Evaluation of ELISA and fecal culture strategies for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Johne's Disease (JD) is a chronic, infectious, enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). In the clinical stage of this disease, the infection causes severe diarrhea and wasting of the affected animal. The subclinical infection with *Mptb* also has a detrimental effect on productivity of cattle. In ELISA-positive cattle, there is a 2.4 times increase in the risk of culling, and lactational 305d milk production is decreased by at least 370 kg. Reduced slaughter value and premature culling accounts for losses of CD$ 1330 per year per infected 50-cow herd. Research has failed to show a consistent association between *Mptb* test status and reduced fertility or risk of clinical/subclinical mastitis. Host level factors for becoming infected include age of exposure and level of exposure, along with source of exposure such as manure, colostrum or milk. Agent factors involve the dose of infectious agent and strains of bacteria. Environmental management factors influence the persistence of the bacteria and the level of contamination in the environment. Emphasizing a risk factor approach is the design of various control strategies at a national level in many countries throughout the world, specifically Australia, The Netherlands and the United States.

Control of JD historically has been based upon identification of infected animals and either segregation or removal from the herd. The focus of this study was to investigate two diagnostic methods for detection of *Mptb*: ELISAs and culture. The first goal was to compare the diagnostic characteristics of various ELISAs for *Mptb* using the most appropriate gold standard. Then a comparison was done on the two commonly used gold standards: tissue and fecal culture. Additionally, pooled fecal culturing was examined to determine sensitivity of various pools sizes and use that information to estimate herd sensitivity.

A commonly used diagnostic modality is an ELISA to test for antibodies to *Mptb*, however, the licensed assays in North America have poor sensitivity for sub-clinically infected cattle that are not heavily fecal shedding. A European ELISA has shown some improvement in sensitivity in testing done in Germany. In order to test this ELISA against the North American ELISAs, a slaughterhouse study was conducted to obtain serum from culled dairy cows along with tissue and feces to be cultured as a gold standard comparison.

The European ELISA had a slight advantage in sensitivity over one of the North American ELISAs, but overall there was no dramatic improvement in test accuracy and agreement among the three assays was poor. Comparison of tissue culture and fecal culture indicated that fecal culture was not as sensitive as tissue culture and therefore may be a poor gold standard for JD test evaluation.

The feces collected allowed for a unique opportunity to investigate the validity of pooled fecal culture as a herd level diagnostic tool in conjunction with control programs. This testing modality is already in place in many countries and would be a component of the proposed Canadian Voluntary Johne's Control Program. The results of this research support the validity of utilizing pooled fecal culture as a herd level testing modality and also provides some credibility to minimizing the use of ELISA testing in control programs.

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Finally, and most importantly, I want to express my sincere thanks love and appreciation to my wife and daughter, Heather and Emily. Without your love and tolerance dealing with me moving you back to PEI and occasionally putting the needs of this project in front of other more important issues, I never would have been able to finish. I love you both dearly and hope to make it up to you in the future.

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List of Abbreviations

AMB	Amphotericin B
AUC	Area Under Curve
BHI	Brain Heart Infusion
CAB	Commonwealth Animal Bureaux
CFU	Colony Forming Units
DHI	Dairy Herd Improvement
ELISA	Enzyme Linked Immunosorbant Assay
HEY	Herrold's Egg Yolk
JD	Johne's Disease
LAM	Lipoarabinomannan
Mptb	*Mycobacterium avium* subsp. *paratuberculosis*
NAL	Nalidixic Acid
PPV	Positive Predictive Value
ROC	Receiver Operator Characteristic
SCC	Somatic Cell Count
VAN	Vancomycin

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CHAPTER 1. INTRODUCTION TO JOHNE'S DISEASE IN CANADA: DISEASE IMPACTS, RISK FACTORS AND CONTROL PROGRAMS FOR DAIRY PRODUCERS

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Submitted to Canadian Veterinary Journal as Part II of a Two Part Series on a Literature Review of Johne's Disease. Part I is included in this thesis as Appendix A.

1.1. Introduction

Johne's Disease (JD) is a chronic, infectious, enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). In the clinical stage of this disease, the infection causes severe diarrhea and wasting of the affected animal. The clinical aspects, pathophysiology, and the currently available diagnostic tests for JD were discussed in the initial paper of this two part series. In this second and final part of the series, the impact of the disease is discussed, including economic consequences on dairy production. Additionally, risk factors that are associated with the spread of the organism and the incidence of new infections on dairy farms are outlined. A review of control strategies that can be used to avoid new infections on a dairy farm is included. The proposed Canadian Voluntary Johne's Disease Control Program is presented here and is compared to current national control programs in other countries.

Canada has had success in controlling other mycobacterial diseases, specifically tuberculosis. Modern disease control programs for pathogens without clear zoonotic potential will have to be driven by economics of the disease at the individual and national herd level.

1.2. Methods

Due to substantial differences in management, production, and related control options between dairy and beef cattle, and the extensive literature on JD for both dairy and beef

cattle, this paper focuses on dairy cattle. Also, because the intended audience for this paper is primarily Canadian veterinarians, we have emphasized Canadian references as much as possible.

Medline (accessed via PubMed from 1950 to present), The Commonwealth Animal Bureaux (CAB) (accessed via VetCD and ParasiteCD sets from 1973 to present) and Agricola, produced by the National Agricultural Library of the U.S. Department of Agriculture (from 1970 to present) were used to collect the references. The keywords used in the search of the databases were *Mycobacteria*, paratuberculosis, Johne's, Canada, Canadian, dairy and cattle. In addition, a small number of papers were identified from the reference lists of other papers, or personal knowledge of reports or conference proceedings.

All relevant material collected from the above process was included in the review, provided that it was pertinent to the methods of production within the Canadian dairy industry. Exclusion of material was only done if information was redundant or outdated and had been directly refuted. Otherwise, all available information was included.

1.3. Impacts on Productivity

Cattle that develop clinical JD have thickened intestinal mucosa, resulting in malabsorptive diarrhea and subsequent decreased intestinal absorption of nutrients. Reductions in milk production, infertility, premature culling, and lower slaughter value due to poor body condition are the common causes of economic loss associated with JD (1).

Losses associated with subclinical JD, defined as infected without overt clinical signs, have been more difficult to quantify because of the difficulty of accurately detecting subclinical paratuberculosis with the diagnostic tests currently available. Studies on

subclinical impacts, as determined by identification of the organism through fecal culture, are less susceptible to misclassification bias than those using identification of an immune response to the organism (eg. enzyme linked immunosorbent assays - ELISAs), as explained below. However, due to the cost and long time lag between submission of samples and results for fecal culture, ELISAs are frequently used in the field to identify subclinically infected animals. Therefore, observational studies on impacts based on positive ELISA results have been conducted and are also critically evaluated in the following section. An estimate of subclinical economic losses is necessary for assessing the cost-effectiveness of control strategies at the farm, region, and national levels.

Reported economic losses attributable to subclinical JD include decreased milk production (1,2), decreased milk fat and protein yield (3,4), reduced slaughter weight at culling (5), and premature culling (6). Decreased fertility (6) and increased incidence of clinical and/or subclinical mastitis (7) may also be associated with *Mptb* infections. In the following sections, each of these areas of loss is critically evaluated and summarized in Table 1-1.

1.3.1. Milk production, fat and protein yield

In one of only two published Canadian studies looking at the impacts of subclinical infection in dairy cattle, records from 2395 randomly selected dairy cattle in 90 randomly selected herds were examined (8). Overall, 305-day milk production for ELISA-seropositive cows was lower than for seronegative cows. There was significant lower production in their first and fifth lactation of ELISA-seropositive animals, producing 573 and 1273 kg less than seronegative cows, respectively. Similar milk production losses were found in ELISA-based

studies in Wisconsin (7) and Colorado (9). The study from Wisconsin found positive cows had a 4% (376 kg) decrease in 305-day mature equivalent milk production (305ME) (7), and the study from Colorado found ELISA-positive cows had a 551 kg decrease in 305ME (9). Only two studies have reported an association between paratuberculosis and decreased milk fat and protein yield (3,4), costing approximately US$ 205 per cow per lactation (3). In a study based in Ontario (10), associations between results from an experimental ELISA and milk production were inconsistent, perhaps owing to the experimental nature of the test utilized.

When culled dairy cows were diagnosed positive by histopathology and culture of tissues, cows that showed no clinical signs but were positive had a 16% decrease in milk production in their last lactation compared to the lactation two years prior, and a 6% decrease in milk production compared with the lactation one year prior (1). However, there was no extensive look at production in uninfected cows for comparison. Assuming a lactation which produces 8000 kg of milk, these estimates would equate to 1280 and 480 kg, respectively. Similar results of milk production losses associated with subclinical paratuberculosis were determined for cattle that were positive on fecal or tissue culture (1,3,6). In a dairy herd of 210 cows in the USA with a high prevalence of *Mptb* infection, fecal culture-positive cows produced 590 kg and 1270 kg less milk in their third and fourth lactations, respectively, as compared to their fecal culture-negative herdmates (2).

There is potential for bias in each of these studies. In the cull cow study it is possible that cull cows would likely include older cows, which if infected, are close to entering the clinical stage of infection. Using cull cows instead of a random sample of cows in milking herds, potentially overestimates the overall impact on milk production among the general

population. Similarly, cows from a herd with a high prevalence of infection (fecal culture study) would also more likely include cows entering the clinical stage of infection due to increased exposure to *Mptb* as compared to the average farm.

However, there is a potential that this estimate of productivity loss may be an underestimation. The specificity of fecal culture is generally considered to be 100% and, therefore, all culture-positive cattle are assumed to be infected (no false-positives). However, because sensitivity is less than 100% (estimated at 50% (11)) there are false-negatives in the culture-negative group. It is likely that these false-negatives lower the average milk production of this group, bringing the mean productivity of the two groups closer, leading to an underestimation of the difference between test positive and test negative animals. Conversely, a herd with a high prevalence of infection may overestimate the milk production effects for the industry because heavy exposure to calves in this herd could lead to earlier clinical signs than would be seen for the rest of the industry.

As shown by the above studies, the level of reduction in milk production in subclinically infected cows depends on a number of factors, including: stage of subclinical infection (1), parity, with infection in older cows having a larger negative impact (2), sensitivity and specificity of the test utilized for identifying infected cattle, which varies with the stage of infection (12), and farm management (e.g. cow comfort, concurrent infections) (8). Further studies are needed to identify the onset, progression and extent of milk production effects associated with JD in culture-positive animals, controlling for these and other confounding variables at the cow, herd and regional level. However, it is evident that current knowledge supports the fact that subclinical JD has a considerable negative effect on milk production and udder-health, the only difference being the magnitude of this effect.

1.3.2. Premature culling and reduced slaughter value

Premature culling associated with paratuberculosis is one of the major economic burdens of this disease (13). In the only Canadian study estimating culling risk based on serological testing, results from randomly sampled cattle among randomly selected Maritime Canadian dairy herds indicated that after controlling for parity, 305-day milk production and somatic cell count (SCC), the odds of being culled during the 3 years after testing was 2.3 times greater in ELISA-seropositive cows as compared to seronegative cows (14). While the owners in this study were informed of test results 1 year after sampling, the difference in odds of culling between seropositive and seronegative cattle was not significantly different before and after the results were communicated, indicating that the difference was for biological reasons, not simply due to the test results. Similar results were found in a study comparing fecal culture-negative and culture-positive cows in one herd from New York, where the estimated loss due to premature culling for *Mptb* for that herd was US$ 75 per cow per year (15). Age of culling in tissue culture-positive cows showing clinical signs, tissue culture-positive cows not showing clinical signs, and non-infected cows have been demonstrated to be 4.3, 4.9 and 7.7 years, respectively (16).

In addition to premature culling, slaughter value has also been shown to be affected by JD. Clinical JD has resulted in a reduced slaughter value of 20 to 30% in culled cattle (1). In addition, fecal culture-positive cattle without clinical signs have been shown to weigh approximately 59 kg less at slaughter, a loss of US$ 48 per head (5). Using economic modeling techniques, a loss of CD$ 1330 Canadian (CND) per 50 cow herd (assuming an

average apparent prevalence of 7%) due to reduced cull value and premature culling associated with subclinical *Mptb* seropositive cows was reported in Maritime Canadian dairy cattle (17). Further analysis is needed to verify that this estimate is appropriate for all of Canada.

1.3.3. Reduced fertility

Research has failed to show a consistent association between *Mptb* test status and reduced fertility (18). In a study performed in a 900-cow Guernsey herd spanning a total of 10 years, data were collected on reasons for culling along with fecal, terminal ileum and associated lymph node culture (6). A greater percentage of infected cows (68.8% or 106/155) were culled for infertility than culture-negative cows (60.2% or 797/1324). The reliability of producer-reported reasons for culling have been questioned due to the inability to identify more than one reason for culling in most dairy herd improvement (DHI) systems when multiple parameters often contribute to a culling decision (19). In the case of this 10-year study, however, they were not restricted to picking one reason for culling and multiple reasons were listed.

In 90 dairy herds from the Canadian Maritime provinces, seropositive first lactation heifers had an increase of 49 days open as compared to seronegative first lactation heifers, but no association was found in other parities (20). In a study done in Michigan, seropositive cows had a 28-day increase in days open; however, there was no significant association when infected cows were identified with fecal culture (18). Furthermore, studies done in six herds in New Zealand with fecal culture testing (21), and 304 herds from Ontario with ELISA testing (10) found no association between subclinical paratuberculosis and calving interval.

Overall, four studies (2 ELISA and 2 culture based) found JD had a negative effect on reproduction and three studies (1 ELISA and 2 culture based) found no negative effect on reproduction. As a result, it is difficult to draw strong conclusions regarding the impact of JD on fertility. From the available literature, seropositive cows appear to have some degree of diminished fertility; however this impairment is not evident when the classification of disease status is by fecal culture. Therefore, the implications of reduced fertility due to JD may not have anything to do with whether the cow is actively shedding bacteria.

If there truly is an impact of subclinical paratuberculosis on fertility, it has been hypothesized that the mechanism may be related to the impaired immunological and gastrointestinal absorptive capacity and accentuated negative energy balance sustained by infected cattle (18). Preliminary work has been performed looking at the effect of energy on metabolic and immune status of cows with JD around the time of parturition (22). In this study, fecal culture-positive cows were supplemented with a higher energy diet by means of "force feeding" through rumen cannulae. This study found that although supplemented cows had the same periparturient decline in neutrophil function, there was an increased *in vitro* immunoglobulin production and a less protracted return to normal lymphocyte proliferation activity. However, the major limitation of this study was that there were no uninfected cattle included for comparison.

1.3.4. Mastitis

There has also been an inability to show a consistent association between *Mptb* test status and risk of clinical or subclinical mastitis. In 304 herds in Ontario, when an experimental ELISA was used to identify cattle with subclinical paratuberculosis (10),

seropositivity was associated with higher SCC at the cow and herd level. Conversely, a study done in New Zealand (NZ) of six dairy herds found that subclinical, fecal culture-positive cows had significantly lower SCC compared to culture-negative cows (21). The differences in testing methodologies make it difficult to make direct comparisons between the two studies. However, the results from NZ may be specific to the six herds that were sampled, which are unlikely to be comparable to most dairy farms in Canada due to major differences in climate, management, and productivity between the two countries.

Results from a study in Maritime Canadian dairy cattle indicated that, after controlling for parity, 305-day milk production and linear score SCC, the odds of being culled because of either decreased milk production, mastitis or reproductive inefficiency was 2.9 times greater in *Mptb* ELISA-positive cows as compared to ELISA-negative cows (14). These findings support previous reports where cull rates due to mastitis were higher for fecal culture-positive cows compared to negative cows (6). In the previously mentioned culling study using a Guernsey herd, the risk of culling due to mastitis in the culture-positive cows was 22.6% (45 of 199 cows) versus 3.6% (49 of 1361 cows) in the culture-negative cows (6). However, due to the potential unreliable nature of producer-reported reasons for culling in dairy herd improvement (DHI) data, mastitis-related culling evidence should be interpreted with caution. Nevertheless, in this case where there are such dramatic differences, there is likely a real association present.

Overall, there appears to be more evidence for than against the theory that JD negatively affects udder health (Table 1-1). However, further research is needed to clarify and quantify this impact. The pathophysiology of how subclinical paratuberculosis could affect mastitis remains unclear and also requires further research. Speculation includes

negative energy balance and reduced cellular immunity, which have been shown to occur at least in periparturient cows with JD (22).

1.3.5. Total economic losses at the farm, regional and national level

Studies of annual economic losses associated with clinical and subclinical JD among dairy farms have indicated that there are substantial effects at the farm, regional, and national levels (15,17,21,23). As part of a survey by the USDA National Animal Health Monitoring System (NAHMS) conducted in 1996, it was estimated that, averaged across all herds in the United States (US), JD cost the US dairy industry US$ 200-250 million annually (23). Subsequently, a study was conducted in Canadian Maritime region (17) which found that based on ELISA testing, the losses were CD$ 2472 per infected herd per year (average herd size of 50 cows with an average apparent prevalence of 7%) and CD$ 0.84 million per year for the Canadian Maritime provinces. Direct production losses included decreased milk production, increased culling risk, reduced cull value, mortality, treatment costs and reproductive loss. Assuming the prevalence of JD in the Maritimes were the same as the rest of Canada, the national cost of JD could be estimated at CD$ 15 million annually. However, these calculations most likely underestimated the actual losses associated with JD because of misclassification of infected cattle by the ELISA and the authors' decision to not recognize other potentially relevant economic effects, such as mastitis, decreased feed efficiency, and restrictions on market access. Further data and improved diagnostic methods are needed in order to accurately determine economic losses associated with JD for the Canadian dairy industry.

1.4. Risk Assessment

Various factors such as host susceptibility and environmental factors (mode of transmission) interact to determine the prevalence and severity of *Mptb* infection (23).

1.4.1. Host factors

Level of exposure (dose of organisms) and age at the time of exposure are major factors in determining whether an animal eventually becomes infected with JD. Although there is a paucity of scientific evidence on these factors, there is consensus that younger animals require a lower infective dose than older animals (24-26), and it is quite unlikely for an adult animal to become infected, unless there is extreme environmental contamination (27). Poor nutrition, stress related to transport, lactation, parturition and immunosuppression by agents like bovine viral diarrhea virus have been proposed as biologically plausible factors accelerating or precipitating the onset of the clinical phase of infection (28).

Milk or colostrum may serve as the source of *Mptb* organisms for neonates in two ways. Fecal contamination of these fluids may occur, allowing the milk or colostrum to act as the vehicle for infection. Additionally, *Mptb* has been isolated from sterile collections of milk and colostrum from infected cows (29), indicating that there is potential for direct transmission through colostrum and milk from an infected dam (30). Infection can also occur directly across the placenta, as tissue-positive fetuses have been found in culled tissue-positive cows, although this occurrence is quite infrequent (31). This happens more often in cows displaying advanced clinical signs of JD, however it can occur in cows that are heavy fecal shedders, yet not displaying clinical signs of disease (32).

It is suspected that on rare occasions, certain animals that are exposed to *Mptb* can generate a protective immune response resulting in full clearance of the *Mptb* (27). It is unclear whether this capacity, if it exists, is restricted to mature animals, or whether some young animals also have this capability.

Some studies have shown higher susceptibility of Jersey and Shorthorn cows for paratuberculosis (33,34). However, these observed differences were biased by the fact that breed differences were actually linked to husbandry practices in specific regions (i.e. herds with these breeds in these particular studies, had worse hygiene). Due to these circumstances, genetics and breed appear to be minor factors.

1.4.2. Agent factors

The specific number of organisms required to establish infection for specific age groups has not been determined. Infection can occur in calves with a dose of $1.6x10^7$ organisms, which would easily be surpassed in a 2-gram sample of heavily infected feces (35). However, this number is likely to increase with increasing age, as the resistance to infection of the animal increases (24).

Infection of animals may cause clinical disease, but this is not necessarily advantageous or essential to the organism. To survive, *Mptb* only needs to colonize, replicate and be shed so that the rate of recruitment of new bacteria is equal to or greater than the loss of bacteria from the population. The presence of obvious clinical disease is not required for the spread of the organism in the animal population system (30). It has been shown that, although the risk of individual cows being infected is higher on farms with clinical JD, there are still many herds that are infected, yet display no clinical signs of JD (36).

Different strains of *Mptb* exist depending mainly on the species infected. Infections in cattle and sheep are considered to be caused by separate strains of *Mptb* and are sometimes classified as type C and type S, respectively. There has been some evidence of cross-infection of animals between these species (37), but more research is needed to determine the degree to which these two *Mptb* types can actually cross the species barrier. There is also evidence that wildlife species, particularly rabbits (38), may play a role in dispersion of *Mptb* throughout the environment and cause contamination of feed for cattle (39).

1.4.3. Environmental Factors

The primary method of *Mptb* transmission is believed to be a direct fecal-oral cycle. The process is quite similar to the transmission of other enteric infections whereby any exposure to manure from shedders can potentially lead to new infections. However, there is still a possibility of indirect transmission, such as through manure contamination of water bowls and machinery used for feed delivery. Therefore, any management activities that directly or indirectly lead to exposure of manure from shedding animals to susceptible animals could be considered risk factors of infection, and will be reviewed in detail in the section on disease control strategies. The efficiency of transmission by these pathways depends upon factors such as number of organisms shed in the feces and the organism's survival characteristics in the environment (40).

Factors that influence survival of the organism include substrate (feces, water, milk), temperature and pH. The *Mptb* organism can persist in the environment for at least a year (40), but does not replicate in the environment. Although hardier than most other pathogens, the bacterium is susceptible to long-term desiccation, repeated freeze-thaw cycles, exposure

to sunlight, and soils with alkaline pH or low iron (41). *Mptb* is more thermal resistant than other *Mycobacteria*, making pasteurization of milk and milk products somewhat problematic (42). Viable *Mptb* was found in 2.1% of the pasteurized milk samples in Great Britain (43), but studies done in North America, including one using samples collected from retail stores and dairy plants in south-western Ontario, did not find viable *Mptb* in pasteurized milk (44). This may be due to differences in pasteurization methods and temperature (45). However, recently, low numbers of viable organisms were found in 2.8% of 702 samples tested in commercially pasteurized milk purchased from stores in California, Minnesota and Wisconsin (46). While these data have not been published in peer-reviewed journals, these occurrences have called into question the validity of feeding pasteurized milk products to calves as a possible means of lowering the risk of *Mptb* infection.

Although relationships are not well defined among the numerous combinations of host, agent, and environment factors found on different farm situations, known risk factors and routes of transmission must be recognized and addressed for implementation of a control program.

1.5. Dignostic Tests

The diagnostic tests to detect infection with *Mptb* can be categorized into those that identify the organism, and those that identify an immunological reaction to the organism. Evaluation of the performance of diagnostic tests is typically done by comparing estimates of sensitivity (Se – ability of the test to detect infected cattle) and specificity (Sp – ability of the test to identify healthy cattle) based on a "gold standard" that has identified animals as truly infected and truly non-infected.

Due to delays of 2-10 years between time of infection and measurable immune system reactions and shedding, various "gold standards" have been utilized for *Mptb* infection status in the past. Tissue culture of *Mptb* is considered the ideal gold standard test because, even before fecal shedding or an immune response is present, it can detect growth of *Mptb* in multiple organs, including the intestinal mucosa and submucosa, and regional lymph nodes (41). However, fecal culture has been used as the gold standard in many studies (42) in the past due to the high cost and logistical difficulties of sampling for tissue culture.

The delays in immune response or shedding also mean that tests utilized on animals with clinical JD will have a better Se than when they are testing animals for subclinical JD (43,49) because the clinically affected animals are much more likely to be shedding bacteria or have developed a detectable immune response (19). False-negative test results are common, particularly in calves, heifers, and even 1st lactation cows (cattle in silent and subclinical stages) (50). Test results from animals with subclinical paratuberculosis can be a challenge to interpret because clinical signs are not present to assist in their interpretation (42,49,51). More details concerning diagnostic test parameters are discussed in Appendix A.

1.6. Herd Level Control Strategies

In general, disease control programs have three main objectives: decrease the number of new infections; decrease the number of clinically diseased or shedding animals, and decrease the duration of disease or its infective period. Understanding the transmission and pathogenesis of the bacterium is crucial for achieving these objectives. For JD, the prolonged pre-clinical phase of the life-long infection and subsequent poor performance of diagnostic tests makes identification of subclinical shedders difficult. This in turn makes

exposure of sub-clinical cows to the susceptible population the main risk factor for spreading infection (47). Furthermore, the ability of the bacterium to survive for more than a year in the environment makes it difficult to stop transmission within herds without stringent manure management control for young stock. Therefore, effective JD control programs involve two main objectives: to implement best management practices in order to decrease calf exposure to all manure (decrease incidence of new infections), and to reduce the number of infected animals that may shed bacteria in their manure (decrease prevalence of existing infections). We will review each in turn.

As with other herd health activities, a JD control program for a farm needs to be customized to the goals and resources of the farm. While all farms should have a plan for implementing best management practices for reducing fecal-oral transmission due to the challenges associated with the tests to identify infected animals, the intensity and focus of that plan will depend on the goals and resources of the farm. Table 1-2 summarizes recommended management practices for minimizing fecal-oral transmission of JD, and they are adapted from numerous sources (33, 48-52) including the USDA Johne's Disease website (www.aphis.usda.gov/vs/nahps/johnes).

Due to the many possible ways that calves can become exposed to *Mptb*-infected manure, and the long interval between exposure and detectable disease, it is both very difficult and costly to conduct clinical trials to test and quantify the importance of recommendations related to minimizing calf exposure to infected manure. However, it is universally accepted that poor manure management and hygiene around calves will lead to exposure and infection in herds with JD (51,52). Assessing and improving management practices that minimize calf exposure to infected manure will be cost-effective on most

farms, not only for reducing the impacts of JD, but also for reducing the impacts of other fecal-orally transmitted diseases of cattle (e.g. *Escherichia coli, Campylobacter, Salmonella, Rotavirus, Coronavirus,* and *Cryptosporidium*).

The type of testing strategies utilized on a farm will depend not only on the farm goals and resources, but on which of three types a dairy farmer is categorized: 1) those that know they have a JD problem and want to decrease the prevalence; 2) those that have confirmed or suspected that they have JD but do not think it is present at a high prevalence; and 3) those that do not suspect they have JD and want it to remain that way.

Herds that have had at least one cow with clinical signs of JD and a positive fecal culture, likely have an infection prevalence of at least 15% (47). This assumption is based on the premise that the cow was not recently purchased (i.e. was born on the farm), and therefore at the time this cow became infected, there was a high likelihood that others were also infected. Additionally, the clinical cow has likely had heifer calves of her own that are still in the herd and could have infected those calves along with other in-contact heifer calves. This statement, though highly generalized and not accounting for herd size, compels one to consider the true implications of one clinically infected cow. For those farms with a moderate to high prevalence (>30%), regular testing of the herd is likely warranted to identify cows that are shedding and dramatically increasing the environmental load of *Mptb*. On these farms, fecal culturing will identify a substantial number of shedders. However, such farms should also consider using ELISA testing because the number of ELISA false-positives will not outweigh the number of true-positives, producing a good predictive value of a positive test.

The most appropriate testing strategy to use in high prevalence herds depends upon the goals of the producer and the time frame during which they want to accomplish those goals. If the goal is reduction of prevalence to almost zero within a few years, an aggressive approach of annual fecal culture testing of all cows 2 years and older may be warranted. However, if the herd size is large (>300 animals), more strategic measures may be necessary due to limitations of cost, time, and lab-space for fecal culture. For example, ELISA testing the whole herd and then fecal culture testing all ELISA-positives plus all cows with S/P ratios higher than one standard deviation below the cut-off value (cows that are more likely to be fecal culture-positive but ELISA-negative) may be an alternative approach (53). However this latter, less expensive approach will miss cows that are shedding *Mptb* but have not mounted much of an immune response yet. With the improvements in fecal culture techniques during the last few decades, it is unlikely that fecal culture-negative cows are shedding significant numbers of bacteria in their feces. Simulation studies have reported that successful and simultaneous implementation of best management practices and strategic testing and culling leads to the largest and fastest reductions in infection levels (49).

In the past, the Dutch have attempted to decrease the number of clinically diseased animals on farms with high prevalence by use of a vaccination program using a killed vaccine on some farms so that they would not have to rely on imperfect tests to detect the subclinical animals (48). However, their observations with this program are that producers often become less vigilant with other management-related control measures and too reliant on the vaccine. For this reason, if vaccination is considered, it should be restricted to high prevalence farms that have numerous clinical cases of JD.

If there has not been a confirmed clinical case of JD on a farm, and it is suspected, based on herd history, husbandry practises or testing, that the herd has a low to moderate prevalence (<30%), then individual fecal cultures will seem quite costly for the low number of shedding cows detected. Furthermore, using an ELISA with less than optimal specificity will result in interpretation difficulties of test positives. The likelihood of a positive test being a true positive is low in herds with low prevalence, and therefore a confirmatory test, such as a fecal culture, should always be performed. This approach can become quite costly in large herds and can be viewed as unrewarding, especially if the herd truly is negative and all confirmatory tests are negative. One viable strategy would be to perform pooled fecal cultures of the mature cows. The major benefit of pooling is the decreased testing cost, however diluting the sample with too many cows may lead to false-negatives. Additionally, there is need for subsequent confirmation testing of individual cows in positive pools, taking additional time before culling can be implemented. The use of broth media for cultures has reduced the time required for incubation from 16 to 6 weeks, which is one reason why this is becoming a viable option. The ideal number of cows per pool has not been well established, but available research would suggest pools of five would likely be adequate (54,55). This strategy has been shown to identify 87% of positive animals where individual culture found 96% of positive animals (54). However, cows with low level or intermittent shedding could be missed with pooled fecal cultures, and therefore management changes should be implemented as well to minimize transmission between the missed cows and susceptible youngstock.

Another alternative for moderate to low prevalence herds would be to forego testing and focus on controlling the spread of the disease within the herd, focusing on the young

animals. Implementing strict control measures along with a high turnover of cows should lead to a lower within herd prevalence after approximately five years. Considering the performance of the tests available, individual testing may be more difficult and costly than the benefits from such a program. Simply implementing control procedures will decrease the risk associated with a low number of cows spreading the disease within the herd, and eventually lead to a decreased prevalence.

For herds that have never identified a cow with clinical signs of JD on the farm, have had at least some of the herd tested for JD, and all tests produced negative results for JD, the assumption would be that the prevalence of infection on these farms is either zero or very low. However, with the poor sensitivity of current tests for identifying *Mptb*-infected cattle, there is no method to definitively state that a farm is free of JD. With repeated negative tests over many years, producers and their health advisors may assume that they have disease-free status, leading to the temptation to relax within farm management practices and concentrate on keeping the disease out. However, because JD-free status cannot be guaranteed, continued vigilance is needed to minimize calf exposure to manure to avoid unknowingly spreading this insidious disease (52).

For those farms where the data suggest a high likelihood of being JD-free, along with on-farm biosecurity measures, it is likely more important to focus efforts onto keeping the disease out of the herd (56). This could be accomplished by implementing all of the control points previously mentioned in the risk factor section (Table 1-2), and applying methods for keeping manure from cattle from other farms away from the herd, including: avoiding community or shared pasture; and restricting application of manure from other farms on the farm (49-52).

With the currently available tests for JD, the common movement of animals/equipment between herds, the difficulty of completely eliminating fecal-oral exposure of youngstock on a dairy farm, and the long incubation period, it is difficult to eradicate JD from an infected farm.

1.7. National / Regional Control Programs

A number of countries have developed national, government-funded, animal health programs to provide logistical, administrative and funding support to control JD. National, government-funded, animal health programs are typically directed at exotic diseases and to the control of specific diseases of widely recognized economic or public health importance. Johne's Disease has emerged as a disease requiring a national control program due to mounting evidence and concern over production losses, and due to the possible restrictions to international movement of cattle as some countries require testing. In addition, it has been suggested that because DNA from *Mptb* has been found in 69% of patients with Crohn's disease, that *Mptb* may be a factor in the causation of the disease (57), although *Mptb* may just be an opportunistic organism found in the intestines of Crohn's patients. If the relationship between JD and Crohn's Disease were confirmed, government funding of a control program would likely be forthcoming. A review into this potential relationship is beyond the scope of this paper. For further information, the reader is referred to the following website: http://www.crohns.org/.

Recently, in Canada a proposal for a nation-wide Voluntary Johne's Disease Control Program for dairy cattle has been developed by the authors of this review paper in response to a request by the Johne's Disease Consultation Group sub-committee of the Production Limiting Disease Committee. The proposal has received support in principle from the

Canadian Animal Health Consultative Committee, Dairy Farmers of Canada and the Canadian Cattlemen's Association (58). The program builds on the strengths of national JD control programs developed in other countries. The current Alberta voluntary JD control program is similar to the proposed national program and can be easily modified to reflect the proposed national program. The proposed program will be reviewed here, following brief reviews of the most recent editions of other established national control programs in Australia, The Netherlands, and the United States. The proposed Canadian program is described in some detail to demonstrate its strengths and weaknesses for Canadian veterinary practitioners and others.

1.7.1. Australian National Voluntary JD Control Program

Australia was among the first countries to implement a national JD control program, although many countries have a long history in JD education and extension. In 1996, the Australians launched the National Johne's Disease Market Assurance Program for Cattle (59,60). In this program, herds progress through levels of assurance on the basis of annual negative herd tests from Monitored Negative 1 (MN1) to Monitored Negative 3 (MN3), the highest level. The actual method of testing (ELISA, fecal culture, etc.) is not specified but is assessed for validity by the Chief Veterinary Officer (CVO). Annually, the supervising approved veterinarian also uses auditing procedures to monitor critical herd management aspects to control the spread of JD. Herds can opt to stay at a level by carrying out a maintenance test every two years where the entire herd, up to a maximum of 100 animals, is tested. Herds not participating in testing can be classified as a Non-Assessed Herd (NA), which is a herd with no history of JD or where any suspicion of infection has been resolved

to the satisfaction of the CVO. A herd may be classified as a Suspect herd (SU) for numerous reasons including violations of the annual management audit; however, no diagnostic confirmation of positive animals has occurred in these herds. Infected herds (IN) are herds with a confirmed infected animal. Restricted herds (RD) are herds that were previously IN herds, but are currently undertaking an approved test and control program under supervision of the CVO. In addition, RD herds have achieved one or more negative herd tests commencing at least 12 months after the last known infected animal was removed from the herd.

There has been a linear increase in herds participating in Australian JD control program from approximately 180 at the end of 1996, to approximately 1000 herds in 2000 (60), and by December 2003, 1623 herds were participating (61). There are some desirable components of this program which have been included in the proposed Canadian program, including maintenance testing every two years. However, the numerous different categories of herds were felt to be too confusing, and therefore, simplification of the categories was sought.

1.7.2. The Dutch National Voluntary JD Control Program

The original JD control program in The Netherlands began in 1991. It evolved into a pilot program in 1997 based on fecal culture of 125 herds that were tested every six months. After five rounds of testing (24 months), only 58 herds (46%) remained clear of infection (62). The lessons gained from this pilot program were that although these herds had no clinical signs in the last 5 years, more than half were infected. Secondly, fecal culture, regarded as the "gold standard" was not sensitive enough to detect all infected animals.

Finally, producers were disappointed to find out their herds were truly positive and labelled as such. As a result, highly motivated producers felt they were penalized for participating.

The current Dutch JD control program, initiated in 1998, has an extensive program based on management assessment only and an intensive program based on pooled fecal cultures and management assessment. The extensive program was developed for the dairy industry, but has not yet been accepted by the industry as a whole (63). The rules of the management assessment are aimed at reducing the spread of infection to young calves. For example, pooled colostrum must not be used, milk replacer is required, and cows should calve separately in clean calving areas. There are also strict rules governing the purchase of animals and grazing practices, along with contact of animals of different species that may carry *Mptb*.

In the intensive program, there are 10 levels of classification of herd status. The program is categorized to certify herds as free (level 10) or unsuspected (level 6-9) and provides a control program for infected herds. The program employs annual testing of adult cattle. Fecal samples are pooled in batches of five for status advancement and the ELISA is used at various levels individually for maintenance of levels. Positive ELISA results confirmed by positive fecal culture results lead to a decrease of a status level. There is a well-defined program to assist farms that have been identified as infected, which encourages farmers to participate. Additionally, there are funds provided to assist with the high cost of repetitive testing for farmers to re-enter the certification program (62).

From the original 350 infected herds, half are now unsuspected-free herds. There are now 1000 herds in the certification program for unsuspected-free herds and 250 are classified as infected herds (63). Again, there are some specific desirable components of this program

which have been included in the proposed Canadian program, including the use of pooled fecal culturing. However, due to limited funds currently available for the program in Canada, no financial assistance for testing costs will be provided.

1.7.3. United States National Voluntary JD Control Program

In order to address disparities between existing programs among states, and encourage non-participating states to participate, in April 2002, the USDA Veterinary Services section published the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program (64). This program recommended an advisory committee in each State to assist the State veterinarian in establishing and operating a JD program. By the end of 2002, 40 states had established advisory committees for JD with federal representation on each committee (65).

The structure of the program has three parts. Part 1 is education of the producers using any means that is at the discretion of the state advisory committee. Part 2 is an assessment of on-farm risk and herd management plans. Part 3 of the program involves herd testing and classification into four levels. Under normal circumstances 10 months must pass before a herd can advance to the next level. If a herd does not test after 14 months, it reverts to a herd of unknown status or in some states a maximum risk herd. Testing in the initial stage is done on 30 randomly selected animals 36 months of age or older. The test used is specified as a screening test and is determined by the state administrator. At a recent US Animal Health Association-Johne's Committee meeting, a resolution was passed to include environmental sampling as a potential screening test available to state administrators (66). The idea behind this is to decrease the cost of identifying positive herds without loss of herd

sensitivity (67). However, if a herd is found positive, all animals must then be tested with an individual screening test. If an animal is found positive on the screening test, an appeal can be made to have that animal tested with an official Johne's test (either PCR or fecal culture, upon the discretion of the state administrator). If the official test is negative, the herd regains its test-negative status, but the animal that was retested must be submitted for testing at the next assessment if the animal still resides in the herd. If the appeal test is positive, the owner can request another appeal in which the animal must be either necropsied for further testing or undergo surgical biopsy of the ileum and lymph nodes. At the end of the testing and appeals process, if it is found that the animal is positive, the herd is assigned a positive status.

There is a Fast Track option in part 3 of the program that allows a herd to reach level 4 in two years with three tests, which was added at the insistence of the livestock industry. With this option, Level 1 is skipped with a signed declaration that no cows were seen or diagnosed with JD in the last five years (65).

At the end of 2002, approximately 2,675 herds were enrolled in JD control programs with herd management plans and/or risk assessments filed with State programs (65). As of the end of 2003, there were 4722 herds enrolled in JD control programs (68). Approximately 543 herds were enrolled in State-specific herd status programs in 2003, herds that test negative and are considered less likely to have JD than untested herds.

Again, there are some specific desirable components of this program that have been included in the proposed Canadian program, primarily the simple four-level system. However, the fast-track system was not included due to the experience of a Dutch study showing that more than half of herds that have never reported seeing clinical JD, in fact end up being infected. Additionally, due to the performance of ELISAs, particularly in low

prevalence herds, the U.S. program's reliance on ELISA testing is less likely to be applicable to the majority of low prevalence herds in Canada (69) due to low herd level specificity. Herd-level specificity (HSp) is the probability that an uninfected herd yields a negative herd-test result, while herd-level sensitivity (HSe) is the probability that an infected herd yields a positive herd-test result. With 30 cows tested in a herd, HSe will be 66% and HSp will be 49% using the ELISA (assuming test Se and Sp of 45% and 98%, respectively). However, for fecal culture, the HSe will be 66% but the HSp will be 100% (assuming test Se and Sp of 45% and 100%, respectively). In 11 Dutch dairy herds, the reported HSe for fecal culture and pooled fecal-culture were 64 and 73%, respectively (54).

1.7.4. Alberta JD Control Program

In September 2001, Alberta Agriculture Food and Rural Development implemented a Voluntary Johne's Disease Herd Status Program. The testing protocol used in Alberta's program is similar to the American program, but is more specific about what test can be used at each level (70). There are also strong similarities between Alberta's program and the proposed Canadian program, and therefore Alberta's program is not described here. This congruence was done intentionally so that the Alberta program could seamlessly be modified to fit within the Canadian program. However, some additional modifications have been made based on recent scientific information, such as, to not include ELISA testing for level advancement beyond level 1 due to the recent clearer understanding of the inaccuracies of the ELISA.

1.7.5. Proposed Canadian National Voluntary JD Control Program

The proposed Canadian National Voluntary Johne's Disease Control Program contains two major components (58). The first aspect of the program is a Best Management Practices (BMP) Assessment based upon a herd risk analysis of critical control points in the avoidance of transmission of *Mptb*. The questions included in the assessment are a detailed evaluation of a herd's level of management with respect to the recommendations for decreasing the risk of new *Mptb* infections. Table 1-2 summarizes BMPs for dairy herds, and recommendations and a BMP Assessment will be developed in the future that is specific to cow-calf operations. Herds may participate in this aspect of the program without taking part in the second component, the Voluntary Johne's Disease Herd Status Program.

The proposed Herd Status Program will have a total of five levels, zero through four (Figure 1-1). All herds entering the status program begin at level 0. In order to advance to level 1, a herd must have completed a recent BMP assessment and ELISA testing of 30 animals in their second or higher lactation. If all animals are ELISA-negative, the herd then advances to level 1. If some animals are ELISA-positive, the producer may elect to perform fecal cultures on those animals. If all fecal cultures are negative, the herd can advance to level 1. However, if any fecal cultures are positive or the producer does not elect to do fecal culturing following positive ELISA results, the herd remains as Status Level 0.

There is a waiting period of a minimum 10 months before a producer can re-apply for Status Advancement. For a herd to advance from Level 1 to Level 2, a recent BMP assessment must be completed and pooled fecal cultures (currently pools of up to five animals will be accepted) are performed on all cows in their second or higher lactation. If all

pools are negative, the herd advances to the next level. However, if at least one pool is positive, the producer can elect to: 1) do individual cow fecal cultures on animals contributing to the positive pool(s), and cull the infected cow(s) and remain at level one, or 2) allow the cow(s) in the positive pool(s) to remain in the herd and drop to level zero.

A producer that is at level one may opt to follow the Status Maintenance strategy rather than attempt to progress through the Status Advancement strategy. In order to maintain a herd status (i.e. not drop down to the next lower level), a producer must have a BMP assessment performed and have 30 cows in their second lactation or higher ELISA tested once during the two-year period subsequent to their previous test and BMP assessment. If all cows are negative, the herd maintains its status. If any cows are ELISA-positive, the producer can fecal culture those cows. If the fecal cultures are negative, the herd maintains its status. If a fecal culture is positive, the cow(s) must be culled in order to maintain status, otherwise the herd drops to level zero.

Advancement from Levels 2 to 3 and 3 to 4 follow the same protocol of a recent BMP assessment and pooled fecal culture. However, even if all positive cows are culled, the herd drops to Status Level 1. If the individual cows are not tested and positives culled, the herd drops to level zero.

For Status Maintenance at levels 2, 3 and 4, the same process applies, as described above for maintenance of level 1. The difference for maintaining these three levels versus level 1 is that if the culture is positive and the producer culls the infected cow(s), the herd still drops to level 1. If the producer does not do a fecal culture confirmation, or does not cull the test-positive cow, the herd is assumed to be infected and drops to level zero.

One strength of the proposed Canadian control program will be its ability to aid in the future research of JD. The program will provide data to monitor the proportion of enrolled herds with the disease, and the impacts of implementation of control measures in infected herds, enabling modifications as needed. It will also provide some indication on the prevalence of the disease in various regions of the country, which will be a vital aspect in understanding the epidemiology of the disease. The data will also allow on-going evaluation of the JD program and identification of strengths and weaknesses of the program and recommend modifications for maximum cost-benefit.

One weakness of the proposed program is that there are currently no funding mechanisms in place to subsidize testing and indemnification of fecal culture-positive cattle. Also, there is a need for market-based incentives to engage producers (i.e. higher prices for heifers from JD-negative certified herds) to participate in the program. Increased involvement of the producers and their governing organizations is going to be critical to any control program success.

The focus of this thesis is to better define the diagnostic parameters of ELISAs and pooled fecal culture for Johne's disease using the best available gold standards available. To achieve this, tissue culture of cows sampled at slaughter will be the standard for sensitivity evaluation. Testing the feces of cows in negative herds with long standing participation in control programs would allow for accurate estimation of specificity for ELISAs. The impact this information may have on control programs will also be discussed.

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Table 1-1. Affects of paratuberculosis on health, production, and culling.

Parameter	Diagnosis criteria	Affect of paratuberculosis	Reference
Milk Production	Tissue positive	16% decrease in last lactation compared to two years prior and 6% decrease compared to one year prior	1
	Fecal positive	590 kg and 1270 kg less milk in third and fourth lactations, respectively	2
	ELISA positive	4% (376 kg) decrease in 305ME production	7
		551 kg decrease in 305ME production	9
		573 and 1273 kg less milk in first and fifth lactations, respectively	8
Risk of Culling	Fecal positive	Greater culling rate cost infected herd US$ 75 per cow per year	15
	ELISA positive	Odds Ratio= 2.34 for risk of culling	14
Reduced Slaughter Value	Clinical JD	Reduced slaughter value of 20 to 30%	1
	Fecal positive	59 kg less weight at slaughter, for a loss of US$ 48 per head	5
	ELISA Positive	Estimated losses of CAN$ 1330 per infected 50 cow herd	17
Fertility	Tissue Positive	Higher fertility cull rate overall	6
	Fecal positive	No difference in fertility	21
	ELISA positive	49 day increase in days open in first lactation heifers	20
		No difference in fertility	21
Mastitis	Fecal positive	Lower Somatic Cell Count	21
		Cull rate for mastitis was 22.6% versus 3.6% in culture-negative cows	6
	ELISA positive	Higher somatic cell count at the cow level and herd level	10
		Odds Ratio = 2.90 for risk of culling due to mastitis	14

Table 1-2. Recommendations for decreasing the risk of new infections of *Mycobacterium avium* subsp. *paratuberculosis* in a dairy operation.

Protect young stock from feces of mature cattle and feces-contaminated feed and water	Reduce the number of infected animals that may be shedding bacteria
a. Clean and disinfect maternity and calf pens after each use b. Calve cows in clean, dry, dedicated maternity pens c. Immediate removal of calves from maternity pen (while calf still wet) d. Collect colostrum from cleaned udders e. After colostrum feeding, use pasteurized milk or milk replacer f. Raise calves separate from the adult herd for first year of life g. Do not allow shared feed/water between adult cows and young stock h. Use separate equipment for handling feed and manure i. Feed-bunk and waterers should have no risk of fecal contamination j. Do not spread manure on grazing or hay land for young-stock	a. Immediate cull of animals with clinical signs of JD. b. Consider testing adult cows with ELISA or fecal culture; positive ELISA should be confirmed with fecal culture in clinically normal cows c. Cull fecal culture-positive cows; they are active shedders and are increasing the environmental challenge on the farm d. Maintain a closed herd or purchase animals only from source farms that have implemented similar or better control programs than purchasing farm (management practices and testing)

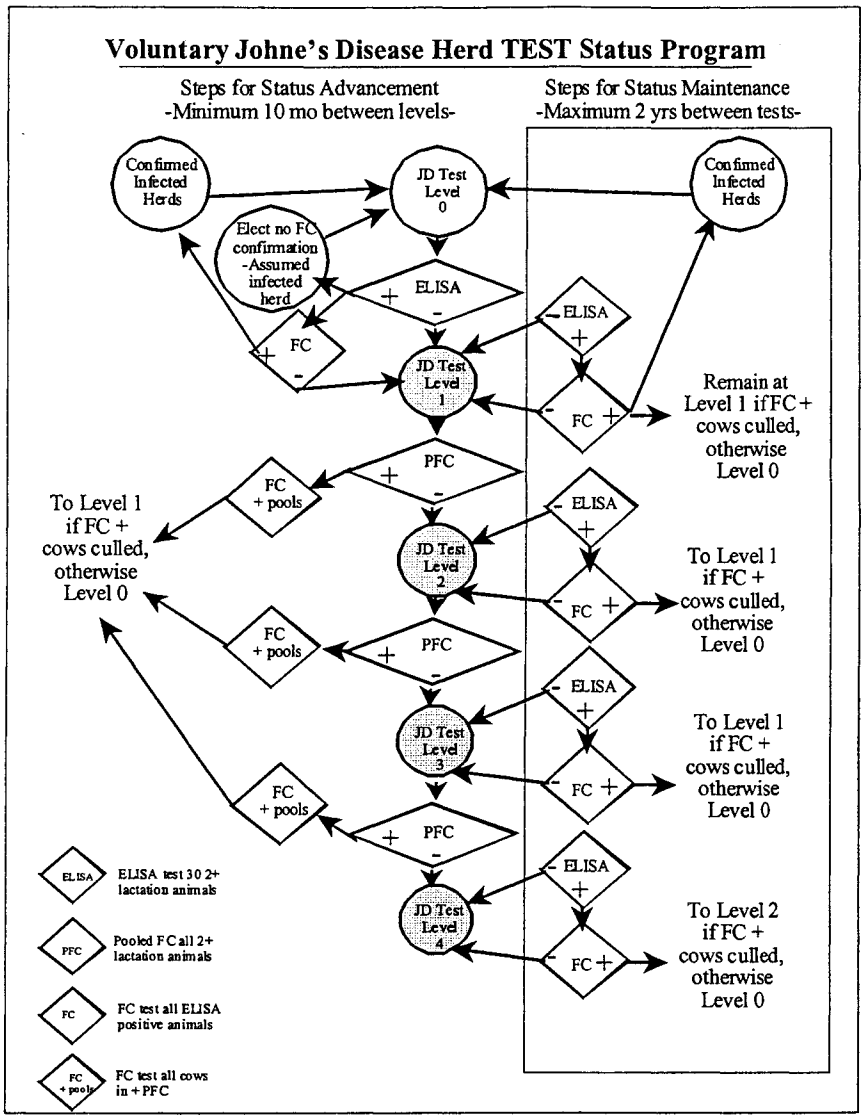

Figure 1-1. Schematic diagram of proposed Canadian Voluntary Johne's Disease Control Program for dairy cattle.

CHAPTER 2 . COMPARISON OF ELISAS FOR *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*: A NON-ABSORBED EUROPEAN ELISA AND AN ABSORBED NORTH AMERICAN ELISA

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2.1. Abstract

The commonly used ELISAs to detect subclinical disease have relatively low sensitivity. The inclusion of an absorption phase, while improving specificity, potentially decreases sensitivity. Sera and feces of 383 adult dairy cows in eight dairy herds were used to compare the test characteristics of an absorbed and a non-absorbed indirect ELISA for the detection of Johne's disease in cattle. The potential advantage for the non-absorbed ELISA is that it may be less specific and more sensitive. Two herds certified free of Johne's disease were used to compare the specificity of the ELISAs. The other six herds used to compare sensitivity were either infected with *Mycobacterium avium* subsp. *paratuberculosis* or had a previous unknown status. Using fecal culture as a gold standard, the diagnostic specificity for the absorbed and non-absorbed ELISAs were 98.4% and 87.9%, respectively. The diagnostic sensitivity was 72.4% and 65.5% for the absorbed and the non-absorbed ELISA, respectively. A comparison using a positive fecal DNA probe as the gold standard resulted in both ELISAs having a sensitivity of 60.3%. Agreement between the two ELISAs was only moderate, with a kappa statistic of 0.58. The non-absorbed ELISAs did not have a higher sensitivity and did have a lower specificity than the absorbed ELISA. Therefore, in this population of animals, there was no advantage gained from using the non-absorbed ELISA.

2.2. Introduction

Johne's Disease (JD) is a disease of ruminants that causes a chronic granulomatous enteritis. The causative organism is *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). This disease has become of great concern to North American cattle owners in recent years due to evidence of economic losses associated with the disease and possible trade barriers. Another emerging issue is the association between *Mptb* and Crohn's Disease in humans. The DNA from *Mptb* has been found in 69% of Crohn's patients suggesting that it may be a factor in the causation of the disease. Alternatively, *Mptb* may just be an opportunistic organism found in the intestines of Crohn's sufferers (1).

Efforts are being made to manage JD through herd-level and national control programs. With other diseases, it is common to utilize testing procedures to identify infected animals and limit the spread of disease. Similar efforts have been made to control JD, but have had limited success, partly due to the performance of diagnostic tests available.

Test characteristics for diagnostic procedures include sensitivity and specificity and, in the target population, the subsequent negative and positive predictive values (NPP and PPV). Current diagnostic tests for JD have many pitfalls. Fecal culture techniques yield results with 100% specificity (and subsequently 100% PPV), but with traditional culture methods, livestock owners must wait up to three months for results (2). Therefore, more rapid test results, which are subsequently cheaper, are more desirable. As a whole, immunodiagnostics have become more favourable due to the degree of speed in which results can be generated. However, these tests have low sensitivity in subclinically affected animals.

Depending on the stage of disease, the sensitivity of ELISAs have been reported to range from 15 to 88% (3, 4, 5) with a specificity as high as 98.9% (6)

Initially, serologic testing for JD suffered from a lack of diagnostic accuracy due to a low diagnostic specificity, which resulted in a high proportion of test positive animals having false-positive results (7). Inclusion of an absorption step, where serum was pre-absorbed with *Mycobacterium phlei* to remove potential cross-reacting antibodies, improved specificity (8) and was validated using Western blotting (9). The absorption procedure was then validated by using two runs of an identical ELISA; only one had the addition of the absorption phase (10). The results demonstrated an increased specificity in the absorbed ELISA at various cut-points.

Current commercial ELISAs for *Mptb* have been developed to include an absorption phase, thereby making the test more specific. However, there is a potential decrease in sensitivity, due to absorption of antibodies present for antigens of *Mptb*. Therefore, the potential benefit of removing the absorption phase would be to increase test sensitivity. As part of many JD control programs, it is recommended that cattle first be tested with an ELISA, followed by testing ELISA-positive cattle with a confirmatory fecal test. In a program where all ELISA-positive cattle are confirmed with fecal culture, using an ELISA screening test with a higher sensitivity would be beneficial. In a study performed in Germany with a non-absorbed ELISA[a] modified for milk, the sensitivity was reported to be 60.9% with a specificity of 94.6% (11). However, there were no comparisons with absorbed ELISAs in the same population of animals.

The objective of the present study was to compare the test characteristics of the ELISA developed in Europe without an absorption phase with the absorbed ELISA commonly used in North America, using fecal culture and PCR test results on North American cattle as gold standard tests.

2.3. Material and methods

2.3.1. Herds and Cows

In this study, sera and feces were used from 383 adult dairy cows from eight Wisconsin dairy herds (Table 2-1). Two of the herds (Herd A and B), which contributed 116 animals to the study, were certified free of JD as part of the State of Wisconsin Voluntary Johne's Disease Control Program. Prior to joining the program, both herds had no history of clinical JD or any *Mptb* fecal culture-positive cows. The other six herds either had unknown *Mptb* status or infected herd status and their contribution is listed in Table 2-1. Four of the remaining herds (Herds D, E, H and G) had submitted serum samples of the whole adult herd previously for absorbed ELISA evaluation. These herds then re-submitted serum samples from test-positive cows along with a fecal sample for re-testing and confirmation, and the re-test data were used for this study.

2.3.2. Laboratory Analysis

Fecal samples were collected at the same time the serum was collected. Serum samples were split and *Mptb* sero-status was determined using the absorbed ELISA[b] at the

Wisconsin Veterinary Diagnostic Laboratory in Madison, Wisconsin, and the non-absorbed ELISA[a] at the Svanova research facility in the Uppsala Science Park, Sweden. These two ELISAs are both indirect ELISAs but they use different antigens; the absorbed ELISA uses a protoplasmic antigen and the non-absorbed ELISA uses a purified extract of Lipoarabinomannan (LAM). Fecal cultures were performed on Herrold's Egg Yolk media at the Wisconsin Veterinary Diagnostic Laboratory using the method described previously (12). For 196 samples for which there was still feces available (Herds C, D, F, and H), a PCR assay was performed using a DNA Probe as described by the manufacturers instructions[c] (13).

2.3.3. Statistical analyses

Analysis of data was done using Stata 8.0™[d]. Specificity and sensitivity calculations along with exact confidence intervals were generated through the "diagt" command. Agreement of the two ELISA tests was tested using a kappa statistic using the "kapgof" command. Estimations of PPV based on various levels of apparent prevalence were calculated using Excel[e].

2.4. Results

Fecal cultures for *Mptb* on the two JD-free herds were all negative (Table 2-1). Of the 267 cattle tested in the other six herds, 87 (32.6%) yielded a positive culture for *Mptb*. The PCR analyses were performed on 196 fecal samples, and of those 63 (32.1%) were positive for *Mptb*. When these results were combined in a parallel manner, 104 cows

(39.8%) were positive on either culture or PCR, out of 267 tested in the six JD infected herds. There were 17 cows positive only on PCR, 46 cows positive on both, and 41 cows positive only on culture (Table 2-1).

In the JD-free herds (Herds A and B), the absorbed ELISA identified 110 of the 116 cows tested as negative, resulting in a specificity of 94.8% (95% CI 89.1, 98.1), while the non-absorbed ELISA identified 102 cows as negative resulting in a specificity of 87.9% (95% CI 80.6, 93.2).

When calculating the sensitivity of the ELISAs, the results varied somewhat depending on the standard of comparison, culture versus PCR. Using the 104 cows that were positive on either PCR or culture as truly disease-positive, the absorbed ELISA identified 65 cows as positive for a sensitivity of 62.5% (95% CI 52.5, 71.8), and the non-absorbed ELISA identified 59 cows for a sensitivity of 56.7% (95% CI 46.7, 66.4). The sensitivity results for the ELISAs compared to the 87 fecal culture-positive cows were 72.4% (95% CI 63.0, 81.8) for the absorbed ELISA, and 65.5% (95% CI 55.5, 75.5) for the non-absorbed ELISA. The absorbed and non-absorbed ELISA had the same sensitivity when compared to PCR, 60.3% (95% CI 47.2, 72.4) by identifying 38 of 63 positive cows (Table 2-1).

In Table 2-2, a summary is given of all relevant kappa statistics. The kappa statistic comparing the absorbed ELISA and the non-absorbed ELISA was 0.58 (95% CI 0.09, 0.66), meaning that the two tests had a 58% agreement on the status of the samples beyond agreement by chance alone. The agreement determined between the ELISAs and fecal culture was lower, with the kappa value for the non-absorbed ELISA being 0.34 (95% CI – 0.03, 0.42) and the absorbed ELISA of 0.44 (95% CI –0.01, 0.52). The agreement was even

lower when cattle were classified using results from culture or PCR (testing parallel) with a kappa value for the non-absorbed ELISA of 0.28 (95% CI –0.04, 0.37) and the absorbed ELISA of 0.38 (95% CI –0.02, 0.47). Fecal culture and PCR had moderate agreement with a kappa of 0.54 (95% CI 0.03, 0.66).

Using the specificity and sensitivity estimates derived from using parallel testing (fecal or PCR-positive), values were calculated for PPV of the two ELISAs at various levels of apparent prevalence. The PPV was then plotted against apparent prevalence to demonstrate the effect that the lower specificity of the non-absorbed ELISA would have on PPV. At low apparent prevalence, the non-absorbed ELISA had a lower PPV than the absorbed ELISA, and did not meet the PPV of the absorbed ELISA until an apparent prevalence of 63% (Figure 2-1).

2.5. Discussion

In order to implement a reliable eradication program that has a testing component, a test is needed with high sensitivity to ensure identification of infected animals at all stages of disease. In the absence of a confirmatory test, a high specificity is also needed to achieve a high PPV at low prevalence to ensure that cattle identified are truly positive. In control programs, it is also desirable for tests to be performed quickly and inexpensively. Therefore, it is imperative to determine if there is an advantage to using a non-absorbed ELISA such as the one tested here, to an absorbed ELISA which is commonly used in North America.

Two major components make the two study ELISAs different. One ELISA uses an absorption step, whereas the other does not, and they use slightly different antigens.

Attributing strengths and weaknesses between the ELISA to one rather than the other of these differences is difficult. In a recent study an ELISA using a polysaccharide antigen in the 32-42 kDa range consistent with LAM provided enhanced sensitivity in comparison to an ELISA based on protein antigens (14). In that case, both ELISAs contained an absorption phase. It has been established by previous research that ELISAs without an absorption phase were associated with decreased specificity; therefore absorbed ELISAs became more common (7). Although it is difficult based on this current data to determine which component affects the performance, it is important to identify if one ELISA may have an advantage over the other.

When evaluating diagnostic accuracy on any test, it is important to include samples from individuals that represent the entire spectrum of disease. In reference to JD, it is difficult to ensure that animals in all stages of the disease are represented based on the limited ability to accurately classify infected animals. The use of animals from farms that have had a series of negative test results over a period of time is the usual source of negative animals to assess specificity. There is the potential for inclusion of animals infected with *Mptb* in this test population, however the use of animals with repeated negative culture results (19) or animals that have had a long-standing history in a negative herd certification program are commonly used as negative gold standards (15).

To analyze specificity in the case of this dataset, 116 animals from two JD-free herds were used. The data from these herds provided a good estimate of the test specificity in this particular population. The absorbed ELISA identified more animals as negative in a negative herd with a specificity of 94.8%, while the non-absorbed ELISA had a specificity of 87.9%.

This difference in diagnostic specificity can be accounted for by the exclusion of an absorptive stage in the non-absorbed ELISA. The absorbed ELISA has an absorption stage, which absorbs antibodies that share epitopes common to all mycobacteria by using *M. phlei*, as demonstrated by Western Blot analysis (16). The non-absorbed kit does not use *M. phlei* antigens to absorb non-specific antibodies, therefore it would be expected that it should have a lower diagnostic specificity. However, it would also be expected that with a decrease in specificity there would be a gain in sensitivity, or at least a significant increase in the number of positive results, which was not demonstrated with these data.

The two ELISAs evaluated had similar sensitivities ($p>0.05$, overlapping 95% CI) when based on fecal culture and PCR alone, or parallel testing of both fecal culture and fecal PCR. When PCR was the sole comparison standard, the two ELISAs had the exact same sensitivity. These estimates of sensitivity were slightly higher than those in previous studies (15, 17), but would be similar to estimates of sensitivity found in more recent studies for animals that were more likely to be fecal shedders (5, 18). Furthermore, with 32.6% of tested cows being culture positive, the six test herds would have to be categorized as high prevalence herds. High prevalence herds are known to produce higher estimates of sensitivity for ELISAs due to the likelihood of more cows in those herds being in more advanced stages of infection compared to low prevalence herds (5).

Inclusion of animals that are only fecal culture or PCR-positive for sensitivity estimation of a serological test is not ideal because it is possible for animals to be serologically positive but not shedding detectable numbers of the organism in a fecal sample (19). It would be better to compare a test against individuals in various stages of JD, not

simply fecal shedders. Unfortunately, due to the nature of this disease and its long incubation phase, that would be difficult to accomplish. When one serologic test appears to identify more animals that are fecal culture-positive than another test, it does not guarantee that it is finding more cattle that have *Mptb*, but rather it is better at identifying cows that have detectable organisms in the feces. In this report, the absorbed ELISA found 65 of 104 fecal culture or PCR-positive animals for a sensitivity of 62.5 % whereas the non-absorbed ELISA found 59 for a sensitivity of 56.7%. However, there was no statistically significant difference in sensitivity of the two tests, and the non-absorbed ELISA did identify more cattle as positive. Some of the animals classified as test positive by the non-absorbed ELISA may in fact be in the earlier stages of disease, not yet fecal shedding, or simply false positives. It was impossible to differentiate between these two possibilities.

It would be advantageous to identify animals that may have *Mptb* antibodies but are not fecal shedding. From preliminary results of a slaughterhouse study (20), it appears that animals can be lymph node culture - positive, yet are negative on ileum culture and have no evidence of infection based on histology, indicating these animals are in the early stages of disease. If the organisms were in the lymph system at a level detectable by culture methods, antigens may be present for the immune system to develop antibodies. Therefore, these animals may be serologically positive but fecal culture-negative. It would be advantageous to identify these animals before they become fecal shedders and remove them so that there is less shedding of the bacteria ito the environment, potentially infecting other cattle.

Another interesting finding was how the tests performed when compared in various ways. Overall, the only combinations that showed moderate agreement were the two

ELISAs compared to each other and the fecal culture/PCR comparison. In this report, the two ELISAs had a kappa value of 0.58, which is a moderate level of agreement. The absorbed ELISA identified 127 samples as positive and the non-absorbed ELISA identified 133. They agreed on 94 samples as being positive and disagreed on 72 samples. There were four samples that were fecal-positive that only the non-absorbed ELISA detected and 10 fecal-positive samples only the absorbed ELISA detected. Neither ELISA performed well in comparison to fecal culture or PCR, either interpreted in series or parallel, with kappa's which were not significantly greater than zero. This means that the amount of agreement between the ELISAs and PCR was very close to what one would predict based on just chance alone. The culture results and PCR data had moderate agreement with an observed agreement of 79.6% and a kappa of 0.54.

Since two JD free herds were used to determine specificity, the ELISA specificity results are accurate. The non-absorbed ELISA had a lower specificity that the absorbed ELISA. This has implications for the utilization of the tests in the field and their PPV. The value of a positive non-absorbed ELISA result on an individual animal is diminished, especially in lower prevalence herds. In Figure 2-1, this point is illustrated. At low to moderate apparent prevalence, the non-absorbed ELISA has a lower PPV than the absorbed ELISA, and in fact it is not until extremely high apparent prevalence that the PPVs becomes equal. The differences at low prevalences are not large, however if testing a low prevalence herd, you may want to minimize the risk of false positive ELISA results. The fact that the non-absorbed ELISA lacks specificity means that it will result more positive samples, but there is a high likelihood that many are false positives.

Although the non-absorbed ELISA identified more animals as test-positive animals, it had a lower sensitivity than the absorbed ELISA when fecal culture or PCR positive results were used as the gold-standard for comparison. The non-absorbed ELISA also had lower specificity than the absorbed ELISA, based on the presumed negative herds. Based upon these fecal results, the higher numbers of positive animals identified by the non-absorbed ELISA would appear to be animals that are erroneously called positive. However, better gold-standard data for *Mptb*-positive animals is needed for accurate comparison of sensitivities and to more accurately assess the potential differences due to using different antigens and absorption phases. It can be concluded from this study that the non-absorbed ELISA had a lower specificity compared to the absorbed ELISA.

2.6. Acknowledgements

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2.7. Sources and manufacturers

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b. Herdchek® ELISA, IDEXX Laboratories, Westbrook, ME.

c. HerdChek® DNA Probe, IDEXX Laboratories, Westbrook, ME.

d. Stata Corporation, College Station, Texas

e. Excel, Microsoft Corporation, Redmond, WA.

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Table 2-1. Distribution of animals for each herd along with *Mycobacterium avium* subsp. *paratuberculosis* status and ELISA test results ([a] = fecal culture and PCR-positive, percentages based number of cows tested, [b]= ELISA-positive based on whole herd)

Farm	No. of test cows	Herd size	No. *Mptb* positive cows (%)[a]	No. *Mptb* PCR positive cows (%)[a]	No. *Mptb* fecal culture positive cows (%)[a]	Absorbed ELISA positive (%)[b]	Non-absorbed ELISA positive (%) [b]
A	67	67	0 (0)	- -	- -	1 (1.5)	6 (9.0)
B	49	49	0 (0)	- -	- -	5 (10.2)	8 (16.3)
C	73	73	46 (63.0)	38 (52.0)	34(46.5)	23 (31.5)	27 (37.0)
D	28	111	12 (42.8)	8 (28.6)	12(42.5)	17 (15.3)	16 (14.4)
E	20	218	1 (0.05)	- -	1(5.0)	13 (6.0)	11 (5.0)
F	78	78	18 (23.1)	10 (12.8)	13(16.7)	13 (16.7)	24 (30.1)
G	51	282	17 (33.0)	- -	17(33.3)	42 (14.9)	26 (9.2)
H	17	66	10 (58.2)	7 (10.6)	10(58.8)	13 (19.6)	15 (22.7)
Total	383	944	104 (11.0)	63 (19.2)	87(22.7)	127 (12.8)	133 (13.7)

Table 2-2. Comparison of Kappa values and agreement for various combinations of diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis*.

	Observed Agreement	95% CI for Agreement	Kappa	95% CI for Kappa
Non-absorbed ELISA vs Absorbed ELISA	81.2%	76.9, 85.0	0.58	0.09, 0.66
Non-absorbed ELISA vs PCR	64.8%	57.7, 71.5	0.25	0.06, 0.38
Non-absorbed ELISA vs Culture	72.3%	67.6, 76.8	0.34	0.00, 0.42
Absorbed ELISA vs PCR	73.0%	66.2, 79.0	0.39	0.03, 0.52
Absorbed ELISA vs Culture	77.0%	72.5, 81.1	0.44	0.00, 0.52
PCR vs Culture	79.6%	73.3, 85.0	0.54	0.03, 0.66
Non-absorbed ELISA vs PCR+Culture	68.9%	64.0, 73.5	0.28	0.04, 0.37
Absorbed ELISA vs PCR+Culture	73.6%	68.9, 78.0	0.38	0.00, 0.47

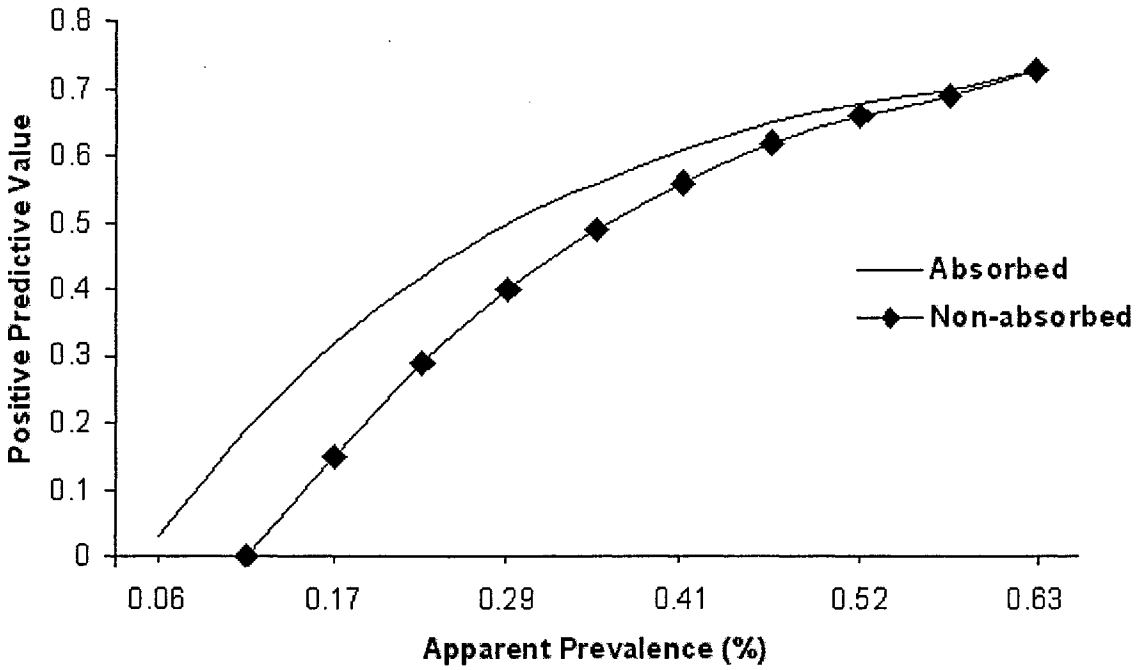

Figure 2-1. Estimated positive predictive values of two ELISAs with increasing apparent herd prevalence for *Mycobacterium avium* subsp. *paratuberculosis*, based on calculated sensitivity and specificity.

CHAPTER 3 . COW-LEVEL PREVALENCE OF PARATUBERCULOSIS IN CULLED DAIRY COWS IN ATLANTIC CANADA AND MAINE

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3.1. Abstract

The prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) in culled dairy cattle in Eastern Canada and Maine was determined to be 16.1% (95% confidence interval 13.8 to 18.3%) based on a systematic random sample of abattoir cattle. Mesenteric lymph nodes and ileum from 984 cows were examined by histologic and bacteriologic methods. Histological testing was far less sensitive than bacteriologic methods for detecting infected cattle. A seasonal pattern of positive cows was also detected, with the highest proportion of cows being *Mptb*-positive in June (42.5%). Overall, body condition score was not associated with prevalence of *Mptb* isolation.

3.2. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Mptb*) is the bacterium that causes Johne's disease, a chronic wasting disease of ruminants associated with infection of the animal's gastrointestinal tract. The effect this infection has on health and productivity has been studied and one estimate of cost for Johne's positive herds that had 10% of cull cows with clinical signs was over US$200 per cow (1). In another study within the region this study was performed, using serologic data and partial-budget modeling the annual cost for an average, infected, 50 cow herd was CDN$2472 (2). Although strategies exist in many places to control this disease, it still continues to affect the health and productivity of cattle worldwide.

Before a control and prevention program for Johne's diseases can be designed, both herd and cow level prevalence of *Mptb*-infection should be known. Several regional and

national surveys have been conducted to estimate the prevalence of *Mptb*-infection. However, different methods were used to estimate the prevalence of *Mptb*-infection including: 1) serological testing; 2) bacteriological culture of fecal samples; 3) bacteriological culture of tissue samples from slaughterhouses; and 4) polymerase chain reaction (PCR) on tissue, blood or bulk milk. Moreover, sampling strategies differed among the studies. Thus, it is difficult to directly compare the results of different studies. During the early nineties, the herd-level prevalence of Johne's disease in countries with a significant dairy industry had been calculated to be approximately 10% (3). However, recently, in Belgium (4), The Netherlands (5), Denmark (6), Canada (7), and USA (8) herd-level prevalence has been estimated to be 30-50%, based on an observed increased frequency of clinical disease, occurrence of Johne's disease in areas where the disease was formerly unknown, and serological prevalence studies. Due to the relatively low sensitivity of serological tests, they are more accurate at determining herd-level rather than cow-level prevalence. Serologic studies performed to determine cow-level prevalence have indicated a prevalence between 1.2 and 8.8% (4; 5; 9).

In comparing different studies involving prevalence of disease, part of the difficulties arise from variation in the definition of what constitutes a positive case. In some cases, positive serologic result is the case definition and others, such as this study, a positive result is presence of bacteria. A previous study done in Atlantic Canada using serologic testing of dairy cows reported a cow level prevalence of 2.6%, with an estimated true herd level prevalence of 30% (7). This study used an enzyme-linked immunosorbent assay (ELISA) for classification of cow status on a stratified (cows, farms) random sample. The ELISA test

was assumed to have a sensitivity of 45.5% (10) but more recent work has estimated the sensitivity to be closer to 15.4% in low fecal shedders (1-10 colony forming units (CFU)) to 88.1% in high fecal shedders (>100 CFU) (11).

Most estimates of ELISA performance are based upon culture methods as the comparison gold standard (most often being fecal culture). The relationship between fecal culture sensitivity and ELISA sensitivity depends highly upon the stage of the disease in an individual animal. In the earlier stages of disease, the lower the sensitivity for both methods (12). However, tissue culture in comparison to repeated fecal culture reveals that animals that are fecal culture negative may actually be tissue culture positive, therefore resulting in a higher sensitivity for tissue culture (13).

Body condition scores (BCS) of cattle are believed to be linked to the prevalence of *Mptb* because clinical Johne's disease results in an emaciated, debilitated cow with diarrhea. This has been demonstrated in Australian sheep. Animals with low BCS (<2 out of five) had a *Mptb*-prevalence of 51.5% compared to the sheep with a higher BCS (2-4 out of five) where the prevalence was only 19.3% (14). Additionally, BCS in sheep has an affect on serologic test sensitivity. In evaluating test sensitivity in sheep, animals with lower BCS ($\leq$2) resulted in test sensitivity of 53% and sheep with a body condition $\geq$3 resulted in a calculated test sensitivity of 16% (15). Therefore, interpreting BCS and prevalence becomes difficult due to the effects of changes in sensitivity. The apparent link of BCS and prevalence could be due to its effects on sensitivity or an association between BCS and stage of infection. In either case, an association between BCS and infections could be used to select cows for

laboratory diagnosis of *Mptb*. No data on the association between *Mptb*-infection and BCS in the bovine are currently available.

The goals of this study were: 1) to estimate slaughtered cow-level prevalence of *Mptb* in Atlantic Canada and the state of Maine (USA), and 2) to study the association between BCS and prevalence of infection with *Mptb*. Testing for *Mptb* usually consists of serology (ELISA), culture (fecal or tissue), or use of DNA detection methods such as polymerase chain reaction techniques (PCR). In this study, culture of infected tissues was used to determine infection with *Mptb* because its sensitivity may be greater than that of fecal culture, since some repeatedly fecal culture negative animals will be tissue positive (13). However, this may be influenced by stage of disease. Because culture of tissue is impractical to perform on a large number of live animals, *Mptb*-prevalence was estimated in slaughterhouse cows.

3.3. Materials and Methods

3.3.1. Sample Population

For the period from January to October 2001, excluding April and May in which a labor dispute at the slaughterhouse halted collection of samples, a weekly visit was made to a local slaughterhouse for the dairy cow-processing day. The slaughterhouse was located in Moncton, New Brunswick, and slaughtered cows were primarily from the four Atlantic Canadian Provinces: New Brunswick, Nova Scotia, Prince Edward Island, and Newfoundland. Additionally, this slaughterhouse occasionally processed animals purchased

from buyers located in the state of Maine (USA). These animals were identified as American cattle for accurate data analysis.

The sample size required was estimated using seroprevalence data from a previous study done in the area, which resulted in a cow level apparent prevalence of 2.6% (7), with a 95% level of confidence and a desired accuracy of the prevalence to be within 1% of the true value. This yielded a number of 973 cows needed for the study (Win Episcope 2.0). Animals that were not of dairy breeds were not included in the study. In addition, animals that did not appear to have udder development consistent with previous lactation were excluded, to ensure that cows were all at least 20 months old. Sampling was conducted using a systematic random sample, in which every third slaughtered cow was selected. Slaughterhouse staff were the only individuals who had any control over the order and grouping of cows that entered the production line. Using this methodology, 984 cows were sampled over the 10-month period. Holstein-Friesian was the predominant breed, making up 961 of the 984 cows.

3.3.2. Sample Collection

Three people were involved with sample collection. As animals entered the kill line, the first person recorded the cow's body condition score (BCS) on a five-point scale with quarter point divisions (16). The same individual recorded breed and BCS on every visit to the slaughterhouse in an attempt to avoid inter-person variation between visits. For consistency, all assessments of body condition were performed immediately after the cow

was stunned and hanged, because this was often the first time the scorer would see the animal.

Upon exsanguination, a free catch blood sample of approximately 80 ml was obtained. The animal was then identified with numbered colored tags to track the carcass to the evisceration area. At the evisceration table, a second person identified the gastrointestinal tract of each study animal with a separate tag containing a unique numeric identifier. At a subsequent station, a third person collected at least two lymph nodes in the mesenteric chain in the region of the ileum, with focused efforts to retrieve the lymph node in the ileo-cecal region, similar to methods described by Benedictus and Bosma (17). Additionally, a 5 to 10 cm segment of terminal ileum was removed approximately 25 cm proximal to the ileo-cecal junction. Samples were placed in individual containers labeled with the numeric identifier for each animal and transported fresh in a cooler with ice packs to the Atlantic Veterinary College.

After returning from the slaughterhouse, samples were processed for storage within 8 hours of collection. From each sample of ileum and lymph node, a section was preserved in 10% neutral buffered formalin and the remainder was frozen at –80°C. Physical characteristics of samples were recorded, indicating whether or not there was gross thickening of the ileum, loose feces, or enlarged lymph nodes.

3.3.3. Histological Preparation

After formalin fixation, samples were trimmed and embedded in paraffin wax. They were subsequently sectioned and Ziehl-Neelsen staining was performed (18). All

histopathological examinations were performed by a board-certified pathologist (American College of Veterinary Pathology) at the Atlantic Veterinary College Diagnostic Services (Charlottetown, PEI, Canada) for lesions similar to those described by Buergelt et al. (19).

3.3.4. Culture Technique

For each cow, the collected lymph nodes were pooled together to comprise one culture sample, and the ileum was identically processed for a second cultured sample. Tissue sample processing for culture was done using a protocol developed by researchers in Pennsylvania (20). Briefly, samples were thawed and a 2 g piece of sample was weighed out on an individual disposable weight boat to decrease the risk of cross-contamination. Each sample was then placed in a Tekmar bag along with 25 ml of 0.75% hexadecylpyridinium chloride (HPC) solution for decontamination. The sample was placed in a stomacher for a minimum of 1 minute to pulverize the sample. Subsequently, each sample was left undisturbed for 30 minutes to allow for fat and large pieces of tissue to separate out while being bathed in the HPC decontamination solution. A 10-ml sample of the pulverized fluid was transferred into another sterile tube containing a further 10 ml of HPC solution, used for the second decontamination stage in the procedure. After a minimum of 3 hr in the second decontamination stage, samples were centrifuged at 900 x g for 30 min. The supernatant was discarded and the pellet was re-suspended with a combined half strength brain-heart-infusion broth that contained 0.1% Nalidixic acid, 0.1% Vancomycin, and 0.05% Amphotericin B. This mixture was vortexed and placed into sterile cryogenic tubes to be incubated for 12-14 hr at 37°C to allow for *Mptb* growth and to allow an opportunity for the anti-microbials to

more effectively decrease levels of background contaminants. After the incubation phase, the tubes were slowly cooled and then re-frozen at –80°C.

The frozen, processed culture mixture was sent to the Wisconsin Veterinary Diagnostic Laboratory for culturing. At the Wisconsin lab, samples were thawed, and 1 ml from each of the 1968 tubes was inoculated into a TREK ESP® Culture System II broth solution media bottle (TREK Diagnostic Systems, Cleveland, Ohio, USA), supplemented with egg yolk, antibiotics (0.1% Nalidixic acid, 0.1% Vancomycin, and 0.05% Amphotericin B) and Mycobactin J (Allied Monitor Inc., Fayette, Missouri, USA). The inoculated media were incubated for six weeks at 37°C. After six weeks of incubation, each bottle was examined for acid-fast bacteria. For this process, each bottle was vigorously shaken for a minimum of 60 seconds using a Mistral multi-mixer (Lab Line Instruments Inc., Melrose Park, Illinois, USA). A sample (one drop) from each bottle was placed on a microscope slide, with a total of three samples per slide. The slides were air-dried and then heat fixed and an acid-fast staining process was performed on each slide (18). Each slide was then examined at oil-immersion (100X) for the presence of acid-fast bacteria.

Every sample that was positive or suspicious for acid-fast bacteria was sub-cultured onto two slants of Herrold's egg yolk (HEY) media (one tube containing mycobactin and one tube without mycobactin). The HEY tubes were examined weekly for six weeks. Isolates that grew well (ten colonies or more) on tubes that contained mycobactin (mycobactin dependent) and had minimal or no growth on the tubes that did not contain mycobactin were identified as *Mptb*. Isolates that grew well on both HEY slants or had fewer than 10 colonies on the slant containing mycobactin were tested by the PCR.

3.3.5. PCR Technique

For DNA extraction, lysis by the boiling method was used. Briefly, 1 to 2 bacterial colonies from each HEY slant was suspended in 100 µl of lysis buffer containing HPLC grade water with 1% Triton®-X-100 (Fisher Scientific, Pittsburgh, Pennsylvania, USA), 1 mM EDTA and 10 mM Tris-HCL (pH 8.0). This mixture was placed in a heat block (Fischer Scientific, Pittsburgh, Pennsylvania, USA) and heated to 100 °C for 30 minutes to lyse cells. Following centrifugation for 2 min at 13,000-x g in a micro-centrifuge, the cell-free supernatant containing DNA was transferred to a fresh tube and used for PCR.

The oligonucleotide primers used for the IS*900* and F57 genetic elements have previously been described by Vary et al. (21) and Poupart et al. (22). The forward primer designated IS*900/150C* and the reverse primer designated IS*900/921* were used for IS*900*; the forward primer designated F57a and the reverse primer designated F57b was used for F57. The primer pair for IS*900* results in the amplification of a 229-bp fragment and the primer pair for F57 results in the amplification of 439-bp fragment, respectively.

Amplification reactions were performed in a total volume of 50 µl containing 10 mM Tris-HCL (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCL; 0.001% gelatin; 200µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP); 1 µM of each primer; 1.25 U of Amplitaq (Applied Biosystems, Foster City, CA) and 5 µl of the boiled cell lysate for the monoplex PCR. The PCR assay was carried out in a Perkin-Elmer 2400 thermocycler (Perkin Elmer Corp., Norwalk, CT) comprising 5 min of pre-incubation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 minute at 54 °C and 1 min at 72 °C. Final extension was performed for 7 min at 72 °C. A single colony from a HEY culture positive slant was used as

the DNA positive control. The negative control was a reaction mixture containing all reagents but no DNA template. The PCR products were visualized by electrophoresis on a 1% agarose gel following standard procedures. Bacterial isolates positive to both the F57 and IS*900* genetic elements were classified as positive for *Mptb*.

3.3.6. Statistical Analysis

Prior to statistical analyses, observations were checked for unlikely values; no data were excluded for this reason. Missing values (11 of the 984 cows did not have a BCS recorded) routinely caused a record to be excluded if the analysis included BCS. The distribution of BCS was tested for normality using the Anderson-Darling Normality Test. Mean BCS of Canadian and US cows was tested with Student's *t* test. Unconditional associations between prevalence of infection with both month and category of BCS (divided into four categories: < 2, 2-3, 3-4, >4) were evaluated using a chi-square on a contingency table. A logistic regression was performed using *Mptb* culture result as dependent variable and BCS and place of origin as independent variables, with BCS categorized in the same four categories to avoid assumptions of linearity. Statistical significance was defined at $P = 0.05$. All data analysis was done using Stata 7.0 (Stata Corporation, College Station, Texas).

3.4. Results

3.4.1. Prevalence

Overall prevalence of infection with *Mptb* was 16.1% (95% CI: 13.8 – 18.3%). In total, 8.5% of the cows had positive ileum cultures and 11.1% of the cows had positive lymph node cultures. There were 37 cultures that had positive slants without mycobactin that were confirmed positive by both IS*900* and F57 PCR analysis. Only 3.5% of the cows were *Mptb*-positive at both sites (Table 3-1. Prevalence of *Mptb* in the 832 Atlantic Canadian cows was 15.1% (95% CI 12.7 – 17.5%). Prevalence of infection in the 152 cows originating from the state of Maine (USA) was 21.7% (95% CI 15.2 – 28.3%). The odds of a US cow being positive were 1.56 (P = 0.04) times greater than a cow from Canada, when controlled for BCS. There was no detected interaction between BCS and country of origin.

Analysis of the relationship between the physical characteristics of samples (gross thickening of the ileum, loose feces, or enlarged mesenteric lymph nodes) revealed that of the 984 cows, only 134 cows had physical attributes in one of these three categories. Of those 134 cows, only 27 (20.1%) cows were actually culture-positive and only one (0.7%) was histologically positive (Table 3-2). All seven cows that were histologically indicative of Johne's disease were also *Mptb*-positive (Table 3-2).

The monthly proportion of *Mptb*-positive cows varied from 2.4 to 42.5%, with 82.4% of the positive cows identified during three of the eight months the samples were collected from the plant. The highest proportion of culture-positive cows was identified in February, March and June (Figure 3-1; Chi-square = 141.2, 7 df, P = 0.001). In June, prevalence of

Mptb-positive cows was the highest at 42.5%. In all months but June, proportion of *Mptb*-isolation from lymph nodes was higher than from the ileum (Chi-square = 52.0, 1 df, P = 0.001). In June, 34.1% of the ileum samples were *Mptb*-positive, while only 21.2% of the lymph nodes were culture-positive (Figure 3-2).

3.4.2. Body Condition Score

The BCS for all cows in this study was normally distributed (Anderson-Darling Normality Test, $P < 0.001$). Therefore, although BCS is an ordinal scale, using averages is valid. Of the culture-positive cows, 73.0% had a BCS of ≥ 2.75, which is a relatively well conditioned dairy cow. Average BCS was 3.0 (95% CI: 3.0 – 3.1) and for the *Mptb*-positive Canadian cows and 2.8 (95% CI: 2.7 – 2.9) for the *Mptb* positive-cows from Maine. The range of BCS for US cows was 1.5 - 4.25, with quartiles of Q1 = 2.25, median = 2.75, and Q3 = 3.25. The range of BCS for Canadian cows was 1.25 - 4.5, with quartiles of Q1=2.75, median = 3.0, and Q3 = 3.5

Overall, average BCS was higher in the Canadian cows than in cows from Maine (P=0.0003). Prevalence of *Mptb*-infection was not associated with low BCS (Figure 3-3; Chi-square P = 0.33). Ileum was the more prevalent *Mptb*-positive site at lower body condition scores with a prevalence of 16.0% for cows with body condition of ≤ 2.5 compared to the prevalence of 8.9% for lymph nodes (Figure 3-4), but this was not statistically significant (P = 0.18).

3.5. Discussion

The prevalence of *Mptb* found in this study of 16.1% is substantially higher than a previous estimated seroprevalence of 2.6% of randomly sampled cows from a serological study done five years ago of the Atlantic provinces (7). It is unlikely that we would see such a dramatic rise in prevalence in a four year period when dealing with a slow growing pathogen. These comparisons are apparent prevalences, not true prevalences. Likely, much of the difference in prevalence estimates between the two studies is due to an underestimation of the true prevalence based upon the low diagnostic sensitivity of the ELISA used by VanLeeuwen et al. (7). Culture of tissue is expected to be more sensitive, because of detection of earlier stages of infection than an ELISA, therefore the apparent prevalence will be closer to the true prevalence.

The process of thawing and refreezing the samples may have caused the loss of viable organisms. The full magnitude of this loss is not known, but using data from losses accrued during freezing fecal samples, the number of organisms may be reduced by one tenth (23). Conclusions from this study were that it would not change the status of high and moderate shedders, but low shedders may appear as negative cows after two freeze thaw cycles. The impact of this unavoidable need to freeze samples is that there may be cows in this study that are infected with low numbers of *Mptb* that resulted in negative cultures. This would result in biasing our prevalence estimate to be lower than what it truly was.

For some diseases, study of cows sampled at a slaughterhouse may not be representative of the general population. However, for *Mptb*, the estimate of the prevalence

of infection derived from this sample is likely to be a very good estimate of the prevalence in the population of dairy cows for the following reasons. First, the slaughterhouse used in the study was one of only two federally inspected plants in the region. The other plant had a policy of not slaughtering dairy animals. As a result, the great majority of culled dairy cows from the region are killed at the study facility. Second, most *Mptb*-infections occur early in life (24) and are presumably persistent throughout life since studies of repeated ELISA and fecal testing fail to indicate cows that self-cure (25). As a result, the actual prevalence of infection within a birth cohort will remain relatively constant over life. Using less sensitive diagnostic methods (ELISA, fecal culture), which more readily identify late stage disease, may suggest a higher prevalence in certain age groups. Using more sensitive techniques, and limiting the population under study to animals which had calved at least once (> 2 yrs of age), allows the study to closely approximate the true disease prevalence. Finally, Johne's disease appears to have relatively little effect in terms of causing premature culling (26). However, even if it did cause premature culling, the estimate of the prevalence of infection would only be affected if the incidence of new infections was rapidly increasing (or decreasing) across birth cohorts. In the absence of a specific control program in the region, it is unlikely that the incidence is rapidly decreasing and there is no reason to believe the region is experiencing a rapid rise in the incidence of new infections. Consequently, the estimate of the prevalence derived from this slaughterhouse study is likely to be a good estimate of the prevalence of infection in the dairy cow population in the region.

Unfortunately, there was no cattle identification and registration system in place at the time of data collection that would have facilitated tracing each infected animal to herd of

origin. For this reason, no estimate of herd-level prevalence could be made. Such information might have proven to be quite valuable for future monitoring of disease distribution and potentially identifying concentrations of diseased animals within highly infected herds.

Historically, the isolation of a slow growing acid-fast positive bacillus from a cow that required exogenous mycobactin for growth was sufficient to identify a culture isolate as *Mptb* (27). However in this study, 37 of the 158 culture positives (23.4%) failed to demonstrate mycobactin dependence but were positive based on two PCR tests. This was probably due to intracellular stores of mycobactin from the TREK™ media that enabled growth of the organism on HEY slants that did not contain mycobactin. This hypothesis was later validated by sub-culturing acid fast colonies from the 37 questionable HEY tubes without mycobactin onto HEY slants with and without mycobactin, and demonstrating subsequent mycobactin dependence. This observation demonstrated that PCR was superior to sub-culturing onto HEY slants for identifying *Mptb* from acid-fast positive broth media cultures.

Since reports by Englund et al. (28) and Cousins et al. (29) suggest that IS*900* may not be 100% specific for *Mptb*; we elected to also test the questionable samples for the F57 gene product as well as the IS*900*. F57 has been shown to be 100% specific for *Mptb* (22; 30). All 37 positive IS*900* samples were also positive for F57.

Common dogma among farmers with respect to *Mptb* is that infected cows are more likely to be thin and in poor body condition. Animals infected with *Mptb* that are in the latter stages of disease are expected to be in poor body condition due to the onset of clinical signs,

but that change may be gradual (31). Although early disease stage animals would not be expected to have dramatic weight loss, on the continuum of disease, from early to later stages represented in this large sample, some association between condition score and infection status could be anticipated. In our study, there was no association between BCS and infection status, with the majority of the culture-positive cows being in good condition. In fact, 73% of positive cows had a BCS of ≥ 2.75, which is a favorable score for a lactating Holstein-Friesian cow. However, we do not have data on stage of lactation of these cows. Unfortunately, there also is no available data to confirm whether or not these animals were fecal shedders, which might indicate stage of infection. Due to the fact that only 7 of the 160 culture positive animals had histological evidence of infection, the argument could be made that the other 153 animals were in early stage disease and had not reached a level of infection that would result in poor condition. Based upon findings from this study, BCS at slaughter is not a valid method to help find cows with *Mptb*. This study confirms that although cows may seem to be healthy and in good body condition, they may be carrying *Mptb* and are undetectable as affected cows.

The finding of higher prevalence within the spring season was unexpected and difficult to explain. Cetinkaya et al. (32) found a similar trend in the spring compared to the fall, but the difference was not significant. A high proportion of positive samples were collected in June, with a cluster of *Mptb*-positive cows on the third sampling day in June. It could be possible that a herd with a high prevalence of Johne's had just gone through a dispersal and an extraordinary number of positive animals arrived at slaughter the same day. It is unlikely that this high number of positives on a single day was a contamination issue

because the same procedure was used throughout sampling. Also, sample preparation was done on two different occasions so that samples of ileums and lymph nodes were never handled on the same day. No one-day of sample processing had a disproportionately high number of positives.

Peculiarly, in every month, a higher proportion of lymph nodes than ileum were *Mptb*-positive, except for the month of June where the proportion of ileum-positive samples peaked. The higher proportion of *Mptb*-positive ileum samples in June could be due to cows being on pasture for approximately one month (a common practice in Atlantic Canada) and being exposed to higher numbers of *Mptb* microbes and a possible pass through the gastro-intestinal tract effect. This may be due to cow exposure or simple increased stress during changing of feeding practices and environment, which could induce progress of the disease and shedding of *Mptb*. Although it is impossible to determine if this is plausible from this study, further investigation may be warranted to better describe this seasonal finding.

The high prevalence of *Mptb*-infection in slaughter cows found in this study illustrates the need for Johne's disease control program initiatives to be established within this region. Other regions would likely find a similar prevalence if a tissue culture strategy were employed.

3.6. Acknowledgements

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Table 3-1. Prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* in 832 Atlantic Canadian and 152 Maine (USA) cull dairy cows, by sample site.

	Ileum	Lymph nodes	Both	Total
Atlantic Canada	67 (8.1%)	83 (10.0%)	25 (3.0%)	125 (15.1%)
Maine	18 (11.8%)	27 (17.8%)	10 (6.6%)	33 (21.7%)
Total	85 (8.5%)	110 (11.1%)	35 (3.5%)	158 (16.1%)

Table 3-4. Distribution of observed physical characteristics and pathological changes among *Mycobacterium* avium subsp. *paratuberculosis*-positive cull dairy cattle.

Observations in 984 cull dairy cows	Total *Mptb*-positive (n=158)	Atlantic Canada *Mptb*-positive (n=125)	Maine *Mptb*- positive (n=33)
Macroscopic thickened ileum (n=112)	23	18	5
Histological changes (n=7)	7	6	1
Loose feces (n=29)	5	4	1
Enlarged lymph nodes (n=18)	1	1	0

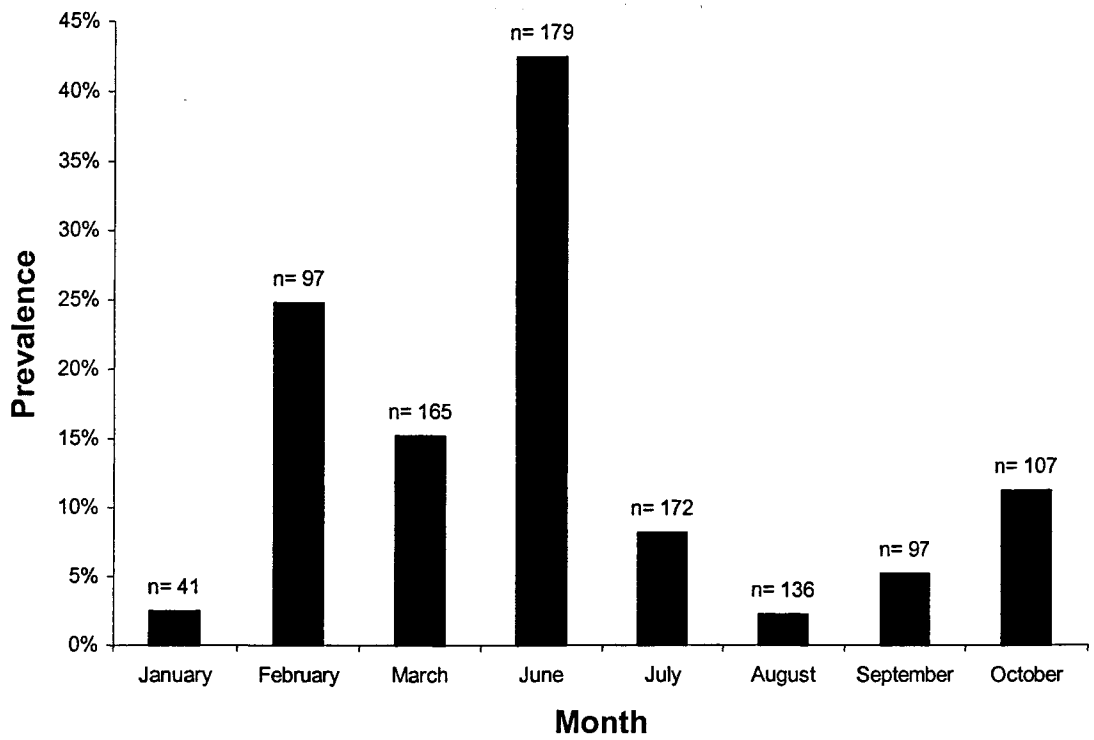

Figure 3-1. Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in 984 cull dairy cattle, by month sampled.

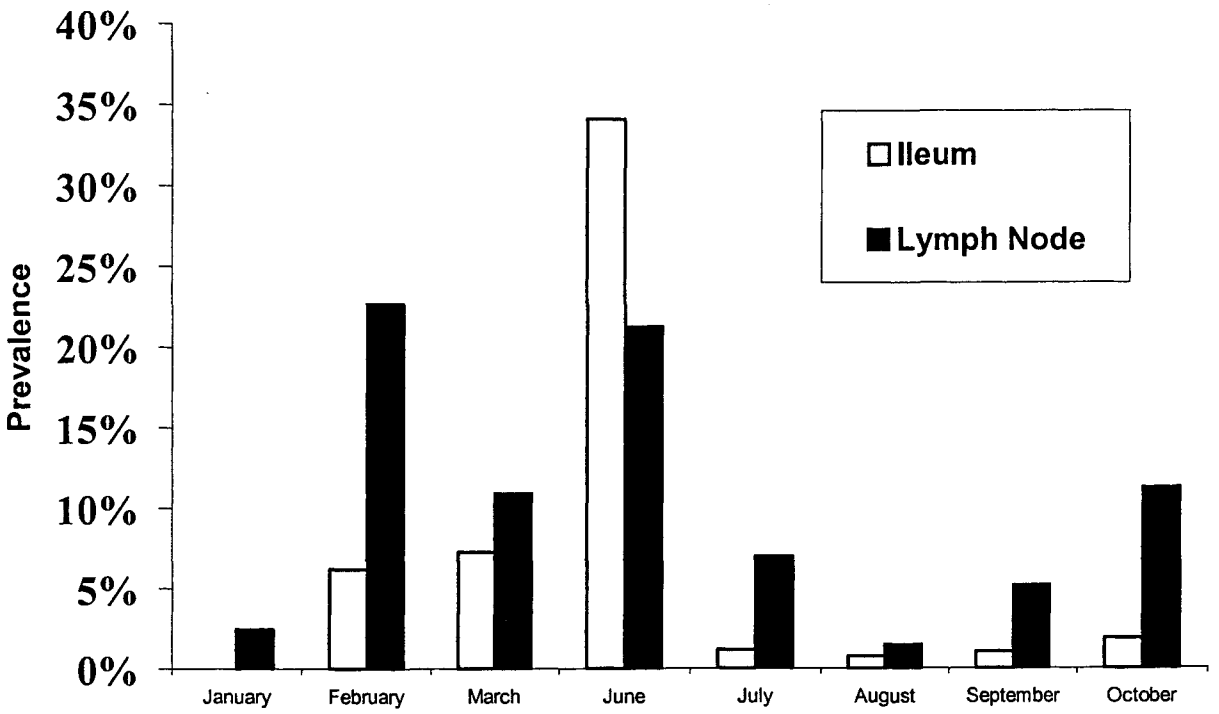

Figure 3-2. Prevalence of isolation of *Mycobacterium avium* subsp. *paratuberculosis* in the two sampled tissue sites, ileum and mesenterial lymph nodes, by month sampled.

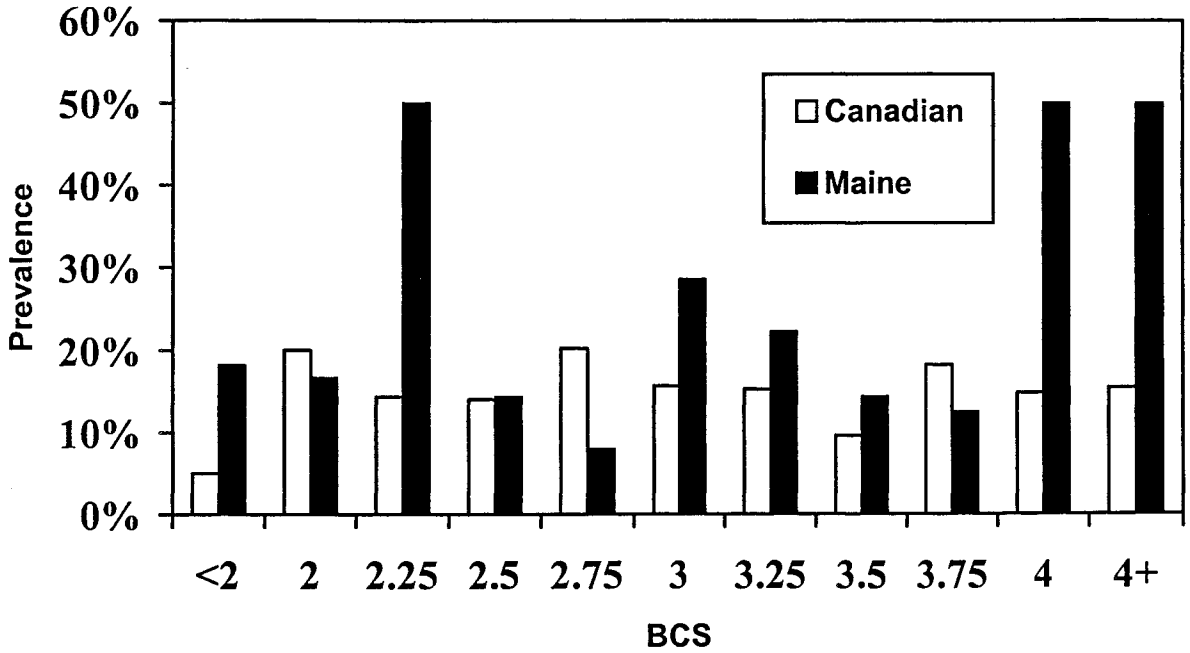

Figure 3-3. Distribution of body conditions score (BCS) and prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* for Canadian and Maine cows.

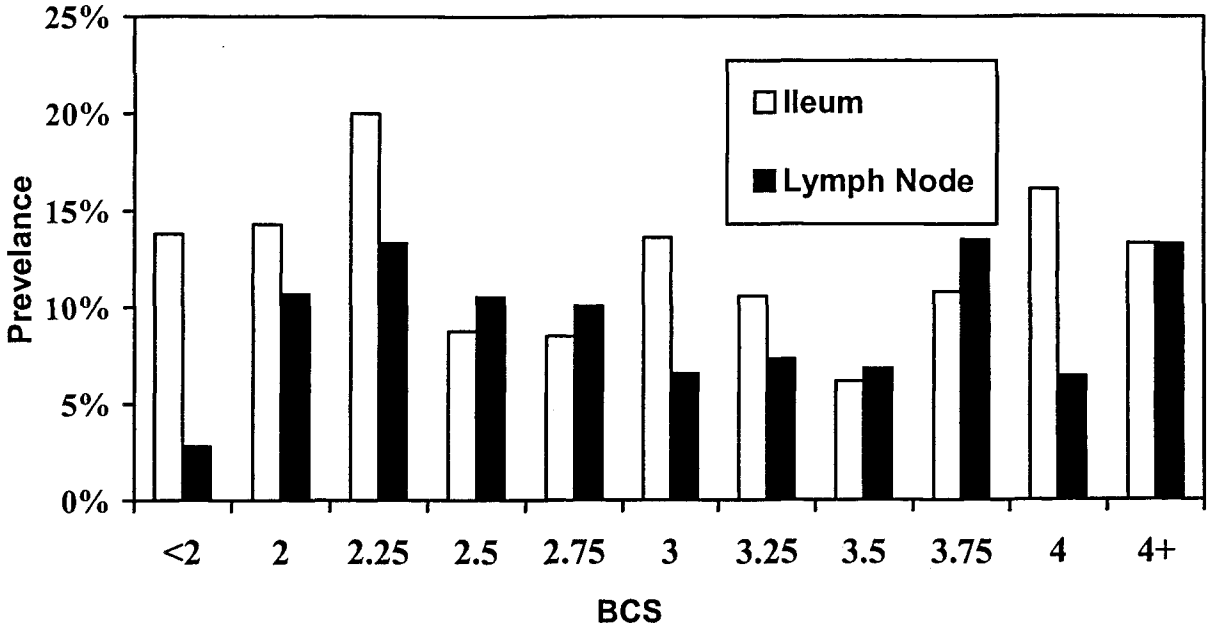

Figure 3-4. Distribution of body conditions score (BCS) and *Mycobacterium avium* subsp. *paratuberculosis* prevalence for two different tissue sites.

CHAPTER 4. COMPARISON OF FECAL CULTURE AND TISSUE CULTURE AS GOLD STANDARDS FOR DETECTION OF INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

Submitted to Veterinary Microbiology.

4.1. Abstract

Johne's Disease is a disease that affects ruminants and causes a chronic granulomatous enteritis. It is caused by *Mycobacterium avium* subsp. *paratuberculosis*. Validation of diagnostic tests for Johne's Disease is problematic due to the difficulty in determining the actual disease status of an individual animal. The use of gold standards for comparison of diagnostic tests is the normal procedure, and in the case of Johne's Disease, fecal culture has been the predominant gold standard used. Fecal culture may not be an appropriate gold standard, since not all infected animals may be shedding. Tissue and feces were collected from 994 dairy cows at slaughter for culture to determine if these animals were infected with *Mycobacterium avium* subsp. *paratuberculosis*. Tissue culture identified 160 animals as positive. Of those tissue culture-positive animals, only 36 (22.5%) were fecal culture-positive. Additionally, of the 160 tissue culture-positive animals, only 7 were histologically positive and 5 of those were fecal culture-positive. Of the 85 animals that were positive on ileum culture, 23 (27.1%) were fecal culture-positive, while of the 110 animals positive on lymph node culture, only 23 (20.9%) were fecal culture-positive. The sensitivity of fecal culture when compared to tissue culture was 19.4%, indicating that tissue culture is a superior gold standard for evaluating diagnostics tests for Johne's Disease.

4.2. Introduction

Validation of diagnostic tests can most readily be done if a gold standard is available for comparison. There are mathematical techniques that can establish diagnostic test attributes without the use of a gold standard (1), but conventionally for Johne's Disease (JD), diagnostic test validation has been done using fecal culture as the gold standard (2 – 11). Fecal culture has been favoured as a gold standard because samples are readily attained and the specificity for fecal culture is assumed to be 100%, because if you isolate the organism *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*), there is no possibility for a false positive result. However, sensitivity of fecal culture is known to be suboptimal for a gold standard. In one extensive study the sensitivity was estimated to be 33% after testing 10 herds repeatedly over four years (11).

In particular, using fecal culture as the comparison standard for interpretation of ELISA results has recently been shown to be problematic. The sensitivity of the ELISA varies depending on the stage of infection; or more specifically the degree of fecal shedding (4, 12, 13). The variation in sensitivity can be from 15% to 88% between low shedders and high fecal shedders (4, 12).

The relationship between fecal culture status and infection of the gastro-intestinal tract has been explored previously. *Mycobacterium avium* subsp. *paratuberculosis* can cause disseminated disease, as the organism has been found in multiple locations throughout infected cattle (14). This finding of multiple organs infected in advanced disease has been substantiated in other studies (15 - 17) and in other species (18, 19). Animals that are tissue

culture-positive can be fecal culture-negative because they are in an early stage of disease and are not shedding *Mptb* in detectable amounts in the feces (15). It is for that reason that the use of fecal culture as a gold standard comparison may inhibit accurate validation of diagnostic methods. Tissue culture cannot readily or economically be done on live animals, so usually it would only be performed on animals on a post-mortem basis.

Previous studies on the association between fecal and tissue culture for *Mptb* utilized animals with known infection status based on previous fecal culture results (17) or used very low numbers of known *Mptb* infected animals (20). A recent study (15) used cattle from high prevalence herds and sampled feces for culture three months prior to slaughter and then used the information gained prior to slaughter for comparison to tissue findings at slaughter. This present study used animals randomly selected from all types of herds of unknown prevalences (low to high), thus increasing the likelihood to identify animals in all stages of disease. This study also uses the TREK ESP® Culture System II broth media system as compared to the solid media used by Pavlik et al. (15).

The goal of this study was to study the association between tissue and fecal culture for *Mptb* and to determine the sensitivity of fecal culture when used on a random sample of cows using the most sensitive culture techniques available.

4.3. Materials and Methods

4.3.1. Sample Population

Sample population and collection has been previously described (21). In short: 994 mature dairy animals were selected at slaughter for collection of blood, ileum, lymph nodes within the region of the ileum, and feces. All were selected based on a systematic random sample, with the exception of 10 cows that were purposively selected based on convenience of sample (line stoppage or extremely thin cows).

4.3.2. Sample Collection

Two mesenteric lymph nodes in the region of the ileum were collected along with a 5 to 10 cm segment of terminal ileum approximately 25 cm proximal to the ileo-cecal junction. Fecal samples were then collected from posterior colon via an incision into the viscera. Samples were placed in individual containers labeled with the numeric identifier for each animal. From each sample of ileum and lymph node, a section was preserved in 10% neutral buffered formalin and the remainder was frozen at –80°C.

4.3.3. Histological preparation

After formalin fixation, samples were trimmed and embedded in paraffin wax. They were subsequently sectioned and Ziehl-Neelsen staining was performed (22). All histopathological examinations were performed by a board-certified pathologist at the Atlantic Veterinary College Diagnostic Services (Charlottetown, PEI, Canada) for lesions similar to those described by Buergelt et al. (23).

4.3.4. Tissue Culture Preparation

Tissue culture preparation has been previously described (21). In short, tissue sample processing for culture was done using a protocol developed by researchers in Pennsylvania (24). Briefly, samples were placed in a Tekmar bag along with 25 ml of 0.75% hexadecylpyridinium chloride (HPC) solution as decontaminate. The sample was placed in a stomacher for a minimum of 1 minute, and then each sample was left undisturbed for 30 minutes to allow for separation of tissue. A 10-ml sample of the pulverized tissue mixture was transferred into another sterile tube containing a further 10 ml of HPC solution, used for the second decontamination stage in the procedure. After a minimum of 3 hr in the second decontamination stage, samples were centrifuged at 900 x *g* for 30 min. The supernatant was discarded and the pellet was re-suspended with a combined half strength brain-heart-infusion (BHI) broth that contained 0.1% NAL, 0.1% VAN, and 0.05% AMB. This mixture was vortexed and placed into sterile cryogenic tubes to be incubated for 12-14 hr at 37°C to allow for microbial growth and an opportunity for the antimicrobials to more effectively decrease levels of background contaminants. After the incubation phase, the tubes were slowly cooled and then re-frozen at –80°C.

4.3.5. Fecal Culture Preparation

For each cow, a two-gram sample of feces was weighed and placed in a 50ml centrifuge tube along with 35ml of distilled/deionized water. This mixture was vortexed and then placed on a shaker-table for 30 min to allow for thorough mixing and agitating of the

mixture. The tubes were then left to stand for 30 min to allow particles to settle out, and then 5ml of sample were drawn off of the top third of the tube and transferred to a second 50ml centrifuge tube where it was mixed with 25 ml of 0.9% HPC solution mixed with half strength BHI broth (final concentration of 0.75% HPC) as decontaminate. The sample tubes were then incubated at 37°C for 24 hr. Then each tube was placed in a new centrifuge tube and was centrifuged at 900 x *g* for 30 min. The supernatant was then discarded and the pellet was re-suspended with a combined half strength BHI broth that contained 0.1% NAL, 0.1% VAN, and 0.05% AMB. This mixture was vortexed and placed into sterile cryogenic tubes, which were subsequently incubated for 12-14 hr at 37°C. After the incubation phase, the samples were slowly cooled and then re-frozen at –80°C.

4.3.6. Culture

All samples were cultured using the TREK ESP® Culture System II broth solution media bottle (TREK Diagnostic Systems, Cleveland, Ohio, USA). Samples were thawed, and 1 ml from each of the tubes was inoculated into a TREK™ broth solution media bottle, supplemented with egg yolk, antibiotics (0.1% NAL, 0.1% VAN, and 0.05% AMB) and a growth supplement containing Mycobactin J (Allied Monitor Inc., Fayette, Missouri, USA). Sample bottles were then placed in the TREK ESP® Culture System II diagnostic incubator. As samples were identified as positive for bacterial growth by the incubator, in turn each bottle was examined for acid-fast bacteria. After 6 weeks, any remaining samples were removed from the incubator and were also examined for acid-fast bacteria. For this process, each bottle was vigorously shaken for a minimum of 60 sec using a Mistral multi-mixer (Lab

Line Instruments Inc., Melrose Park, Illinois, USA). A sample (20 µl) from each bottle was placed on a microscope slide, for a total of three samples per slide. The slides were air-dried and then heat fixed and a cold Ziehl-Neelsen staining method staining process was performed on each slide (22). Each slide was then examined at oil-immersion (100x) for the presence of acid-fast bacteria.

For the tissue samples, all samples positive or suspicious for acid-fast bacteria was sub-cultured onto two slants of Herrold's egg yolk (HEY) media (one tube containing mycobactin and one tube without mycobactin). The HEY tubes were examined weekly for 6 weeks. Isolates that grew well (10 colonies or more) on tubes that contained mycobactin (mycobactin dependent) and had minimal or no growth on the tubes that did not contain mycobactin were identified as *Mptb*. Isolates that grew well on both HEY slants or had fewer than 10 colonies on the slant containing mycobactin were tested by PCR. For the fecal samples, all samples positive or suspicious for acid-fast bacteria were confirmed by the use of PCR.

4.3.7. PCR Technique

The procedure for PCR analysis has been previously described in detail (25, 21). DNA from bacteria harvested from either TREK™ broth or colonies growing on Herrold's egg yolk were prepared as described previously (21, 26). Briefly, DNA was prepared by either, boiling in 100 µl of lysis buffer containing HPLC grade water with 1% Triton®-X-100 (Fisher Scientific, Pittsburgh, Pennsylvania, USA), 1 mM EDTA and 10 mM Tris-HCL (pH 8.0), or by lysis in 600 µl of disruption buffer containing 4.0 M guanidine thiocyanate,

0.025 M sodium citrate, 0.5% sarkosyl, 0.1M 2-mercaptopethanol, and 20 mM EDTA (pH 8.). Bacterial DNA was resuspended in 1 x TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and stored at –20 °C. The oligonucleotide primers used for the IS900 and F57 genetic elements have previously been described by Vary et al. (27) and Poupart et al. (28). The forward primer designated IS900/150C and the reverse primer designated IS900/921 were used for IS900; the forward primer designated F57a and the reverse primer designated F57b was used for F57. The primer pair for IS900 results in the amplification of a 229-bp fragment and the primer pair for F57 results in the amplification of 439-bp fragment, respectively.

Amplification reactions were performed in a total volume of 50 µl containing 10 mM Tris-HCL (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCL; 0.001% gelatin; 200µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP); 1 µM of each primer; 1.25 U of AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA) and either 5 µl or 10 µl resuspended bacterial DNA template. The PCR assay for the broth samples originating from tissue was carried out in a Perkin-Elmer 2400 thermal cycler (Perkin Elmer Corp., Norwalk, CT) and broth samples from fecal culture was performed in a Hybaid PCR Sprint thermal cycler (Thermo Electron Corp., Boston MA). A sample from a TREK™ broth bottle containing an ATCC stain (# 19698) of *Mptb* was used as the DNA positive control. Negative PCR controls included a reaction mixture containing all reagents but no DNA template for the tissue samples and for the fecal samples, ATCC strains of *Mycobacterium avium* subsp. *avium* (# 35715) and *Mycobacterium avium* subsp. *intracellulare* (# 35771). Cycling parameters used were; denaturing temperature of 94 C for 5 minutes followed by 35

cycles of denaturing at 94 C for 60 s, annealing at 54 C for 60 s and elongation at 72 C for 35 seconds, and 1 cycle of final extension at 72 C for 7 minutes. The PCR products were visualized by electrophoresis on a 1% agarose gel following standard procedures. Bacterial isolates positive to either F57 or IS900 genetic elements were classified as positive for *Mptb*.

4.3.8. Statistical Analysis

Prior to statistical analyses, observations were checked for unlikely values; no data were excluded for this reason. All data analysis was done using Stata 8.0 (Stata Corporation, College Station, Texas) and Win-Episcope 2.0 (Wageningen University, Wageningen, The Netherlands).

4.4. Results

Of the 994 cows cultured, 160 (16.1%) were tissue culture-positive. The distribution of tissue culture results was 85 (8.5%) animals with positive ileum cultures and 110 (11.1%) animals with positive lymph node cultures (Table 4-1). Of the 160 tissue positive animals, only 7 (0.7%) were identified as histologically positive.

In comparison, 36 (3.6%) of the cows were fecal culture-positive. Of the 85 animals that had positive ileum culture, 23 (27.1%; 95% CI: 17.6-36.5%) were fecal culture-positive. Of the 110 animals that had positive lymph node culture, 23 (20.9%; 95% CI: 13.3-28.5%) were fecal culture-positive. Of the 7 histologically positive animals, 5 (71%) were fecal culture-positive. Five (3%; 95% CI: 0.4-5.6%) animals that were tissue culture-negative were fecal culture-positive.

The proportion of fecal culture-positive samples in animals that were tissue culture-positive at both sites was higher than that of individual sites. Of the samples that were only positive at the ileum (50 samples), 8 (16.0%) were fecal culture-positive and 42 were negative, which was significantly different from being positive at both sites (Chi-square=7.82, 1 df, P=0.006). Of the samples that were only positive at the lymph nodes (75 samples), 8 (10.7%) were fecal culture-positive, which again was significantly different from being positive at both sites (Chi-square=14.95, 1 df, P=0.0001).

Using tissue culture-positive results as the gold standard, it is possible to determine the sensitivity of fecal culture. In this population of animals, fecal culture would have a sensitivity of 19.4% (95% CI: 13.3-25.5%).

4.5. Discussion

This study indicated that of the 952 animals with negative fecal cultures, 13.6% were tissue culture-positive. Similar results were found by Pavlik et al. (15) and Whitlock et al. (29), who reported that 25.6% and 28.6%, of fecal culture negative animals to be tissue positive, respectively. This would indicate that these are animals in the early stages of disease, in which they have not yet begun shedding. These animals are the ones that would elude the designation of a positive animal when fecal culture would be used as a gold standard. These animals are truly infected, and need to be designated as such when evaluating a diagnostic test such as an ELISA. Undoubtedly the likelihood of an ELISA identifying these cows not shedding is low, since it is known that as the shedding of bacteria in feces decreases, so does the ELISA sensitivity (11). However, these animals need to be

included as positive animals in the gold standard group, and present the true effect they have on the ability of a diagnostic test to accurately identify infected animals. In addition, the finding that the diagnostic sensitivity of fecal culture is quite low in animals in the early stages of *Mptb* infection means that ELISA positive but fecal culture negative animals may be infected with *Mptb*. Sockett et al. (8) reported that approximately 10% (17/177) of *Mptb* infected animals were ELISA positive but fecal culture negative.

The culture of lymph nodes resulted in more positive cultures than did ileum. This is different from what was previously reported (20, 15, 29). In the other studies, ileum was the predominant tissue that was infected. This finding suggests that the majority of positive animals were in the early stage of disease and not actively shedding into the gastro-intestinal tract, therefore not fecal culture-positive (21).

The fact that only 7 animals were histologically positive also lends support that the majority of the positive animals were in early stage of disease. Results from Huda and Jensen (20) indicated that tissue culture was more sensitive than histological examination, but only slightly. It was not as substantial as this current study and was only looking at 12 animals from infected herds and 4 from non-infected herds. However, in animals with disseminated disease, histology was a sensitive method, indicating that histology is more sensitive in later stage disease. From that work, it was suggested that tissue samples from cattle with subclinical infections should be processed for both histopathological examination and tissue culture to enhance the diagnostic sensitivity (20). In that study, there were a few cases in which samples were histologically positive and culture-negative.

In order to accurately describe the sensitivity of any diagnostic test for JD, it is imperative to first define the population being considered. It has been established that ELISAs perform differently based on fecal-shedding patterns (4, 12, 29), or essentially, the stage of disease. Therefore, it is only logical that the same could be assumed for the sensitivity of fecal culture based on stage of disease. The sensitivity of fecal culture as a diagnostic test in this population of cattle was determined to be 19%. This value is lower than what had been reported in the past (11), but is similar to estimates found by maximum-likelihood estimations (1). When using maximum-likelihood estimation to determine fecal culture sensitivity, the sensitivity was reported to be in the range of 60-70% when used as a confirmatory test on ELISA-positive animals (1). However, the same study indicated that if fecal culture were used as a first screening test, its sensitivity would more likely be in the range of 20-25%.

The critical implication of these findings is that many animals infected with *Mptb* are indeed fecal culture-negative. When assessing other diagnostic tests, fecal culture is an inferior gold standard compared to tissue culture. Truly, tissue culture should be the standard for comparison of diagnostic tests for *Mptb*. The difficulty in doing so is the lack of practicality of performing tissue culture on live animals. Examination of supra-mammary lymph nodes for the presences of *Mptb* has been performed, with results that indicated higher prevalence of positive tissue cultures with heavy shedders than light shedders (17). However, more research may be warranted on peripheral lymph nodes as possible diagnostic modalities using advanced tissue culture techniques. The low diagnostic sensitivity of fecal culture in animals in the early stages of *Mptb* coupled with the ability of the organism to

persist in the environment for months has caused considerable debate in the livestock community as to whether JD can be cost-effectively eradicated in a timely manner (~5 years) from livestock herds using current diagnostic tests. The answer to this question is not known at this time but experience with bovine tuberculosis, another mycobacterial infection of cattle where cattle are the natural hosts, suggests that whole herd depopulation is the only viable method to eradicate the disease in known infected herds.

Certainly diagnostic testing for control of JD in high prevalence herds may be warranted. In those cases, yearly or twice yearly fecal culture of individual animals may be the diagnostic test of choice. The main purpose would be to detect animals that are actively shedding *Mptb* into the environment. However, animals not shedding at the exact moment of testing would not be detected, due to intermittent shedding of cows not yet clinical. In some cases, it would be advantageous to be able to identify all animals infected, by a method that would identify infected cows that are not shedding. The negative aspect of that approach is that if all animals that were tissue culture-positive would be detected, the number of animals that would need to be replaced would be too high in a high prevalence herd.

Additionally, if fecal culture as a screening test has only a slightly higher sensitivity than tests such as ELISAs, yet consumes voluminous amount of space in laboratories, fecal pooling of samples maybe the most logical and cost effective manner to screen herds. That is of course assuming that the number of animals included in the pool does not affect the ability to correctly identify a fecal culture-positive animal. Also, the sensitivity of fecal pooling using the TREK ESP® Culture System II culture methods is not known. However, it has been shown that broth-media solutions do expedite the generation of results and has similar

performance to solid media (30). Pending research on that aspect of fecal pooling on broth media should provide insight on the validity of such methods.

4.6. Conclusions

From these data, if fecal culture were used either as a diagnostic screening test or as a gold standard comparison test, the majority of truly infected animals would erroneously be classified as negative. The impact this has on its use as a gold standard is that test performance parameters such as sensitivity may be misconstrued. It is necessary to state or at least comprehend, that fecal culture sensitivity is correlated to stage of disease, and using a gold standard that only works proficiently on latter stage disease may not give a true understanding of test performance on all stages of disease. However, due to current difficulties in using tissue culture on live animals and the economic challenges associated with using individual fecal cultures as screening test, pooled culture of feces may be the most viable and reliable option available.

4.7. Acknowledgments

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Table 4-1. Culture results for *Mycobacterium avium* subsp. *paratuberculosis* from tissue and fecal samples, with location of actual site indicated.

	Ileum	Lymph nodes	Both	Total (n=994)
Tissue culture-positive	85 (8.5%)	110 (11.1%)	35 (3.5%)	160 (16.1%)
Fecal culture-positive	23 (27.1%)	23 (20.9%)	15 (43%)	31 (19.4%)
Fecal culture-negative	62 (72.9%)	87 (79.1%)	20 (57%)	129 (80.6%)
Histologically positive	6 (7.1%)	7 (6.4%)	6 (17%)	7(4.4%)
Fecal culture-positive	5 (83%)	5 (71%)	5 (83%)	5 (71%)
Fecal culture-negative	1 (17%)	2 (29%)	1 (17%)	2 (29%)

CHAPTER 5. EVALUATION OF THREE ELISAS FOR *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* USING TISSUE AND FECAL CULTURE AS COMPARISON STANDARDS

Submitted to Veterinary Microbiology.

5.1. Abstract

Three serum ELISAs for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) were evaluated against culture of tissue and fecal samples from 994 dairy cows collected at slaughter. Culture of ileum and associated lymph nodes for *Mptb* were positive for 160 (16.1%) of the 994 cows and 36 (3.6%) were fecal culture-positive for *Mptb*. Two of the ELISAs evaluated were absorbed indirect assays and the third was a non-absorbed indirect assay. Estimated sensitivities of the absorbed ELISAs when compared to tissue culture were 8.8% and 6.9 %, while the unabsorbed ELISA had a sensitivity of 16.9%. Specificities were 97.6%, 96.0% and 90.8% respectively. When compared to fecal culture, the sensitivities of the absorbed ELISAs were 16.6% and 13.9%, respectively, and the sensitivity of the unabsorbed ELISA was 27.8%. Specificities were 97.1%, 95.9% and 90.1%, respectively. Area under the curve (AUC) of receiver operator characteristic curves for the absorbed ELISAs, when tissue culture was the standard, were 0.553 and 0.547, while the unabsorbed ELISA had an AUC of 0.540. When fecal culture was the comparison standard, the AUC of the absorbed ELISAs was 0.575 and 0.574, while the unabsorbed ELISA was 0.529. Overall, the sensitivities of the ELISAs when compared to tissue culture were low. The apparent advantage of the unabsorbed ELISA with respect to sensitivity is at the cost of lowered specificity and test accuracy.

5.2. Introduction

Accurate detection of subclinical cases of bovine Johne's disease (JD) has been viewed as a critical step in the reduction of disease prevalence in infected dairy herds (1). In

order to achieve that goal, numerous diagnostic tests have been used focusing mainly on detection of serum antibodies and excretion of *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) in the feces.

For improved control of JD, a test is needed that accurately detects infected animals in the earlier stages of infection, preferably before animals start fecal shedding. In order to achieve this, ELISAs have been utilized in the past with the goal of detecting antibodies to *Mptb* before they become heavy shedders and overt clinical cases. Reports of diagnostic test performance differ among publications in various ways, such as methodology and target population, making comparison of test performance difficult. In one report based on fecal culture of cows from known infected herds, the *Mptb* ELISA tested had a sensitivity as high as 94% (2). Other estimates of test sensitivity, however, range from 59% to 43%, while the reported specificity ranges from 95% to 99% (3;4) in fecal culture-positive cows. More recent estimates of *Mptb* ELISA performance based on fecal culture results have been categorized based upon the fecal shedding pattern. The basis of this strategy is that as animals progress into later stages of the disease, they shed a higher number of organisms, and will have a higher probability of being positive on an ELISA (5;6). Estimates for ELISA sensitivity based on this methodology are 15% for low fecal shedders (< 10 cfu/g) to 88% for high fecal shedders (> 100 cfu/g) (7). The discrepancies seen in these estimates are due to differences in case definition of what is a true positive or the use of imperfect "gold standards" to define the infection status of an animal (3).

The use of an ELISA is not typically reserved for late stage disease animals or animals that are solely high fecal shedders. Based on samples collected in dairy herds

participating in the Australian JD program, ELISA test sensitivity on first round testing in 2, 3, and 4-year-old animals was estimated at 1.2, 8.9, and 11.6% respectively, but were between 20 and 30% in older cattle (9). The *Mptb* ELISAs are marketed to be used as a herd-level test, and all age cohorts of animals can be tested. However, most control programs recommend second or greater lactation animals be tested. Estimates of test sensitivity should be based on samples that reflect all animals that are infected with *Mptb*, not simply those that are fecal shedding at high numbers. This report describes the evaluation of three ELISAs for *Mptb* using two different "gold standards": tissue culture and fecal culture.

5.3. Materials and Methods

5.3.1. Sample Population

Sample population and collection procedures have been previously described (10). In short: 994 mature dairy animals were selected at a local slaughterhouse for collection of blood, ileum, lymph nodes within the region of the ileum, and feces. All cows were selected based on a systematic random sample, with the exception of 10 cows that were purposively selected based on convenience of sample (line stoppage or extremely thin cows). Cows were from the four Atlantic Canadian provinces along with some cows from auction houses in Maine, USA.

5.3.2. Sample collection

Blood was collected free-catch upon exsanguination of the animal in two 50 ml conical centrifuge tubes (Fisher Scientific, Ottawa, ON, Canada). The blood was then transferred to the Atlantic Veterinary College (Charlottetown, PEI, Canada) where the blood was centrifuged at 1000 x g for 10 minutes, and the serum was collected from each sample. Aliquots were pipetted off and kept frozen at –20°C until the time of analysis at the individual laboratories participating in the study. Two mesenteric lymph nodes in the region of the ileum were collected along with a 5 to 10 cm segment of terminal ileum approximately 25 cm proximal to the ileo-cecal junction. Fecal samples were then collected from posterior colon via a stab incision into the viscera. Samples were placed in individual containers labeled with the numeric identifier for each animal. All tissue samples and fecal samples were stored frozen at –80°C within 5 hours of collection, until further processing was initiated.

5.3.3. Tissue culture preparation

Tissue culture preparation has been previously described (10), using a protocol developed by researchers in Pennsylvania (11). Briefly, samples were placed in a Tekmar bag along with 25 ml of 0.75% hexadecylpyridinium chloride (HPC) solution as decontaminate. The sample was placed in a stomacher for a minimum of 1 min, and then each sample was left undisturbed for 30 minutes to allow for separation of tissue. A 10-ml sample of the pulverized fluid was transferred into another sterile tube containing a further 10 ml of HPC solution, used for the second decontamination stage in the procedure. After a

minimum of 3 hr in the second decontamination stage, samples were centrifuged at 900 x *g* for 30 min. The supernatant was discarded and the pellet was re-suspended with a combined half strength brain-heart-infusion (BHI) broth that contained 0.1% nalidixic acid (NAL), 0.1% vancomycin (VAN), and 0.05% amphotericin B (AMB) (Allied Monitor Inc., Fayette, Missouri, USA). This mixture was vortexed and placed into sterile cryogenic tubes to be incubated for 12-14 hr at 37°C to allow for microbial growth and an opportunity for the antimicrobials to more effectively decrease levels of background contaminants. After the incubation phase, the tubes were slowly cooled and then re-frozen at –80°C.

5.3.4. Fecal culture preparation

From each sample, two-grams of feces was weighed and placed in a 50ml centrifuge tube along with 25ml of distilled/deionized water. This mixture was then vortexed and placed on a shaker-table for 30 min to allow for thorough mixing and agitating of the mixture. The tubes were then left to stand for 30 min to allow particles to settle out, and then 5ml of sample were drawn off of the top third of the tube and transferred to a second centrifuge tube where it was mixed with 25 ml of 0.75% HPC solution as decontaminate which also contained BHI broth. The sample tubes were then incubated at 37°C for 24 hr. Then each tube was placed in a new centrifuge tube and was centrifuged at 900 x *g* for 30 min. The supernatant was then discarded and the pellet was re-suspended with a combined half strength BHI broth that contained 0.1% NAL, 0.1% VAN, and 0.05% AMB. This mixture was vortexed and placed into sterile cryogenic tubes to be incubated for 12-14 hr at 37°C, then slowly cooled and then re-frozen at –80°C.

5.3.5. Culture methodology

All samples were cultured using the TREK ESP® Culture System II broth solution media bottle (TREK Diagnostic Systems, Cleveland, Ohio, USA). Samples were thawed, and 1 ml from each of the tubes was inoculated into a TREK ESP® broth solution media bottle, supplemented with egg yolk, antibiotics (0.1% NAL, 0.1% VAN, and 0.05% AMB) and Mycobactin J (Allied Monitor Inc., Fayette, Missouri, USA). Sample bottles were then placed in the TREK ESP® Culture System II diagnostic incubator. As samples were identified as positive for bacterial growth by the machine, in turn each bottle was then examined for acid-fast bacteria. After 6 weeks, any remaining samples were removed from the incubator and were also examined for acid-fast bacteria. For this process, each bottle was vigorously shaken for a minimum of 60 sec using a Mistral multi-mixer (Lab Line Instruments Inc., Melrose Park, Illinois, USA). A sample (one drop) from each bottle was placed on a microscope slide, for a total of three samples per slide. The slides were air-dried and then heat fixed and an acid-fast staining process was performed on each slide (12). Each slide was then examined at oil-immersion (100 x) for the presence of acid-fast bacteria.

For the tissue samples, all samples positive or suspicious for acid-fast bacteria were sub-cultured onto two slants of Herrold's egg yolk (HEY) media (one tube containing mycobactin and one tube without mycobactin). The HEY tubes were examined weekly for 6 weeks. Isolates that grew well (10 colonies or more) on tubes that contained mycobactin (mycobactin dependent) and had minimal or no growth on the tubes that did not contain mycobactin were identified as *Mptb*. Isolates that grew well on both HEY slants or had

fewer than 10 colonies on the slant containing mycobactin were tested by PCR. For the fecal samples, all samples positive or suspicious for acid-fast bacteria were confirmed by the use of PCR.

5.3.6. PCR technique

The procedure for PCR analysis has been previously described in detail (10;13). For DNA extraction, lysis by the boiling method was used by heating a mixture of sample with 100 µl of lysis buffer containing HPLC grade water with 1% Triton®-X-100 (Fisher Scientific, Pittsburgh, Pennsylvania, USA), 1 mM EDTA and 10 mM Tris-HCL (pH 8.0). The oligonucleotide primers used for the IS900 and F57 genetic elements have previously been described (14;15). The forward primer designated IS900/150C and the reverse primer designated IS900/921 were used for IS900; the forward primer designated F57a and the reverse primer designated F57b were used for F57. The primer pair for IS900 results in the amplification of a 229-bp fragment and the primer pair for F57 results in the amplification of 439-bp fragment, respectively.

Amplification reactions were performed in a total volume of 50 µl containing: 10 mM Tris-HCL (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCL; 0.001% gelatin; 200µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP); 1 µM of each primer; 1.25 U of Amplitaq (Applied Biosystems, Foster City, CA) and 5 µl of the boiled cell lysate for the monoplex PCR. The PCR assay was carried out in a Perkin-Elmer 2400 thermoçycler (Perkin Elmer Corp., Norwalk, CT). A sample from a TREK ESP® broth bottle containing an ATCC strain (# 19698) of *Mptb* was used as the DNA positive control. The negative

control was a reaction mixture containing all reagents but no DNA template. The PCR products were visualized by electrophoresis on a 1% agarose gel following standard procedures. Bacterial isolates positive to either F57 or IS900 genetic elements were classified as positive for *Mptb*.

5.3.7. ELISA methodology

All serum samples were analyzed with three commercial indirect ELISAs, two licensed for the North American market: ELISAs A (Herdchek®, IDEXX Laboratories, Westbrook ME) and B (Parachek™, Biocor Animal Health, Omaha Nebraska, currently owned by Pfizer Animal Health) and one licensed for the European market: ELISA C (SVANOVIR™ ELISA, Svanova Biotech, Uppsala, Sweden). The tests were performed according to the manufacturers' instructions. The assays licensed for North America were performed at the Wisconsin Veterinary Diagnostic Laboratory, while the European licensed assay was performed at the Svanova research facility in the Uppsala Science Park, Sweden. The application of these assays was performed in laboratories in which the assays had either been previously, or was currently, in use at that location.

The two North American ELISAs function similarly and are based on detection of antibodies to protoplasmic antigens for *Mptb*. Both of these ELISAs were absorbed ELISAs, with the use of *Mycobacterium phlei* in the absorption phase. The European licensed assay was based on detection of antibodies to lipoarabinomanan (LAM) and is a non-absorbed ELISA.

The ELISA A reported the observed optical densities (OD) as an s/p ratio (sample OD to positive control OD ratio). The ELISA B reported results as a score value, which is determined in relation to the cut-off that is calculated from the mean of the negative controls plus 0.100. The ELISA C reported a pp-value (percent positive), which was based on a regression analysis of log-log transformed OD values. The calculation involved generating a linear regression of the blanked OD values and "log-log" transformed OD values and using the inverse slope of this line multiplied by the log of the OD of the sample to arrive at the pp-value. This calculation was performed to standardize the linear relationship of the test values with corresponding increases in antibody levels.

5.3.8. Statistical analysis

Statistical evaluation was performed using Stata 8.0™(Stata Corporation, College Station, Texas) and Win-Episcope 2.0 (Wageningen University, Wageningen, The Netherlands). Level of test accuracy was determined by calculating receiver operator characteristic (ROC) curves and the area under the ROC curves (AUC). Estimation of optimized test performance by means of cutoff value alteration was examined by generating two-graph ROC curves.

5.4. Results

5.4.1. ELISA characteristics with tissue culture as gold standard

Tissue culture identified 160 animals out of 994 (16.1%) as positive for *Mptb* infection (Table 5-1). Using those culture-positive animals as a gold standard, ELISA A and B correctly classified 14 and 11 animals as positive for sensitivities of 8.8% and 6.9%, respectively. ELISA C correctly classified 27 animals as positive for a sensitivity of 16.9%. Specificity calculations for ELISAs A, B and C were 97.6%, 96.0% and 90.8%, respectively.

Receiver operator characteristic (ROC) curves for all three ELISAs compared to tissue culture as gold standard are presented in Figures 5-1, 5-2, and 5-3. The area under the curve (AUC) or estimate of test accuracy for ELISA A, B and C were 0.553, 0.547 and 0.539, respectively.

5.4.2. ELISA characteristics with fecal culture as gold standard

Fecal culture identified 36 animals of the 994 (3.6%) as positive for *Mptb* (Table 5-1) and 31 of the 160 (19.4%) tissue positive animals as positive. When fecal culture-positive animals were used as the comparison standard, ELISA A and B correctly identified 6 and 5 of the 36 fecal culture-positive animals as positive for sensitivities of 16.7% and 13.9%, respectively. ELISA C classified 10 of the fecal culture-positive animals as positive for a sensitivity of 27.8%. Specificity calculations for ELISAs A, B, and C were 97.1, 95.9, and 90.1%, respectively. Sensitivity estimates calculated on animals that had a fecal culture

signal positive before 45 days of incubation and therefore higher fecal shedders (8) for ELISA A and B would both be 41.7% (13.8-69.6%) and ELISA C would be 58.3% (30.4-86.2%).

Receiver operator characteristic (ROC) curves for comparisons to fecal culture are presented in Figures 5-1, 5-2, and 5-3. The area under the curve (AUC) or estimate of test accuracy for ELISA A, B and C were 0.575, 0.574, and 0.529, respectively.

5.4.3. ELISA characteristics between tissue culture and fecal culture

Using tissue culture as a gold standard, ELISA A, B and C identified 14, 11 and 27 animals as positive, respectively. Of those tissue culture-positive and ELISA positive animals, fecal culture identified 6, 5, and 9 animals as positive for ELISAs A, B, and C, respectively. That correlates to 57% of ELISA A positive, tissue culture-positive animals being fecal culture negative. The same scenario for ELISA B and C would be 54% and 66%, respectively.

5.5. Discussion

All three ELISAs had lower sensitivity when compared to tissue culture than what has previously been reported (16). Differences in sensitivity between the absorbed and unabsorbed ELISAs were evident, however, all three ELISAs had poor sensitivity. The only significant difference in sensitivity was found between ELISA B and C. The specificities estimated in this study are comparable to those found in other studies (17-19).

The use of tissue culture as a comparison standard represents a wider spectrum of animals in all stages of disease. This is supported by the finding that there was no correlation with body condition scores in the animals tested, as previoulsy reported (10) along with the finding that only 36 animals were fecal shedders. Therefore, the majority of animals that were tissue culture-positive were animals that were in early stage infection and would be considered "diagnostically silent" carriers of disease by more conventional, non-invasive methods (20). Undoubtedly, these non-fecal shedding animals would potentially begin shedding at some time in the future and potentially infect other animals (21).

Relating these findings to fecal culture illustrates how the comparison standard can greatly influence the estimates of sensitivity. The calculated sensitivities based on fecal culture closely reflect reported estimates of low fecal shedding cows (6;7). Furthermore, the sensitivity estimates calculated on animals that had a fecal culture signal positive less than forty-five days (the median time to positive signal) were similar to what have been previously found in moderate to high fecal shedders.

One of the disconcerting factors found was that there are ELISA-positive/tissue culture-positive animals that were fecal culture negative, as evident by the differences in sensitivity. This illustrated that a proportion of ELISA positive/fecal culture negative cows are truly infected. The other disturbing factor was that the ELISA sensitivity values for cows in all stages of disease were much lower than what have been stated in earlier literature (2-4). The use of tissue culture as a gold standard results in a lower estimate of sensitivity of ELISAs than when fecal culture is the gold-standard. However, the ROC analysis indicated

that, with the cattle tested here, it did not matter which standard you used because ultimately the tests were inaccurate with either standards as the comparison.

Implementing ELISA tests to identify cattle infected with *Mptb* will produce many false negative and some false positives. Implications of using these tests on a herd level do vary depending on prevalence of infection and degree of bacteria shedding within a herd. With such low levels of sensitivity, the usefulness of these tests on low prevalence herds is questionable. In high prevalence herds, the tests will be reasonably more effective at identifying high shedding cattle, and not the low to intermittent shedding cattle. However, for infected cattle that are either not fecal shedding or shedding in low to moderate levels, the accuracy of these three ELISAs was very poor and would be of questionable value in most herds.

5.6. References

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Table 5-1. Results of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* evaluated based upon tissue or fecal culture status.

	Sensitivity (95 % CI)	Specificity (95 % CI)
ELISA A		
Tissue culture[1]	8.8 % (4.4 – 13.1 %)	97.6 % (96.6 – 98.6 %)
Fecal culture[2]	16.7 % (4.5 – 28.8 %)	97.1 % (96.0 – 98.1 %)
ELISA B		
Tissue culture[1]	6.9 % (3.0 – 10.8 %)	96.0 % (94.7 – 97.4 %)
Fecal culture[2]	13.9 % (2.6 – 25.2 %)	95.9 % (94.6 – 97.2 %)
ELISA C		
Tissue culture[1]	16.9 % (11.0 – 22.7 %)	90.8 % (88.8 – 92.7 %)
Fecal culture[2]	27.8 % (13.1 – 42.4 %)	90.1 % (88.2 – 92.0 %)

[1] 160 tissue culture-positive and 834 tissue culture-negative samples
[2] 36 fecal culture-positive and 958 fecal culture-negative samples

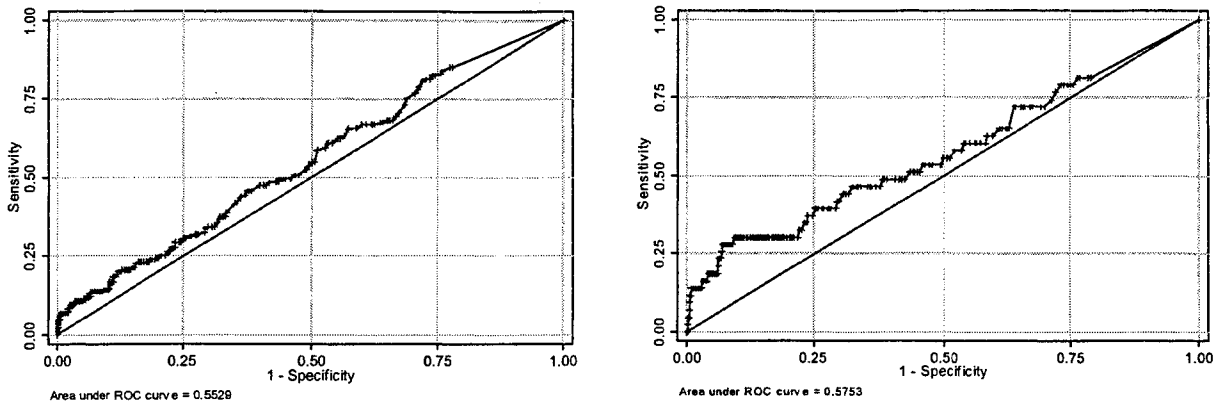

Figure 5-1. Receiver operator curves for ELISA A for *Mptb* using tissue culture (left) and fecal culture (right) as a gold standard.

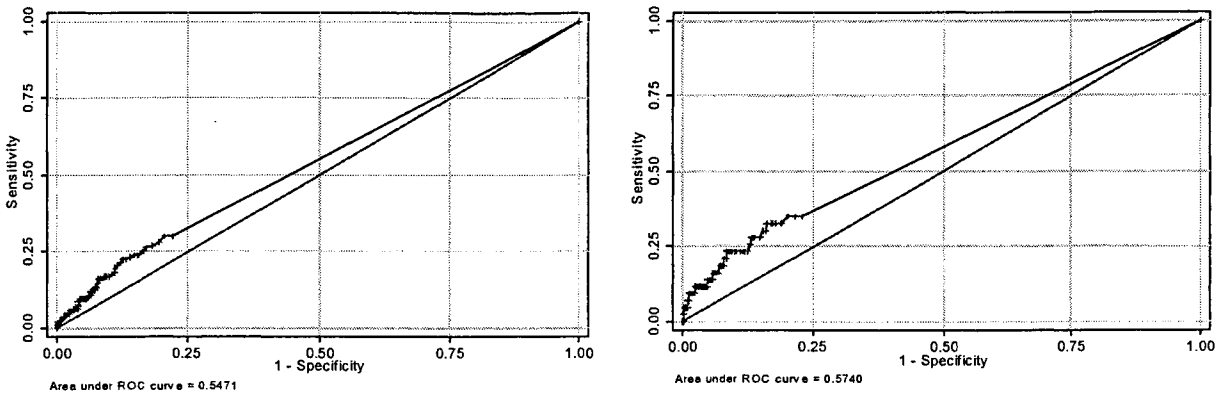

Figure 5-2. Receiver operator curves for ELISA B for *Mptb* using tissue culture (left) and fecal culture (right) as a gold standard.

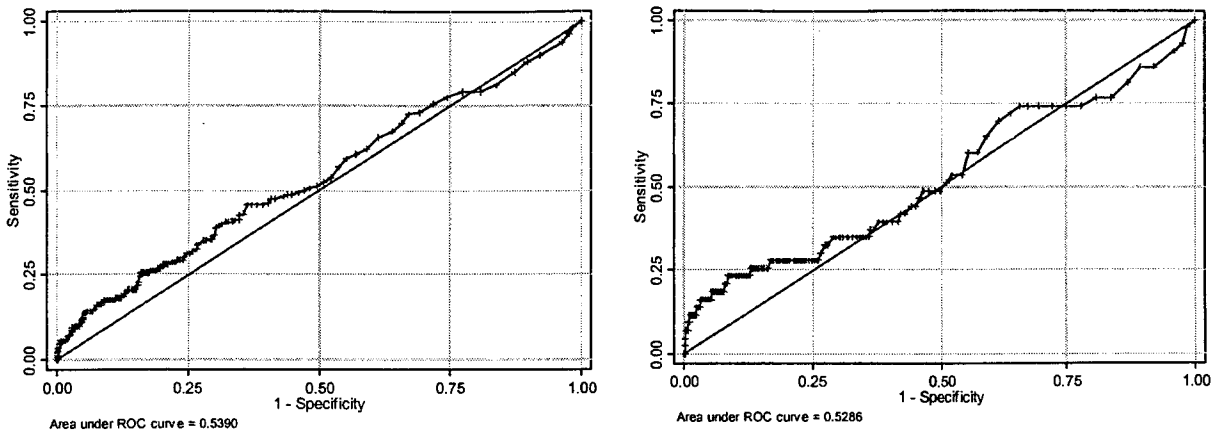

Figure 5-3. Receiver operator curves for ELISA C for *Mptb* using tissue culture (left) and fecal culture (right) as a gold standard.

CHAPTER 6. AGREEMENT BETWEEN THREE ELISAS FOR *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN DAIRY CATTLE

Submitted to Veterinary Microbiology.

6.1. Abstract

During a ten-month period in 1999, 994 serum and tissue samples were collected from dairy cows at slaughter in eastern Canada. The sources of these cattle were from all four Atlantic Canadian provinces along with some cows from the state of Maine. The sera were used to assess the agreement of three commercially available ELISAs for *Mycobacterium avium* subsp. *paratuberculosis*. Two ELISAs were indirect absorbed ELISAs licensed for use in North America, the third was an indirect non-absorbed ELISA licensed for use in Europe. Overall, there was poor agreement between the three ELISAs. The highest and lowest kappa values were 0.33 and 0.18, which is fair and poor agreement, respectively. However, when only tissue culture–positive cattle were compared, the ELISAs had better agreement (kappa = 0.37 to 0.51). The proportions of positive tests, however, were significantly different among the three ELISAs. The poor agreement among the three ELISAs is as conerning as the fact that these tests have low sensitivity. The implications are greatest when the tests are used at the cow level to make individual animal decisions, which is not the recommended method on the product labels. At the cow level, if the result obtained from one ELISA is positive, using a different ELISA in a pre-clinical animal has a high likelihood of giving a different result due to low predictive values of positive test results.

6.2. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Mptb*) is the cause of Johne's disease (JD), which is an important disease of cattle. The relative importance of this disease to cattle

has recently been elucidated with respect to direct costs (1-5) as well as a possible link to Crohn's disease (6). These facts along with others have highlighted the importance of controlling the spread of *Mptb* in numerous countries and have resulted in the formation of various control strategies.

As part of most control strategies, whether at the national level or at the farm level, there is an aspect of testing for infected cattle. Sometimes the testing is used as a tool to estimate prevalence, and other times it is used to diagnosis the status of the individual animal. Diagnosis of infection with *Mptb* in individual live animals can be made by various methods. The advantage that the ELISA has over fecal culture is the accessibility to diagnostic labs, quick reporting of results, and low cost per test sample. However, the ELISA for *Mptb* has the disadvantage of low sensitivity in low fecal shedding cows (7,8).

There are multiple commercial ELISAs available for *Mptb*. In North America, there are three companies that manufacture kits to diagnosis *Mptb* that are USDA approved[a,b,c]. Despite the fact they are marketed in North America as herd-level diagnostic tools, they are commonly utilized as cow-level tests. The wide availability of these various ELISAs within diagnostic laboratories throughout North America provides the means to utilize whichever test one prefers.

Numerous comparisons of ELISAs have been performed in various locations around the world, but the primary focus has been the diagnostic sensitivity and specificity of the tests (9-16). The repeatability and reproducibility has also been examined (17-20), however, assessment of multiple test agreement have not been documented. The aim of this study was to compare agreement between three existing ELISAs for *Mptb* and investigate if it is

possible to use these tests interchangeably. In order to achieve that, agreement between tests was calculated using a kappa statistic for all ELISA results and a subset of ELISA results from tissue culture positive cows. The ELISAs tested were two ELISAs that are currently licensed for use in North America (ELISA A[a] and B[b]), along with a third ELISA (ELISA D[d]) that is licensed for use in Europe. The North American ELISAs are indirect absorbed ELISAs, while the European ELISA is an indirect non-absorbed ELISA.

6.3. Materials and Methods

6.3.1. Collection and storage of samples

A panel of 994 serum samples was collected from animals at a slaughterhouse located in Eastern Canada. The collection methodology has been previously described (21). In short, animals were selected based upon a systematic random sample of mature dairy cows, with the exception of ten cows purposively selected. Blood was collected free-catch upon exsanguination of the animal in two 50 ml conical centrifuge tubes (Fischer Scientific, Hampton, NH, USA). Concurrently, tissue samples were collected from the terminal ileum and associated mesenteric lymph nodes for gold standard comparison of ELISA results. The blood was then transferred to the Atlantic Veterinary College (Charlottetown, PEI, Canada) where the blood was centrifuged at 1000 x g for 10 minutes, and the serum was collected from each sample. Aliquots were pipetted off and kept frozen at –20°C until the time of analysis at the individual laboratories participating in the study.

6.3.2. Tissue culture

Tissue samples cultures were performed on sections of ileum and associated lymph nodes, as previously described (21;22). Briefly, samples were processed in a stomacher for a minimum of 1 minute along with 25 ml of 0.75% hexadecylpyridinium chloride (HPC) solution as decontaminate. Each sample was then left undisturbed for 30 minutes to allow for separation of tissue. A 10-ml sample of the pulverized fluid was transferred into a sterile tube containing a further 10 ml of HPC solution, used for the second decontamination stage in the procedure. After a minimum of 3 hr in the second decontamination stage, samples were centrifuged at 900 x *g* for 30 min. The supernatant was discarded and the pellet was re-suspended with a combined half strength brain-heart-infusion (BHI) broth that contained 0.1% NAL, 0.1% VAN, and 0.05% AMB. This mixture was vortexed and placed into sterile cryogenic tubes to be incubated for 12-14 hr at 37°C to allow for *Mptb* growth and to allow an opportunity for the antimicrobials to more effectively decrease levels of background contaminants. Incubation of prepared samples was performed using the VersaTREK™[e] broth solution media with PCR confirmation (21).

6.3.3. Analysis of serum samples

The samples were analyzed with three commercial indirect ELISAs, two licensed for the North American market (ELISAs A[a] and B[b]), and one licensed for the European market (ELISA D[d]). The tests were performed according to the manufacturers' instructions. The ELISAs licensed for North America were performed at the Wisconsin Veterinary Diagnostic

Laboratory while the European licensed assays were performed at the at the Svanova research facility in the Uppsala Science Park, Sweden.

6.3.4. Comparison of test features

The two North American ELISAs functioned similarly and were based on detection of antibodies to protoplasmic antigens for *Mptb*. Both of these ELISAs were absorbed ELISAs, with the use of *Mycobacterium phlei* in the absorption phase. The application of these assays was performed at a laboratory in which both assays had either been previously, or was currently, in use at that location, and staff were familiar with both assays. The European licensed assay functions quite differently. It was based on detection of antibodies to lipoarabinomanan (LAM) and was a non-absorbed ELISA. Both laboratory facilities were blinded to the outcome of tissue culture status of cattle and the outcome of ELISAs performed in the other facility.

6.3.5. Test outputs

The ELISA A reported the analyzed optical densities (OD) as an s/p ration (sample OD to positive control OD ratio). The ELISA B reported a score value, which is assessed in relation to the cut-off that is determined by the mean of the negative controls plus 0.100. The ELISA D reported a pp (percent positive) value, which was based on a regression analysis of log-log transformed or normalized OD values. The calculation involved generating a linear regression of the blanked OD values and "log-log" transformed sample OD values and using the inverse slope of this line multiplied by the log of the OD of the sample, then inverse

logged to arrive at the pp-value[d]. This calculation was performed to standardize the linear relationship of the test values with corresponding increases in antibody levels. Scatterplots were generated to visualize the correlation between the test results of the three ELISAs (Figures 6-1 – 6-3).

6.3.6. Statistical Analysis

Statistical analysis was performed using Stata 8.0™[f]. Level of agreement beyond that expected due to chance alone was determined between the three ELISA by calculating kappa statistics. The McNemar X^2 test was used to compare paired population proportions of results for each of the possible combinations of ELISA results. A significant McNemar test indicated serious disagreement between tests, producing a biased kappa. A kappa statistic was calculated for a subset of tissue culture-positive cattle. This was done to enable a more accurate assessment of kappa statistics, due to the difficulties associated with interpreting kappa values when there is low prevalence (23). Due to large differences in scale and lack of a linear relationship between the outputs for the different ELISAs, a Pearson correlation coefficient was not calculated.

6.4. Results

The frequency of positive results among the 994 samples for ELISA A, B, and D were 3.4% (34/994), 4.4% (44/994), and 10.5% (104/994), respectively. The agreement on positive or negative classification between the tests varied from fair to poor. Comparisons made between ELISA A and D resulted in the highest agreement with a kappa value of 0.33

(95% CI, 0.25 – 0.41). Comparison of ELISA B to D had the lowest agreement (kappa = 0.18; 95% CI 0.09 – 0.27) and the agreement between ELISA A to B was intermediate (kappa = 0.25; 95% CI 0.11 – 0.39).

There were highly significant McNemar test results for all three comparisons ($p<0.001$). Therefore, kappa statistics were also calculated for the subset of tissue culture-positive cattle (Tables 6-1– 6-3). The level of agreement between the various ELISAs on tissue culture-positive cattle ranged from fair to moderate (kappa = 0.37 to 0.51). In all combinations of ELISAs, proportions of positive results between each pair of tests were all still significantly different (all p values < 0.001).

Probabilities of predictive values of one positive test versus another positive test from combinations of ELISAs can also be calculated from Tables 6-1– 6-3. The probability of a tissue culture-positive animal testing positive on ELISA A given it was positive on ELISA B was 55% (Table 6-1). The probability of a tissue culture-positive animal testing positive on ELISA B given it was positive on ELISA D was 30% (Table 6-2). The probability of a tissue culture-positive animal testing positive on ELISA A given it was positive on ELISA D was 44% (Table 6-3).

Figures 6-1– 6-3 demonstrate graphically the proportion of agreement and disagreement of the various combinations of ELISA results. The addition of vertical and horizontal lines at the recommended cut-off levels for each ELISA aid in the interpretation of agreement and disagreement between the tests by quadrants. The ELISAs had best agreement on ELISA negative samples. However, in Figures 6-1 and 6-2, there are numerous test results in the lower right quadrant, indicating that ELISA D was positive, yet

ELISAs A and B respectively, were strong negatives. In Figure 6-3, the plotted coordinates of ELISA A and B in the upper left and lower right quadrants were found mainly around the cut-off values and not at extreme values. This indicates that the disagreement is largely among samples that were marginally positive for either test.

6.5. Discussion

The agreement beyond that due to chance between the three ELISAs presented here ranged from fair to poor when the full dataset of samples is used and ranged from fair to moderate when tissue culture-positive samples were used. Although it is essential to estimate diagnostic performance variables such as sensitivity and specificity for diagnostic tests, it is equally important to assess the agreement between tests for practical application of the assays. That is especially true when access to various ELISAs is readily available. The ELISAs licensed for the North American market are specified to be used as herd–level tests. However, they are often used to make decisions on the individual cow level. It is at this level of use, which is not recommended on the product labels, that there is the greatest potential for confusion when two ELISAs are used on the same animal, and arrive at discrepant results.

Additionally, even when used as a herd–level test, this poor agreement and low predictive values on positive test results can still have a detrimental effect. In low prevalence or negative herds these tests potentially will provide erroneous and conflicting information that would be used to estimate herd prevalence. This may be cause for confusion and dissatisfaction with participants in herd control programs.

The two ELISAs marketed for North America (A and B) utilize similar antigens and are both absorbed ELISAs, yet they had quite poor agreement. The European ELISA D was based on a different antigen and is a non-absorbed ELISA, yet it had the highest agreement when compared to ELISA A. The relative importance of the kappa statistics, however, needs to be examined based upon the distribution of agreement or prevalence of positive samples. It is for that reason that Tables 6-1to 6-3 included only animals that were tissue culture–positive, to increase the prevalence of positive samples in reasonable manner. Under this scenario, the kappa values improved dramatically.

The scatter-plots illustrate that there were a large number of cattle that had negative results on two ELISAs, although there were cases where an animal had an extremely low value on one ELISA and a high value on a second ELISA. Overall, this relationship on negative cattle reinforces the point that these tests have a relatively good specificity (8,24,25) and therefore adequate positive predictive values in moderate to high prevalence herds. In herds with a low prevalence, however, the positive predictive value can still be poor in apparently healthy cows due to the vast majority of animals not being infected (26). However, due to their lower sensitivities, especially for low fecal shedders (7,8), the negative predictive value of the ELISAs are poor, especially in high prevalence herds. In low prevalence herds, the negative predictive values of ELISAs are considered to be acceptable because there are very few truly infected cattle. In reality, for ELISA positive samples, the likelihood of getting a different result if a different ELISA is used is actually high due to the relative lack of agreement on positive samples, as shown by Tables 6-1 to 6.3. This is

particularly true for samples positive with ELISA D, and for ELISA A and B samples that fall close to the cut-off for being positive.

The cattle in this dataset were also non-clinical cows in good body condition (21). The ELISAs are potentially useful in estimating prevalence within herds or discriminating between high and low prevalence herds, keeping in mind that their sensitivity is low in non-fecal shedding animals (7). In conclusion, agreement beyond that due to chance between tests was limited on tissue culture-positive animals and was even lower on tissue culture-negative animals. Therefore, the use of a combination of tests to identify infected cattle is of limited value. Using these herd level tests in combination as a decision tool on the individual animal gives poor agreement results and makes accurate interpretation of disjoint results impossible.

6.7. Sources and Manufacturers

[a] Herdchek® ELISA, IDEXX Laboratories, Westbrook, Maine

[b] Parachek® ELISA Biocor Animal Health, Omaha Nebraska, currently owned by Pfizer Animal Health

[c] Synbiotics, San Diego, California, USA

[d] SVANOVIR™ ELISA, Svanova Biotech, Uppsala Sweden

[e] TREK Diagnostic Systems, Cleveland, Ohio, USA

[f] Stata Corporation, College Station, Texas

6.7. Acknowledgements

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Table 6-1. Cross-classification of results of ELISA A and ELISA B for *Mycobacterium avium* subsp. *paratuberculosis* from serum samples of 160 cows that were tissue culture-positive for *Mycobacterium avium* subsp. *paratuberculosis*.

	ELISA B		
ELISA A	Positive	Negative	Total
Positive	6	8	14
Negative	5	141	146
Total	11	149	160

Kappa = 0.45 (95% CI, 0.23 to 0.67), P – value for McNemar test < 0.001

Table 6-2. Cross-classification of results of ELISA B and ELISA D for *Mycobacterium avium* subsp. *paratuberculosis* from serum samples of 160 cows that were tissue culture-positive for *Mycobacterium avium* subsp. *paratuberculosis*.

	ELISA D		
Results for ELISA B	Positive	Negative	Total
Positive	8	3	11
Negative	19	130	149
Total	27	133	160

Kappa = 0.37 (95% CI, 0.19 to 0.55), P – value for McNemar test < 0.001

Table 6-3. Cross-classification of results of ELISA D and ELISA A for *Mycobacterium avium* subsp. *paratuberculosis* from serum samples of 160 cows that were tissue culture-positive for *Mycobacterium avium* subsp. *paratuberculosis*.

	ELISA A		
Results for ELISA D	Positive	Negative	Total
Positive	12	15	27
Negative	2	131	133
Total	14	146	160

Kappa = 0.51 (95% CI, 0.34 to 0.68), P – value for McNemar test < 0.001

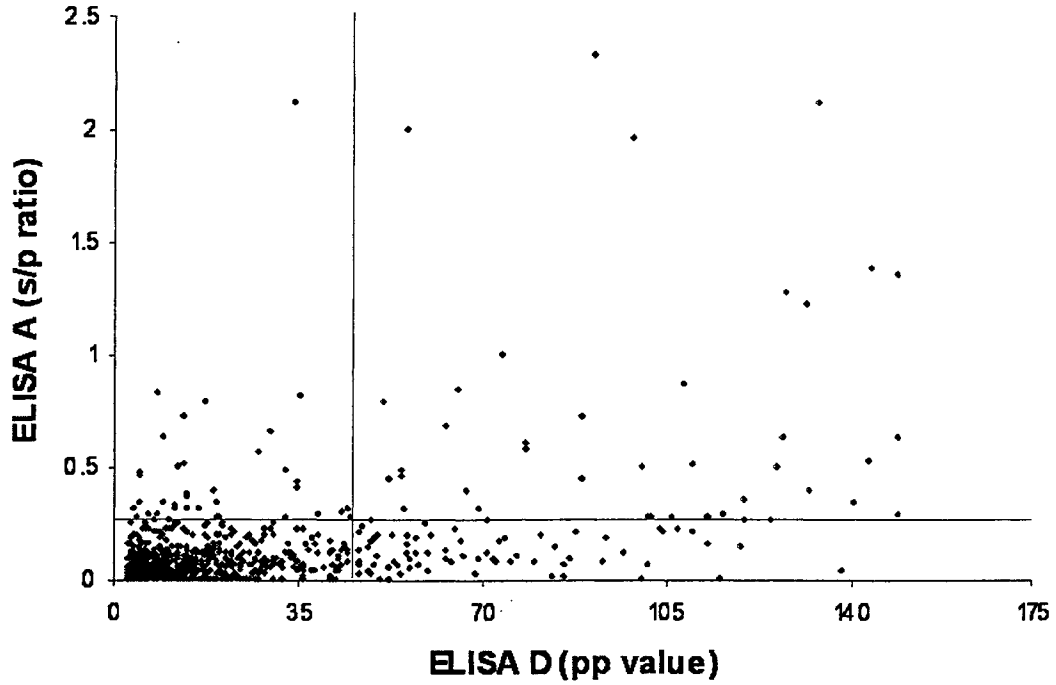

Figure 6-1. Scatterplot of s/p ratios from ELISA A compared to pp values of ELISA D, overlaid by horizontal and vertical lines indicating the recommended cut-off values for each ELISA, for 994 cows.

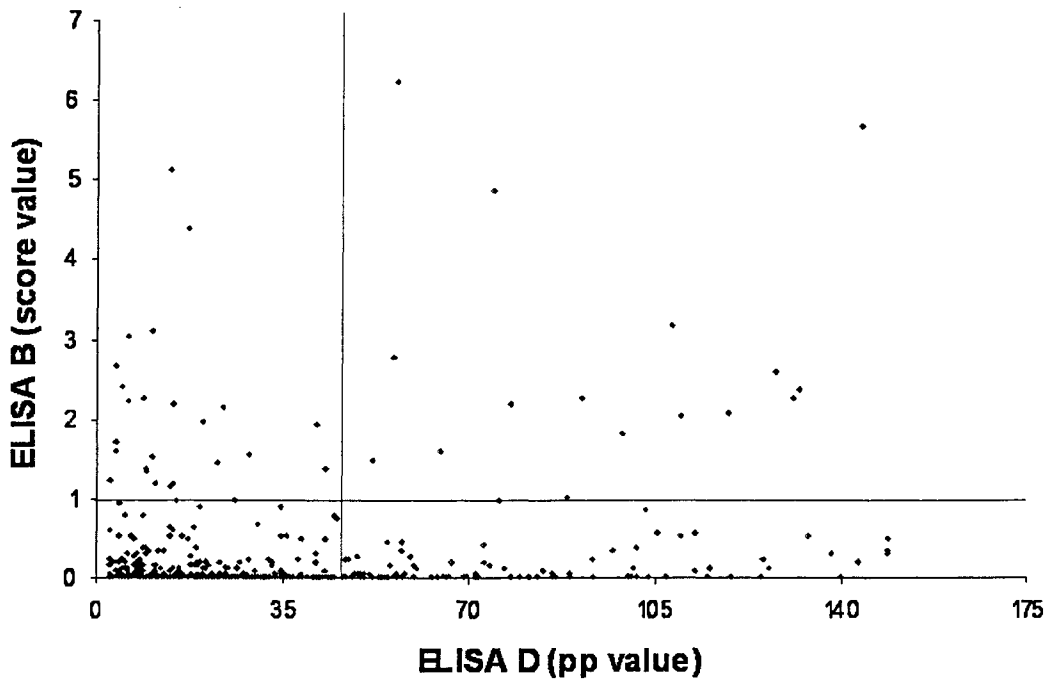

Figure 6-2. Scatterplot of score values from ELISA B compared to pp values of ELISA D, overlaid by horizontal and vertical lines indicating the recommended cut-off values for each ELISA, for 994 cows.

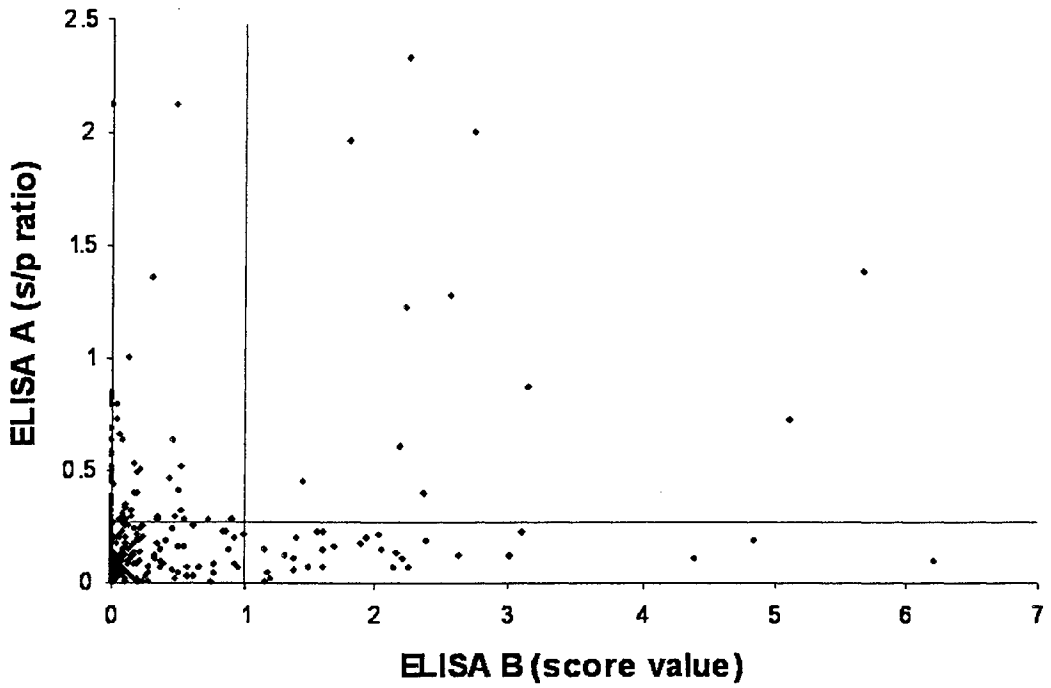

Figure 6-3. Scatterplot of s/p ratios from ELISA A compared to score values from ELISA B, overlaid by horizontal and vertical lines indicating the recommended cut-off values for each ELISA, for 994 cows.

CHAPTER 7. EXAMINATION OF FECAL POOLING STRATEGIES FOR DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

7.1. Abstract

Feces collected from 994 dairy cows sampled at slaughter were cultured for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*), both individually and as composite pooled samples using the TREK ESP® Culture System II broth media. The composite samples consisted of pools containing 3, 5, 8, 10 and 15 cows. The number of individual fecal culture-positive animals within each pool varied from 1 to 4. Pooling was performed by mixing fecal samples from animals that were tissue-positive and therefore presumed to having a high probability of being fecal-positive. Culture of individual fecal samples detected *Mptb* in 36 (3.6%) of the 994 cows. In total, 840-pooled fecal samples were examined for presence of *Mptb* and of that, 272 pools actually contained feces from fecal culture-positive animals. The overall crude sensitivity (proportion of pools positive that contained a positive animal) for pools of 3, 5, 8, 10, and 15 were 47, 67, 44, 59, and 39%, respectively. The sensitivity for pools of 3 containing 1 positive or 2 positive samples was 35 and 100%, respectively and for pools of 5 it was 62 and 90%. The sensitivity for pools of 8 containing 1, 2, or 3 positive samples was 38, 63, and 100%, respectively. For pools of 10, the sensitivity of pools with 1, 2, 3, or 4 positive samples was 52, 77, 67, and 50% while pools of 15 was 38, 33, 50, and 67%, respectively. By dichotomizing the pools into those consisting of individuals that were early detected (<40 days) and those late detected (>40 days) by the TREK ESP® Culture System II, the sensitivity of pools changed significantly. The sensitivity for pools having early detected individuals for pools of 3, 5, 8, 10, and 15 were 88, 93, 66, 100, and 95% and for late detected individuals it was 19, 44, 33, 16 and 3%, respectively. All pool sizes were sensitive at detecting pool containing cows that were early

detected individually. A low prevalence herd simulation indicated that using pools of 5 or 10 had similar diagnostic capabilities, allowing for a cost savings by utilizing pools of 10.

7.2 Introduction

Johne's disease (JD) in cattle is an infectious, chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). This disease typically infects cattle in the early stages of life without overt clinical signs, but can lead to decreased milk production, loss of body condition, and intermittent diarrhea later in life. This disease is widespread in cattle populations around the world. Besides the economic losses incurred by decreased production, there have also been associations to early culling of infected animals (1). Evidence to an association between Crohn's disease and JD has also been found (2).

Besides being an important disease of the individual animal, in most cases, JD is generally a herd problem. Identifying infected herds and implementing strategies to decrease the prevalence and minimize the spread of disease in these herds is the basic goal of control programs for JD. Many countries have implemented control programs for JD and the majority of the programs currently use fecal culture testing for *Mptb*. A disadvantage of fecal culture to diagnosis positive herds is the cost. For that reason, pooling fecal samples in aggregates of various sizes has been considered as an option.

Early investigation of culturing pooled fecal samples reported a decrease in the sensitivity of detecting infected cattle in pools of 10 (3). However, more recent studies have indicated that the sensitivity of a pooled fecal sample can be adequate. The sensitivity for pools of 10 animals was 69% (25 of 36 pools) (4) from cows pre-selected based upon

positive ELISA results. The sensitivity for pools of 5 animals was 81% (26 of 32 pools) using a modified Jorgensen culture method that is different from culture techniques widely used in North America (5).

Herd level sensitivity using pooled samples of 5, based on age clustering was 73% (8 of 11 herds) using a modified Jorgensen culture method (5). In a study conducted in the United States, culture of 5 fecal samples per pool revealed a sensitivity of 94% for detection of *Mptb*-infected dairy herds (6). The herd level sensitivity using pools of 10 samples has been estimated to range from 90 to 100%, depending on the prevalence in the population (4). Only one previous study investigated the use of the TREK ESP® Culture System II as a viable method to culture pooled samples (4). In that study, however, it could not be determined whether recovery of positive pools was different between the broth system and solid media, because that study was limited to 87 samples per method.

The purpose of this study was to determine the sensitivity of fecal pooling using the TREK ESP® Culture System II, and compare the sensitivity of various pool sizes using the same fecal material and technique concurrently. Additionally, a simulation model was constructed to compare the use of pools of 5 and 10 in two herd sizes with varying degrees of low prevalence to determine if the herd was actually positive.

7.3. Materials and Methods

7.3.1. Sample Population

The sample population and sample collection methods have been previously described (7). In short: 994 mature dairy animals were selected at slaughter for collection of

tissue samples and feces. Two lymph nodes in the mesenteric chain in the region of the ileum were collected along with a 5 to 10 cm segment of terminal ileum approximately 25 cm proximal to the ileo-cecal junction. Fecal samples were collected from the posterior colon via an incision into the viscera. Samples were placed in individual containers labeled with the numeric identifier for each animal. Tissue cultures were performed initially and fecal samples were kept frozen at -80°C until tissue culture results were available so that fecal pools could be assembled such that all fecal pools (except 50 of 840) contained at least one tissue culture positive cow. This process ensured that there would be sufficient numbers of pools with fecal culture positive cows to have sufficient power to detect differences in herd level sensitivity, while still maintaining the representativeness of the pooling process compared to what would be done on a farm. Strategic pooling by age cohorts, another pooling process that has been recommended (5), was not possible in this study due to the lack of age data from the slaughter cows.

7.3.2. Tissue preparation

Tissue preparation for culture has been previously described (7; 8). Briefly, samples were placed in a Tekmar bag along with 25 ml of 0.75% hexadecylpyridinium chloride (HPC) solution as decontaminate. The sample was placed in a stomacher for a minimum of 1 minute, and then each sample was left undisturbed for 30 minutes to allow for separation of tissue. A 10-ml sample of the pulverized tissue mixture was transferred into another sterile tube containing a further 10 ml of HPC solution, used for the second decontamination stage in the procedure. After a minimum of 3 hr in the second decontamination stage, samples

were centrifuged at 900 x *g* for 30 min. The supernatant was discarded and the pellet was re-suspended with a combined half strength brain-heart-infusion (BHI) broth that contained 0.1% nalidixic acid (NAL), 0.1% vancomycin (VAN), and 0.05% amphotericin B (AMB) (Allied Monitor Inc., Fayette, Missouri, USA). This mixture was vortexed and placed into sterile cryogenic tubes to be incubated for 12-14 hr at 37°C to allow for *Mptb* growth and to allow an opportunity for the antimicrobials to more effectively decrease levels of background contaminants. After the incubation phase, the tubes were slowly cooled and then re-frozen at -80°C.

7.3.3. Fecal preparation

For each cow, a two-gram sample of feces was weighed and placed in a 50 ml centrifuge tube along with 35 ml of distilled/deionized water. This mixture was vortexed and then placed on a shaker-table for 30 min to allow for thorough mixing and agitating of the mixture. The tubes were then left to stand for 30 min to allow particles to settle out, and then 5 ml of sample were drawn off of the top third of the tube and transferred to a second 50 ml centrifuge tube where it was mixed with 25 ml of 0.9% HPC solution mixed with half strength BHI broth (final concentration of 0.75% HPC) as decontaminate. The sample tubes were then incubated at 37°C for 24 hr. Then each tube was placed in a new centrifuge tube and was centrifuged at 900 x *g* for 30 min. The supernatant was then discarded and the pellet was re-suspended with a combined half strength BHI broth that contained 0.1% NAL, 0.1% VAN, and 0.05% AMB. This mixture was vortexed and placed into sterile cryogenic tubes,

which were subsequently incubated for 12-14 hr at 37°C similar to the tissue samples. Finally, the samples were slowly cooled and then re-frozen at -80°C.

7.3.4. Pooled fecal sample preparation

Based upon results from the tissue culture that was initially performed, pools were formulated in batches of 3, 5, 8, 10 and 15. The assumption made was that tissue culture-positive animals would more likely be fecal culture-positive. This allowed for individual and pooled samples to be processed simultaneously, avoiding additional freezing and thawing of samples and subsequent potential loss of viable organisms. The distribution of tissue culture-positive animals among pools varied from 0 to 5 (Table 7-1).

In constructing the pools, 2 grams of feces from each animal designated in the pool was mixed using disposable plastic sticks and disposable weigh-boats. After thorough mixing to a homogenous matrix, 2 g was weighed out for the pooled fecal culture. The same culture procedure was then used as described for the individual fecal samples.

7.3.5. Culture Technique

All samples were cultured using the TREK ESP® Culture System II broth solution media bottle (TREK Diagnostic Systems, Cleveland, Ohio, USA). Samples were thawed, and 1 ml from each of the tubes was inoculated into a TREK® broth solution media bottle, supplemented with egg yolk, antibiotics (0.1% NAL, 0.1% VAN, and 0.05% AMB) and a growth supplement containing Mycobactin J (Allied Monitor Inc., Fayette, Missouri, USA).

All sample bottles were then incubated for 56 days, unless they were identified as positive by the TREK ESP® Culture System II diagnostic incubator. As samples were identified as positive for bacterial growth by the incubator, in turn each bottle was then examined for acid-fast bacteria. After 56 days, any remaining samples were also examined for acid-fast bacteria, to identify samples that contained low numbers of bacteria unable to trigger the system. For this process, each bottle was vigorously shaken for a minimum of 60 sec using a Mistral multi-mixer (Lab Line Instruments Inc., Melrose Park, Illinois, USA). A sample (20 µl) from each bottle was placed on a microscope slide, for a total of 3 samples per slide. The slides were air-dried and then heat fixed and a cold Ziehl-Neelsen staining process was performed on each slide (9). Each slide was then examined at oil-immersion (100x) for the presence of acid-fast bacteria. All samples that were acid-fast positive were then tested using a polymerase chain reaction technique (PCR).

7.3.6. PCR technique

The procedure for PCR analysis has been previously described in detail (7;10). For DNA extraction, lysis by the boiling method was used by heating a mixture of sample with 100 µl of lysis buffer containing HPLC grade water with 1% Triton®-X-100 (Fisher Scientific, Pittsburgh, Pennsylvania, USA), 1 mM EDTA and 10 mM Tris-HCL (pH 8.0). The primers used for the IS900 and F57 genetic elements have previously been described (11;12). The forward primer designated IS900/150C and the reverse primer designated IS900/921 were used for IS900; the forward primer designated F57a and the reverse primer designated F57b were used for F57.

Amplification reactions were performed on a total volume of 50 µl containing 10 mM Tris-HCL (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCL; 0.001% gelatin; 200µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP); 1 µM of each primer; 1.25 U of Amplitaq (Applied Biosystems, Foster City, CA) and 5 µl of the boiled cell lysate for the monoplex PCR. The PCR assay was carried out in a Perkin-Elmer 2400 thermocycler (Perkin Elmer Corp., Norwalk, CT). A sample from a TREK ESP® broth bottle containing an ATCC strain (# 19698) of *Mptb* was used as the DNA-positive control. The negative control was a reaction mixture containing all reagents but no DNA template. The PCR products were visualized by electrophoresis on a 1% agarose gel following standard procedures. Bacterial isolates positive to either F57 or IS900 genetic elements were classified as positive for *Mptb*.

7.3.7. Statistical analysis

Statistical evaluation was performed using Stata 8.0™ (Stata Corporation, College Station, Texas) and Excel (Microsoft Corporation, 2000). Confidence intervals for proportions were calculated according to the efficient-score method based on the Wilson estimate of population proportion, due to the situation of small proportions (13). Significance of proportion comparisons was tested using a two-sided Z test. For interpretation of results, a positive pool was defined as a pool that contained feces from at least one cow that was individually fecal culture-positive. A positive pool test result was a pooled sample from which *Mptb* was isolated. Herd sensitivity was defined as the ability to detect at least 1 fecal culture positive animal in an infected herd. The herd simulation was

performed in Stata 8.0™ by creating a simulation loop to place in turn either 1, 2, or 5 infected cows into either pooled fecal samples of 5 or 10 and count the number of times each possible pattern of results would occur. This simulation was set to contain 1000 iterations and was tested for herds of 50 and 100, assuming all cows in the herd would be tested. The simulation determined the probability that the shedding cows would be individually spread out among the pools or clustered within differing numbers of pools. For each of the possible pooling patterns with differing numbers of shedding cows per pool, the probability of a herd with that pooling pattern testing positive was also determined. A weighted mean herd probability was calculated for 1, 2 and 5 shedding cows to determine the average probability that the herd would have at least one test positive pool.

7.4. Results

7.4.1. Culture of individual fecal samples

Out of the 994 animals tested, 160 (16.1%) were tissue culture-positive, while 36 (3.6%) were fecal culture-positive. Of the 36 fecal culture-positive cows, 5 (3.1%) were tissue culture-negative.

7.4.2. Culture of pooled fecal samples

In total, 840 pools were created of which 790 contained one or more tissue culture-positive cows. Of these, 272-pooled samples contained feces from fecal culture-positive cows. The overall sensitivity of fecal culture pools, averaged over all combinations within pool size, varied from 67% in pools of 5 to 39% in pools of 15 (Table 7-2). The sensitivity

of pools of 3, 8, and 15 was lower compared to pools of 5 and 10. Comparing the sensitivity of pool size to identify one positive animal within each pool, pools of 5 (62%) and 10 (52%) had the highest sensitivity.

Sensitivity within pool size increased as the number of fecal culture-positive cows increased (Table 7-2). Increasing from 1 to 2 positive cows in pools of 3, the sensitivity increased from 35 - 100%. A similar, but non-significant pattern was seen in pools of 5, 8 and 10 but the same pattern was not seen in the pools of 15. Time to positive detection in the individual samples was used to classify the pooled samples containing one positive sample (Table 7-3). This was done to correct for pools having a potential high shedder increasing the probability of a positive pool over the probability of a pool containing a low shedder. Pools containing early-detected individual samples had a higher probability of resulting in a positive pool in all pool sizes. The sensitivity to detect high shedders was highest in pools of 10 (100%), which was not different from pools of 3, 5, and 15 (88, 93, and 95%, respectively), but was different from pools of 8 (66%).

Pools of 8 had a significantly higher proportion (85%) of late-detected individual samples compared to pools other than the pools of 3 (80%). Pools of 3 in which there were individuals that triggered positive between 41-50 had a significantly lower sensitivity than other pools of that time category. In that time category, 5 of the 8 samples that tested negative in pools of 3 included a sample from the same individual cow. The fecal sample of this particular cow was included in 5 other pools. None of these pools were culture-positive for *Mptb*.

7.4.3. Herd level estimations

The sensitivity for having at least one positive pool as determined by the herd test simulation using the sensitivity estimates for individual pools, was found to be similar whether it was a 50 or 100-cow herd, assuming all cows would be tested. As a result, only the 100-cow simulation is displayed (Table 7-4). Using pools of 5 with 1, 2 or 5 fecal culture-positive animals, the weighted herd sensitivity would be 62, 86.1, and 99.3%, respectively. Using pools of 10 with 1, 2 or 5 fecal culture-positive animals, the sensitivity for having at least one positive pool would be 52, 76.1, and 97.0% in a 100-cow herd, respectively (Table 7-4). If fecal pooling were performed to screen a potentially negative herd of 100 animals that had one true fecal culture-positive animal, testing with fecal pools of 5, the probability of detecting the herd as positive would be 62%. Testing the same herd with fecal pools of 10, the probability of detecting the herd as positive would be 52%. However, if there were two animals positive, they could either be in the same pool or two pools. Then the probability of determining the herd as positive in pools of 5 would be 86% if they were in two separate pools or 90% if they were in the same pool. The probability of determining the herd as positive in pools of 10 would be 77% if they were in two separate pools or 76.9% if they were in the same pool.

7.5. Discussion

Pooled fecal culture was found to be a reasonalbley sensitive method of detecting fecal shedding cattle in various pool sizes. In this manner, sensitivity is defined as the ability

of a fecal culture positive animal within a pool resulting in a positive pooled sample. This does not mean that all pools with infected animals will have a fecal culture positive animal. The difference in sensitivity across pool sizes was surprising small, ranging from 39% in pools of 15 to 67% in pools of 5. Although this difference was statistically significant, overall pool sizes did not affect the sensitivity as much as was expected. In fact, although it would seem counter-intuitive, there was no statistical difference in overall sensitivity between pools of 5 and 10.

Detection sensitivity of pooled fecal culture has also been associated with concentration of bacteria in the infected sample. Results from pooling of feces from 10 cows indicated that, compared with concurrent bacterial culture of individual infected samples, 37 to 44% of pooled samples with low bacterial concentrations yielded positive culture results and 94% of pooled samples with high bacterial concentrations yielded positive results (8). It would appear that pre-culture techniques to concentrate the bacteria are effective.

There were some divergent results among pool sizes when pools contained only one individually fecal culture positive sample. The surprising low sensitivity of pools of 3 cows with one fecal culture positive sample included could be explained if the positive samples were low fecal shedders and less likely to yield a positive pool, as seen previously (8). The sensitivity of pools of 8 was also slightly lower than what would be expected, especially compared to pools of 5.

Using time until detection as a proxy for heavy shedders has not been extensively researched, however, it has been established that there is a high correlation (14). In the pools of 8, 85% of the samples included late-detected positive samples, which was significantly

more that the pools of 5. The significantly different sensitivity in the pools of 3 (samples triggered between 40 and 50 days), was likely influenced by the fact there was one positive cow present in 5 of the 8 negative pools. This cow was also in 5 other pools (2 in pools of 8, 1 in 10 and 2 in 15), yet never triggered a positive pool. Across all pools there was dramatic increases in sensitivity when only pools containing early detectors were considered. Pools of 10 and 15 had excellent sensitivity for early detectors that was not different from pools of 5 or 3.

An integral part of attempts to control JD at the herd level is the application of diagnostic tests (15). Herd-level diagnosis is problematic in low prevalence herds using ELISAs (16), however, progress can be made in higher prevalence herds (17). That in part, is the rationale in using pooled testing strategies when *Mptb* prevalence is low. Maximizing the economic benefit without sacrificing diagnostic sensitivity is the ultimate goal of pooled sample testing. This study provided a unique opportunity to examine various pool sizes on samples that were randomly allocated and not strategically constructed based on either age or colony counts. This provides a valid model to resemble herd testing. Since the process of pooling fecal samples seems to be validated with current diagnostic methods (4-6), it adds credibility to the economic benefits of these methods, even if there would be a slight decrease in diagnostic sensitivity. A simulation study to estimate costs and benefits of whole herd testing in low prevalence herds by use of pooled fecal samples in cattle revealed that pooling reduced costs by 43% in a 100-cow herd and 71% in a 1000-cow herd (18). Similar results have been documented in the application of pooled fecal culture in sheep flocks where it has

been reported that the laboratory cost of pooled samples applied as a flock test is approximately 30% that of serologic testing (19).

The main impact of the results seen here is that the probability of detecting low prevalence herds as positive is high whether pools of 5 or up to 10 are used. There is an economic benefit of using pools of 10 over 5 in a herd of 100 cows with two positive cows. Based on the probabilities of occurance found in Table 7-4 and assumining cost of a culture is 40$, there is an approximate 30% reduction in the cost of testing using pools of 10 over pools of 5. However, if ultimately the goal is identification of all positive individual cows, using pools of 5 is just as economically viable due to the lower number of follow-up individual pools that would be necessary in a low prevalence herd (18).

Based upon the results of this study, if the goal is detecting cows that are heavy shedders, than using pools of up to 15 results in a high probability of detecting those pools. The estimate of sensitivity for pools of 3 found here would indicated that there appears to be no substantial diagnostic benefit of using pools of 3 and therefore it would be more economical to use larger pool numbers.

The data presented here also indicate that overall, using pools of 5 or 10 have similar diagnostic sensitivity. The major differences then come down to the size of herd and expected prevalence and whether the ultimate goal is to determine herd status or identification of all infected cattle. This in conjunction with economic studies (18) would suggest that it would be justifiable to utilize pools of 10 in herds that are deemed unsuspected herds as a confirmation tool, and pools of 5 in herds of higher risk of being positive.

Considering no diagnostic test is infallible at the herd level, pooled fecal culture performs similarly to what is seen with other diagnostic modalities such as individual culture. However, the economic benefits of pooled culture along with its similar diagnostic abilities make it a reasonable economic choice for herd level testing.

7.6. References

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Table 7-1. Distribution of pools indicating the number of tissue culture-positive animals for *Mycobacterium avium* subsp. *paratuberculosis* included in each pool sample (n=840).

No. tissue culture-positive	Pool size				
	3	5	8	10	15
5	-	-	-	-	10
4	-	-	-	-	10
3	-	10	10	10	20
2	50	50	50	40	50
1	100	100	100	100	80
0	10	10	10	10	10
Total	160	170	170	160	180

Table 7-2. Distribution of positive pooled fecal samples for *Mycobacterium avium* subsp. *paratuberculosis*, the number of fecal culture-positive pools and corresponding sensitivities by poolsize.

	Pools of 3			Pools of 5			Pools of 8			Pools of 10			Pools of 15		
# pos[a]	# pools	% pos.	(95% CI)	# pools	% pos.	(95% CI)	# pools	% pos.	(95% CI)	# pools	% pos.	(95% CI)	# pools	% pos.	(95% CI)
1	40	35[b A1]	(21 - 52)	42	62[B1]	(46 - 76)	39	38[A1]	(24 - 55)	31	52[B1]	(33 - 69)	55	38[A1]	(26 - 52)
2	9	100[BC2]	(63 - 100)	10	90[BC1]	(54 - 100)	8	63[A12]	(26 - 90)	13	77[AB1]	(46 - 94)	12	33[A1]	(11 - 65)
3	-	-	-	-	-	-	1	100[A2]	(46 - 100)	3	67[A1]	(13 - 98)	4	50[A1]	(9 - 91)
4	-	-	-	-	-	-	-	-	-	2	50[A1]	(3 - 97)	3	67[A1]	(13 - 98)
Total	49	47[A]	(33 - 62)	52	67[B]	(53 - 79)	48	44[A]	(30 - 59)	49	59[B]	(44 - 73)	74	39[A]	(28 - 51)

[a] Pools containing various numbers of individually fecal culture positive samples, as indicated.
[b] One positive pool of 3 did not contain an individual fecal culture-positive animal.
[A-C] Within a row, estimates without common superscript were different ($p<0.05$).
[1-2] Within a column, estimates without common superscript were different ($p<0.05$).

Table 7-3. Distribution of pooled fecal samples (and sensitivity) for *Mycobacterium avium* subsp. *paratuberculosis* for pool with one positive sampl, by pool size and time to positive dectection of individual samples.

	Pool size														
Days until	3			5			8			10			15		
positive	+	-	%[b]	+	-	%[b]	+	-	%[b]	+	-	%[b]	+	-	%[b]
< 25	5	1	15	9	-	21	4	2	15	5	-	16	6	-	11
25-30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31-40	2	-	5	5	1	14	-	-	-	8	-	26	14	1	27
Subtotal	7	1	20^{AB}	14	1	35^{A}	4	2	15^{B}	13	-	42^{A}	20	1	38^{A}
Sensitivity			88^{AB1}			93^{AB1}			66^{A1}			100^{B1}			95^{B1}
41-50	5	8	33	12	3	36	9	5	35	3	2	16	1	3	7
51-55	-	8	21	-	2	5	2	7	23	-	4	13	-	14	25
56[a]	1	9	26	-	10	23	-	10	27	-	9	29	-	16	30
Subtotal	6	25	80	12	15	65	11	22	85	3	15	59	1	33	62
Sensitivity			19^{A2}			44^{B2}			33^{AB1}			16^{AC2}			3^{C2}
Total	13	26		26	16		15	24		16	15		21	34	

[a] Indicates pools that were not detected by the TREK system but by acid-fast staining and PCR

[b] Indicates proportion of pooled samples within each pool size

[A-C] Within a row, estimates without common superscript were different ($p<0.05$).

[1-2] Within a column, estimates without common superscript were different ($p<0.05$).

Table 7-4. Simulation of possible outcomes of testing for *Mycobacterium avium* subsp. *paratuberculosis* in a 100 cow herd in fecal pools of 5 and 10, assuming there are truly either 1, 2 or 5 positive cows.

Pool size	# pos cows		Probability of occurrence (%)	Probability of a positive test (%)[c]	Weighted mean herd probability (%)
5	1	1 per pool	100	62	62
	2	1 per pool	96	86	86.1
		2 in one pool	4	90	
	5	1 per pool	64.3	99.2	99.3
		2+1+1+1	32.5	99.4	
		2+2+1	2.4	99.6	
		3+1+1	0.7	99.9[b]	
		2+3	0.1	99.9[b]	
		4+1	-[a]	99.9[b]	
		5	-[a]	100[b]	
10	1	1 per pool	100	52	52
	2	1 per pool	83	76	76.1
		2 in one pool	17	77	
	5	1 per pool	33.4	97.2	97.0
		2+1+1+1	51.4	97.4	
		2+2+1	9.3	97.5	
		3+1+1	4.7	92.3	
		2+3	0.9	92.4	
		4+1	0.3	97.2	
		5	-[a]	97.2[b]	

[a] Missing values occurred where the probability was essentially zero
[b] Assuming the probability is equal to the next lowest available estimate
[c] Probabilities determined from sensitivity estimates in Table 7-2.

CHAPTER 8: SUMMARY AND GENERAL DISCUSSION

8.1. Introduction

The initial objective of this research, as an MSc. project, was to evaluate the diagnostic characteristics of a non-absorbed European ELISA for Johne's Disease (JD). It was anticipated that this ELISA would have higher sensitivity than two of the currently licensed absorbed ELISAs in North America. Based on the results from this project and the apparent inability of all tested ELISAs to detect infected animals, a new focus was created as the research was expanded for a PhD.

In the initial stages, the specificity of the three diagnostic tests was compared. Although it was anticipated that there would be a decreased specificity of the non-absorbed ELISA, the lack of a significant increase in sensitivity was unexpected (Chapter 2). While collecting slaughterhouse samples for a more rigorous gold-standard, it was noted that the prevalence of JD in Atlantic Canada was higher than any previous estimate (Chapter 3). Also, there was no indication that sub-clinical JD had any impact on body condition of the infected cattle. It was also determined that fecal culture as a "gold-standard" was significantly less sensitive than tissue culture (Chapter 4). When using these gold-standard comparisons, all three ELISAs performed poorly and were unable to accurately predict culture status (Chapter 5). More importantly, the agreement between the three ELISAs was remarkably poor (Chapter 6). The most encouraging findings with respect to diagnostic test evaluation was the fact that fecal pooling appears to be a valid herd level test, that is economically feasible (Chapter 7).

8.2. ELISA diagnostic characteristics

Absorption procedures have been added to ELISAs for JD to decrease the amount of non-specific reactions and increase specificity, yet potentially decrease their sensitivity (1). The effect on specificity was demonstrated when the non-absorbed ELISA was used on fecal culture-negative cows from negative herds in Wisconsin (Chapter 2). The specificity of the non-absorbed ELISA (87.9%) was lower than the comparison absorbed ELISA (98.4%). Previously, there was an indication that the non-absorbed ELISA would have a higher sensitivity when compared to typical absorbed ELISAs (2); this was, however, not clearly demonstrated in two comparisons done here. When compared to fecal culture of cows from positive herds in Wisconsin, the sensitivity of the non-absorbed ELISA (65.5%) was actually lower than the absorbed ELISA (72.4%) (Chapter 2). Comparing the non-absorbed to the two absorbed ELISAs using tissue culture results from slaughterhouse samples, the sensitivity of the non-absorbed ELISA (16.9%) was only significantly different from one of the absorbed ELISAs (6.9%), yet not the other (8.8%) (Chapter 5). Test accuracy, as determined using ROC curves, indicated that all three ELISAs tested lacked the ability to accurately identify animals as positive or negative when compared to tissue culture results, with AUC of 0.54, 0.55, and 0.55 for the non-absorbed and two absorbed ELISAs, respectively (Chapter 5).

8.3. ELISA agreement

Despite the poor sensitivity at the individual cow-level, and the fact that they are marketed as herd-level diagnostic tests, the ELISAs will continue to be used as individual cow tests by a number of individuals. Part of the reasoning for this is the fact that some

individuals promote their use and report sensitivities of different tests that make them more appealing than other tests, depending on the standard of comparison. A major problem, however, is that although there are different ELISAs available on the market with various estimates of reported sensitivity, rarely are they evaluated simultaneously on the same samples (Chapter 6). If they do not agree, the value of application of these tests on the individual cow decreases dramatically. If the result from one ELISA is unsatisfactory, simply retesting with another ELISA will likely result in a different conclusion. That is the case with the ELISAs evaluated in this thesis, with the highest agreement beyond that due to chance being a kappa of 0.51 and the lowest agreement was 0.37 (Chapter 6). This could have a major effect in the perception of test and control programs in the dairy industry.

The low agreement between ELISAs is as important if not more important than which test has the highest sensitivity, because sensitivity numbers fluctuate depending on the definition of what constitutes a positive cow (Chapter 5). In other words, the sensitivity changes dramatically based on the gold-standard comparison. Clinical and high fecal shedding subclinical animals will likely have a positive ELISA result, while subclinical and low/intermittently shedding cows may not. Using tissue culture over fecal culture as a gold-standard encompasses a higher percentage of infected animals and consequently yields a more revealing estimate of sensitivity (Chapter 4).

8.4. Pooled Fecal Testing

Pooled fecal testing has been considered an intriguing potential method of evaluating herds for presence of *Mptb*, however, the full implications of this strategy have not been investigated (Chapter 7). Multiple fecal pool sizes have never been simultaneously investigated, only pool sizes of 5 or 10 have been evaluated (3-5). By comparing different pool sizes with various numbers of fecal culture-positive animals, pools of higher number of animals performed satisfactorily depending on the goal of testing (Chapter 7). Based on our findings, if the primary goal was to detect pools containing high fecal shedders, pools of 15 would likely be adequate. If the goal was to find all positive cows, regardless of shedding pattern, pools of 5 would be desired. Prevalence of infection also would affect the choice of pooling if the goal is to identify positive herds. If 5 or more fecal culture-positive cows were suspected in a herd of 50 – 100 cows and the goal was to determine if the herd was infected, pools of 10 or 15 would be adequate. In reality, there may not be one ideal pool size, that is, pool size perhaps should be determined based on pre-test assumption of prevalence in the herd tested and the primary goal of testing (i.e. herd classification or identifying cows infected).

8.5. Impacts on Canadian National Johne's Disease Control Program

The Canadian National Johne's Disease Control Program is proposed to be voluntary in nature and to contain two major components. The first aspect of the program will be a Best Management Practices (BMP) assessment based upon a herd risk assessment of critical control points in the avoidance of transmission of *Mptb*. The second component is the Voluntary Johne's Disease Herd Status Program. The proposed Herd Status Program will

have a total of 5 levels, zero through four (Chapter 1). In order to advance to level 1, as it stands now, a herd must have completed a recent BMP assessment and ELISA testing of 30 animals in their second or higher lactation. Part of the rationale for this testing is that it is inexpensive and feasible to perform. However, based on the findings of this thesis, this approach seems to have very low validity. The sensitivity of the ELISAs and agreement between ELISAs is low. As a result, using this as the entry point for a program may result in conflicting or erroneous results, which may later be contradicted by culture methods. The counter argument is that ELISA testing is convenient and will increase the likelihood of producers initiating participation in the program. However, based on findings of this thesis, utilizing fecal pooling will result in adequate herd sensitivity, along with avoiding the frustrations of false-positives in negative herds.

Pooled fecal culture allows for herds to progress from Status Level 1 to Level 4 with the current proposed program. It would be reasonable to institute pools of 5 to attain Status Level 1 in place of ELISA testing, which would provide similar assurances of even low prevalence herds would be deemed positive. As it stands now, only 30 cows are tested with an ELISA. If you tested the same cows using pooled fecal culture, there would be no loss or gain in herd sensitivity. The probability of detecting a herd with 10% prevalence as positive would be the same as using the ELISA

8.6. Future Directions

Although the research within this thesis does provide some further insight into the control of JD, there is still need for additional research. One aspect that was not discussed directly in this thesis was the process of animals becoming infected. To combat a disease in

which the understanding of how an animal truly becomes infected is difficult. It has been stated that cattle are most susceptible to infection at the early stages of life due to uptake of *Mptb* by M cells in the Peyer's patches of the ileum (Chapter 1). However, it is not known exactly when an animal is no longer at risk and if that risk truly is gone when the M cells regress as the animal ages. This is important because Best Management Practices in control programs are focused at protecting the young stock until the age of 6 months, without truly knowing if this is sufficient (Chapter 1). Attempts have been made to model the infectious process in rabbits (6), with limited success in creating infections in that species. There has been more success in infecting young calves (7;8), but these attempts have not focused on when the animal is susceptible to infection, but instead, on the immune response post-infection.

Additionally, there are still many unanswered questions as to the mechanism responsible for the progression of animals through the various stages of disease. There is some indication that interleukin enhancement may play a role in this process (9;10). However, more extensive infection models will be needed to elaborate on these findings. Ancillary information from those studies will also provide insight into the earlier detection of infected animals. Infection modeling has also confirmed that mycobacteria-specific antibodies are detectable early in the course of experimental JD, even preceding the development of specific cell-mediated responses (7). Although this indicates that the immune system does recognize the invasion of *Mptb* into the macrophages, so far it seems unlikely that this progresses to a level sufficient to support a reliable diagnostic method.

The main advantage of testing for antibodies and cell-mediated responses is the ease of sampling and potentially lower cost over fecal culture. Efforts have been made to look at

different antigenic components in an effort to improve on diagnostic tests (11;12). Again, even though advances have been made and more antigenic components of the bacteria have been found, there is still no evidence that they will provide a more sensitive diagnostic test (12).

More extensive research should be done on fecal culturing systems. Regardless of what antigen is discovered, it is unlikely that any diagnostic test will be as specific as culture. More extensive work needs to be done with broth media systems to elucidate the correlation to time of detection in the machine and colony counts on solid media to allow identification of high and low shedders. This may be important because it will shorten the incubation time required in these broth systems, which would increase throughput of the machines and make them more economically feasible for diagnostic laboratories.

Additionly to culturing, more efforts need to be made into exploring the validity of environmental sampleing for JD. Sampling of the animals environment may lead well be useful tool in diagnosising positive herds. Monitoring of water contamination around infected herds should also be investigated as a source of transfer of disease within the herd and a potenial means of spreading the bacteria to other sources via a contaminated water source.

Part of the future in controlling JD will need to be changing our traditional ways of thinking to combat this disease. The focus should shift away from diagnostic tests towards prevention of the spread of disease by implementing management control factors. Control of JD in dairy cattle and efforts towards minimizing prevalence will not likely be feasible, so long as we don't focus more on management and understanding the infection process (13). Part of that reasoning is for the most part, work done on JD focuses heavily on dairy herds,

and not beef producers. This could leave beef production systems as a great source of *Mptb* for re-introducing it back into dairies either by human activities or by wildlife species (14). As a result, total eradication is unlikely. However, control is possible with a focus on management and understanding the epidemiology of infection. What is really needed is a critical evaluation of control programs. There are many countries that have had programs in place for a number of years, many without any real investigation to find evidence that they are decreasing the prevalence of disease.

8.7. Reference List

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APPENDIX A: JOHNE'S DISEASE: CLINICAL SYMPTOMS, PATHOPHYSIOLOGY, DIAGNOSIS AND PREVALENCE IN DAIRY HERDS

Submitted to Canadian Veterinary Journal as Part I of a Two Part Series on a Literature Review of Johne's Disease

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Abstract

Recent international developments in the area of infectious disease control and non-tariff trade barriers, along with possible zoonotic concerns, have provoked a revival of interest in Johne's disease (JD) in Canada and elsewhere. The bacteria causing JD, *Mycobacterium avium* subspecies *paratuberculosis*, is distributed worldwide and causes chronic granulomatous enteritis, also known as paratuberculosis, in domestic and exotic ruminants, including cattle. The subclinical form of this disease results in progressive weight loss, reduced milk production, lower slaughter value and premature culling, with possible impacts on fertility and udder health. Eventually, infection can lead to the clinical form that manifests as chronic diarrhea, emaciation, debilitation and eventual death. Currently, available tests to detect infected animals produce many false-negative results and some false-positives, particularly in subclinically infected animals, thus making their interpretation and utilization challenging in control programs.

The objective is to critically review the literature about Johne's disease in dairy cattle for Canadian bovine practitioners. By reviewing the scientific literature about JD, control of the disease could be pursued through informed implementation of rational biosecurity efforts and the strategic use of testing and culling.

Introduction

Paratuberculosis, or Johne's disease (JD), is a chronic infectious enteritis of domestic and wild ruminants. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Mptb*), a hardy, slow growing, Gram-positive and acid-fast bacterium (1,2). Despite having 99% DNA homology (3), *Mptb* can be differentiated phenotypically from *M. avium* subspecies *avium* and *M. avium* subspecies *sylvaticum* by its dependence on mycobactin (4), and genotypically by the presence of multiple copies of an insertion element, IS900 (5,6).

Restriction endonuclease analysis has identified variations in 2 principal types of *Mptb*, a cattle type (C) and a sheep type (S) that were first identified by Collins et al. (7). Other variations have also been identified, although their importance is unclear (8-10). Paratuberculosis in cattle, goats, deer and camelids is mainly caused by type C whereas sheep are usually infected by type S. However, the cattle type can infect sheep and vice versa (11).

The natural hosts for *Mptb* are wild and domesticated ruminants including: dairy and beef cattle, sheep, goats, red deer, cervids, and camelids (12). However, other non-ruminant wildlife, such as the fox, weasel, crow, rat, wood mouse, rabbit, hare and badger, have also been found to harbour *Mptb* (13). Calves inoculated with *Mptb* from a free living rabbit developed typical histological lesions consistent with Johne's disease, demonstrating that wild animals other than ruminants may also contribute to the spread of the disease (14). However, calves are more likely to be exposed to manure from other mature cattle than wildlife, and therefore, the major sources of infection on most farms are likely infected domesticated ruminants that shed the bacteria in their feces. The route of infection is usually through ingestion, be it contaminated water, milk or feed.

The purpose of this first part of a two-part series of review articles is to critically review the literature on clinical stages of JD, pathophysiology, diagnostic and screening tests, and prevalence estimates of infection in Canada to enable Canadian bovine practitioners to successfully implement control strategies.

Methods

Due to substantial differences in management and production between dairy and beef cattle, and the extensive literature on JD for both dairy and beef cattle, this paper focuses on dairy cattle. Also, because the intended audience for this paper is primarily Canadian veterinarians, we have emphasized Canadian references as much as possible.

Medline (accessed via PubMed from 1950 to present), The Commonwealth Animal Bureaux (CAB) (accessed via VetCD and ParasiteCD from 1973 to present), and Agricola, produced by the National Agricultural Library of the U.S. Department of Agriculture (accessed via the National Agricultural Library from 1970 to present) were used to collect the majority of the references that were used in this paper. The keywords used in the search of the databases were Johne's disease or paratuberculosis, Canada or Canadian, dairy and cattle. In addition, a number of papers were included from the reference lists of other papers, or personal knowledge of reports or conference proceedings, where the literature search did not identify papers with salient information for this review.

All relevant material collected from the above process was included in the review, provided that it was pertinent to the methods of production within the Canadian dairy industry. Exclusion of material was only done if information was redundant or outdated and had been directly refuted. Otherwise, all available information was included.

Pathophysiology

Ingested *Mptb* bacteria enter the intestinal wall through the small intestinal mucosa, primarily in the region of the ileum, via M cells (specialized absorptive mucosal cells) residing in the Peyer's patches (15). The bacteria are eventually phagocytosed by sub-epithelial macrophages where they are resistant to intracellular degradation (16). While the bacteria are in the mucosal tissue and submucosal macrophages, there is little or no detectable reaction to the infection. This delayed detectable humoral immune response is one reason for the poor Se of serological diagnostic tests for *Mptb*, as explained in detail later.

Eventually, the infected macrophages migrate into local lymphatics (17,18) spreading the infection to regional lymph nodes. In the regional lymph nodes, the organisms are capable of stimulating inflammatory and immunological responses (19). The immune response towards *Mptb* resembles that of other mycobacterial infections. Most animals mount a cellular immune response involving a variety of cells, most importantly T lymphocytes (20). Cytokines produced by T helper cells also contribute to the protective response against mycobacterial infections, especially the cytokine gamma interferon (IFN-γ). Production of IFN- γ has been recognized as a key step in resistance against mycobacterial diseases in general, and may provide a means to help monitor early infection in some animals (21). In some cows, the cellular immune response has been shown to be able to control the infection, with the cows never developing clinical signs, but remaining subclinically infected for life (22). In those animals in which the cellular immunity is unable to control the disease, a detectable humoral immune response will develop, along with increased shedding of bacteria (22).

Typically, the organism proliferates slowly in the ileal mucosa and regional lymph nodes. However, poor nutrition, stress related to transport, lactation, parturition and immunosuppression by agents like bovine viral diarrhea virus have been proposed as accelerating or precipitating the onset of the clinical phase of infection (23).

Early reports on the physiological mechanism for development of diarrhea in clinically affected animals was thought to be related to antigen-antibody reactions in infected tissue, with subsequent release of histamine (24-26). Macroscopic lesions, if present, are seen primarily in the intestine and draining mesenteric lymph nodes, more specifically in the region of the ileum, although they can occur throughout the whole length of the intestinal tract. The intestinal wall is thickened and edematous and the mucosa has exaggerated transverse folds, mimicking the appearance of corrugated cardboard. The serosal and mesenteric lymphatic vessels are dilated and thickened. Subsequent muscle atrophy, emaciation, alopecia, renal infarcts, anemia and leukopenia are thought to be mediated by cytokines (23,27). There is no evidence to suggest that the pathophysiology or progression of disease differ between infected cattle in Canada and elsewhere.

Clinical effects and stages of paratuberculosis

Infection with *Mptb* can be divided into four stages as described by Whitlock & Buergelt (28), depending on the severity of clinical signs, potential for shedding organisms into the environment, and the ease with which the disease may be detected using current laboratory methods.

Silent infection

This stage generally includes young stock up to 2 yrs of age and is called "silent" because: 1) there are no clinical signs of infection; 2) there are no measurable subclinical effects of infection; and 3) there are no cost-effective diagnostic tests that can detect infection. The only means of detecting infected cattle at this early stage is by demonstration of the established organism in the intestinal tract, either by culture or by histologic demonstration of microgranulomas in the intestine or regional lymph nodes, a cost-prohibitive procedure if multiple animals require testing. Other diagnostic tests, such as Johnin skin testing and gamma-interferon tests which utilize the cell mediated response (CMI), have also been used to detect this stage of the disease. However, there are common antigens between *Mptb* and other environmental *Mycobacteria* species, resulting in low Sp for these tests (29,30), making them ineffective as a routine screening test. Infected animals in this stage may shed infectious organisms into the farm environment at levels below the threshold of detection (31).

Sub-clinical infection

Animals with subclinical *Mptb* infection do not have clinical signs of infection yet, but may be detected as infected using cost-effective diagnostic tests and may begin to have measurable effects of infection (as discussed in part 2) (32,33). Some of these infected cattle may be detected by fecal culture and subsequently removed from the herd. However, focal lesions, variable rates of disease progression and shedding, and dilution of organisms in large volumes of intestinal content result in intermittent detection of fecal shedding (34). Therefore, the remainder of these infected animals test negative using current fecal culture

techniques, yet they may be shedding low numbers of organisms in the manure, which contaminate the environment and pose a threat to other animals on the farm. Some animals may have detectable antibodies to *Mptb* and/or an altered cellular immune response, particularly if they are getting close to entering the next stage of the disease (clinical phase) (12). However, the *Mptb* fecal shedding usually occurs before a detectable antibody response (35).

Clinical infection

Initial clinical signs follow a prolonged incubation period of 2 to 10 years, depending on the exposure level and the capacity of an animal to fight the infection (36,37). The first apparent sign is gradual weight loss despite a normal or, occasionally, increased appetite. During a period of 3-6 months, concurrent with the weight loss, the manure consistency becomes more fluid. The diarrhea may be persistent, or intermittent at first, with periods of normal manure consistency. Thirst is usually increased and milk production is decreased. However, appetite and vital signs (i.e., heart rate, respiratory rate, and temperature) remain normal (28).

Most animals at this stage have a positive fecal culture and have increased serum antibody levels detectable by the commercial enzyme linked immunosorbant assay (ELISA) and agar gel immunodiffusion (AGID) tests. It is estimated that only 10-15% of infected animals survive to this stage of infection because they are often culled due to reduced productivity earlier in the subclinical stage (38). One study showed that out of 113 herds who did not have a clinical case of JD in the previous five years, only 32 herds were completely test-negative during 9 pooled fecal-culture tests done every 6 months (39).

Advanced clinical infection

Clinically affected animals, if not culled, become increasingly lethargic, weak, and emaciated. "Water-hose"or "pipestream" diarrhea, hypoproteinemia and intermandibular edema (bottle jaw) characterize the advanced stage of the disease. In the last stage of JD, cows become cachectic, anemic and too weak to rise (40). Most animals are culled from the herd before this time due to the chronic/intermittent diarrhea, decreased milk production and/or weight loss in the earlier stages of disease (28).

Diagnosis

The diagnostic tests to detect infection with *Mptb* can be categorized into those that identify the organism, and those that identify an immunological reaction to the organism. Evaluation of the performance of diagnostic tests is typically done by comparing estimates of sensitivity (Se – ability of the test to detect infected cattle) and specificity (Sp – ability of the test to identify healthy cattle) based on a "gold standard" that has identified animals as truly infected and truly non-infected. However, comparisons of Se and Sp of diagnostic tests for *Mptb* should be interpreted with great caution, because there are a number of factors that have a major impact on these estimates, including: 1) the type of gold standard used; 2) the stage of infection of the study animals; and 3) the type of farms utilized to source animals for testing. Each of these will be discussed in turn.

Due to delays of 2-10 years between time of infection and measurable immune system reactions and shedding, various "gold standards" have been utilized for *Mptb* infection status in the past. Tissue culture of *Mptb* is considered the ideal gold standard test

because, even before fecal shedding or an immune response is present, it can detect growth of *Mptb* in multiple organs, including the intestinal mucosa and submucosa, and regional lymph nodes (41). However, fecal culture has been used as the gold standard in many studies (42-48) in the past due to the high cost and logistical difficulties of sampling for tissue culture. The methods for fecal and tissue culture are identical and are described below.

The delays in immune response or shedding also mean that tests utilized on animals with clinical JD will have a better Se than when they are testing animals for subclinical JD (43,49) because the clinically affected animals are much more likely to be shedding bacteria or have developed a detectable immune response (19). False-negative test results are common, particularly in calves, heifers, and even 1st lactation cows (cattle in silent and subclinical stages) (50). Test results from animals with subclinical paratuberculosis can be a challenge to interpret because clinical signs are not present to assist in their interpretation (42,49,51).

Regarding farm type, on farms with a known history of clinical JD, there will likely be more bacteria in the environment and higher exposure to *Mptb* of the youngstock compared to farms without history of clinical JD. If youngstock undergo higher exposure to *Mptb* bacteria on a particular farm, they are more likely to develop a detectable immunological reaction or begin shedding bacteria earlier in life (24,25,52), leading to higher detectable prevalence of infection on that farm and higher shedding of bacteria in those infected cattle on those farms, compared to farms where there is lower exposure to *Mptb*. Therefore, evaluation of test performance using cattle on these farms will lead to higher estimates of Se and Sp than farms without history of clinical JD.

As a result, in Table 1, the test sensitivities of the most widely used diagnostic tests for *Mptb* infection are categorized with respect to testing for clinical versus subclinical infection, and for subclinical infections, testing in farms with high prevalence (≥25%) of *Mptb* infection versus low prevalence (<25%) herds.

Identification methods

Culture on tissue or feces (individual and pooled samples)

A number of different media have been used to culture *Mptb*. The standard culture procedure utilized in Canada is Herrold's egg yolk medium, however culture time is 16 weeks before the observable growth is seen for this slow growing bacterium (28). A radiometric system has been developed that reduces the culture time by half because detection of growth is not visual but through the detection of metabolized radioisotopes in the media (53). However, because the system requires expensive safety equipment to handle the radioisotopes, at the time of publication, only laboratories in British Columbia (Animal Health Monitoring Laboratory, Abbotsford, BC) and Ontario (Animal Health Laboratory, Guelph, Ontario) are offering this system on a commercial basis, with the Manitoba provincial lab (Veterinary Services Branch, Winnipeg, Manitoba) conducting research and development with the system. Recently, a specialized broth media system has been developed which has reduced the detection time to 6 weeks, without loss in test Se, through the detection of alterations in oxygen, CO_2, or pressure within a sealed bottle (54). At the time of publication, the only Canadian laboratory currently offering broth culture testing is the Atlantic Veterinary College in Charlottetown, PEI. The Agri-Food Laboratories Branch lab in Edmonton, Alberta does *Mptb* cultures using the standard HEYM methods, but only

for research purposes and for some of the samples submitted under the Alberta Johne's Control Program.

If bacterial growth is detected, the bacterium is isolated and its identity is confirmed through morphologic characteristics and mycobactin dependency of the bacterial colonies, acid fast staining, and sometimes, through the detection of the insertion sequence IS900 by polymerase chain reaction (see description below). With identification of *Mptb*, the animal is considered infected. However, laboratory error could occasionally lead to cross-contamination and false-positive test results (55). Also, the phenomenon of "pass-through" of bacteria through the gastrointestinal tract could lead to other false-positives, but this remains a hypothesis and has not been fully substantiated. Therefore, Sp of fecal culture is described as being virtually 100% (49) and fecal culture is considered an excellent confirmatory diagnostic test of paratuberculosis for animals that test positive using immunological tests (see Table 1).

Due to the lengthy testing duration and specialized equipment and media required for culturing *Mptb*, the cost per sample tested is high (ranges from $35-60/sample). Therefore, pooling fecal samples has been utilized to test large numbers of animals for less cost per animal (56), while still maintaining reasonable Se to detect infected animals (57). In comparing conventional culture to pooled culture on a herd level basis, 94% of pooled samples with moderate to high numbers of *Mptb* yielded positive culture results (58). Pooled fecal culturing has been shown to have a herd level Se of 73% (56) meaning that 73% of infected herds were detected with a single set of strategically (by age cohorts) pooled samples. However, the maximum number of negative animals that can be mixed in with a sample from a positive animal (while still getting a positive pooled test) needs to be

determined, and this number needs to be appropriate to infection prevalence, severities and shedding levels seen in Canada. Based on initial results from outside of Canada, 3-5 samples in a pool may be the optimal number (56,58,59). Any Canadian laboratory that is equipped to conduct *Mptb* cultures should also be able to conduct pooled fecal cultures.

Polymerase chain reaction on feces

Since the discovery of the IS900 insertion sequence, attempts have been made to perform polymerase chain reaction (PCR) techniques directly on clinical samples (60,61). Through amplification of this piece of genetic material, PCR is able to provide a much faster result in comparison to the culture techniques with a turnaround time of a four days for most laboratories (55). However, PCR is less sensitive than culture due to the presence of inhibitory substances in fecal specimens (62). Another concern is that IS900 may not be as specific for *Mptb* as once believed. The IS900 element has been detected in other mycobacterial strains isolated from the feces of ruminants (63). At the time of publication, PCR testing is only being offered in Canada commercially at BIOVET Inc. in St. Hyacinthe, Québec, and at the Faculté de Médecine Vétérinaire in Montréal, Québec. It is being used as a confirmation to positive cultures where culturing is being done. If PCR were to be done on a large scale, laboratory error could lead to false-positive test results without very strict adherence to preventing even minute cross-contamination.

Immunological methods

Enzyme linked immunosorbant assays on serum and milk

The main type of immunological test that is widely available and commonly used is the enzyme linked immunosorbant assay (ELISA) which detects an optical density in serum (64) or milk (65,66) that correlates with an antibody response to *Mptb*. The ELISAs have been desirable tests to use because of their ease of sample collection (blood or milk), rapid test results (within a week), and relatively low cost (approximately $10 per sample). However, for several reasons, results from ELISAs need to be interpreted with caution. Due to the long delay between infection and presentation of bacteria to the immune system in sufficient quantity to develop a detectable immune response, the reported sensitivities of the serum ELISAs for detecting subclinically infected cattle are much lower than the sensitivities of fecal cultures, leading to many false-negative test results (42,45,46,65). In fact, many studies have estimated the Se of the serum ELISA on the basis of the proportion of fecal shedders that were seropositive, with these estimates ranging from 15 to 75% (42,43,49).

Milk ELISA testing has recently been introduced as another immunological test for detecting subclinically infected cattle, with obvious practical advantages of ease of sample collection. However, there is still limited independent, peer-reviewed evaluation of the operating characteristics of ELISA testing of milk. A recent study in Ontario (67) reported a 12% lower relative Se for a milk ELISA versus a serum ELISA when compared to fecal culture results, and only moderate agreement between the serum and milk ELISAs. These findings make biological sense considering that antibody concentrations in milk depend not only on levels in serum, but also on milk production (67), parity and days in milk (68). The

added variability in antibody levels in milk (70) makes interpretation of milk ELISA results even more challenging at the animal level than serum ELISA results, which have inherent laboratory variability (71). Further research may identify a role for the milk ELISA as a practical method of monitoring *Mptb* infection at the herd level.

Another caution regarding ELISA tests is that, in low prevalence herds, false-positive test results can also be a problem when a large number of cattle are tested. Similarly, this same study has reported a more conservative estimate of 96.8% for Sp of the IDEXX™ ELISA (42). While some studies have reported Sp estimates of 99% (43,49,69), a recent study (42) likely provides a more realistic estimate of Sp because it utilized multiple sources of samples (more representative of the North American dairy cattle industry as a whole) providing more possibility of cross reactivity with other micro-organisms to give false-positive test results. Using an ELISA test on 100 uninfected cattle would therefore likely produce at least 3 false-positive test results. In a low prevalence herd where there may only be 2-3 truly infected animals, these false-positive test results would lead to predictive values of the positive test results of lower than 50%, making it difficult to know how to interpret and act on these test results. Therefore, apparently healthy cows that are ELISA-positive should be fecal cultured to confirm infection status, particularly in herds suspected to have a low prevalence of infection. If the fecal culture is negative, these ELISA-positive cows should be retested in 6-12 months because the owner does not know if these non-shedding ELISA-positive cows are truly uninfected or just not shedding in detectable numbers at the time of sampling.

One additional caution regarding the interpretation of ELISA results relates to the form in which they are reported. Interpretation of results has generally been made on a single

cut-off value that allows for dichotomous test results, positive or negative. Although this would appear to make results easier to interpret and allowing Se and Sp calculations, valuable information is lost because the likelihood of true infection is much higher in cattle with a high optical density, particularly in herds that have a history of clinical JD infection (84). As a result, some laboratories are appropriately utilizing a three-level result system - negative, suspect and positive (72), or four-level result system – negative suspect, weak positive and strong positive, based on categorizations of likelihood ratios generated from the optical densities (55).

At the time of publication, there are only 2 serum ELISAs currently offered on a commercial basis in Canada, with different provinces using different ELISAs. It is unlikely that there is a large and significant difference in the test performance of the two ELISAs; both tests have difficulty detecting, subclinically infected cattle.

Agar gel immunodiffusion

There is one other immunological test that is available in Canada, the agar gel immunodiffusion (AGID) test. It was developed as a quick test for animals that were showing clinical signs of JD. Some reports estimate that when AGID results are positive, there is a 95% chance of actual *Mptb* infection in a clinically affected cow (28). However, the Se of the AGID for subclinical cows is poor, with one report of a Se of 18.9% (73). Therefore, use of the AGID is restricted to animals showing clinical signs of JD. The ELISA tests are equally sensitive at detecting *Mptb* in clinically affected cattle, and therefore it is unlikely that the AGID offers any advantage over the ELISA.

Prevalence

Table 2 shows the results of seroprevalence studies done recently in dairy cattle in Canada. Seroprevalence at the animal level in dairy cattle ranged from 1.3% (PEI) (74) to 7.0% (Alberta) (75). At the herd level, 9.8% (Ontario) to 40.0% (Alberta) (75) of herds had at least 2 seropositive cows. The provincial differences in seroprevalence may represent real differences in the distribution of the organism, due to variations in management or other risk factors for transmission. Conversely, these seroprevalence differences may be due to variations in sampling and testing protocols. Normally estimated true prevalences of infection can be calculated to adjust for differences in testing protocols, however, for JD, there is little consensus on the test Se and Sp adjustments required to calculate true prevalence estimates. Therefore, even comparisons of estimated true prevalences should be interpreted with caution.

All of the above provincial studies were conducted using the same number of animals per herd, with the same IDEXX ELISA test (except for Alberta), using the same cut-off value for interpretation of test-positives. However, comparisons between these seroprevalence estimates should be interpreted with caution for a number of reasons. Sera from Manitoba were tested at a separate laboratory from other provinces, possibly impacting on the survey results. Even the sera that were tested at the same laboratory were tested at different points in time using different lots of test kits, also possibly impacting on survey results. Furthermore, the Ontario study consisted of herds that were purposely chosen to monitor disease (mastitis) incidence, and is likely to be an underestimate of the true prevalence of paratuberculosis in Ontario dairy herds. A 1986-89 survey of 14,932 cows in 304 dairy herds in Ontario found 15.2% of the animals tested seropositive using a LAM-ELISA (76). It is unlikely that all of

the difference between these two estimates is due to differences in test performance, due to similarities in the two tests used. Furthermore, with a control program for JD not existing in Ontario during the time between the two studies, it is unlikely that the *Mptb* infection prevalence would have declined between 1989 and 1998.

While comparisons in seroprevalences between provinces were possible with the above provincial studies, they are likely underestimates of true prevalences of infection at the animal level due to the low sensitivities of ELISAs for *Mptb*. There have been very few Canadian studies conducted to determine *Mptb* infection prevalence in dairy cattle based on fecal culture. An Albertan study (75) collected fecal samples from cows in 50 dairy herds, cultured them as pools of 3 samples each. They found 3.4% of pools were culture-positive, meaning that 3.4 to 10.2% of cattle were test positive (individual cattle results were not reported). They concluded that the estimated true herd-level prevalence ranged from 28% to 57%, depending on whether 1, 2, or all 3 individual fecal samples in the positive fecal pools were culture-positive.

While fecal culture testing has a better Se than ELISAs for *Mptb*, it still produces many false-negatives, particularly in young infected cattle that have not started shedding yet. A recent prevalence estimate from tissue culture testing of ileocecal lymph nodes and ileum from dairy cows at a slaughterhouse in New Brunswick found that 16.1% of dairy cows were culture-positive for *Mptb* (41). This prevalence estimate is likely to be a close approximation of the true infection prevalence because culturing the ileum and ileocecal lymph node of the selected animals is better than fecal culturing at detecting cows that are infected but not yet shedding bacteria in their feces. Furthermore, the sample was randomly selected, cull dairy

cows, and a very high percentage of all dairy cows are eventually sent to slaughter, usually at a mature age when tissue culture will likely detect infected cattle.

Many other prevalence surveys have been carried around the world, with Table 3 providing a brief summary of recent international seroprevalence estimates from representative samples. While comparisons between countries should be conducted with caution, there does appear to be a large variation in the reported seroprevalences between countries and even within countries. Seroprevalences at the animal level are similar in Canada to other countries, ranging from 0.8% in Belgium (77) to 17.1% in Florida (78). At the herd level, the proportion of herds with 2 or more seropositive cows in Canada (74) was also similar to other countries, ranging from 17% for the 20 tested states in the US (NAHMS 1996) (79) to 44% in Michigan (80). Some Scandinavian countries have very low levels of *Mptb* seroprevalence, leading those countries to seriously consider eradication efforts (81,82).

It is difficult to determine if the prevalence of *Mptb* infection is increasing because of the significant advances in the quality of the diagnostic tests used to detect *Mptb*. There are very few, if any, data from random samples of the same area over time, using similar diagnostic tests. Before the evolution of ELISAs in the late eighties, most of the initial reports of JD prevalence were limited to slaughterhouse data. Perhaps future studies will address this paucity of data and clarify whether *Mptb* is becoming more prevalent or not.

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Table 1. Characteristics of currently available diagnostic tests for Johne's disease in Canada

		Sensitivity			Specificity
		Sub-clinical cow		Clinical cow	
		Low prevalence herds and/or low shedders[a]	High prevalence herds and/or high shedders[b]		
Fecal culture	Estimate	19%[c]	53%[d]	>90%[e]	Approaching 100%[e]
	Reference	(83)	(83)	(49)	(49)
Serum ELISA	Estimate	12%[f] - 15%[f]	40%[g] - 75%[g] - 95%[h]	87%[i] - 88%[i]	96.8%[j] - 99%[i]
	Reference	(84) - (42,43)	(84) - (42,43) - (67)	(43) - (42)	(42) - (83)
Cow Milk ELISA	Estimate	-[k]	51%[l] - 84%[h]	-[k]	92%[i] - 96%[i]
	Reference		(67) - (67)		(85) - (85)

[a]Low prevalence herds (<25%) and/or low shedders (<10CFU)
[b]High prevalence herds (≥25%) and/or high shedders (≥10CFU)
[c]Tissue culture was gold standard in low prevalence herds
[d]Tissue culture was gold standard in high prevalence herds
[e]Tissue culture was gold standard
[f]Fecal culture was gold standard in low shedders
[g]Fecal culture was gold standard in high shedders
[h]Fecal culture was gold standard in high shedders in high prevalence herds
[i]Fecal culture was gold standard
[j]Tissue culture or fecal culture or history of herd was gold standard
[k]No published reports found
[l]Fecal culture was gold standard in low shedders in high prevalence herds

Table 2. Seroprevalence estimates of bovine paratuberculosis in Canadian dairy herds (from East to West)

Province (Reference #)	Number of Herds	Number of animals	Animals test +[a] (%)	Herds with 1 test +[b] (%)	Herds with 2 test +[c] (%)	Animals test + in herds with 1 test +[d] (%)	Animals test + in herds with 2 test +[e] (%)
Nova Scotia (74)	30	814	3.3	53.3	16.7	5.3	8.1
Prince Edward Island (74)	30	816	1.3	33.3	16.7	5.0	6.3
New Brunswick (74)	30	804	2.9	43.3	16.7	6.4	11.0
Ontario (86)	60	-	2.2	37.0	9.8	4.6	8.4
Manitoba (87)	40	1204	4.5	68.4	43.1	6.6	8.7
Saskatchewan (88)	51	1530	2.7	43.6	24.2	6.3	8.6
Alberta (75)	50	1500	7.0	74.0	40.0	- [f]	- [f]

[a]Animals testing positive
[b]Herds with at least 1 animal testing positive
[c]Herds with at least 2 animals testing positive
[d]Animals testing positive in herds with at least 1 animal testing positive
[e]Animals testing positive in herds with at least 2 animals testing positive
[f]Published report did not include these figures

Table 3. Seroprevalence estimates of bovine paratuberculosis in dairy herds from major dairy producing countries and states outside of Canada (from high prevalence to low prevalence at the animal level)

Country (Reference #)	Areas	Number of Herds	Number of animals	Animals test +[a] (%)	Herds with 1 test +[b] (%)	Herds with 2 test +[c] (%)	Animals test + in herds with 1 test +[d] (%)	Animals test + in herds with 2 test +[e] (%)
U.S.A. (78)	Florida	452	4491	17.1	-	-	-	-
U.S.A. (89)	Wisconsin	158	4990	7.3	50	-	20	
U.S.A. (80)	Michigan	121	3886	6.9	66	44	8	12
U.S.A. (90)	20 states	967	31,745	2.5	41	17		
Netherlands (91)	National	378	15,822	2.5	54	28	-	-
Austria (92)	National	2757	11,028	1.9	7	-	-	-
Sweden (12)	National	-	4000	1.2	-	-	-	-
Belgium (77)	National	556	13,317	0.8	18	-	3	-

[a]Animals testing positive
[b]Herds with at least 1 animal testing positive
[c]Herds with at least 2 animals testing positive
[d]Animals testing positive in herds with at least 1 animal testing positive
[e]Animals testing positive in herds with at least 2 animals testing positive