calves, will not only require more sensitive gold standard tests for detection of Map in feces, but also a method to differentiate active from passive fecal shedding of Map.

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3.8 Tables

Table 3-1 Percentage of ELISA Negative, Positive, and Suspect calves by age and by herd

Age	Herd A			Herd B		
	Negative	Positive	Suspect	Negative	Positive	Suspect
2 months	95.2	0.9	3.9	91.3	1.7	7
4 months	99.2	0.4	0.4	100	0	0
6 months	94.8	1.7	3.5	100	0	0
8 months	93.1	1.3	5.6	98.3	0	1.7

Table 3-2 Percentage of skin test positive, suspect and negative heifers by herd and by age

Age	Herd A			Herd B		
	Negative	Positive	Suspect	Negative	Positive	Suspect
2 months	93.8	3.5	2.7	89.7	1.7	8.6
4 months	83	8.7	8.3	89.7	3.4	6.9
6 months	71.3	11.0	17.7	89.6	5.2	5.2
8 months	81.5	7.5	11.0	69	8.6	22.4

Table 3-3 Percentage of negative and positive results to the γ-IFN test by age and by herd

Age	Herd A		Herd B	
	Negative	Positive	Negative	Positive
2 months	75	25	57	43
4 months	83	17	82.4	17.6
6 months	87	13	98.1	1.9
8 months	89	11	94	6

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Table 3-4 Bayesian analysis: Comparison of γ-IFN (test 1), skin test (test 2) and ELISA (test 3) – 8 month old heifers

	Prior Means	Posterior Means	Posterior 2.5%	Posterior 97.5%
γ-IFN SE	0.33	0.32	0.03	0.79
γ IFN SP	0.9	0.89	0.66	0.99
ELISA SE	0.1	0.10	0.003	0.33
ELISA SP	0.98	0.98	0.86	1.0
SKIN TEST SE	0.33	0.33	0.03	0.77
SKIN TEST SP	0.9	0.89	0.66	0.99
PREVALENCE	0.5	0.49	0.14	0.85

Table 3-5 Kappa coefficient for agreement between γ-IFN, skin test and ELISA by age

Age	Skin test vs. G-IFN		Skin test vs. ELISA		ELISA vs. γ-IFN	
	Kappa	95 % CI	Kappa	95 % CI	Kappa	95 % CI
2 mo	-0.053	-0.081 0.071	-0.028	-0.045 -0.010	-0.012	-0.050 0.026
4 mo	0.0408	-0.073 0.155	0.031	-0.028 0.090	-0.006	-0.018 0.006
6 mo	-0.0038	-0.103 0.096	0.009	-0.036 0.054	-0.018	-0.037 0.0010
8 mo	0.0081	-0.096 0.112	0.015	-0.042 0.071	-0.017	-0.035 0.001

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3.9 Appendix A: Bayesian Analysis

We acknowledge the assistance provided by Dr Ian Gardner, UC Davis, in the evaluation of tests in the absence of gold standard by using Bayesian analysis and the Win BUGS software package.

Initially, Bayesian analysis was used to estimate herd prevalence, as well as the SE and SP of the skin test, γ-IFN test, and ELISA in 8-month old heifers from both study herds. The two-population model of Hui and Walter,[5] modified by others to accommodate the possibility of test dependency, was considered. The γ-IFN test and the skin test could be considered conditionally dependent from one another since they are both based on the same biological phenomenon, the cell- mediated immunity. The ELISA, instead, is based on the detection of antibodies, which are produced by a different biological pathway. Hence, the ELISA was considered independent form the other two assays. The Hui and Walter model assumes constant test accuracy across populations. Because this assumption was not fulfilled, (both herds had different prevalence of disease), and the data from Herd B were sparse, another modelling strategy was explored. The selected modeling strategy consisted of the use of three tests (two dependent and one independent) in one population. This model relies heavily on prior data, but it has been shown that as the third test becomes closer to a gold standard, it is possible to obtain accurate estimates of the two dependent tests (Dr. Ian Gardner, personal communication). The modeling for test dependence was done using the Dendukuri and Joseph parameterization)[2] that specified uniform priors for sensitivity and specificity covariances, as described in Gardner et al.[3] In addition, the same model was utilized to

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determine prevalence as well as Se and Sp of fecal PCR, fecal culture and ELISA in the adult cows from herd A, as the data in this age group had less sparse cell.

Prior distributions: Prior information about the sensitivity and specificity of diagnostic tests was modeled using independent beta prior distributions[4]. Beta (a,b) distributions provide a flexible means of modeling uncertainty about parameters ranging from 0 to 1. The magnitude and relative values of a and b determine how peaked and how skewed the distributions are respectively. Detailed descriptions of beta distributions are given elsewhere.[1]

The prior distributions for sensitivity and specificity were constructed as follows: A collaborator (Dr.Ian Gardner, University of California, Davis) provided best guesses (i.e. mode or most likely value) for the sensitivity and specificity of the ELISA and skin tests in animals less than one year of age. In addition, he provided estimates of the values that he was 95% sure that the values did not exceed. The values were used to compute the corresponding a and b values for the beta distributions for each parameter.

Categorization and cross-classification of the data incorporated in the model: ELISA test results were categorized as positive if the SP value > 0.25, otherwise negative. Skin test results were categorized as positive if any type of inflammation, induration, swelling or redness was present in the skin fold, otherwise negative. Gamma interferon results were classified as positive or negative as described in the methods section. For each herd, results from the last test (8 months) were cross-classified in 2x2x2 tables. Table 3-6 shows the counts for each test combination. Similar 2x2x2 tables were constructed for PCR, fecal culture and ELISA in adults.

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Table 3-6 Frequencies of the 8 possible combinations of ELISA, skin test and γ-IFN results in 8 months old heifers by herd.

Combinations	ELISA	SKIN TEST	GAMMA IFN	HERD A	HERD B
1	P	P	P	0	0
2	N	N	N	203	50
3	P	N	N	2	0
4	P	P	N	1	0
5	P	N	P	0	0
6	N	P	P	4	0
7	N	N	P	21	0
8	N	P	N	10	5

The selected modeling strategy was the use of 3 tests (2 dependent and one independent of the other) in a single population. The modeling of test dependence was done using the Dendukuri and Joseph parameterization[2] that specified uniform priors for sensitivity and specificity covariances, as described in Gardner et al.[3]

The codes for the evaluation of tests in the heifers (appendix A.1) as well as in the adult cows (appendix A.2) was written in WinBUGS. Inferences were based on 10,000 iterations after discarding an initial burn-in of 1000 iterations.

3.10 Appendix A.1

WinBUGS code to run 3 tests in one population using Dendukuri and Joseph parameterization model for data from herd A - test 1 is γ-IFN, test 2 is skin test, and test 3 is ELISA; data are from 8 month- old heifers

```
model
{
y[1:k, 1:k, 1:k] ~ dmulti (p[1:k, 1:k, 1:k], n)
p[1,1,1] <- pi*se3*(se1*se2 + covDp) + (1-pi)*(1-sp3)*((1-sp1)*(1-sp2) + covDn)
p[1,2,1] <- pi*se3*(se1*(1-se2) -covDp) + (1-pi)*(1-sp3)*((1-sp1)*sp2 - covDn)
```


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```
p[1,1,2] <- pi*(1-se3)*(se1*se2 + covDp) + (1-pi)*sp3*((1-sp1)*(1-sp2) + covDn)
p[1,2,2] <- pi*(1-se3)*(se1*(1-se2) -covDp) + (1-pi)*sp3*((1-sp1)*sp2- covDn)
p[2,1,1] <-pi*se3*((1-se1)*se2 - covDp) + (1-pi)*(1-sp3)*(sp1*(1-sp2) - covDn)
p[2,2,1] <- pi*se3*((1-se1)*(1-se2) + covDp) + (1-pi)*(1-sp3)*(sp1*sp2 + covDn)
p[2,1,2] <- pi*(1-se3)*((1-se1)*se2 - covDp) + (1-pi)*sp3*(sp1*(1-sp2) - covDn)
p[2,2,2] <- pi*(1-se3)*((1-se1)*(1-se2) +covDp) + (1-pi)*sp3*(sp1*sp2 +covDn)
ls <- (se1-1)*(1-se2)
us <- min(se1, se2) - se1*se2
lc <-(sp1-1)*(1-sp2)
uc <- min(sp1, sp2) - sp1*sp2
rhoD <- covDp / sqrt(se1*(1-se1)*se2*(1-se2))
rhoDc <- covDn / sqrt(sp1*(1-sp1)*sp2*(1-sp2))
pi ~ dbeta(3, 3)
se1~ dbeta(1.5,3)
sp1 ~ dbeta(9,1)
se2 ~ dbeta(1.5,3)
sp2 ~ dbeta(9,1)
se3 ~ dbeta (1,9)
sp3 ~ dbeta (10,0.2)
covDp ~ dunif (ls, us)
covDn ~ dunif(lc, uc)
}
list(n=241, k=2)
pi = 0.5, se1 = 0.3, sp1 = 0.9, se2 = 0.3, sp2 = 0.9, se3=0.1, sp3 = 0.98)
y[,1,1] y[,1,2] y[,2,1] y[,2,2]
80 40 0 2
1 2 2 114
END
```

Comparison of γ- IFN (test 1), skin test (test 2) and ELISA (test 3) – 8 month old heifers

node	mean	sd	MC error	2.5%	median	97.5%	start	sample
covDn	0.01635	0.02479	2.483E-4	-0.01312	0.008333	0.08458	1000	9001
covDp	-0.1719	0.201	0.002232	-0.6213	-0.1419	0.1391	1000	9001
pi	0.4994	0.19	0.002028	0.1431	0.4976	0.8508	1000	9001
se1	0.328	0.1995	0.002336	0.03008	0.3024	0.7595	1000	9001
se2	0.334	0.2003	0.001884	0.03175	0.3087	0.7665	1000	9001
se3	0.1003	0.08953	0.001056	0.002977	0.0755	0.3348	1000	9001
sp1	0.8999	0.09037	8.01E-4	0.6627	0.9248	0.9972	1000	9001
sp2	0.8993	0.09123	0.00117	0.6647	0.9253	0.9972	1000	9001
sp3	0.9808	0.04084	4.292E-4	0.8577	0.9979	1.0	1000	9001

Posterior densities for the sensitivity for each of the 3 tests are shown below:

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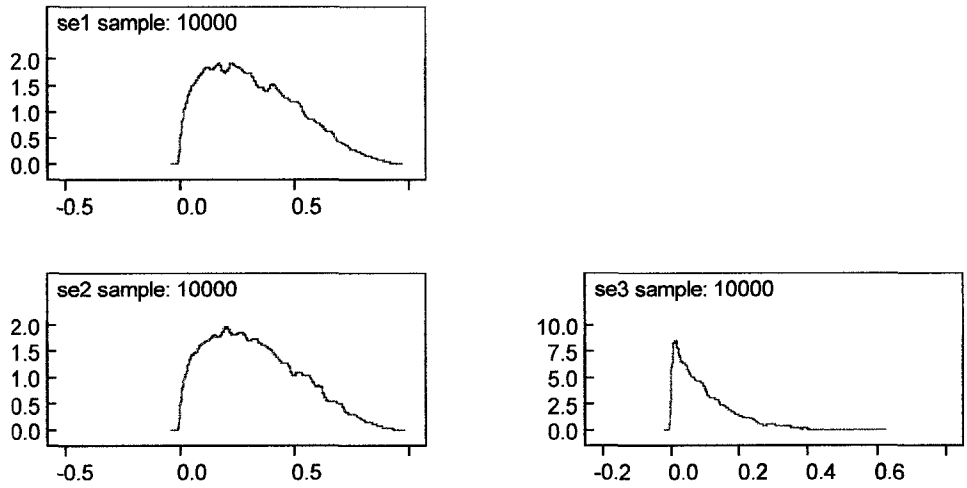

3.11 Appendix A. 2

WinBUGS code to run 3 tests in one population using Dendukuri and Joseph parameterization model for data from herd A- test 1 is PCR, test 2 is culture, and test 3 is ELISA; data are from adults cows

```
model
{
y[1:k, 1:k, 1:k] ~ dmulti (p[1:k, 1:k, 1:k], n)
p[1,1,1] <- pi*se3*(se1*se2 + covDp) + (1-pi)*(1-sp3)*((1-sp1)*(1-sp2) + covDn)
p[1,2,1] <- pi*se3*(se1*(1-se2) -covDp) + (1-pi)*(1-sp3)*((1-sp1)*sp2 - covDn)
p[1,1,2] <- pi*(1-se3)*(se1*se2 + covDp) + (1-pi)*sp3*((1-sp1)*(1-sp2) + covDn)
p[1,2,2] <- pi*(1-se3)*(se1*(1-se2) -covDp) + (1-pi)*sp3*((1-sp1)*sp2- covDn)
p[2,1,1] <-pi*se3*((1-se1)*se2 - covDp) + (1-pi)*(1-sp3)*(sp1*(1-sp2) - covDn)
p[2,2,1] <- pi*se3*((1-se1)*(1-se2) + covDp) + (1-pi)*(1-sp3)*(sp1*sp2 + covDn)
p[2,1,2] <- pi*(1-se3)*((1-se1)*se2 - covDp) + (1-pi)*sp3*(sp1*(1-sp2) - covDn)
p[2,2,2] <- pi*(1-se3)*((1-se1)*(1-se2) +covDp) + (1-pi)*sp3*(sp1*sp2 +covDn)
ls <- (se1-1)*(1-se2)
us <- min(se1, se2) - se1*se2
lc <-(sp1-1)*(1-sp2)
uc <- min(sp1, sp2) - sp1*sp2
rhoD <- covDp / sqrt(se1*(1-se1)*se2*(1-se2))
rhoDc <- covDn / sqrt(sp1*(1-sp1)*sp2*(1-sp2))
pi ~ dbeta(1, 5)
se1~ dbeta(2,3)
sp1 ~ dbeta(9,1)
se2 ~ dbeta(1.5,3)
sp2 ~ dbeta(999,1)
```


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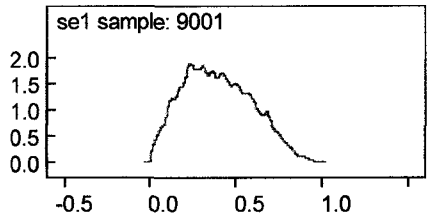
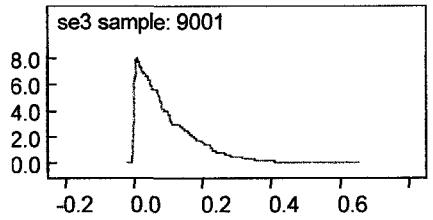
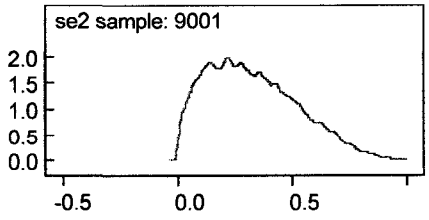
se3 ~ dbeta (1,9)
sp3 ~ dbeta (10,0.2)
covDp ~ dunif (ls, us)
covDn ~ dunif(lc, uc)
}
list(n=163, k=2)
list(pi = 0.15, se1 = 0.3, sp1 = 0.9, se2 = 0.3, sp2 = 0.999, se3=0.2, sp3 = 0.98)
y[,1,1] y[,1,2] y[,2,1] y[,2,2]
6 3 4 6
4 2 19 119
END

Comparison of PCR (test 1), culture (test 2) and ELISA (test 3) - adults

node	mean	sd	MC error	2.5%	median	97.5%	start	sample
covDn	3.98E-4	5.792E-4	6.329E-6	-1.046E-4	1.908E-4	0.002023	1000	9001
covDp	-0.1442	0.1892	0.001955	-0.5729	-0.1145	0.1473	1000	9001
pi	0.1664	0.1414	0.001457	0.004908	0.1277	0.5221	1000	9001
se1	0.3973	0.1993	0.002176	0.06645	0.3803	0.7968	1000	9001
se2	0.3327	0.1976	0.001989	0.03318	0.3078	0.7583	1000	9001
se3	0.1004	0.08997	8.967E-4	0.002621	0.0741	0.339	1000	9001
sp1	0.8995	0.08974	7.822E-4	0.6685	0.9238	0.9973	1000	9001
sp2	0.999	0.001009	9.73E-6	0.9964	0.9993	1.0	1000	9001
sp3	0.9802	0.0417	4.422E-4	0.8527	0.9979	1.0	1000	9001







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3.12 Appendix A.3

Table 3-7 shows the posterior probabilities for the prevalence of infection in 8-months old heifers from herd A, as well as the SE and SP of the γ-IFN test, ELISA , and skin test. Table 3-8 shows similar information regarding the application of fecal culture, ELISA, and fecal PCR in adult animals. The broad intervals for the estimates indicated that the prior information on test accuracy and prevalence in the heifers was weakly informative. Similar results were obtained regarding the accuracy of tests and prevalence in the adult cows with the exception of fecal culture, which was assumed to have a very low false-positive rate (1 in 1000) with high probability.

Table 3-7 Bayesian analysis: Comparison of γ-IFN (test 1), skin test (test 2) and ELISA (test 3) – 8 month old heifers

	Prior Means	Posterior Means	Posterior 2.5%	Posterior 97.5%
G-IFN SE	0.33	0.32	0.03	0.79
G IFN SP	0.9	0.89	0.66	0.99
ELISA SE	0.1	0.10	0.003	0.33
ELISA SP	0.98	0.98	0.86	1.0
SKIN TEST SE	0.33	0.33	0.03	0.77
SKIN TEST SP	0.9	0.89	0.66	0.99
PREVALENCE	0.5	0.49	0.14	0.85

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Table 3-8 Bayesian analysis: Comparison of PCR (test 1), culture (test 2) and ELISA (test 3) in adult cows

	Prior Means	Posterior Means	Posterior 2.5%	Posterior 97.5%
PCR SE	0.4	0.39	0.07	0.80
PCR SP	0.9	0.89	0.67	0.99
CULTURE SE	0.33	0.33	0.03	0.76
CULTURE SP	0.99	0.99	0.99	1.0
ELISA SE	0.1	0.10	0.003	0.34
ELISA SP	0.98	0.98	0.67	0.99
PREVALENCE	0.16	0.17	0.005	0.52

3.13 References

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5. Hui SL, Walter SD. Estimating the error rates of diagnostic tests. *Biometrics* 1980;36:167-171.

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CHAPTER 4 RISK FACTORS ASSOCIATED WITH POSITIVE GAMMA AND JOHNIN SKIN TEST RESULTS IN 8-MONTH-OLD DAIRY HEIFERS

4.1 Preface

This chapter addresses the second objective of the study: To determine the association between the Cell Mediated Immune (CMI) response in 8-month old heifers exposed to Paratuberculosis (PTB) and risk factors related to their dams, risk factors related to the environment, and risk factors related to the heifers themselves. A manuscript for publication will be prepared from this material; co-authors will be Salman MD and Garry F.

4.2 Structured Abstract

Objective: To determine the association between the cell-mediated immune (CMI) response in 8-month old heifers (measured by the Gamma Interferon and skin test) and risk factors related to the dams, to the environment, and to the heifers themselves.

Design: Prospective cohort study.

Sample population: The heifer cohort was selected from two well-characterized dairy farms with 11% (farm A) and 2% (farm B) sero-prevalence of paratuberculosis (PTB) infection determined by a commercial ELISA. A total of 300 dam/heifer pairs from farm A and 64 from farm B were enrolled in the study based on the dams' serological status.

Procedure: Heifers were tested with a commercial test for detection of Gamma Interferon (γ-IFN), a Johnin skin test, and a commercial ELISA at 8 months of age. In addition, paired fecal samples were collected from the rectum and tested by conventional

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fecal culture in Herrold egg yolk with mycobactin (HEYM), and a nested Polymerase Chain Reaction (PCR). Serum and fecal samples were collected from the dams at 60 days in milk (DIM) +/- 15 days to be tested by fecal culture, fecal PCR, and ELISA. Historic ELISA records from the dams were available from a whole herd testing performed a year prior to their enrollment in the cohort study. Logistic regression analysis was used to determine the association between risk factors and the CMI-response in the 8-month old heifers. Risk factors evaluated were fecal shedding detected by culture or PCR, ELISA results, results from other CMI-based test in the heifers, fecal shedding in the dams detected by culture or PCR, ELISA results at 60 DIM, old ELISA records and farm of origin.

Results: A statistically significant association was found between a positive γ-IFN test at 8 months of age in the heifers and the detection of ***Mycobacterium avium*** subsp ***paratuberculosis*** (Map) in fecal samples from the dams at 60 DIM.

Conclusions and Clinical Relevance: Heifers born from dams shedding Map in feces at 60 DIM are 2.7 times more likely to have a positive γ-IFN test at 8 months of age than those born from non-shedders.

4.3 Introduction

Economic losses attributed to Paratuberculosis (PTB) in dairy cattle include premature culling with consequent increased replacement costs, reduced feed efficiency,[1;2] reduced milk production,[3;4] increased incidence of mastitis,[5]and reduced body weight at slaughter.[1;2] Results from the dairy survey conducted by the National Animal Health Monitoring System (NAHMS) in 1996 indicated that herds with a PTB

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prevalence higher than 10% produce US$ 200 less annual income per cow compared with herds in which PTB was not detected.[6]

In addition to the economic impact on the livestock industry, PTB generates a human health concern due to its suspected role in the etiology of Crohn's disease. The Voluntary Johne's Disease Herd Status Program (VJDHSP) was created in 1997-1998 and publicized by the US Animal Health Association as a response to both animal and public health issues related to PTB.[7] This program is based on the use of ELISA and fecal culture (FC) in adult animals (older than 2 years) for control of PTB within infected herds by testing and culling cattle with ELISA positive and FC positive test results, as well as for identification of PTB-free herds. One of the limitations of the VJDHSP is that the screening is only performed in adult animals. However, the onset of PTB infection in cattle seems to occur mainly during the first months of life. Moreover, fecal shedding of ***Mycobacterium avium*** subsp ***paratuberculosis*** (Map) in calves has been reported to occur under experimental and natural conditions.[8;9]

The detection of young shedders spreading infection becomes relevant in advanced stages of PTB control in cattle herds. Cell mediated immunity (CMI) has been identified as the primary response to most mycobacterial infections, including PTB.[10] Diagnostic and screening tests for identification of animals in the initial phases of PTB infection should be aimed at detecting CMI. Unfortunately, the evaluation of CMI-based tests for detection of PTB in young animals has been hampered by the lack of accurate reference tests for comparison. The identification of risk factors associated with the transmission and establishment of PTB infection in young calves could contribute to predicting and/or partially explaining the presence of CMI, particularly when reference tests do not provide

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information about the true status of the individuals. Vertical transmission of PTB takes place by ingestion of contaminated milk, colostrum, or by exposure to contaminated manure from the dam or from other adult cows.[11] Other mechanisms of vertical infection transmission such as in-utero infection or transmission attributable to the sire have been either described or suspected, but their magnitude has not been fully explored.[11]

Horizontal transmission is suspected to occur mostly by the fecal oral route when calves are exposed to a contaminated environment. One study exploring the proportion of PTB transmission attributable to the dam in dairy cattle concluded that dam effects could explain a significant variation (approximately 7.7%) of the antibody response in the offspring.[12] It was assumed that the influence of the dam on the offspring's serological profile was due to vertical transmission (either in-utero infection or post-partum contact with an infected dam). However, discrimination between vertical transmission and genetic susceptibility could not be determined in this study. Another study regarding the importance of genetic susceptibility suggested that genetic susceptibility might play a more significant role than vertical transmission on the PTB status of the offspring.[13]

The present study was intended to explain the occurrence of a CMI response to PTB in 8 month old heifers by determining the association with risk factors related to the dams (vertical transmission) and risk factors inherent to the calves themselves (horizontal transmission). The main objective was to determine the association between positive γ-IFN or skin test results and risk factors related to their dams such as fecal shedding or historic ELISA records, in 8-month old heifers from farms with low and moderate seroprevalence of PTB. An additional objective was to evaluate the results of other tests

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performed in the calves were associated with a positive γ-IFN or skin test result at 8 months of age.

4.4 Hypothesis

There are risk factors related to the dam and to the calf that could influence the γ-IFN or skin test results in 8-month-old heifers exposed to PTB infection

4.5 Materials and Methods

Selection of cattle and herds: The study heifers belonged to a cohort that had been monitored for detection of PTB infection from the time the heifers were 2 months old until they reached 8 months of age as part of a study for evaluation of diagnostic tests for PTB. The cohort was selected from two well-characterized dairy herds with 2% and 11% sero-prevalence of PTB infection determined by a commercial ELISA. Both herds had participated in a serological monitoring program for PTB together with 18 other dairy herds of Northern Colorado. The farm with 11% seroprevalence (hereafter referred to as farm A) had approximately 2500 milking cows and a history of PTB infection confirmed by fecal culture and presence of clinical cases. The farm with 2% seroprevalence (hereafter referred to as farm B) had approximately 1000 milking cows and no history of clinical cases. One decisive criterion for the selection of these herds was the willingness of the owners to participate in a long-term cohort study. A total of 300 dam/heifer pairs from farm A and 64 from farm B were enrolled in the study based on the dams' serological status. The enrollment was performed on a monthly basis, when the female calves were 2 months +/- 15 days old (and the dams were approximately 60 days in milk). A total of 100 seropositive, 100 suspect, and 100 negative dams (and their female

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calves) from farm A, and 64 sero-negative dams (and their female calves) from herd B were enrolled in the study.

At eight months of age, the heifer cohort was tested with a commercial test for detection of gamma interferon (γ-IFN), a Johnin skin test, and a commercial ELISA. In addition, paired fecal samples were collected from the rectum and tested by conventional fecal culture in Herrold egg yolk with mycobactin (HEYM), and a nested PCR. Serum and fecal samples were collected form the dams at 60 days in milk +/- 15 days to be tested by fecal culture, fecal PCR, and ELISA. Historic ELISA records from the dams were available from a whole herd testing performed a year prior to their enrollment in the cohort study.

4.5.1 Tests

4.5.1.1 γ-IFN test

Whole blood samples from each calf were collected in heparinized Vacutainer tubes. Samples were transported to the Animal Population Health Institute (APHI) laboratory at Colorado State University within six to eight hours after sampling in a cooler (with no cooling). Upon arrival, 1.5 ml aliquots were pippetted into each of three wells of a 24-well tissue culture plate[a]. Aliquots from each blood sample were stimulated with 100 ul of phosphate-buffered saline (PBS, negative control), Map PPD, and ***M avium*** PPD (Maa PPD), respectively. Map PPD from NVSL (1mg/ML) was used diluted in PBS to a concentration of 0.3 mg/ml and Maa PPD was provided by the γ-IFN kit[b] and applied undiluted, at a concentration of 0.3 mg/ml as well. After an average incubation of 18

[a] Costar Corning Inc, Corning NY, 14831

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hours at 37° C, and 5% CO_2, the plates were centrifuged at 300 xg for 10 minutes. The supernatant plasma was collected and shipped to the Colorado Department of Agriculture -Rocky Mountain Regional Animal Health Laboratories (RMRAHL) where they were examined with a commercial test for detection of γ-IFN. Results were reported from the RMRAHL in crude absorbances for each antigen. The classification of test results was performed according to the following formula, which was the most conservative and similar to the one indicated by the manufacturer of the γ-IFN kit:

Positive = (OD of blood stimulated with Map PPD – OD of blood stimulated with PBS ≥ 0.100) AND (OD of blood stimulated with Map PPD – OD of blood stimulated with Maa PPD ≥ 0.100), *Negative* if otherwise.

4.5.1.2 Intradermal Skin Test

The skin test was performed simultaneously to the collection of blood in order to avoid any interference with the immune response detected by the other methods. Cervical (left/right) and caudal fold (left/right) injection sites were randomized a-priori for every calf. One tenth of Map PPD (NVSL, 1 mg/ml) was injected intradermally. The absence of reaction 72 hours post injection of Map PPD were categorized as "negative" and any other sign of inflammation or evident delayed type hypersensitivity reactions were categorized as "positive".

4.5.1.3 ELISA

Non-heparinized blood samples were centrifuged at 1000 xg for 20 minutes and serum was transferred into 1 ml aliquots and stored at –70°C until tested. The test was

[b] BOVIGAM, Bovine Gamma Inteferon Test, CSL, Parkville, Australia

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performed according to the manufacturer's instructions[c]. Results were classified according to the sample-positive (S/P) ratio (S/P ratio= Sample OD at 650 - Neg. control mean /Pos. control mean - Neg. control mean). S/P ratios $\geq$ 0.25 were categorized as positive and those <0.24 were categorized as negative.

4.5.2 Analysis

Descriptive statistics were obtained for all tests performed in the heifer cohort at eight months of age by farm. In addition, descriptive analyses from variables related to the dams, such as fecal culture, fecal PCR, ELISA results at 60 days in milk (DIM), and past ELISA records from a year prior to enrollment were obtained for each farm. The apparent prevalence of disease, measured by the presence of Map in fecal samples detected by FC and PCR, was calculated in the heifers for each of the farms included in the study. This information was used to explore the possibility of horizontal transmission among 8-month old heifers. Factors related to the dams, such as shedding of Map in feces or the presence of a humoral response detected by ELISA, were considered indicators of infection in the parental cohort and were utilized to explore their association with the CMI response in the heifers. Univariable logistic regression was used to evaluate the associations between (suggesting horizontal infection transmission), factors related to the heifers (suggesting vertical transmission), and the response to the γ-IFN and skin tests.

Risk factors evaluated were fecal shedding detected by culture or PCR, ELISA results, results from other CMI-based tests in the heifers, fecal shedding in the dams

[c] IDEXX *Mycobacterium paratuberculosis* antibody test kit, IDEXX Laboratories Inc, Westbrook, ME

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detected by culture or PCR, ELISA results at 60 DIM, old ELISA records and herd of origin. Factors associated with a positive skin test or γ-IFN test in the univariable analysis (variables with $P < 0.25$), were included in a multivariable logistic regression model. Only variables determined via the Wald F statistic to be significant at the 10% level (i.e. $P \leq 0.1$) were allowed in the final model. The predicted probability (Pred PR) of a calf responding positively to the skin test of the γ-IFN test was calculated from the logistic regression model with the following formula:

$PredPr = e^{logit} / (1+e^{logit})$, where the *logit* is the sum of the coefficients of the model including the constant term, multiplied by the presence or absence of the factors.

The final model was evaluated for fit with the Hosmer-Lemeshow Goodness of fit statistic. The statistical analysis was done in SAS version 8.02.[d]

4.6 Results

4.6.1 Descriptive Statistics (Table 4-1)

4.6.1.1 Adult Cows

Historic ELISA records were available for 299 dams from farm A and 44 dams from farm B. The proportions of seropositive cows based in historical records that were enrolled in this study were 33.1% from farm A and 0% from farm B. By the time of enrollment (60 DIM), the seroprevalence of disease in our dam cohort had changed to 16.5% in farm A and 4.9% in farm B. Fecal shedding of Map was detected by conventional culture in 10% of the dams in farm A and in 0% of the dams from farm B at 60 DIM. Similar proportions of shedders were detected by PCR in fecal samples from

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both farms. However, the Kappa statistic for agreement between these two tests was moderate (Kappa= 44% , 95% CI= 26%, 62%, data not shown).

4.6.1.2 Heifers

Map was detected by conventional FC in 1.5% of the heifers from farm A and by fecal PCR in 1.2% of the heifers. PCR and FC positive results did not agree in the heifer's cohort. Map was not detected in any of the heifers from farm B by any of these methods. Results from G-IFN, skin test and ELISA in 8-month old heifers did not show any association with the presence of Map in feces. As expected, few heifers (0.9%) were ELISA positive at 8 months. A total of 18.5% of the heifers from farm A reacted to the skin test while 10.9% of the heifers demonstrated detectable γ-IFN levels in plasma.

4.6.2 Factors

Factors associated with a positive γ-IFN test result in 8-month old heifers: Univariable analysis of factors related to the calves, such as presence of Map in feces detected by FC or PCR, ELISA and skin test results at 8 months of age were not associated with a positive γ-IFN test result (Table 4-2). Herd of origin was not found to be statistically significant either, and therefore, it was deleted from the model. On the other hand, variables related to the dams, such as fecal PCR positive (P= 0.10) and fecal culture positive results at 60 DIM (P= 0.06) were found to be associated with a positive γ-IFN test in the 8-month old heifers. However, when these two variables were included in a multivariable model, no statistically significant associations with a positive γ-IFN test were found (P-value for Fecal PCR = 0.15 and P-value for fecal culture = 0.63). An

[d] SAS version 8.02, SAS Insititute, Cary, NY,14831

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Odds Ratio (OR) of 2.7 (90% CI= 1.1, 6.7) indicated that heifers born from fecal culture positive dams were 2.7 times more likely to have a positive γ-IFN test at 8 months of age.

Table 4-3 shows factors associated with a positive skin test result in eight-month old heifers. Univariable analysis of factors related to the calves, such as the calves' ELISA, γ-IFN test, fecal culture and fecal PCR, revealed that none of these variables were associated with a positive skin test result in 8-month old heifers. Among the factors related to the dam, the historic ELISA positive records (P= 0.14) and a positive fecal culture results (P= 0.12) were associated with the outcome. Herd of origin was also associated with the outcome at P= 0.10. However, when these variables were included into a multivariable model, none of them were found to be statistically significant. None of the odds ratios for the explanatory variables was statistically significant and, therefore, the skin test response in 8-month old heifers could not be explained by any of the variables considered in this study. The Hosmer Lemeshow goodness of fit test did not provide any valuable information about model fit due to the low percentage of positive test results available and poor agreement between them.

4.7 Discussion

To our knowledge, this is the first study exploring the associations between the CMI response in 8-month old heifers, the infection status of their dams, herd of origin, and the results from other tests performed in the calves at 8 months.

Due to the scarce information provided by FC, fecal PCR, and ELISA in the heifers, no association was found between these tests, the γ-IFN, or the skin test at 8 months of age. Contrary to our expectations, no association was found between G-IFN and skin test

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results at 8 months of age either. Even when both tests measure the host's CMI, there are differences inherent to each test that could explain the lack of association between them. The skin test is based on the detection of a delayed hypersensitivity reaction type IV after the exposure to an antigen for which the host has immune memory. If the test is positive, a reaction characterized by induration, redness, and swelling of the skin fold where the antigen was injected develops 72 hs post injection. This reaction is an accumulation of activated macrophages and lymphocytes recruited by the cytokines released by stimulated T cells (CD4+).[10] On the other hand, the γ-IFN test detects the levels of γ-IFN released by T cells sensitized by previous exposure to the antigen. Recent studies, however, have reported the production of non-specific levels of γ-IFN in young animals.[14;15] It is believed that innate γ-IFN levels detected in young animals are produced by certain NK cell populations or δγ cells, and tend to disappear with age.[14;15] It is possible that the lack of association between skin test and γ-IFN test was due to the detection of non-specific γ-IFN production in the heifers. It is also possible that the skin test responses were non-specific since the antigenic structure of Map PPD has a high degree of homology with other mycobacterial antigens, particularly within the ***M avium*** Complex.

Herd of origin did not show any significant association with the positive γ-IFN test or the skin test at 8 months of age. Although the 90% and 95% CI indicated that the OR between farm A and a positive γ-IFN test is not statistically significant, there seems to be a trend (indicated by the skewness of the CI) suggesting that farm A is more likely to have a higher proportion of positive G-IFN test results than farm B. This would be an expected finding since results from fecal culture suggested that there was a higher

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prevalence of Map shedders in farm A compared to farm B, which could impact on the heifers' exposure and immune response to PTB.

No associations could be determined between the status of the dams (indicated by ELISA results and fecal shedding) and the occurrence of a positive skin test in their heifers. Most of the information regarding the dam's status was either collected when the dams were 60 DIM or corresponded to historical ELISA records. Hence, it is possible that the status of the dams around calving, when calves were most susceptible to PTB exposure, was different than the status reflected by the results of tests performed at 60 DIM.

A significant association (P= 0.1) was found between the occurrence of a positive γ-IFN test and the presence of Map in the dam's feces collected at 60 DIM. Fecal shedders could be shedding continuously or intermittently. Dams shedding Map at 60 DIM could have been shedding continuously or intermittently during the first two months of the calf's life, thereby exposing the newborn to Map when they are most susceptible to PTB infection. Although the OR between the heifers' γ-IFN test and the fecal PCR result in the dams at 60 DIM was not statistically significant, the skewness of the confidence interval suggests that there is potential for an association between these two factors given larger sample size.

In summary, this study has shown that heifers born from dams shedding Map in feces at 60 DIM are 2.7 times more likely to have a positive γ-IFN test at 8 months of age than those born from non-shedder dams. Even when this finding does not imply causality of PTB infection, it supports the recommendation of separating dams from their newborn

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calves as soon as they give birth in order to prevent infection transmission to the offspring.

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4.9 Tables

Table 4-1 Results of tests for PTB infection in adult and 8 month old heifers (proportion [%]) from farm A and B

Age	*Variable*	*Farm A*	*Farm B*	*Total*
8-month old heifers	Skin test positive results	48/260 (18.5)	16/57 (28.0)	64/317(20.2)
	γ-IFN positive results	31/283 (10.9)	4/59 (6.8)	35/342 (10.2)
	ELISA positive results	3/264 (1.1)	0/59 (0)	3/323(0.9)
	Fecal culture positive results	4/263 (1.5)	0/56 (0)	4/319(1.3)
	Fecal PCR positive results	3/262 (1.2)	0/53 (0)	3/315 (0.9)
Adult cows	Historic ELISA positive results	99/299 (33.1)	0/44 (0)	99/343 (28.9)
	Positive ELISA results at 60 DIM	49/297 (16.5)	3/61 (4.9)	52/358 (14.5)
	Fecal culture positive results	25/250 (10.0)	0/59 (0)	25/309 (8.1)
	Fecal PCR positive results	28/268 (10.5)	0/55 (0)	28/323 (8.7)

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Table 4-2 Factors potentially associated with a positive γ-IFN test in 8-months old heifers

Independent Variables	*Category*	G-IFN test result *(Dependent variable)*		*P-value*	*OR*	*95% CI* *90% CI*
		Positive	Negative			
Farm	A	31	252	0.34	1.7[a]	(0.6, 4.9)
	B	4	55			(0.7, 4.2)
Skin test	Positive	7	57	0.87	1.1	(0.4, 2.6)
	Negative	26	227			(0.5, 2.3)
ELISA calves	Positive	0	3	0.74[c]	NA[b]	NA[b]
	Negative	31	289			NA[b]
Fecal culture Calves	Positive	0	4	0.64[c]	NA[b]	NA[b]
	Negative	33	282			NA[b]
Fecal PCR Calves	Positive	0	3	0.72[c]	NA[b]	NA[b]
	Negative	32	280			NA[b]
Dam's ELISA Historic	Positive	12	79	0.28	1.5	(0.7, 3.2)
	Negative	21	209			(0.8, 2.8)
Dam's ELISA 60 DIM	Positive	3	44	0.33	0.6	(0.2, 1.9)
	Negative	32	258			(0.2, 1.5)
Dams' Fecal PCR	Positive	5	20	0.10[d]	2.4	(0.8, 6.9)
	Negative	27	260			(0.9, 5.8)
Dams' Fecal Culture	Positive	5	18	0.06[d]	2.7	(0.9, 7.9)
	Negative	18	245			(1.1, 6.7)[e]

[a] Farm A vs. Farm B
[b] NA= not applicable with logistic regression due to sparse cells
[c] Based on Fisher's exact test
[d] Significant in univariable analysis, included in multivariable model
[e] Significant at alpha= 0.1

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Table 4-3 Factors potentially associated with a positive skin test response in 8-months old heifers

Independent Variables	*Category*	Skin test results *(Dependent variable)*		*P-value*	*OR*	*95% CI 90% CI*
		Positive	Negative			
Farm	A	48	212	0.10[d]	0.60[a]	(0.3, 1.1)
	B	16	41			(0.3, 1.0)
G-IFN test	Positive	7	26	0.87	1.072	(0.4, 2.6)
	Negative	57	227			(0.5, 2.2)
ELISA calves	Positive	1	2	0.53	2.134	(0.2, 23.9)
	Negative	56	239			(0.3, 16.2)
Fecal culture Calves	Positive	1	2	0.58	1.966	(0.2, 22.1)
	Negative	59	232			(0.3, 14.9)
Fecal PCR Calves	Positive	0	3	0.5 [c]	NA[b]	NA[b]
	Negative	61	231			NA[b]
Dam's historic ELISA	Positive	13	73	0.14	0.605	(0.3, 1.2)
	Negative	48	163			(0.3, 1.1)
Dam's ELISA 60 DIM	Positive	9	33	0.83	1.091	(0.5, 2.4)
	Negative	54	216			(0.7, 2.1)
Dams' Fecal PCR	Positive	4	20	0.61	0.750	(0.2, 2.3)
	Negative	56	210			(0.3, 1.9)
Dams' Fecal Culture	Positive	1	19	0.12[b]	0.203	(0.0, 1.6)
	Negative	51	197			(0.1, 1.1)

[a] Farm A vs. Farm B

[b] NA= not applicable with logistic regression due to sparse cells

[c] Based on Fisher's exact test

[d] Significant in univariable analysis, included in multivariable model

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CHAPTER 5 EVALUATION OF A ONE TUBE SEMI-NESTED PCR FOR DETECTION OF PARATUBERCULOSIS IN YOUNG AND ADULT DAIRY CATTLE: COMPARISON TO OTHER DIAGNOSTIC AND SCREENING TESTS

5.1 Preface

This chapter addresses the third objetive: To optimize and apply a One Tube Semi Nested PCR (OTSN-PCR) on fecal samples from adult (> 2 years) and young (8 months) dairy cattle from two herds with moderate and low prevalence of PTB. A related objective was to compare results of OTSN-PCR in feces with those of conventional fecal culture, and a commercial ELISA in the same study animals.

A manuscript for publication will be prepared from this; co-authors will be Boegli-Stuber K, Hirst HL, Triantis J and Salman MD.

5.2 Structured Abstract

Objectives: One objective of this study was to optimize and apply a One Tube Semi-Nested Polymerase Chain Reaction (OTSN-PCR) in spiked fecal samples, reference samples of known cultural status, and field samples from adult (> 2 years) and young (8 months) dairy cattle from two herds with moderate and low prevalence of Paratuberculosis (PTB). An additional objective was to compare results of OTSN- PCR in feces with those of both a conventional fecal culture and a commercial ELISA in the same study population

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Design: Paired fecal samples were collected from adult cows and from their 8-month old calves to be tested by conventional fecal culture and OTSN-PCR. Serum samples from the same animals were collected to be tested by a commercial ELISA.

Sample population: Three hundred cows and their female calves from a dairy farm with 12 % seroprevalence of PTB infection and 59 dam-calf pairs from a herd with 2% seroprevalence of infection were included in the study.

Procedure: A study was conducted in which adult cows and their 8- month old female calves were sampled to be tested by fecal culture, fecal PCR and ELISA. Results from paired fecal samples from each animal were compared with McNemar's Chi-square test, and Kappa scores were calculated to determine the agreement among the three tests under evaluation

Results: The lowest detection limit for the OTSN-PCR when used on spiked samples ranges between 62-125 Map cells per gram of feces. When applied to feces from adult cows OTSN-PCR detected more shedders than fecal culture in the high prevalence herd. The coefficient of agreement between OTSN-PCR and culture in adult cows was 42%. Slight agreement (Kappa 0.17) was observed between ELISA and OTSN-PCR in feces from adult cows. The proportion of fecal shedders detected by OTSN-PCR and culture among 8-month-old heifers did not differ significantly. However, there was no agreement among any of the tests applied to the young heifers. All heifers and adult cows from the low prevalence herd were negative to OTSN-PCR.

Conclusions and clinical relevance: Moderate agreement was observed between OTSN-PCR and culture in adult animals. Detection of low numbers of Map in feces is a challenging goal and it might require the combined use of OTSN-PCR and culture. The

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major advantage of PCR is its rapidity when compared with culture. Further testing of tissues is necessary to differentiate microorganisms passing through from those that represent established infection

5.3 Introduction

Johne's disease or Paratuberculosis (PTB) is a chronic granulomatous enteritis caused by ***Mycobacterium avium*** subsp ***paratuberculosis*** (Map), which affects domestic ruminants and other wild species. PTB is important to dairy producers because it causes reductions in milk production, weight loss, decreased fertility, and high replacements costs, all of which damages the economic efficiency of the dairy production system.[1;20] Experimental infection studies in which PTB was confirmed by fecal culture and histopathology of intestinal tissues have shown that cattle develop resistance to Map as they grow older.[3] Young animals seem to be more susceptible to infection than adult cattle, particularly within the first months of life.[14] However, the disease only becomes evident in adult animals, which might show clinical signs of infection, such as intermittent diarrhea and weight loss. Currently, the screening and diagnosis of PTB is performed with tests that work at their best accuracy level in cattle older than 2 years of age. The ELISA is the most conventional screening technique for identification of PTB infection due to its low cost and rapid results. A combination of ELISA and whole-herd fecal culture (either from individual or pooled fecal samples) is the presently most recommended strategy to estimate apparent herd prevalence.[19;20] The ELISA combined with individual fecal culture is also used to confirm disease status in individual animals. This strategy, which is recommended for adult animals, has limited value in young cattle populations since the humoral response appears to develop in association with age and

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severe disease.[4;4] Although fecal shedding is most commonly present in adult cattle, studies involving the experimental infection of calves detected Map in feces from calves as young as one year old.[2;6;14] Although the mechanism by which Map is excreted to the intestinal lumen remains unknown, Iw Lugton, has suggested that phagocytes containing intracellular Mycobacteria probably migrate back onto the mucosal surface to shed bacilli.[15] According to Whitlock and Buerguelt, calves with natural infection may shed significantly lower amounts of Map in feces than adult livestock.[23] Hence, the currently available fecal culture techniques might not be sensitive enough to detect young fecal shedders. Since the rapid identification and culling of fecal shedders is central to PTB control in cattle populations, the need for a sensitive assay to identify fecal shedders, not only among adult cattle, but also among young stock, becomes a priority.

Molecular techniques, such as PCR, offer several advantages over bacteriological methods with rapidity being on top of the list. In addition, PCR does not depend on bacterial viability, which increases the probability of detecting the organisms in feces. PCR also has acceptable sensitivity compared to fecal culture. Several studies on the use of PCR for detection of Map in clinical specimens concluded that the diagnostic accuracy of PCR depends on both the type of PCR system and the procedure used for DNA extraction.[12] Nested and real-time PCR systems combined with commercial DNA extractions were reported to reach the highest sensitivity on samples with know amounts of Map DNA.[9;12] Compared to the real-time PCR system, the OTSN-PCR presents the advantage of having a lower cost of required equipment with a similar degree of sensitivity. A study comparing three PCR systems (a nested, a commercial and a real-time PCR) and two DNA extractions concluded that the DNA extraction is the key in the

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performance of any PCR system.[7;8;11;12] One of the advantages of the nested PCR is that the analytical sensitivity of the system is enhanced by the use of two sets of primers and two amplification cycles, which increase exponentially the sensitivity of the method. Another benefit of OTSN-PCR is the fact that, similar to real time PCR, the DNA amplification is performed in a closed lid tube, which impedes cross-contamination between samples.

One objective of this study was to optimize and apply a OTSN-PCR in spiked fecal samples, reference samples of known cultural status, and field samples from adult (> 2 years) and young (8 months) dairy cattle from two herds with moderate and low prevalence of PTB. An additional objective was to compare results of OTSN-PCR in feces with those of conventional fecal culture, and a commercial ELISA on the same study animals.

5.4 Material and Methods

5.4.1 OTSN-PCR Characteristics and Conditions

Primers [a] for the DNA target sequence IS 900 were adapted from Englund et al.[8] The reaction is called semi-nested because there is one primer that is used for both external and internal amplifications:

External Primers

BS 749 (10 uM): CGC GGC ACG GCT CTT GTT

BS 204 (10 uM): TGA TCT GGA CAA TGA CGG TTA CGG A

Product size : 563 bp

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Internal Primers

BS 204 (100 uM): TGA TCT GGA CAA TGA CGG TTA CGG A

BS 535 (100 uM): AGC GTC TTT GGC GTC GGT CTT G

Product size: 353 bp

Conditions:

⇒ **External PCR**: The PCR was performed in 0.675 ul microcentrifuge tubes. The first reaction mix was prepared and dispensed into the bottom of the tubes. Each 12.5 µl reaction consisted of 5% of DMSO, 1 X Amplitaq Buffer I, 3 uM Mg Cl, 150 µM of each dNTP, 0.3 µM of each external primer, 1.25 U of Amplitaq Gold Polymerase[b], and 2.5 ul of template. The reaction was overlayed with 50 µl of wax to prevent evaporation and premature mixing of the two reactions. The internal semi-nested reaction (described below) was prepared first and dispensed into the lids of each tube.

⇒ **Internal (Semi-Nested) PCR**: The volume of the internal (nested) reaction was 50 µl and consisted of 3 mM MgCl, 150 µM of each dNTP, 2 µM of each external primer, 2 U of Amplitaq Gold Polymerase, 5% DMSO, 1 X Amplitaq buffer II, within a 1% suspension of agarose. The reaction mix was briefly heated up at 85° C to melt the agarose, and 50 µl were directly dispensed into the tube lids.

Thermalcycling parameters:[c]

⇒ **External PCR**: Following a 10 minute hot start at 95°C, the first PCR reaction was performed as follows: Thirty five cycles of DNA denaturation at 95°C for 1.5

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[a] Operon Technologies Inc, Alameda, CA.
[b] Perkin Elmer Applied Biosystems, Foster City , CA.

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minutes, annealing at 65°C for 1.5 minutes, and extension at 72°C for 1.5 minutes, followed by one additional cycle of 1.5 minutes at 95° C and 4 minutes at 72°C. The tubes were held at 4°C until the internal PCR was performed.

⇒ **Semi-Nested PCR**: After completion of the external PCR, the tubes were briefly centrifuged to allow contact between the 563 base pairs (bp) product of the external PCR and the semi-nested reaction mix. Tubes were heated briefly to melt the agarose, gently vortexed to mix components, and briefly centrifuged. After a hot start at 95°C for 10 minutes, the second PCR reaction consisted of DNA denaturation at 94°C for 1 minute, annealing at 65°C for 2 minutes and extension at 72°C for 1.5 minutes, sequentially repeated for 35 cycles. The tubes were held at 4°C until PCR products were separated by gel electrophoresis.

Product analysis: After thermalcycling reactions were completed, tubes were heated at 95°C to melt the agarose and enable products to be transferred to gels elecrophoresis. Elecrophoretic separations were performed in a 1.5 % agarose gel stained with 1 μg/ml ethidium bromide solution, and observed under UV light. The final product size was of 352 base pairs.

5.4.2 OTSN-PCR optimization on purified DNA

This technique was initially optimized on pure Map DNA extracts and spiked fecal samples. The specificity of the system was also tested on DNA from other related mycobacterias, such as ***M avium*** subsp *avium* serovars 1- 2- 14, ***M. smegmatis***, ***M fortuitum***, and ***M. intracellulare***.

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[c] M.J. Research, Incline Village, NV.

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5.4.3 Optimization of OTSN-PCR in spiked fecal samples

The OTSN-PCR system was tested on fecal samples spiked with serial dilutions of a Map strain American Type Culture Collection (ATCC) 19698 to final concentrations of 5 x 10^6, 5 x 10^5, 1 x 10^5 1 x 10^4, 1 x 10^3, 250, 125, 62, and 31 cells per gram of feces. The feces-Map cells mix was vigorously vortexed for 1 minute to obtain a homogeneous distribution of Map within the sample. Each spiked fecal sample was tested approximately 8 times to explore the reproducibility of the technique at each cell concentration. Fecal dilutions of Map strain ATCC 19698 were made from a stock culture media containing 4 x 10^8 cells/ ml of media. Quantification of the stock was made by spectrophotometer readings of undiluted culture media, and from 1:2, 1:10, and 1:20 dilutions of the stock in 0.02% Tween 80. Feces used for spiking were collected from a cow with no history of PTB or clinical signs of disease, negative ELISA results, and negative fecal culture. The cow belonged to a dairy herd with low seroprevalence of PTB infection. The absence of Map in the fecal sample used for spiking was confirmed by the lack of amplification products obtained after repeated testing of several sample aliquots.

Map DNA extraction from feces (adapted from Garrido et al.):[10] Fecal samples of approximately 1 gram were thawed at either room temperature or with a cold water bath, were mixed with 3.5 ml of 10% sodium dodecyl sulphate (SDS), and vortexed for 1 minute. After allowing for 30-minute sedimentation of a pellet, 5 ml of supernatant were collected into a 15 ml tube and centrifuged at 12000 g for 20 minutes in order to pellet mycobacterial cells. The supernatant was removed and discarded, and the pellet was thoroughly mixed with 200 µl of Phosphate Buffer Saline (PBS). The PBS-pellet was

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collected with a wide orifice tip and transferred into a 2 ml cryo-tube in which 13.5 µl of Proteinase K (1.135 mg/ml)[d] were added. The feces-Proteinase K suspension was vortexed and incubated for twenty minutes in a heating block at 72° C. After treating the samples with proteinase K, 200 ul of a commercial binding buffer[e] were added, and the samples were incubated at 72°C for 10 minutes. Three freeze-and-boil cycles of 5-minutes each were alternated to disrupt the mycobacterial cell wall and release the DNA. A centrifugation step of 10 minutes at 10,000 g was done to bring the DNA into the upper layer. The supernatant was then collected and transferred into a sterile microcentrifuge tube, and the rest of the DNA extraction and purification was performed with a commercial DNA extraction kit following the manufacturer's instructions.[e] Extracted DNA was tested undiluted by OTSN-PCR.

5.4.4 OTSN-PCR evaluation on reference fecal samples with known culture status

The PCR system was evaluated on 19 fecal samples of known culture status. Fifteen of these samples were reference samples for fecal culture proficiency testing (fourteen of which were positive and one negative) provided by the Colorado Department of Agriculture-Rocky Mountain Regional Animal Health Laboratories (RMRAHL). The remaining 4 samples corresponded to clinically infected animals, which were culled and necropsied at the Colorado State University's Veterinary Teaching Hospital. All four cows presented clinical signs of disease, macroscopic thickening of the terminal small intestine, lymphadenopathy of mesenteric lymph nodes, and positive fecal culture.

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[d] Boeringher Mannheim, Indianapolis, IN

[e] High Pure PCR Template Preparation Kit, Roche diagnostics Corporation, Indianapolis, IN

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5.4.5 OTSN-PCR Evaluation on Field samples

This section discusses the OTSN-PCR evaluation on field samples from cattle with unknown fecal culture status.

Cattle and Herds: Two dairy farms with 11% (farm A) and 2% (farm B) seroprevalence of PTB infection were the source of the study animals. Fecal and blood samples were collected from 300 dams and their respective 8-month-old female calves in farm A, and from 59 dams and their 8-month old heifers in farm B. Feces were collected with a clean obstetric glove and transported to the lab in an iced cooler. In the laboratory, each fecal sample was aliquoted into a pair of sterile whirl pack bags (one for PCR and the other for culture). Feces for culture were stored at –70°C and shipped within one month to the RMRAHL. Feces for OTSN-PCR testing were stored at –70°C until processed.

Classification of OTSN-PCR Results in Fecal Samples : Each fecal sample was tested at least twice. When results from both repetitions were discordant, the sample was tested a third time and the final classification of results was as follows:

NEGATIVE: if all repetitions negative

POSITIVE: at least two repetitions positive

SUSPECT: if one test positive and the result could not be reproduced

Confirmation of PCR Products by Sequencing: The identities of the amplicons obtained from positive samples were confirmed by sequencing. Suspect samples could not be re-amplified and confirmed by sequencing; thus, they were finally merged within the negative category for analysis.

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ELISA: Approximately 10 ml of blood were collected from the jugular or tail vein from each cow or heifer in a vacutainer tube for ELISA testing. Serum was obtained after centrifugation of the samples for 20 minutes at 12,000 xg and was shipped the RMRAHL to be tested by a commercial ELISA.[f]

5.4.6 Analysis

The analytical sensitivity (lowest detection limit) and specificity of OTSN-PCR were determined on pure Map DNA and fecal culture samples of known status. Results from conventional culture and OTSN-PCR on paired fecal samples from each animal were compared with a McNemar's Chi square test for paired samples. The observed agreement and the Kappa statistics among tests calculated using formulas described elsewhere.[18] The statistical significance level used in this study was 0.05.

5.5 Results

5.5.1 Analytical Accuracy of OTSN-PCR

The lowest detection limit (LDL) for OTSN-PCR on pure Map DNA was 2 fg per ul of template, which is less than one mycobacterial cell. No amplification products were observed when the system was tested on purified DNA of mycobacteria other than Map. Table 5-1 shows the LDL in spiked feces. Feces spiked with doses ranging between $5x10^6$ and $1x\ 10^4$ cells per gram of feces were all positive by this PCR. OTSN-PCR detected 125 cells per gram of feces in all samples tested, and 62 cells per gram of feces in 90% of the samples tested. Below this limit (62 cells/gram) the detection capabilities of OTSN-PCR became unreliable. When OTSN-PCR was tested on reference fecal

[f] IDEXX Laboratories, Westbrook, ME.

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samples of known culture status, Map was detected in all samples containing 31-70 cells/tube (moderate shedders) and in all 4 animals with clinical disease (heavy shedders).

However, only 50% of the low shedders were detected by this PCR system as shown in Table 5-2 . The lack of amplification of sample # 1, which was classified as "too numerous to count" (TNTC) by fecal culture, lead to the hypothesis that excess DNA could have caused inhibition of the PCR reaction. To prove this hypothesis the template was tested in 1:10 and 1:100 dilutions, all of which yielded in a positive amplification product. This sample belonged to a cow with experimental PTB infection, severe signs of disease, and high numbers of Map in feces. Since the field samples tested in this study corresponded to subclinically infected animals, PCR inhibition due to excess DNA seemed unlikely. Hence, only undiluted templates were included during routine testing of field samples. The addition of an arbitrary number of template dilutions per sample would not only increase the cost and labor of the PCR, but also it would fail to ensure the absence of inhibition if all (diluted and undiluted) templates yield negative results.

The detection limit for the currently available culture methods has been determined to be between 10 to 50 viable Map cells per gram of feces.[21] Our results showed that the lowest detection limit for OTSN-PCR was similar to that if culture with the additional advantage of providing results in a significantly shorter time.

5.5.2 OTSN-PCR, ELISA and Culture from Field Samples

Table 5-3 and Table 5-4 show results from OTSN-PCR in adult and young cattle, respectively. Map was detected by OTSN-PCR in feces from 11.5% of the adult cows and from 1.2% of the 8-month-old heifers from farm A. Fecal samples from dams and heifers from farm B were negative for the presence of Map by OTSN-PCR. Fecal culture

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results were positive in 10.1% of the adult cows from farm A and in 0% of the cows from farm B. Map was detected in feces from 1.2% of the heifers from farm A and in none of the heifers from farm B by culture. The sensitivity of the ELISA relative to fecal culture in adult cows was 56%, while the sensitivity of ELISA relative to OTSN-PCR was 35.7% (Table 5-5 and Table 5-6).

5.5.3 Association Between Tests

Adult Cows: No statistically significant differences were observed between the proportion of positive OTSN-PCR results and fecal culture positive results in farm A (Table 5-3). The observed agreement between OTSN-PCR and culture in adult cows from farm A was 88.9%, whereas the agreement beyond chance between culture and OTSN-PCR given the available data was moderate (Kappa = 0.42 , 95% CI= 0.24, 0.61). The proportion of ELISA positive results in farm A was significantly higher than that of OTSN-PCR ($P \leq 0.02$) and fecal culture. The observed agreement between culture and ELISA, and PCR and ELISA, was 84.7% and 80.5%, respectively. The agreement beyond chance between PCR and ELISA, and between culture and ELISA was poor (Kappa values of 17% and 34%, respectively). None of the cows from farm B were positive to either culture or OTSN-PCR.

Eight Month-Old Heifers: There was no statistical difference between the proportion of positive culture results and the proportion of positive OTSN-PCR results in heifers from farm A (Table 5-4). However, these two tests did not agree in young cattle. Negative agreement beyond chance is expected between OTSN- PCR and fecal culture in 8-month old heifers (Kappa= -0.0141, 95% CI=-0.02,-0.0032). Similar kappa values were obtained for fecal culture and ELISA (Kappa= -0.0143, 95% CI= - 0.0254, -0.0033)

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and for OTSN-PCR and ELISA (Kappa= –0.0123, 95% CI= -0.0221, -0.0025). All three tests were negative in the 8-month-old heifers from farm B.

5.6 Discussion

In this study, we evaluated a one tube semi-nested PCR for detection of Map in fecal samples from adult and young dairy cattle. Our results on purified Map DNA showed that the analytical sensitivity of the OTSN-PCR is satisfactory (1 mycobacterial cell per reaction) and comparable with more advanced systems such as the real-time PCR.[13] Although a previous study reported that the primers were specific for Map,[8] we confirmed this finding by showing the absence of amplification products when Mycobacteria genetically similar to Map were tested with our OTSN-PCR system.

The LDL of our PCR and DNA extraction procedure in spiked fecal samples oscillated between 90% to 100% at cell concentrations ranging between 62 to125 cells per gram of feces. Below this threshold, the analytical sensitivity of the technique fell significantly (20%). The OTSN-PCR combined with our DNA extraction procedure demonstrated similar sensitivity to conventional culture for detecting Map in bovine feces 11;22

The lack of amplification of the reference fecal sample #1 (Table 5-2), which was classified as "Too numerous to count" (TNTC) in culture, led to the hypothesis that an excess of Map DNA could have generated false negative OTSN-PCR result. Therefore, all reference fecal samples with a negative or suspect OTSN-PCR result were re-tested in 1/10 and 1/100 dilutions. As a result of this, amplification products were observed in samples 1 (1/10 and 1/100 dilutions) and 11 (1/10 dilution). However, since the study

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animals selected for validation of OTSN-PCR belonged to herds with moderate or low seroprevalence of PTB infection and without clinical evidence of the disease, it was assumed that excess load of Map in feces would not be an issue. On the contrary, it was suspected that the major challenge of the OTSN-PCR would be to have enough sensitivity to detect low numbers of Map in feces. Therefore, all field samples were tested undiluted. The authors were aware that by choosing this approach there was potential for false negative results during the validation of this assay in field specimens. This would lead to higher prevalence estimates of fecal shedders than those presented in Table 5-3 and Table 5-4 .

The prevalence of fecal shedders among adult cows detected by OTSN-PCR was 11.5% in herd A and 0 % in herd B. The identity of all positive results from OTSN- PCR were confirmed as Map by DNA sequencing of the amplicons. Recent studies on the molecular epidemiology, strain diversity, and strain sharing of Map in the US suggested that the diversity of Map in the US is limited.[16] Thus, the confirmation of Map amplicons by sequencing is a suitable methodology to confirm the specificity of our results. Interestingly, the prevalence of fecal shedding in farm A determined by conventional culture was 10.1%, similar to the prevalence determined by OTSN-PCR. However, the Kappa statistic for agreement between culture and OTSN-PCR was moderate (Kappa= 42%). It is relevant to mention that Kappa scores are highly influenced by prevalence, and therefore we could expect higher agreement between these methods in farms with higher prevalence of infection.[17] The lack of perfect agreement between OTSN-PCR and culture methods applied on the same samples could be explained by several factors: [1] Non homogeneous distribution of Map in the sample could have led to differences in

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results, [2] Flaws in the sensitivity of both methods when the number of CFUs present in feces is low, and [3] Lack of bacterial viability could lead to a positive PCR result with a negative paired culture results.

The sensitivity of the ELISA related to fecal culture in adult cows from farm A was 56%. This value fell approximately within the previously documented range for ELISA related to conventional culture (45 to 50%). The sensitivity of ELISA related to OTSN-PCR in the same cows from farm A was 35.7%. A study by Stabel et al. suggested that the sensitivity of the commercial ELISA compared to conventional fecal culture might be significantly lower than the values reported in the literature.[19] Her conclusions support the low sensitivity estimate of the ELISA related to OTSN-PCR reported in our study. Since the majority of the subclinically infected cattle are either intermittent shedders or non-shedders, the low sensitivity of the ELISA related to fecal shedding (as detected by either culture or PCR) could be an expected finding.[22]

All adult cows in farm B were negative to fecal culture. The absence of positive OTSN-PCR results in this farm enhances our confidence in the specificity of the test. The presence of almost 5% ELISA positive/culture negative cows in this farm, could be indicating a flaw in the specificity of the ELISA. Although the specificity of the commercial ELISA has been reported to be of approximately 99%,[5] a recent study reported a lower specificity estimates of 94%.[19]

Results from fecal culture indicated that 1.6 % of the 8-month-old heifers from farm A were shedding low numbers of Map in feces (between 1 and 7 colony forming units per slant of media). A similar percentage of heifers (1.2%) was identified as fecal shedders by OTSN-PCR; however, there was no agreement between results from both tests. The

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lack of agreement between culture and OTSN-PCR in young heifers is not unexpected since the reliability of tests for PTB is poor when it comes to the identification of low shedders. Results from ELISA in this farm, indicated that 1.2% of the heifers showed a detectable humoral response. None of the heifers in farm B were identified as fecal shedders by either culture or OTSN-PCR. ELISA results were negative for all heifers in this group. Several studies have described the presence of Map in feces from 8 month-old calves and younger after experimental infection with Map,[14 2;6]however, without histopathological confirmation it is impossible to determine whether the presence of Map in feces is the consequence of established infection or passive shedding. Despite this, differences in results from ELISA, OTSN-PCR and culture between farms A and B seem to be indicating that different epidemiological conditions exist in these two farms, and the tests under evaluation appear to reflect this phenomenon by showing dissimilar results in both categories.

In summary, some limitations of fecal culture are the loss of cell viability (due to presence of toxic materials, sample handling, and presence of competing bacteria), the long time required for development of colonies, and poor sensitivity in individuals shedding low numbers of bacteria. OTSN-PCR overcomes these limitations by providing a rapid and sensitive method for detection of Map in field samples. However, in animals shedding low numbers of Map, the combination of both tests might be necessary to reach a more accurate estimate of prevalence. The discrimination between presence of Map due to infection or "pass through" is a topic that requires further research.

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5.7 Acknwledgements:

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5.8 Tables

Table 5-1 Results of OTSN-PCR in spiked fecal samples

	Cell dilutions in spiked samples				
Cells/gram of feces	1000	250	125	62	31
% of samples with a positive PCR result	100 (8/8)	100 (9/9)	100 (9/9)	90 9(/10)	20 (2/10)

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Table 5-2 OTSN-PCR results on fecal samples of known culture status

Sample	PCR rep 1	PCR rep 2	PCR final result	fecal culture
1	N*	N*	N*	TNTC
2	N	N	N	N
3	P	P	P	2+
4	P	N	S	1+
5	N	P	S	1+
6	N	N	N	1+
7	P	P	P	3+
8	P	P	P	1+
9	P	P	P	3+
10	P	P	P	1+
11	N	N	N	2+
12	P	P	P	3+
13	P	N	S	1CFU
14	P	P	P	1+
15	N	N	N	1+
16	P	P	P	CS&ML
17	P	P	P	CS&ML
18	P	P	P	CS&ML
19	P	P	P	CS&ML

* This sample was positive after a 1:10 and 1:100 dilution of the template

TNTC: too numerous to count
N: negative
P= positive
S= suspect
CS=clinical signs
ML= macroscopic lesions
1+ = 1-7 CFU/tube, low shedder
2+ = 8-30 CFU/tube, moderate shedder
3+ = 31-70 CFU/tube, moderate shedder
4+ = >70 CFU/tube, heavy shedder

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Table 5-3 OTSN-PCR, culture and ELISA results in adult cows from farms A and B

FARM	TESTS	CATEGORY	OTSN-PCR		P- VALUE
			Positive	Negative	
A	Culture	Positive	12	11	> 0.5
		Negative	14	190	
	ELISA	Positive	10	34	> 0.02
		Negative	18	205	
B	Culture	Positive	0	0	NA*
		Negative	0	53	
	ELISA	Positive	0	3	NA*
		Negative	0	52	

* Not applicable

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Table 5-4 OTSN-PCR, culture and ELISA results in 8-month-old heifers from farms A and B

FARM	TESTS	CATEGORY	OTSN-PCR		P- VALUE
			Positive	Negative	
A	Culture	Positive	0	4	> 0.7
		Negative	3	242	
	ELISA	Positive	0	3	> 1.0
		Negative	3	241	
B	Culture	Positive	0	0	NA*
		Negative	0	50	
	ELISA	Positive	0	0	NA*
		Negative	0	53	

* Not applicable

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Table 5-5 Combined culture and ELISA results from adult cows in farms A and B

FARM A	CULTURE POSITIVE	CULTURE NEGATIVE	TOTALS
ELISA POS	14	27	41
ELISA NEG	11	197	208
TOTALS	25	224	249
FARM B	CULTURE POSITIVE	CULTURE NEGATIVE	TOTALS
ELISA POS	0	3	3
ELISA NEG	0	56	56
TOTALS	0	59	59

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Table 5-6 Combined OTSN-PCR and ELISA results from adult cows in farms A and B

FARM A	OTSN-PCR POSITIVE	OTSN-PCR NEGATIVE	TOTAL
ELISA POS	10	34	44
ELISA NEG	18	205	223
TOTALS	28	239	267
FARM B	OTSN-PCR POSITIVE	OTSN-PCR NEGATIVE	TOTAL
ELISA POS	0	3	3
ELISA NEG	0	52	52
TOTALS	0	55	55

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CHAPTER 6 COMPARISON OF TWO FECAL CULTURE SYSTEMS FOR THE DETECTION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN ADULT AND YOUNG DAIRY CATTLE

6.1 Preface

This chapter addresses objective 4: To determine the apparent prevalence of fecal shedding of ***Mycobacterium avium*** subsp ***paratuberculosis*** (Map) in adult cows and in 8-month old heifers from a dairy farm with 12% seroprevalence of Paratuberculosis (PTB) infection, and to compare the performance of the conventional fecal culture (HEYM) and the non radiometric liquid Extra Sensing Power (ESP) Culture System II in feces from adult cows and from 8-month old heifers.

A manuscript for publication will be prepared with this material; the co-authors will be Hirst H., Brigner T., and Salman M.D.

6.2 Structured Abstract

Objectives: To determine the apparent prevalence of fecal shedding of Map in adult cows and in 8-month old heifers from a dairy farm with 11% seroprevalence of PTB infection, and to compare the performance of the conventional fecal culture (FC) system in Herrold Egg Yolk with Mycobactin (HEYM) and the non radiometric liquid Extra Sensing Power (ESP) Culture System II in feces from adult cows and from 8 month old heifers

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Design: Paired fecal samples were collected from adult cows and from 8-month old calves to be tested by conventional FC culture and non radiometric liquid ESP.

Sample population: Paired fecal samples were collected from 107 adult cows and 138 eight-month old heifers from a herd with 12% seroprevalence of PTB, history of clinical disease and history of fecal culture positive adult animals.

Procedure: A study was conducted in which paired fecal samples were collected from adult cows and young 8-month old heifers to be tested by HEYM and ESP culture systems. Results from paired fecal samples were compared with McNemar's Chi-squared test and Kappa scores were calculated to determine the agreement among both culture methods.

Results: No statistically significant differences were found in the proportion of adult cows with presence of Map in feces. Map was detected in 16.8% of the adult cows by HEYM and in 12.1% by ESP. The agreement beyond chance between both methods in adult cows was moderate (Kappa 66% , 95% CI = 45.8%, 86.5%) While HEYM detected a high proportion of low shedders, ESP detected mostly heavy shedders. The proportion of young heifers detected by both culture systems were the same, however, no agreement is expected between these two methods in young calves (Kappa approximately zero). The prevalence of Map in feces from 8-month old heifers was 2.9% when both culture systems used in parallel. Both methods revealed that the calves were shedding low numbers of Map.

Conclusions and clinical relevance: In this study HEYM detected a few more shedders than ESP; however, moderate agreement is expected between both methods in adult animals. The percentage of expected agreement is lower when the sample contains

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few microorganisms. Hence, the detection of low shedders might be improved by the simultaneous use of both culture systems.

6.3 Introduction

The National Johne's Working Group (NJWG) has identified several research priorities related to Johne's disease (Paratuberculosis; PTB), "the development of a more sensitive, efficient, cost-effective test for detection of PTB in subclinically infected animals" being at the top of the list. Furthermore, the NJWG has expressed: "Currently, our diagnostic tests are appropriate for whole herd screening but are sorely inadequate for individual animal detection".[12] The evaluation of new diagnostic methods requires the comparison of test results with the true disease status of the individual, most generally determined by a reference test or "gold standard". This comparison allows estimating the accuracy of the new test in detecting disease, which is measured by its sensitivity, specificity, and predictive values. Most of the published studies on screening and diagnosis of PTB in livestock, considered the isolation of the causal agent, *Mycobacterium avium* subsp *paratuberculosis* (Map), from feces as the gold standard method for confirmation of disease/infection status.[2;13] One of the major constraints in Johne's research is that the gold standard is so imperfect that some research groups prefer to consider it almost nonexistent.[4] Among the limitations to the use of fecal culture (FC) as a gold standard are its low sensitivity, intermittent shedding patterns, and the fact that the presence of Map in feces does not always indicate established infection but simply the passing of microorganisms through the intestinal tract.[16] Yet, with all these imperfections, FC seems to be the only feasible way of confirming infection in live animals. The sensitivity of the cultivation methods appears to vary depending upon the culture system,

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procedures for concentration of Map and decontamination of pellets, sample size and inoculum volume, and the number of microorganisms present in the sample.[6;14] Conventional FC using Herrold Egg Yolk with the addition of Mycobactin J (HEYM) has been suggested as the standard method for cultivation of Map.[13] Required time to detection of colonies with this culture is between 8 and 16 weeks.[10] The reported sensitivity of FC in HEYM ranges between 30% to 50% depending on the procedures involved.[1;2;7;16].

Both time to detection and sensitivity of fecal culture have improved with the development of radiometric culture systems, such as the BACTEC 460TB commercialized by Beckton and Dickinson (BD). This system uses a radiometric liquid media and relies on the detection of C_{14} present in CO_2 generated during the metabolism of palmitic acid (which is the source of labeled C_{14}). Time to detection of organisms is reduced to half compared to the time required by HEYM.[3] Although BACTEC 460TB demonstrated to be the most sensitive culture system for isolation of Mycobacteria spp, including Map, rules regulating the use of radiolabeled material hinder the widespread use of this method.[3;17]

To overcome the inconveniences of handling radioactive material, a new non radiometric systems, the Extra Sensing Power (ESP)[a] Culture system II was released to the market. The ESP technology detects mycobacterial growth in a liquid media by continuous monitoring of pressure changes generated during consumption of oxygen.

[a]ESP II; TREK Diagnostics, Inc, Cleveland OH

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The ESP system has been adopted as one of the methods for confirmation of disease status for the National Animal Health Monitoring System (NAHMS) Dairy 2002 study.[b]

A study comparing the ESP and BACTEC 460TB systems for detection of Mycobacterial species, in which culture in solid media was used as a reference method, indicated that false positive results could be expected in approximately 1.27 % of the ESP signals.[11] The BACTEC 460TB culture system, however, is labor intensive and requires laboratories to comply with safety procedures and regulations associated with the use of radioisotopes. Compared to HEYM, both radiometric and non radiometric liquid culture systems are limited in their ability to provide information on the phenotypic characteristics of the colonies or a prompt recognition of contaminants and mixed cultures, which makes more difficult to assess the specificity of the growth.[11] The possibility of microscopic examination or PCR confirmation by detection of IS 900 in early positive vials is an advantage of the non–radiometric ESP over the radiometric BACTEC system, in which these procedures are hampered by the presence of radioisotopes.[11] BD has released another non-radiometric system to the market, the BACTEC MGIT960 but its use in veterinary diagnostic has not been reported until the present.

In humans, the detection and typing of Mycobacterial species from clinical specimens has been improved by the combined used of conventional and non radiometric culture systems. It is possible that a similar approach be needed to improve the confirmation of PTB in the field of veterinary diagnosis.

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[b] Dr Brian McCluskey, personal communication

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The objectives of this study were to determine the apparent prevalence of Map in feces from adult cows and 8-month old heifers from a dairy farm with 12% seroprevalence of PTB infection, and to compare the performance of FC in HEYM and in the non radiometric liquid ESP Culture System II in samples collected from the same animals.

6.4 Materials and Methods

The study animals belonged to a herd that was previously tested using a whole herd commercial ELISA[c], and found to have an apparent seroprevalence of 12%.[5] This herd also had cases of clinical disease confirmed by fecal culture and histopathology on samples collected at post mortem. The cows and heifers selected for this study were part of a cohort that had been closely monitored since the year 2000 as part of graduate study at Colorado State University. The study animals were selected based on the dam's serological results because ELISA is considered the most conventional, affordable, and rapid technique for screening of PTB infection. Forty percent (40%) of the dams selected for this particular study were ELISA positive, 18% were suspect and, 40% were negative, according to a classification described elsewhere.[5] The 8-month old heifers were all negative to ELISA. Fecal samples from adult cows (n=107) and from 8 month old heifers (n=138) were collected from the rectum with a clean and dry obstetric glove and transported to the Animal Health Population Institute (APHI) laboratory at Colorado State University in a controlled temperature box. In the APHI laboratory, each sample was divided into two whirl-pack bags and stored at –70° C for 2-3 weeks before being transported to testing laboratories. One sample set of each pair was sent to the Rocky

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Mountain Regional Animal Health Laboratory (RMRAHL) in Denver to be tested by conventional culture in HEYM media. At the same time, the other sample set of each pair was shipped to the Veterinary Diagnostic Laboratory at Cornell University to be tested by the non radiometric ESP culture II system.

Conventional Culture in HEYM: Each fecal sample was processed with the method recommended by the National Animal Disease Center (NADC),[10] which consists of centrifugation of the total fecal sample supernatant and a 2-step decontamination protocol. Results were reported as negative, positive, atypical, or contaminated. In addition, positive results were scored according to the number of Colony Forming Units (CFUs) present in the slant. The reported results were as follows:

4+	> 70 CFU
3+	30 – 70 CFU
2+	8-30 CFU
1+	1-7 CFU
Negative	No growth (this category was assigned value 0)
Contaminated	Overgrowth of other microorganisms
Atypical	Acid fast positive, non-PTB

Scores 1+ and 2+ could also be considered low shedders, 3+ moderate shedders, and 4+ heavy shedders.[d]

Non Radiometric ESP Culture II : Fecal samples were decontaminated by the Cornell double-incubation process (detailed elsewhere)[10] prior to culture in ESP MYCO bottles. Samples were incubated at 37°C in the ESP bottles until a positive signal was detected or for a total of eight weeks. Every culture bottle with negative signal was tested by the acid-fast stain. If acid fast bacilli were present, further confirmation was

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[c] IDEXX *Mycobacterium Paratuberculosis* antibody test kit, IDEXX Laboratories Inc, Westbrook, Me.

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performed by PCR targeting the IS 900, specific for Map.[e] All culture bottles with positive signals were tested with IS 900-PCR for confirmation of Map. Results were reported in categories and days to detection. The categories were as follows:

Negative:	not detected
Few:	1-30 CFU/0.1g (low shedders)
Moderate:	31-299 CFU/0.1 g (moderate shedders)
Many:	>299 CFU/0.1 g or too numerous to count (heavy shedders)
Suspicious:	unable to confirm identity
AFB:	acid fast bacillus detected, confirmation needed
AFBNC:	acid-fast bacillus detected, not confirmed as Map
Contaminated:	overgrowth of other microorganisms

Analysis : Results from HEYM and ESP cultures were analyzed by McNemar's Chi-square test to determine if there were significant differences between results obtained by both culture systems in adult and young cattle. The percentage of observed agreement as well as Kappa coefficients for expected agreement beyond chance were calculated between the culture systems under evaluation. The statistical significance level used in this study was 0.05. The apparent prevalence of fecal shedders among adults was determined as the proportion of adult animals with a positive culture result (to ESP, HEYM or both) over the total adult animals tested. The prevalence of fecal shedders among young stock was calculated by substituting the numerator and denominator for the results in the 8-month old heifer population.

6.5 Results

Adult Cows: The prevalence of Map in feces from adult cows detected by either one or both culture systems was18.7%. While HEYM results indicated that 16.8% of the

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[d] Tiffany Brigner, RMRAHL, personal communication
[e] Dr S Shin , Veterinary Diagnostic Laboratory, Cornell University, personal communication.

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adult cows in this study were shedding Map in feces, ESP II identified Map in only 12.1% of the samples. Application of McNemar's Chi square and Fisher's exat probability test found no statistically significant differences between the proportions of positive results detected by HEYM and ESP. Table 6-1 shows the distribution of shedding patterns detected by HEYM and ESP. According to these results, most of the fecal shedders identified by HEYM were categorized as low shedders (9.3%) whereas the majority of the positive results reported by ESP (11.1%) were categorized as heavy shedders. While HEYM did not identify any atypical mycobacteria in the adult cows, ESP detected one animal (0.93%) with acid fast bacilli lacking IS 900, which could have been atypical mycobacteria.

Agreement between HEYM and ESP Results: The observed percentage of agreement between the HEYM and ESP results in fecal samples from adult cows was 91.5%. However, the agreement expected beyond chance calculated by the Kappa score was 66% (95% CI= 45.8%, 86.5%) (Table 6-2).

As observed in Table 6-2 , there were 9 pairs of discordant results, 7 of which were HEYM +/ESP- and 2 were ESP+/HEYM-. Within the first combination (HEYM+/ESP-), 5 of the 7 HEYM positive results corresponded to samples with low numbers of CFUs per tube(1+), 1 of the 7 was classified as a 3+ by HEYM and the other was a 4+. Within the second combination of results (ESP+/HEYM-), there were 2 animals classified as heavy shedders ("MANY") by ESP and negative by HEYM.

It is important to notice that Map was detected by both culture methods in feces from 11 adult cows. Of those, 5 cows were categorized as heavy shedders by both methods. The remaining 6 animals detected by both culture systems were however categorized

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differently regarding their shedding pattern. Thus, 4 out of 6 were categorized as HEAVY shedders by ESP and LOW shedders by HEYM; 1 out of 6 was categorized as MODERATE by ESP and LOW by HEYM and 1 out of 6 was HEAVY according to ESP and MODERATE when tested by HEYM.

Eight Month-Old Heifers: The prevalence of Map in feces from 8 month old heifers detected by either or both fecal culture systems was 2.9% . Results from HEYM indicated that Map was present in 1.4% of the heifers tested (2/138). Same percentage of positive results was reported by ESP, therefore no differences were found between HEYM and ESP when applied on feces from 8-month old heifers. Although the observed agreement between the two culture systems under evaluation was approximately 97% (reflected by high agreement in negative results), the agreement beyond chance based on this study was nonexistent (–1%) (Table 6-4). Positive results from both culture systems classified these heifers in the category of "low shedders" (Table 6-5). Times to detection of growth with the ESP system in samples from both adult and young animals were similar to those reported in the literature (Table 6-6).[8]

6.6 Discussion

To our knowledge, this is the first study focusing, not only on the shedding patterns of adult cows, but also on the amounts and frequency of fecal shedding among young stock in a natural setting.

The prevalence of Map in fecal samples from adult cows was 18.7% (20/107) when both systems were used combined. When considered separately, ESP and HEYM detected Map in 16.8% and 12.4% of the samples, respectively. Although the

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seroprevalence among the selected dams in this study was 40%, the prevalence of Map in feces was nearly half. It has been mentioned that the sensitivity of fecal culture progresses according to the severity of the disease. It is possible that some of the dams were not correctly identified by any of the FC systems evaluated in this study if they were in the initial stages of the disease.[16]

The prevalence of Map in feces from 8 year-old heifers was 2.9% when both systems were combined. The recovery rates were the same for both cultures systems (1.4%) when applied to feces from 8-month old heifers. Fecal shedding of Map in young calves has been described only in studies involving experimental infection. Differentiation between active shedding of Map in feces as a consequence of established infection and passive shedding of Map, also referred to as "pass through", is important in subclinically infected animals shedding low numbers of Map. This differentiation, however, could only be achieved after a complete post mortem examination including histopathology of intestinal tissues and mesenteric lymph nodes. Current programs for control of Johne's disease are based on screening for PTB among adult animals. Extending the screening among young stock could result in a more effective and rapid identification of sources of infection to the rest of the herd. According to Whitlock and Buerguelt, the amounts of fecal shedding in naturally infected calves are expected to be low. This hypothesis leads to the conclusion that more sensitive diagnostic strategies are needed in order to identify fecal shedders among young stock.

Although no statistically significant differences were found in the proportion of animals with presence of Map in feces detected by each system, HEYM identified a few more fecal shedders than ESP among adult cows. Among young heifers, however,

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HEYM and ESP identified equal percentage of fecal shedders. The majority of the concordant positive results between both culture systems were obtained in adult cows shedding high numbers of microorganisms in feces. On the other hand, the majority of the discordant results corresponded to samples with low numbers of CFU's per tube (1+). A possible explanation for this finding is that Map cells aggregate in clumps, and when these clumps are not abundant then their distribution in the fecal samples could be heterogeneous.

Of the two CF systems compared in this study, conventional FC in HEYM is the least expensive currently available method for isolation and growth of Map. Its cost runs at approximately US$ 12.00, including labor, and it requires between 8 to 12 weeks for a result.[9] Although HEYM is commercialized by BD, most diagnostic laboratories prepare a home made HEYM, which contributes to its low cost. A report presented in the 7th International Colloquium of Paratuberculosis comparing the "in house" and the commercial HEYM media indicated that in 60 % of the tested samples the "in house media" was superior than the commercial, and in 30% of the samples both media performed equally.[15] In addition to the low cost, HEYM allows for the easy visualization of growth and colony characteristics, which enhances the specificity of the system. However, the major downside of HEYM is the long time required for results.

The ESP system, on the other hand, runs at a cost of approximately US$ 33 per sample (Cornell Diagnostic laboratory), including labor. This system is particularly suitable for large-scale sample processing, since one unit has a capacity of 384 bottles, which supports the processing of approximately 60 samples per week. In addition, it provides a fully automated monitoring of specimens, which allows for the semi-

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quantification of Map fecal shedders. The ESP system can detect growth 2 to 3 weeks earlier than conventional methods and has the ability to report negative results in 6 weeks compared to 12 weeks required by conventional culture. Moreover, a study conducted at Cornell University reported a mean time to detection of 14.9 days, 22.7 days, and 34.5 days for heavy, moderate, and low shedders, respectively.[8] Time to detection of Map by ESP in this study coincided with the times reported by the researchers at Cornell University.

The detection of infected animals shedding low numbers of bacteria in feces has always been a challenge and results from this study suggest that the identification of low shedders might require more than just one method. Experts from the Center for Disease Control have recommended the use of the ESP culture method as a complement to the conventional culture on solid media for confirmation of mycobacterial infections from human specimens.[17] Results from our study support this recommendation by considering this strategy of high value in animals shedding low numbers of Map. However, it is important to consider that the presence of Map in feces (especially in light shedders) is not necessarily an indicator of infection. Further studies should focus on the estimation of the expected proportion of cattle shedding Map and those in which Map is passing through, at any given prevalence of disease in the herd.

6.7 Acknowledgements

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6.9 Tables

Table 6-1 Frequency distribution of fecal culture results by HEYM and ESP system II from adult cows

HEYM			ESP II		
Category	Frequency	Percent (%)	Category	Frequency	Percent (%)
Negative	89	83.18	Negative	93	86.92
1+	9	8.41	Few	0	0
2+	1	0.93	Moderate	1	0.93
3+	2	1.87	Many	12	11.21
4+	6	5.61	AFB	0	0
Atypical	0	0	AFBNC	1	0.93
TOTAL	107	100	TOTAL	107	100

AFB: acid fast bacillus detected, confirmation needed
AFBNC: acid-fast bacillus detected, not confirmed as Map

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Table 6-2 Combined results from HEYM and ESP culture II in adult cows for the presence/absence of Map in feces

		HEYM		
		NEGATIVE	POSITIVE	TOTALS
ESP II	NEGATIVE	86	7	93
	POSITIVE	2	11	13
	TOTALS	88	18	106*

* One cow classified as AFBNC (acid-fast bacillus detected, not confirmed as Map) by ESP II was left out for this analysis.

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Table 6-3 Frequency distribution of shedding patterns classified by ESP II and HEYM in adult cows

Frequency	ESP II	HEYM
5	Heavy	Heavy
4	Heavy	Low
1	Heavy	Moderate
2	Heavy	Negative
1	Moderate	Low
1	Negative	Heavy
5	Negative	Low
1	Negative	Moderate

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Table 6-4 Combined results from HEYM and ESP culture II on fecal samples from 8-month old heifers

		HEYM		
		NEGATIVE	POSITIVE	TOTALS
ESP II	NEGATIVE	134	2	136
	POSITIVE	2	0	2
	TOTALS	136	2	138

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Table 6-5 Frequency distribution of fecal culture results by both HEYM and ESP II in 8-month old heifers

HEYM			ESP II		
Category	Frequency	Percent (%)	Category	Frequency	Percent (%)
Negative	135	97.83	Negative	136	98.55
1+	2	1.45	Few	2	1.45
Atypical	1	0.72	AFBNC	0	0
TOTAL	138	100	TOTAL	138	100

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Table 6-6 ESP culture II times to detection

N	ESP Classification	Average time to detection (days)
11	MANY	15.09
2	MODERATE	23
1	FEW	32
2	AFBNC	11

AFBNC: acid-fast bacillus detected, not confirmed as Map

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CHAPTER 7 : GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 Value of the study

To the best of my knowledge, this study differes from any previous study study in the following wayss:

a. The study populations were typical of the naturally-occurring Paratuberculosis (PTB) infection and management conditions in Colorado, whereas most studies on the evaluation of diagnostic tests for detection of PTB in young cattle were performed on either experimentally infected calves or calves from herds with an extremely high prevalence of PTB infection. It is obvious that the disease will have different patterns of expression in experimental vs. natural settings.

b. The selection of the study animals (dam-heifer pairs) was based on ELISA results of the dam, the most commonly used screening test for PTB due to its low cost and rapidity. This study design was employed with the purpose of making recommendations regarding the management of dams and their offspring on the basis of the dams' ELISA status. This selection approach has not been used in any other study.

c. This is the first study attempting to predict the cell-mediated immune (CMI) response to PTB in 8- months old heifers based on the dam's status (measured by ELISA or fecal culture past and current records).

d. This is the first study evaluating three tests (γ-IFN, skin test, and ELISA) simultanously to screen for early infection by following up a cohort of naturally exposed heifers between the ages of 2 to 8 months. Consequently, this study is a

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comprehensive approach to characterize the humoral and CMI response in heifers at 2, 4, 6, and 8 month of age in a natural setting.

e. Lastly, new tests for direct detection of ***Mycobacterium avium*** subsp. ***paratuberculosis*** (Map) in feces from adult cows and 8-month-old heifers were compared with the aim of improving the confirmation of disease status, which is critical for the evaluation of tests in subclinically infected cattle.

7.2 Conclusions and Recommendations

7.2.1 Hypothesis I

Tests based on the detection of CMI could be used for the detection of young dairy cattle exposed to PTB infection.

Due to the lack of a reliable test for confirmation of disease status, the G-IFN test, ELISA, and intradermal skin test could not be fully characterized when applied to heifers at ages ranging from 2 to 8 months. However, relevant conclusions and recommendations could be drawn form this study:

a. In a herd with 11% seroprevalence of PTB infection in adult cows, the proportion of 8-month old heifers with a positive fecal culture (FC) to Map was significantly lower than the proportion of adult cows with positive FC in HEYM (1.5% vs. 10%, respectively). These findings support previous statements by Whitlock and Buergelt, who indicated that young livestock might shed Map to the environment in quantities below the threshold of detectability.[6]

b. Fecal culture was chosen by the Voluntary Johne's Disease Herd Status Program (VJDHSP) as the confirmatory method for disease status on individual animals and

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also at the herd level. Nevertheless, it is questionable to use FC results indicating the presence of Map in low numbers as confirmation of PTB infection. Discrimination between active and passive shedding of Map in feces is an important issue when it comes to culling FC positive animals of high economic value. Further research is required to estimate the percentage of passive shedders that could be expected at different prevalence levels of the disease. The ability to discriminate fecal shedding of Map from "pass through" will help re-assessing the specificity of FC as a diagnostic test for PTB.

c. The results of ELISA at 2, 4, 6, and 8 months of age showed that the humoral response is not relevant in young cattle exposed to PTB. The proportion of positive ELISA results by age were significantly different from the proportion of positive skin test results in both herds (Tables 3.2 and 3.3). In addition, we have shown that the ELISA response remains stable even after the repetitive intradermal injection of Map PPD.

d. Positive skin test reactions were more frequently observed after 2 months of age. The percentage of positive reactors in herds A and B were different at the ages of 4 and 6 months, when most of the reactors were in herd B. In general, the majority of the positive reactions corresponded with the cervical injection site. Although this phenomenon has also been described for bovine tuberculosis, the literature does not address the reason for this finding.[2] It is possible that the use of the caudal fold, instead of the cervical skin, would increase the specificity of the reactions. Unfortunately, the current study could not determine any association between infection and the presence of skin test reactions. However, a small number of 8-

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month old heifers with presence of atypical Mycobacteria classified as Runyon group IV in feces, did not show any cross reactivity with the skin test. Future studies for evaluation of the skin test as a screening test for PTB in young animals should comprise the post-mortem examination of tissues by culture and histopathology.

e. The γ-IFN test showed a steady decline from the time that the cohort was first tested, at 2 months of age, until the last test at 8 months. This study confirms previous findings, which suggested that there is a non-specific production of γ-IFN in young cattle that decreases with age.[5] However, due to the inability to determine infection status in these animals, no conclusions could be drawn regarding the sensitivity or specificity of this assay in 8-month old heifers.

In summary, this study described progression of the humoral immune response measured by ELISA and the CMI response measured by the Johnin skin test and the γ-IFN test in female calves at 2, 4, 6, and 8 months of age. FC from samples collected at 8 months did not provide much information regarding the infection status of the calves. Further evaluation of tests based on the detection of the CMI response in young cattle would require exploring two major issues: [1] A test that would discriminate between shedding and Map "pass though" [2] A test or combination of tests with high sensitivity that would detect low numbers of Map cells per gram of feces.

7.2.2 Hypothesis II

The PTB- status of the dams (measured by ELISA and fecal shedding) is associated with the CMI responses in calves younger than one year old and could be used as a predictor of the calves' CMI response

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To the best of my knowledge, only one study looked at the variations of the humoral response of the offspring attributable to the dams in a dairy cattle herd with PTB.[4] This study took a similar approach and explored the variation in the response to CMI-based tests that could be associated with risk factors related to the dams. Factors such as past or present positive ELISA records and positive FC results of the dams reflect a higher probability of PTB infection. In this study we have found that dams with positive fecal culture results at 60 days in milk (DIM) are more likely to have a heifer that tests positive to the γ-IFN test at 8 months of age. Although this study was unable to prove that a positive γ-IFN test in 8-month old heifers indicates established infection, it would be expected that dams that have been shedding Map in feces during postpartum have exposed their offspring to Map thereby generating a CMI response. The question generated by this finding is whether routine γ-IFN testing of heifers at 8 months of age could provide an indication of fecal shedding in their dams. Such association requires further validation and research.

7.2.3 Hypothesis III

OTSN-PCR used in parallel with fecal culture will increase the accuracy for the detection of fecal shedders more than using fecal culture by itself

The ability of producing results in approximately 48 hours is the most valuable feature of the OTSN-PCR when compared with all currently available culture systems. Kappa values for the expected agreement beyond chance between OTSN-PCR and FC in HEYM was moderate in this study. These two direct diagnostic methods tend to better agree in positive results when the numbers of Map present in feces are high. In general, the scientific community tends to be skeptical regarding the value of PCR-positive

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culture-negative results. Regardless of the system applied, trust in PCR results will require further validation in a larger number of samples of known culture status.

OTSN-PCR, however, is a rapid and affordable test that could detect subclinically infected animals shedding Map in feces earlier than any other any ante-mortem test. In cases where the confirmation of infection on individual animals requires a prompt response, OTSN-PCR could be used in a serial testing strategy with fecal culture.

Particular attention should be paid at the type of PCR system and DNA extraction methods applied. Standardization of reagents required for DNA extraction and amplification seems to provide more consistent and repeatable results than in-house reagents (personal communications with veterinary diagnosticians). Systems such as the real time PCR, which doesn't use agarose gel electrophoresis to evidence amplification products, are reported to improve the interpretation of results by eliminating the subjectiveness of this analytic method.[1;3]

7.2.4 Hypothesis IV

The use of conventional fecal culture (HEYM) in combination with other rapid culture systems, such as the liquid non-radiometric ESP culture system II, will improve the detection of fecal shedders compared with the use of only one of the two fecal culture systems.

This study has demonstrated that the proportion of fecal shedders detected by conventional FC in HEYM and ESP systems in paired samples were not significantly different. The agreement expected beyond chance between these two systems, however, was moderate. The majority of the discordant results occurred in samples in which the

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numbers of Map per gram of feces were low. It is particularly in the category of low shedders that the use of these systems in parallel increases the accuracy in the diagnosis. Even when it is debatable whether the contribution of low shedders to infection transmission is relevant or not, an increase in diagnostic accuracy is needed in final stages of eradication of PTB or to maintain the free herd status certification. The high cost of the ESP culture is somewhat compensated by its ability to detect growth 2 to 3 weeks earlier than conventional FC and to release negative results in 45 days compared to 12 weeks with conventional culture.

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