Epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* Fecal Shedding in Johne's Disease Infected Dairy Herds

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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FEBRUARY- 2012

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Acknowledgements

The information provided in this doctoral dissertation is the result of the hard work of a lot of people. It will be impossible to put in words how thankful I am to them, but I will try.

I will start by acknowledging my advisor, Dr. Scott Wells. His advice and guidance helped me finish all the scientific work involved in this thesis. He helped me polishing my strengths and overcome my limitations. I would like to follow his example as advisor.

I would like to acknowledge my fellow graduate students, João Lima and Amanda Beaudoin. We had interesting scientific and not so scientific conversations and discussions. We helped each other with the scientific challenges during our PhDs.

I would like to thank the dairy farmers who allowed us to take samples from their cows and helped us understand their challenges as producers. I also thank the Minnesota Veterinary Diagnostic Laboratory technicians who helped us with the laboratory work.

Finally, I would like to acknowledge the Minnesota Board of Animal Health directed by Dr. William Hartmann, the Johne's Disease Integrated Program coordinated by Dr. Kenneth Olson, Dr. Charles Fossler from the USDA-APHIS-VS and Drs. Francisco Zagmutt and Huybert Groenendaal from EpiX Analytics LLC. They provided funding for my research, helped with sample and data collection and gave me useful suggestions to improve my research.

To all of them my deepest recognition and my sincere thank you.

Dedication

I would like to dedicate this work to the people who helped me overcome the challenges of this research and shared the joy of the work accomplished.

This dissertation is dedicated to my wife Andrea and my children Isabel and Gustavo. Your support and smiles kept me going until the end and brought out the best in me.

I would also like to dedicate this work to my parents, Horacio and Ema.

Abstract

Johnes's disease, also known as paratuberculosis, is a chronic enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). The disease has a slowly progressing detrimental effect on cow health and production. In the United States most dairy cattle farms are infected, causing an economic impact to dairy herds in the short term by the association of the disease with low milk production, higher culling rates and low reproductive performance. Control of the disease has been focused on implementation of management practices that reduce the *MAP* transmission from infected cows to uninfected young calves, and on culling cattle that shed large amount of *MAP* in feces (heavy fecal shedding cows) as soon as detected. The objectives of these studies were to evaluate the association between use of recommended management practices on Johne's disease incidence and to improve the understanding of the most commonly used diagnostic tests to identify heavy fecal shedders.

The evaluation of the association between use of a standardized control program on the incidence of Johne's disease was conducted in a prospective longitudinal observational study that in 8 dairy herds in Minnesota. Herds were followed during a period of 5 to 10 years. We found a reduction of the incidence of bacterial culture positivity, serum ELISA positivity, heavy fecal shedding status, and clinical Johne's disease associated with higher levels of implementation of the recommended management practices.

The evaluation of the analytical sensitivity of bacterial culture of feces and direct fecal PCR was performed in two separate experiments using MAP negative bovine fecal samples spiked with different concentrations of *MAP*. The analytical sensitivity of the bacterial culture of feces was 10^5 *MAP*/g of feces and the probability of a higher bacterial culture result increased with the concentration of *MAP* in the fecal sample. The analytical sensitivities of the direct fecal PCR in experiments 1 and 2 using different approaches were 10^7 and 10^2 *MAP*/g of feces, respectively.

A latent class model using a Bayesian approach was fitted to estimate the posterior conditional probabilities that the results of the bacterial culture of feces and serum ELISA correctly identified cows as high positive, low positive or negative given

that they were heavy, light and non-fecal shedders, respectively. The estimated conditional probabilities that bacterial culture of feces correctly identified heavy, light and non-fecal shedders were 70.8, 32.2 and 98.5%, respectively. The same values for the serum ELISA were 60.5, 18.8 and 99.5, respectively.

Finally, we conducted a cross-sectional study to evaluate the association between bacterial culture of cow-level and pooled environmental fecal sample results for detection of *MAP* in dairy herds. The sensitivity and specificity of the parallel interpretation of bacterial cultures of pooled environmental fecal samples from the herd to detect at least one heavy fecal shedding cow in the herd was 98.2% and 43.5%, respectively. The sensitivity and specificity of the bacterial culture on pooled individual samples to detect at least one heavy fecal shedding cow in the pool was 100% and 91%, respectively, and these values did not change when pool size increased from 5 to 10 cows per pool.

In summary, these studies shown that implementation of critical management practices are associated with a reduction on the incidence of Johne's disease and diagnostic tests can be used to indentify heavy shedding cows using individual or pooled fecal samples.

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INTRODUCTION

Johnes's disease, also known as paratuberculosis, has been increasing its ranking as one of the most important infectious diseases in the cattle industry during recent decades. In the United States, most dairy cattle farms are infected, representing not only an economical problem but also a welfare problem. In addition, if the etiological agent of Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) is proved to be the cause of Crohn's disease in humans, the cattle industry will need to consider a more aggressive control and eradication program.

Previous experience of the Minnesota Johne's Disease Demonstration Herd Project has revealed that it is possible to reduce the frequency of the disease through good management practices intended to reduce the transmission of the disease among dairy cattle, as well as testing and culling positive cattle. In fact, most of the herds under such enhanced management based intervention programs remain with low disease prevalence. Given this scenario, one would ask why Johne 's disease has not been eradicated yet. Research-based information is needed to characterize the role of these strategies to control and eradicate Johne's disease from the herds. In the present study we addressed the following objectives: 1) the role of management practices on reduction of Johne's disease in the herd; 2) Improve the interpretation of available diagnostic tests; and 3) Find the best way to detect cattle that potentially have the highest probability to spread the disease. In summary, the overall objective of this project is to systematically collect information to better understand Johne's disease and its diagnosis and ultimately improve disease prevention and management.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Johne's disease or paratuberculosis is a chronic enteritis in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*, Gardner et al., 2011). It is a multistage disease characterized by chronic diarrhea, weight loss, and weakness during its clinical stage (Whitlock and Buergelt, 1996). The infection has been described mainly in domestic ruminants; however, wild ruminants, rabbits and carnivores can become naturally infected (Harris and Barletta, 2001; Whittington et al., 2004). The infection is mainly transmitted via the fecal-oral route, although *MAP* shedding through placenta, milk and colostrum has also been documented (Streeter et al., 1995; Sweeney, 1996; Whittington and Windsor, 2009).

In the United States, the prevalence of infection on dairy farms was estimated at approximately 68%, based on environmental samples. This estimation varied depending on herd size with large herds (>500 cows) having a prevalence estimated at 95% (USDA-APHIS, 2008).

1.2. THE PATHOGEN

Mycobacterium avium subspecies *paratuberculosis* is a gram positive acid fast organism (Harris and Barletta, 2001) that it is characterized by aerobic, non-motile, and slow growth, as the other members of the genus. However, *MAP* differs from other mycobateria because it is an obligate intracellular pathogen and it requires mycobactin to grow, which is an iron-chelating protein (Thorel et al., 1990). *MAP*'s capacity to forming endospores has been confirmed recently (Lamont et al., 2010).

A particularity of *MAP* is the presence of multiple copies of insertion segment IS900 (Harris and Barletta, 2001). Although the Insertion Segment IS900 is not unique to *MAP* (Cousins et al., 1999; Englund et al., 2002), it has been suggested that the presence of the IS900 is infrequent in other subspecies of *Mycobacterium avium* (O'Mahony and Hill, 2004).

Even though fingerprint analysis of different *MAP* isolates revealed 2 main strains, cattle (C) and ovine (S) strains, molecular evidence shown that these strains can be shared between these ruminant species (Motiwala et al., 2003). These strains can also

be differentiated by their phenotype on culture media. Cattle strains seem to be pigmented and grow slower on solid media (16 weeks) than sheep strains (Motiwala et al., 2006), which grow even faster on liquid media than on solid media (Whittington et al, 1998).

1.3. STAGES OF JOHNE'S DISEASE

Whitlock and Buergelt (1996) suggested 4 successive stages of the disease based on the presence of clinical signs, and potential of *MAP* fecal shedding:

Stage I: Silent Infection.

Once infected, *MAP* will proliferate slowly in jejunal and ileal mucosa. The infection will gradually spread to the regional lymph nodes in a process that could take 2 or more years. Most cows in this stage are latently infected cows that are rarely diagnosed by conventional tests; and no clinical manifestation of the disease is observed (Whitlock and Buergelt, 1996). Approximately 70 to 75% of infected cattle are in this stage of the disease (Whitlock et al. 2000). Whittington and Sergeant. (2001) indicated that the incubation period in cattle is inversely proportional to the dose of *MAP* received; however, the effect of multiple exposure events to *MAP*, likely to occur on herds with high rate of infection and poor management, is uncertain.

Stage II: Subclinical disease.

Adult cattle will carry *MAP* infection with no visible signs of the disease. They may have an altered immune response and be prone to other diseases. Many of these cows are not going to be detected using diagnostic tests; however, they may shed low but undetectable levels of *MAP* (Visser, 1999). Approximately 15-25% of these cows may shed detectable levels of *MAP* (Whitlock and Buergelt, 1996).

Stage III: Clinical disease.

During this stage, clinical signs of the disease are observed, starting with intermittent diarrhea that becomes chronic, leading to weight loss and significant

reduction in milk production. Most cows at this stage shed detectable levels of *MAP* and have a detectable immune response. In commercial settings, these cows are culled before progressing to the next stage of the disease (Whitlock and Buergelt, 1996).

Stage IV: Advanced clinical disease.

Because of the profuse diarrhea and protein loss, cows become lethargic and emaciated. The hypoproteinemia may be manifested by edema on the submandibular area (bottle jaws) and the cow condition deteriorates rapidly. Death occurs as result of dehydration and cachexia (Whitlock and Buergelt, 1996).

Another classification system used to establish the infection stage of JD in cows based on the diagnostic tests results and clinical signs was suggested by Nielsen and Toft (2008), where *MAP* infected cows are classified as infected, infectious or affected. Infected cows are cattle with an established *MAP* infection in the latent stage that can be detected using diagnostic tests during their life. Infectious cows are cattle that shed detectable levels of *MAP* and represent a risk of transmission to susceptible herdmates. Finally, infected cows are cows with clinical JD. These stages are not mutually exclusive, that is infectious cows are also infected and cows with clinical JD are infected and infectious (Raizman et al. 2007; Nielsen and Toft, 2008).

The association of these stages of JD with test results is not very clear. In terms of avoiding disease transmission, efforts should focus on infectious cattle, which are cows that shed *MAP* in the feces. A study that followed 8 infected cattle up to 22 months found that cows with diarrhea consistently shed large amounts of bacteria in their feces; however not all cows shedding large amounts of bacteria in their feces had diarrhea (de Lisle et al., 1980). Another study using a bigger population, found that 43% of cows with clinical Johne's disease were also heavy fecal shedders (Raizman et al., 2007).

An evaluation of fecal shedding consistency patterns of individual cows becomes important when culling decisions are based on fecal shedding levels of *MAP*. The few available studies indicated that shedding levels in a single cow could vary on a day to day basis (Gay, 1988, Merkal et al. 1968). A longitudinal study with 8 infected cattle showed that cows shedding a low amount of *MAP* in feces would go through periods where *MAP*

was not isolated from their feces; however, it was also shown that cattle shedding large amounts of *MAP* tended to do so consistently during the study period (de Lisle et al., 1980). Similar results were obtained by Whittington et al. (2000) in a short-term study with 7 Merino sheep. They found a daily consistent *MAP* fecal shedding level in those sheep that shed large amounts of *MAP* with feces. Another longitudinal study that followed 112 cows, with samples taken every two months, showed a high Pearson's correlation between fecal culture results (0.9 to 0.94 on concurrent samples and 0.72 to 0.85 on samples taken 4 to 8 months apart). In this study, only 7% of the shedding cows showed negative fecal culture on concurrent tests (van Schaik et al., 2003a).

1.4. DIAGNOSTIC TESTS

There are several different tests to diagnose Johne's disease. They can be classified as tests that detect the pathogen itself and tests that detect the immune response of the host (Collins, 1996; Wells et al., 2006; Collins et al., 2006). Bacterial culture and polymerase chain reaction (PCR) assays are diagnostic tests that detect the presence of *MAP* or *MAP* DNA in the samples. They are normally performed on fecal samples; therefore, positive results are obtained when cows shed the bacteria in feces. Diagnostic tests that detect the specific immune response of the host against the agent are the enzyme-linked immunesorbent assay (ELISA), the complement fixation assay and agar gel immunodiffusion test (Collins, 1996). It is important to take into account that fecal shedding and immune response test results have a high correlation with each other (Chiodini, 1996; Whitlock and Buergelt, 1996); these tests detect associated, but theoretically different groups of cows, which could have implications for the control of the disease. There is a lack of information in the literature about the physiopathological mechanisms involved in the immune response of the host and fecal shedding process, as well as how they are associated.

Another important point to take into consideration when diagnostic tests results are evaluated is the variability of test performance. Higher test sensitivity is observed when cows are closer to clinical stages (Collins et al., 2006). In general, it is assumed that sensitivity and specificity are intrinsic characteristics of the tests and remain constant

among herds; however, if the sensitivity of the tests varied depending on the stage of the disease, then it would be expected that test sensitivity would vary with the proportion of cows in each stage of the disease. Whitlock et al. (2000) suggested that the proportion of cows in each fecal shedding category (low, moderate and heavy shedders) could vary depending on the prevalence of the disease. Currently, there is no published information about Johne's disease test performance and its association with the prevalence of the disease.

The most common tests used to diagnose Johne's disease are bacterial culture of feces, direct fecal PCR assay, serum ELISA and, recently, milk ELISA (Wells et al., 2006; Collins et al., 2006).

1.4.1. Bacterial culture of feces

Isolation of *MAP* from fecal samples is considered a definitive diagnosis of Johne's disease. Classical analysis of sensitivity and specificity of other diagnostic tests have used bacterial culture of feces as reference (Wells et al., 2006). Historically, fecal culture of feces has been considered the ante-mortem test of reference, because it was the most sensitive and specific for diagnosis of fecal shedders. The sensitivity of bacterial culture was estimated at approximately 50% using subclinically infected cattle with previous isolation of *MAP* from feces or tissue and its specificity was estimated at approximately 100% using cattle from negative herds (Collins, 1996).

Currently, there are several standardized procedures for bacterial culture in use for different laboratories (Whittington and Sergeant, 2001). The bacterial culture procedure for detection of *MAP* involves three important steps: concentration and separation of the bacteria from feces, selective decontamination to eliminate other microorganisms while retaining *MAP*, and growing the culture in selective media (Collins, 1996; Clark et al., 2008). The literature describes different procedures for *MAP* concentration, such as sedimentation, centrifugation, or filtration (Reddacliff et al., 2003) as well as different media for culture (Collins, 1996; Eamens et al., 1999; Whittington et al., 1998; Clark et al., 2008).

Test performance is variable with different fecal culture procedures are followed (Whitlock et al., 1988; Whipple et al., 1992; Eamens et al., 1999). Whitlock et al. (1988) compared centrifugation vs. sedimentation fecal culture methods. They found that centrifugation had a higher *MAP* isolation rate. The same findings were reported by Whipple et al. (1992), who compared 3 different methods of concentration and decontamination for bacterial culture: sedimentation, centrifugation and the Cornell method. Although these studies suggest that concentration of *MAP* in fecal samples using the centrifugation procedure may have higher epidemiological and analytical sensitivity than other procedures, this procedure is also associated with increased contamination of samples with other microorganisms (Whitlock et al., 1988; Whipple et al., 1992). Other studies comparing different fecal culture procedures have reported a higher sensitivity of fecal culture performed in liquid media (Whittington et al., 1998; Eamens et al., 1999). Currently, Herrold's egg yolk agar (HEY) is the media most used by U.S. laboratories for *MAP* culturing (Collins, 1996).

A study that followed a cohort of cows tested every 6 months for 4 years indicated that the sensitivity of the fecal culture for the first sampling was 42%. The denominator used to calculate sensitivity was the cumulative number of cows tested positive for fecal culture at the end of the 4 years (Whitlock et al., 2000). The sensitivity and specificity of the centrifugation fecal culture was estimated at approximately 75% and 99.8%, respectively (Wells et al., 2006).

Fecal culture test results may vary as a consequence of *MAP* strain. Different subtypes of *MAP* have led to diverse growth patterns in different culture media (Cernicchiaro et al., 2008). Similarly, Whittington et al. (1998) suggested that *MAP* strains from sheep and goat were more difficult to grow on solid than on liquid media.

One detrimental effect on the bacterial culture specificity is the "passive shedder" phenomena. It has been theorized that cattle exposed to environments with a high concentration of *MAP* could passively shed *MAP* after oral ingestion (Sweeney et al., 1991; Collins, 1996). These passive shedders may increase the false-positive rate of the fecal culture. The proportion of passive fecal shedders in a herd is unknown; however, it

is likely to be inversely associated to the proportion of heavy fecal shedders (Whitlock et al., 2000).

There is also a lack of information in the literature about the minimum detection levels of *MAP* using fecal culture. In spiked fecal samples, 10^2 CFU/g were estimated using culture on Lowenstein-Jensen media (Halldorsdottir et al., 2002). Similar results were obtained by Reddacliff et al. (2003), who reported a minimum detection level of $2.5x10^2$ *MAP*/g using centrifugation to concentrate *MAP* from spiked fecal samples, followed by culturing on liquid medium. Visser (1999) suggested that cows that shed low amounts of *MAP* could not be detected by fecal culture because *MAP* is contained in small clusters rather than homogenously distributed in the fecal sample.

Important disadvantages of fecal culture for detection of *MAP* are the time required for the results and the laborious nature of the test. Fecal culture requires up to 16 weeks of incubation, after which the results are expressed in a 5-level ordered qualitative score based on the number of colonies per slants observed, as follows: 0 = no colonies on the slant; 1 = between 1 and 10 colonies per slant; 2 = between 11 and 50 colonies per slant; 3 = between 51 and 100 colonies per slant; and 4 = more than 100 colonies per slant. Cows with fecal culture result 1 are considered as “light shedders”; cows with fecal culture result 2 are considered “moderate shedders”; and cows with fecal culture results 3 or 4 are considered “heavy shedders”. These classification systems are used to subjectively quantify the risk of transmission (vertical and horizontal transmission) of *MAP* among cows (Wells et al. 2006).

Although classify infected cattle based on the amount of *MAP* shed in feces may be important to estimate the risk of transmission to susceptible cattle, there is limited information about how results from bacterial culture of feces are associated to the actual number of *MAP* shed by cattle.

1.4.2. Direct fecal PCR assay

The performance of the PCR assay for detection of *MAP* on fecal samples depends on the PCR technique (DNA extraction, targeted gene, and DNA amplification) used for diagnosis. The most common gene used to identify *MAP* is the insertion

sequence IS900 (Vary et al., 1990; Collins, 1996), because it is repeated 14-20 times on the *MAP* DNA sequence; however, other studies have demonstrated the PCR test based on this insertion sequence could lead to false positive results (Cousins et al., 1999; Englund et al., 2002). Harris and Baletta (2001) suggesting that the primers originally used to amplify the IS900 of *MAP* (Vary et al., 1990) were also able to amplify the IS1626, which is present in *Mycobacterium avium*.

A study on spiked fecal samples established that a PCR assay based on ground beef samples indicated a differential analytical sensitivity for *MAP* culture and *MAP* PCR. The PCR had a higher analytical sensitivity in these samples. The PCR test led to positive results in 6 of the 6 samples spiked with 10^1 *MAP*, while the culture led to positive results in 2 out of 6 samples spiked with 10^3 *MAP* (Jaravata et al., 2007).

The overall relative sensitivity and specificity of the fecal PCR was estimated at 70.2% and 85.3%, using the fecal culture as reference when the PCR targeted the IS900 (Clark et al., 2008); however, the sensitivity of the fecal PCR assay increases when the levels of fecal shedding increases. The sensitivity of the fecal PCR was 57.1%, 75% and 100% for light, moderate and heavy shedders, respectively (Clark et al., 2008). Wells et al., (2006) estimated the sensitivity of a fecal PCR that targeted the MAV2 gene, at approximately 30%, using a Bayesian approximation; however, when results were compared to fecal culture, the relative sensitivity of the test was estimated at 23%, with 4.1% for light to moderate shedders and 76% for heavy fecal shedders.

1.4.3. Serum ELISA

Several studies have estimated the sensitivity of the serum ELISA test. These studies provide different sensitivity and specificity estimates, mainly due to changes in the ELISA technique, different host populations, different types of analyses, or as it was mentioned earlier in this review, differences on prevalence of the disease across the populations studied.

The specificity of the serum ELISA was estimated at 98.9% using bacterial culture as gold standard by Whitlock et al. (2000). The same study also estimated the sensitivity of the serum ELISA (IDEXX) at 54% using the first result out a series of 3

serum ELISA tests as a gold standard and ignoring the autocorrelation within-results of the same diagnostic test. When bacterial culture was used as gold standard in this study the sensitivity of the serum ELISA decreased to 25%. In another study, Wells et al. (2006) estimated the sensitivity and specificity of the serum ELISA at 27% and 95%, respectively, after using an analytical approach that did not require a gold standard. Similar results were obtained when a classical approach was used to estimate the sensitivity of serum ELISA against fecal culture with a value of 27.8%. An evaluation of another serum ELISA test obtained similar results compared to other studies. The sensitivity and specificity of the test for all shedders was approximately 31% and 98%, respectively (Clark et al., 2008). In contrast, a greater sensitivity value was estimated by Hendrick et al. (2005a), who found the serum ELISA sensitivity to be approximately 73% with respect to bacterial culture. Finally, another study that compared 4 different serums ELISA tests for Johne's disease reported a specificity ranging from 85% to 100%, while the sensitivity ranged from 28 to 45% (Collins et al., 2005).

Throughout the course of the *MAP* infection, cattle will develop different biological responses. Fecal shedding is a consequence of the colonization of *MAP* along the intestinal epithelia. Cellular and humoral immune responses are observed as a consequence of exposure of *MAP* to the immune system (Stabel, 2000). Several of these studies that estimated sensitivity and specificity of serum ELISA have used bacterial culture as a gold standard; however, bacterial culture of feces and serum ELISA are diagnostic tests that measures different biological phenomena in the cows. Even though it may not be completely appropriated to use bacterial culture as gold standard to evaluate the performance of serum ELISA, in practice, both diagnostic tests have been used to classify cattle at different stages of the disease (Collins, 2002; Collins et al., 2005).

As observed for bacterial culture and fecal PCR assays, the sensitivity of the serum ELISA varies depending on the stage of the disease, increasing with the progression of the disease. Whitlock et al. (2000) found that the sensitivity of serum ELISA was 15%, 50%, 75% and 87% for low shedders, moderate shedders, heavy shedders and clinical cases, respectively. Similarly, Wells et al. (2006) found a relative sensitivity of serum ELISA of 12.4% to detect light to moderate shedders and 72.7% to

detect heavy shedders. Clark et al. (2008) also found differences in the sensitivity of the test depending on shedding status, with values of 4.8%, 50% and 88.2%, for light, moderate and heavy fecal shedders, respectively. On the other hand, Hendrick et al. (2005a) reported serum ELISA positive samples in 62%, 100% and 95% of the low, moderate and high shedding cows, respectively.

1.4.4. Milk ELISA

The ELISA assay can also be applied to analyze milk samples, which are often easier to obtain than serum samples from dairy cows, because milk samples can be collected by the Dairy Herd Improvement Association for milk component testing. Results from milk ELISA test have a high correlation with those from serum ELISA. Hendrick et al. (2005a) estimated a Pearson's correlation of 0.79 for the quantitative test results and a kappa agreement of 0.44 for the binomial test results. Although not exactly equivalent, the results of the serum ELISA and milk ELISA are comparable (Collins et al., 2006).

The sensitivity of the milk ELISA compared to fecal culture was estimated at 61.1% for all shedders and 51%, 67% and 84% for light, moderate and heavy fecal shedders, respectively (Hendrick et al., 2005a). Collins et al. (2005) estimated a relative sensitivity of 28.8% for milk ELISA, while the specificity was 99.7% using three different bacterial culture procedures (parallel interpretation) as gold standard to estimate sensitivity and negative herds to estimate specificity. Another study using the same samples of Collins et al (2005) estimated the sensitivity of the milk ELISA in 13.4% and 66.7%, for light to moderate and heavy fecal shedders, respectively, using the average of 3 different bacterial cultures as reference test (Wells et al., 2006).

While diagnostic tests are useful tools to diagnose *MAP* infected cattle, it is important to take into consideration that they are imperfect and their sensitivity and specificity are partially understood. Bacterial culture of feces has been traditionally used as the reference test; however its performance varies depending on the *MAP* strain in the fecal sample, *MAP* concentration technique, and culture media used to identify *MAP* infected cattle. The variability of the performance of bacterial culture of feces and the

arrival of more sensitive diagnostic tests (i.e. direct fecal PCR) have limited its use as reference test.

Another problem when evaluating the performance of diagnostic tests is associated with the stage of infection. The performance of the diagnostic test varies depending on the stage of the *MAP* infection in the cow, and therefore an analysis of the performance of the diagnostic tests by stage of infection is needed. This analysis should also take into consideration that diagnostic tests measure different pathological phenomena within infected cattle (fecal shedding and inmunne response).

1.5. ECONOMIC IMPACT OF JOHNE'S DISEASE

1.5.1. Milk production

Lombard et al. (2005) found that cows with strong serum ELISA reactions against *MAP* infection produced approximately 1400kg less milk (mature equivalent 305) compared to test-negative cows. Another study in Canada found a decrease of 173 to 548 kg in 305-day milk production, depending on which diagnostic test was used. Higher losses were observed for fecal culture positive cows (Hendrick et al., 2005b).

A study in 2 dairy herds in Minnesota found that fecal culture-positive cows tested at the beginning of the lactation that stayed in the herd during the lactation, produced 11% less total milk during the lactation compared to fecal culture negative cows (Raizman et al., 2007). When the analysis was adjusted by other confounders and categorized by *MAP* fecal shedding levels of those cows, they found that the decrease in milk production was -537, -1403 and -1534 kg/lactation, for light, moderate and heavy fecal shedders, respectively (Raizman et al., 2007).

Another study indicated that cows infected with *MAP* produced more milk during the latent period than uninfected cows; however, heavy fecal shedders produced 3.7 and 6.0 kg/day less milk than negative and infected latent cows, respectively (Smith et al., 2009). These authors suggested that there is a high correlation between high production and predisposition to the infection.

A reduction in the total 305-day milk fat and milk protein associated with Johne's disease positivity was observed in a cross-sectional study by Hendrick et al. (2005b). A longitudinal study with a study population 10 times larger than the Hendrick et al (2005b) study, found no association between the percentage of milk fat and protein content and Johne's disease status of the cows (Lombard et at., 2005). The effect of *MAP* infection on milk solids (fat and protein) is likely associated to the decrease of total milk production and not to milk solids content.

No association between *MAP* infection status of the cows and the milk somatic cells linear score has been found (Lombard et al., 2005; Hendrick et al., 2005b; Raizman et al., 2007).

Milk production decreases in *MAP* infected cows and the magnitude of this decrease seemed to be dependent to the stage of the *MAP* infection. Cows in the advanced stage of infection showed a higher reduction of total milk production and therefore total milk solids.

1.5.2. Culling rate

Cows with stronger ELISA reactions against *MAP* were more likely to be culled within a year after the positive test than other cows (Lombard et al., 2005). Hendrick et al. (2005b) found in a survival analysis that cows with Johne's disease test positive results had a greater risk of being culled compared to negative cows and that risk was higher in fecal culture positive cows compared to milk or serum ELISA positive cows. Another study showed that non-fecal shedding cows were less likely to be culled from the herd compared to fecal shedding cows; however the level of fecal shedding was not associated with the likelihood of being culled (Smith et al., 2010). Similarly, Raizman et al. (2007) concluded that *MAP* fecal shedding cows had a lower survival rate and were more likely to leave the herd earlier than non-fecal shedding cows. They found that at 305 DIM, 80% of the fecal culture negative cows remained in the herd compared to 38% of the fecal culture positive cows. They also found that the survival of the cows also depended on the levels of *MAP* fecal shedding. The proportion of light, moderate and heavy fecal shedders that remained in the herd after 305 DIM was approximately 60%,

24% and 15%, respectively. It was likely, however, that after reporting the test results to the producers, heavy fecal shedding cows were prioritized to be included in the culling list, introducing bias to this association. Cows with clinical Johne's disease left the herd 202 days earlier than all the other cows that completed their lactation.

Overall, all studies showed that *MAP*-positive cows were culled from the herd earlier than *MAP*-negative cows, however, it is not clear that the advanced stages of *MAP* infection (measured using levels of *MAP* fecal shedding) were associated to a higher risk of being culled, unless cows were in a clinical stage.

1.5.3. Reproduction

It is challenging to evaluate the effect of subclinical Johne's disease on reproductive parameters in dairy cows, as, recognizing the Johne's disease status of the cows, producers may not include those cows in the breeding list (Raizman et al., 2007). On the other hand, if the subclinical manifestation of the disease have a real detrimental effect on the fertility of the cows, those cows may be culled for reproductive reasons, before they are even tested or diagnosed as *MAP* infected (Johnson-Ifearulundu et al., 2000).

A longitudinal study in Michigan showed that cows that tested ELISA positive had an open period 28 days longer than test-negative cows; however this association was not observed between cows that were fecal culture positive compared to test-negative cows (Johnson-Ifearulundu et al., 2000). Smith et al. (2010) observed an increased calving interval associated with high fecal shedding levels of *MAP* compared with low fecal shedders. In contrast, Lombard et al. (2005) found that cows with strong ELISA positive results had an open period 35 days shorter than negative cows. Smith et al. (2010) also reported that light fecal shedders or serum ELISA positive cows had higher calving rates than test-negative cows. Raizman et al. (2007) also found a detrimental effect of Johne's disease infected cows on the reproductive parameters of 2 dairy herds in Minnesota. They found that fecal culture positive cows were 2.8 times less likely to be inseminated than fecal culture negative cows, and this risk increased up to 9.6 for cows that were heavy fecal shedders.

The effect of *MAP* infection on the reproductive performance of dairy cows seemed to depend on the stage of infection. Cows in advance stages of infection showed the lowest reproductive performance; however, this association may be biased by differential reproductive management practices of the herd between *MAP*-positive or *MAP*-negative cows.

In summary, several studies have shown that JD represents an important economic impact to dairy herds in the short term by the association of the disease with low milk production, higher culling rates and low reproductive performance. However, JD also represents a long-term economic impact because infectious cows are likely to transmit the infection to susceptible herdmates, contributing to maintenance of the infection in the herd (Nielsen and Toft, 2008).

1.6. EPIDEMIOLOGY AND CONTROL

The recommended management practices of the Voluntary Johne's Disease Control Program (USDA-APHIS. 2010) are focused not only on reducing the transmission of Johne's disease from infected shedding cows to uninfected young calves, but also on prioritizing the culling of the most infectious cows, i.e., those cows that shed large amounts of *MAP* (Raizman et al., 2007).

Mathematical simulation models are important tools to understand the transmission of the infection, and evaluate the effect of control measures on the epidemiology of the infection; however, the assumptions behind simulation models are not always evident or easy to understand (Keeling and Rohani, 2008). There are several studies that used simulation models to understand the transmission of Johne's disease (Collins and Morgan, 1992; Groenendaal and Galligan, 2003; Kudahl et al., 2008; Mitchell et al., 2008). These models have studied the transmission of Johne's disease in the herd using different combinations of control strategies, including management, testing and culling. Kudahl et al. (2008) showed that control strategies that included management practices oriented to reduce the spread of the disease were the most effective to reduce prevalence. Similar findings were previously reported by Groenendaal and Galligan (2003) in another simulation model on mid size dairy farms in the United States. These

simulation studies also concluded that testing and culling positive cows alone does not stop the disease transmission and does not reduce the prevalence of Johne's disease in dairy herds.

Although simulation models show that management practices are the most important elements to control the transmission and reduce the frequency of the disease, testing and culling fecal shedders would also help reduce the prevalence of the disease. Farmers prefer to cull only heavy fecal shedders, and that may be effective in herds with good management practices; however, another simulation model concluded that herds with poor management practices would need to cull low and heavy fecal shedders, and test the cows more than once a year using high sensitivity tests in order to reduce the frequency of the disease Lu et al. (2008).

The published scientific literature is scarce about the effect of management practices, testing and culling using real herd data. A longitudinal field study showed a decrease in the frequency of the test-positive cows in 6 dairy herds enrolled in the Johne's Disease Demonstration Herd Project in Minnesota, after 5 years. The reduction of the disease incidence was mainly observed in those herds with lower risk assessment scores (lower risk of transmission). In addition, a reduction of the disease frequency was also observed in cows born after management practices had been implemented. These findings suggested that the reduction in the transmission of the disease was a consequence of well-implemented management practices focused on reducing transmission (Ferrouillet et al., 2009).

Similar observational studies in Pennsylvania and Wisconsin have demonstrated a reduction in the apparent prevalence of JD in cattle herds under a control program (Benedictus et al., 2008, Collins et al., 2010). The study in Pennsylvania included only one herd, and reported approximately a 50% decrease in the within-herd apparent prevalence of disease over an 18 year period after implementation of management practices to reduce transmission along with testing and culling (Benedictus et al., 2008). In the Wisconsin study, an overall decrease of approximately 50% in the apparent ELISA seroprevalence was noted in 9 dairy herds after 6 years in the program (Collins et al., 2010). Although the studies in Pennsylvania and Wisconsin showed a reduction in

apparent prevalence of infection and the study in Minnesota showed a reduction of the incidence of test-positive cows by birth cohort, eradication was not achieved in any of the herds. Mitchell et al., (2008) speculated that the combination of transmission of *MAP* infection among adult cattle as well as vertical transmission could play an important role on maintaining the disease in low prevalence herds.

Longitudinal studies with larger sample sizes have been conducted in Denmark and Australia. The Danish study included approximately 1100 herds and reported a decrease in the sero-prevalence of antibodies against *MAP* associated with specific JD disease management practices, such as culling repeated serum positive cows, feeding waste milk to calves and purchase of cattle from other herds (Nielsen and Toft, 2011). Another longitudinal study with 542 dairy herds participating in the Victorian Johne's disease test and control program in Australia for a period of 10 years indicated that approximately 6% of the herds (32) achieved 3 successive negative annual whole herd tests. Among other requirements, farms participating in this study agreed to cull all cattle that were ELISA positive as well as clinical cases (with compensation), and managed the herds to reduce the risk of transmission of the disease from adults to calves (Jubb and Galvin, 2004).

Vaccination may also help control the disease. In a controlled trial, Kormendy (1992 and 1994) found that calves vaccinated with heat-killed oil-adjuvated bacterin had reduced levels of *MAP* fecal shedding compared to the control unvaccinated group. Similarly, Koets et al. (2006) found a reduction of shedding levels in calves when using a vaccine based on a *MAP* recombinant heat-shock-protein of 70kD. In contrast, Kalis et al. (2001) found no differences in the fecal shedding levels between vaccinated and unvaccinated herds in a cross-sectional and a longitudinal study. Even though, vaccination may reduce the *MAP* fecal shedding levels, Kohler et al. (2001) found positive interference between the immune reaction derived from the vaccination against *MAP* infection and the diagnosis of bovine tuberculosis. In contrast, Santema et al. (2009), in a study that used another type of vaccine (Heat shock protein 70kD, Hsp70/DDA) found no interference with tuberculosis test results. The cost-benefit

analysis of vaccination programs proved to be highly profitable, if interference with the tuberculosis surveillance program was not taken into account (van Schaik et al., 1996).

Another alternative studied by Hendrick et al. (2006) to reduce *MAP* fecal shedding levels is the use of ionopheres. A randomized clinical trial showed that it is possible to reduce the shedding levels of subclinical cows using monensin. Fecal culture positive cows treated with monensin had reduced levels (3.6 colonies per slant) of fecal shedding compared to fecal culture positive cows treated with placebo.

Even though wild animal populations may serve as a reservoir for *MAP* (Harris and Barletta, 2001) an experimental transmission of *MAP* between wild life and sheep has been proven (Williams et al. 1983). In naturally infected herds, Raizman et al. (2005) showed that the common practice of manure spreading on crop fields may be a risk factor for transmission of *MAP* among dairy cattle, deer, and rabbits. Beard et al. (2001) in Scotland found *MAP*-positive and lesion compatible with *MAP* infection in wildlife species (fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare, and badger) using bacterial culture and histopathology. The role of wildlife animals as reservoir of *MAP* need to further investigate.

CHAPTER 2

REDUCTION IN INCIDENCE OF JOHNE'S DISEASE ASSOCIATED WITH IMPLEMENTATION OF A DISEASE CONTROL PROGRAM IN MINNESOTA DEMONSTRATION HERDS

Collaborators: S. Godden, W. L. Hartmann and S. J. Wells

Reduction in incidence of Johne's disease associated with implementation of a disease control program in Minnesota demonstration herds

This prospective longitudinal observational study was conducted to evaluate the effect of a standardized control program on the incidence of Johne's disease in 8 dairy herds in Minnesota. Depending on recruitment year, herds were followed between 5 to 10 years. Program compliance was evaluated using a cohort risk assessment score by birth cohort. Fecal samples from cows in study herds were tested annually using bacterial culture to detect *Mycobacterium avium* subsp. *paratuberculosis (MAP)*, and serum samples from study cows were tested using an ELISA to detect antibody to *MAP*. Clinical Johne's disease were also recorded.

Cohort risk assessment score decreased along birth cohorts. Depending on the follow-up period in each herd, 5 to 8 birth cohorts were followed to describe changes in the time to *MAP* bacterial culture positivity, serum ELISA positivity, *MAP* heavy shedding status, and clinical Johne's disease. The analysis of time to bacterial culture positivity, serum ELISA positivity, heavy fecal shedding status, and clinical Johne's disease using a time dependent Cox regression indicated a reduction of the instantaneous hazard ratio by birth cohorts and by cohort risk score; however, the strength of association between the cohort risk score and each of the 4 disease outcomes decreased over time. The age at which the cows were first tested positive for bacterial culture, serum ELISA and heavy fecal shedding, and the age of the cows at onset of clinical Johne's disease signs remained constant for all birth cohorts. Based on herd risk scores, overall herds complied with the recommended management practices in the program. Results were consistent with a within-herd reduction of Johne's disease transmission and that reduction was associated with the level management practices implemented as part of the control program.

2.2. INTRODUCTION

Paratuberculosis or Johne's disease (**JD**) is a chronic enteritis in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (***MAP***, Gardner et al., 2011) with a long incubation period (Hutchinson, 1996). In 2007, USDA-APHIS estimated that at least 68% of US dairy farms were infected with *Mycobacterium avium* subsp. *paratuberculosis* ***(MAP)***, including 95% of herds >500 cows, based on testing of environmental fecal samples (USDA-APHIS, 2008). Johne's disease causes economic losses directly associated with premature culling as well as reduced milk production (Raizman et al., 2007). Moreover, JD negatively impacts the welfare of cattle, because it has a slowly progressing detrimental effect on cow health, producing protein losing diarrhea and weight loss, which can lead to death. A practical treatment of the disease is not available.

In 1999, the Voluntary Bovine Johne's Disease Control Program (**VBJDCP**) was implemented in the United States and available for all cattle herds. One of the objectives of this program was to recommend implementation of specific herd management changes to reduce the risk of disease transmission (USDA-APHIS, 2010). In Minnesota, the Johne's Disease Demonstration Herd Program (**JDDHP**) started in 2000 and was later established at the national level by USDA in 2003. The JDDHP followed the recommendations of the VBJDCP and focused on evaluation of the effectiveness and feasibility of management-related herd practices to control JD over a period of several years in a selected group of dairy herds, based on the best scientific information available at that time. The objectives of the JDDHP were: 1) to establish management practices in order to minimize transmission of the disease; 2) to reduce environmental contamination from *MAP* fecal shedders; and 3) to prevent introduction of infected cattle from other infected herds (Ferrouillet et al., 2009).

Different strategies to control JD have been evaluated using simulation modeling to assess the effect of varying combinations of control strategies, including herd management, as well as testing and removal. Kudahl et al. (2008) showed that control strategies that included management practices focused on reduction of transmission of the disease were the most effective to reduce prevalence. Similar findings were previously reported by Groenendaal and Galligan (2003) in another simulation model of mid-size

dairy farms in the United States. These authors concluded that testing and culling test-positive cows alone does not stop disease transmission and does not reduce the prevalence of JD in the long term.

In these simulations, management practices were focused on reducing transmission between adult and younger cattle; however, a meta-analysis of 8 publications indicated that adult cattle could also become infected when they are exposed to environments with high levels of *MAP* contamination (Windsor and Whittington, 2010). Similarly, Mitchell et al. (2008) using simulation models found that transmission of *MAP* among adult cattle in combination with vertical transmission could explain the maintenance of herds with low levels of prevalence.

Observational studies in Pennsylvania and Wisconsin have demonstrated a reduction in the apparent prevalence of JD in cattle herds under a control program (Benedictus et al., 2008, Collins et al., 2010). The study in Pennsylvania included only one herd, and reported approximately a 50% decrease in the within-herd apparent prevalence of disease over an 18 year period after implementation of management practices to reduce transmission along with testing and culling (Benedictus et al., 2008). In the Wisconsin study, an overall decrease of approximately 50% in the apparent ELISA seroprevalence was noted in 9 dairy herds after 6 years in the program (Collins et al., 2010). Studies with larger sample sizes have been conducted in Denmark and Australia. The Danish study included approximately 1100 herds and reported a decrease in the sero-prevalence of antibodies against *MAP* associated with specific JD disease management practices, such as culling repetitive serum positive cows, used of wasted milk to feed calves and purchase cattle from other herds (Nielsen and Toft, 2011). The Australian study included cattle from 542 herds under a disease test and control program, and showed a constant decrease in the sero-prevalence of JD and clinical JD during 10 years of follow up (Jubb and Galvin, 2004). Even though prevalence may be useful to study changes on the frequency of the disease in the long term, incidence is a more appropriate parameter to use to study changes in disease transmission, and therefore a better measurement to evaluate the effect of a disease control program.

In the Minnesota JDDHP program, eight dairy herds were enrolled with a follow-up period that varied from 5 to 10 years, depending on the date of herd enrollment. A partial evaluation of the Minnesota project included 6 of the 8 enrolled dairy herds for a 5 year time period from 2000 to 2005. Unlike other studies that focused on the apparent prevalence, the Minnesota study demonstrated a reduction in the incidence of test-positive cattle after 5 years in the program and suggested a reduction in transmission of *MAP* as a consequence of well-implemented management practices focused on reducing transmission, testing and culling positive cattle (Ferrouillet et al., 2009). The disease control program in Minnesota has continued for an additional five years and a complete evaluation of the data was needed to evaluate the long-term effect of the control program on the incidence of JD, taking into account differences among herds.

The objectives of this study were to: i) evaluate the levels of implementation of the recommended management practices in study herds, ii) evaluate changes in JD incidence in study herds, and whether changes in the JD incidence of disease were explained by the level of implementation of the recommended management practices through time, and iii) evaluate if the recommended management practices delayed the onset of test positivity or clinical JD in the cows in these populations.

2.3. MATERIALS AND METHODS

2.3.1. Herd Selection and Characteristics

Eight dairy herds were enrolled in the JDDHP in Minnesota based on history of JD in the herds, assessed by the occurrence of clinical JD prior to study initiation, and owner willingness to participate in the program and cooperate with their herd veterinarian, University of Minnesota researchers and the Minnesota Board of Animal Health. Another criterion for herd enrollment was owner motivation to control and eliminate JD in the long term, including a plan to remain in operation for at least 5 years. All study dairy herds were members of the Minnesota Dairy Herd Improvement Association (**DHIA**). The direct costs of the JDDHP, including herd visits and testing, were funded by the Minnesota Board of Animal Health and USDA-APHIS-Veterinary Services. Herds were enrolled from 2000 to 2004, and remained on the program till 2008

or 2009. Therefore, depending on the year of enrollment and the number of years in the program, herds were followed between 5 to 10 years. Seven of the 8 herds used free stall housing and 1 used tie stall housing as the primary housing system for lactating cows. The mean herd mature equivalent adjusted to 305 days milk production was 11,240 kg (Table 1). Holstein was the predominant breed in all herds.

2.3.2. Recommended Management Practices

Herds owners and herd managers agreed to implement the following recommendations of the disease control program: "1. Clean, dry maternity area protected from manure from other adult cattle, 2. Separation of newborn calves from adult cattle as soon as possible after birth, 3. No use of pooled colostrum; instead, use colostrum from single identified, healthy, low risk or test negative cows, 4. Use of milk replacer or pasteurized milk to feed calves, 5. Minimal exposure of calves and heifers to the manure of mature cattle, 6. Minimal contamination of feed and water fed to young stock by manure from adults and no feeding of adult cattle refusal feeds, 7. Identification and culling of adult cattle contributing most to farm's infection load (*MAP* heavy fecal shedders) by testing or clinical observation in order to market them early or separate them from other cattle, 8. Acquisition of new cattle from low risk herds or testing with a fecal culture and blood for ELISA at the time of purchase, and 9. Use of separate equipment for manure cleaning and feed handling" (Ferrouillet et al., 2009). In this study, the JD disease control program consisted on the incorporation of these recommended management practices, evaluation of their level of implementation using risk assessment score and evaluation of their effectiveness buy measuring changes on the incidence of test positives and clinical JD.

2.3.3. Herd Visit and Risk Assessments

All herds were visited annually by JD certified veterinarians who performed the activities related to the VBJDCP described in more detail in the VBJDCP Program Standards (USDA-APHIS, 2010). Briefly, the VBJDCP Program Standards are focused on education, evaluation of the risk of transmission, and testing. Risk of JD transmission

was evaluated using a risk assessment tool which is a survey to evaluate the risk of JD transmission in six specific production areas: calving area, preweaned heifer calves, postweaned heifers, bred heifers, cows, and replacement and additions to the herd. Numerical scores were assigned to indicate the level of perceived risk in each of the sections of the risk assessment; therefore, the score scale differs in each production section as a reflection of changes in JD perceived susceptibility in cattle by age. The annual risk assessment scores (**ARAS**) for calving area, pre-weaned heifers calves, post-weaned heifers, bred heifers, and cows varied from 0 to 80, 0 to 50, 0 to 30, 0 to 24 and 0 to 16, respectively. The sum of the scores for these 5 sections represented the total ARAS for the herd and can therefore vary from 0 to 200. The official VBJDCP program and the ARAS considered yet another section titled "replacements and addition" that evaluated the risk of introduction cattle infected with JD; however, this study did not consider these data, because it was added to the total ARAS three years after the beginning of the MN JDDHP.

Although guidelines have not been established for ARAS interpretation, higher scores correspond with higher perceived risk, and lower scores correspond to lower perceiver risk (MNBAH, 2005, USDA-APHIS, 2010). Details of the ARAS application within the MN JDDHP have been presented previously (Ferrouillet et al., 2009). As part of the follow up plan, risk assessments were performed by trained veterinarians at the beginning of the program and again each year at approximately the same month.

2.3.4. Sampling and Testing

Sample collection and cattle handling procedures were approved and conducted in accordance with the University of Minnesota IACUC regulations. Individual blood and fecal samples were collected from each adult cow >24 months of age at least once a year in 6 herds on the same day and on approximately the same month each year. In the first 5 years of the program, the other 2 herds scheduled biologic sample collection based on stage of the lactation of the cows, though this changed to annual same day herd testing for the subsequent years of study. Blood samples were obtained from the caudal (tail) vein using a sterile needle and 7 or 10 cc vacuum tube. Blood samples were allowed to

clot and serum was collected for laboratory testing. Fecal samples were obtained directly from the rectum using separate plastic sleeves and a minimum of 10 grams of feces was collected in sample containers. Serum and fecal samples were refrigerated (4°C) and sent to the Minnesota Veterinary Diagnostic Laboratory within 24 hour after collection. Serum samples were tested using a serum ELISA (IDEXX laboratories, Inc, Westbrook, ME). Fecal samples were tested using bacterial culture of feces using sedimentation for 72 h followed by culture on Herrold's egg yolk (HEY) media on 4 slants (Wells et al., 2002). Positive samples for serum ELISA were detected when the Sample to Positive ratio (S/P) was ≥ 0.25. Similarly, fecal bacterial culture positive results were detected when the average of colonies forming units (**CFU**) on the 4 slants was greater than 0. Heavy fecal shedding cows were defined as cows with bacterial culture results ≥ 50 CFU on any of the slants for bacterial culture. Similarly, light fecal shedders cows were defined as cows with bacterial culture results > 0 and < 50 CFU on any of the slants for bacterial culture. Positive test results were used as indication of the JD infection status of the cattle.

2.3.5. Cow Data

Individual cow data, including date of birth, source of the cow (purchased or home-raised), lactation number, mature equivalent 305 milk production and culling information (date and reason) were downloaded from the Minnesota DHIA (DairyComp 305, Valley Agricultural software, Tulare, CA) at least every 6 months, for each herd for the duration of the program. Herd managers were asked to clearly record whether a cow was culled for clinical JD (defined by chronic diarrhea and weight loss with or without a confirmatory diagnostic test result). Based on the date when the program started for each farm, birth cohorts were created. Cows born between 24 and 12 before the program started were defined as birth cohort -2 in the study, and cows born between 12 and 0 months before the program started were defined as birth cohort -1. Similarly, cows born between 0 and 12, 12 and 24, 24 and 36, 36 and 48, 48 and 60, and >60 months after the program started, were defined as cohorts 0, 1, 2, 3, 4, 5, respectively. Therefore 8 birth cohorts (-2,-1, 0, 1, 2, 3, 4 and 5) were created based on the date the program started on

each herd. All cows that were 2 years old or older at time of testing and born after 60 months of the initiation of the program were included in cohort 5. Cows that were more than 24 months of age before the beginning of the program were not included in this analysis. All cattle in the different cohorts were followed for a period up to 60 months of age. For the analysis, cattle were right-censored at 60 months of age to allow enough time to the latent infected cows to become test positive, heavy fecal shedders or clinical JD, and to include a similar number of cows in each birth cohort.

Even though the total ARAS represents an estimation of the exposure to *MAP* of all the cattle in a herd on a specific year, the total ARAS does not allow assessment of the level of exposure to *MAP* of the different birth cohorts. Therefore, Cohort Risk Score (**CRS**) was created to characterize the exposure risk to *MAP* of cows from birth to adulthood. A CRS was calculated for the eight birth cohorts on each farm using the sum of ARAS from 3 consecutive years by stages of life as follows: the ARAS of the calving area, pre-weaned heifer calves and post weaning heifers from the year when calves in that birth cohort were born; the ARAS of bred heifers one year after the cohort was born; and the ARAS of cows and bulls two years after the cohort was born. The ARAS was not performed one year in two herds; therefore the closest risk assessment score by date in the herd was used instead.

2.3.6. Analysis

Different statistical models were performed to: i) evaluate the levels of implementation of the recommended management practices, by evaluating changes in the CRS (Evaluation of program compliance), ii) evaluate changes in *MAP* test-positive and clinical JD incidence, and its association with the level of implementation of the recommended management practices (Association of incidence and recommended management practices), and iii) evaluate if adoption of the recommended management practices delayed the onset of *MAP* test positivity or clinical JD in the cows in these populations (Age at onset of JD).

Evaluation of program compliance. In order to evaluate changes in the level of *MAP* exposure of birth cohorts, CRS was analyzed using a repeated measures exponential

regression. The natural logarithmic transformation of the CRS was used as the dependent variable and birth cohorts as an independent variable. The working correlation structure of CRS within farm was first order autoregressive and chosen using the Akaike's information criterion. The model was fitted using Mixed Procedure of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

Association of JD incidence and recommended management practices. Kaplan Meier survival curves were used to model each of the four primary outcomes of the study: 1) time from 24 months of age to age at first positive from bacterial culture of feces, 2) time from 24 months of age to age at first heavy shedding result from bacterial culture of feces, 3) time from 24 months of age to age at first positive from serum ELISA, and 4) time from 24 months of age to age at culling for clinical JD. Cattle age at first test positive was recorded in months and at the time when fecal and serum samples were collected or when cattle were culled with clinical JD. Analysis was stratified by birth cohorts and differences among strata were tested using the Wilcoxon and Log-rank test. The analysis was performed using the Lifetest procedure of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

The association between the recommended management practices and the incidence of JD was evaluated using a Cox regression model. Four models were built, with a separate model for each of the four primary outcomes of the study: 1) time from 24 months of age to age at first positive from bacterial culture of feces, 2) time from 24 months of age to age at first heavy shedding result from bacterial culture of feces, 3) time from 24 months of age to age at first positive from serum ELISA, and 4) time from 24 months of age to age at culling for clinical JD. Each of these outcomes was modeled using the following predictors: herd, source of the cattle (home raised or purchased), birth cohort, CRS and the 2-way interactions. A main effect model (with no interactions) was fitted for each outcome to obtain Martingale and Shoenfeld residuals; and the baseline hazard functions for the birth cohort -2. Martingale residual were used to confirm the functional form of CRS as a continuous variable and Schoenfeld residuals for each main covariate were obtained to evaluate the proportional hazard assumption. Schoenfeld residuals and baseline hazards were plotted against time using Gplot

procedure of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC). Visual examination of the Schoenfeld residuals showed a trend to decrease by time for the variable CRS in all the models. Similarly, a visual examination of the baseline cumulative hazard functions suggested a decrease of the hazards with time. Finally, the interactions CRS and time showed a statistical significance ($P<0.05$) in all the models. It was concluded that all models violated the proportional hazards assumption; therefore, all models included a time dependent interaction of CRS and time. Because events occurring at the same time were very uncommon, ties were handled using the Breslow's approximation. Birth cohort was included as a categorical variable instead of a continuous variable to allow comparison across birth cohorts and to avoid collinearity of this variable with CRS. A backward elimination model reduction was considered for non-significant variables ($P > 0.05$). Finally, few outliers were found using likelihood displacement; however, they were not found to be influential on the model parameters when deleted. Models were fitted using the PHreg procedure of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC). After model reduction all models included the same variables:

$$h_{ijk}(t) = h_0(t)*exp(B_1x_i+B_2x_j+B_3x_k+B_4(x_k*t))$$

Where:

$h_{ijk}(t)$= hazard ratio of the ijk^{th} case at time t

$h_0(t)$= baseline hazard at time t

B_1x_i = hazard ratio for the i^{th} herd, where i = 1 to 8 herds

B_2x_j = hazard ratio for the j^{th} birth cohort, where j = -2 to 5 birth cohort

B_3x_k = hazard ratio for the k^{th} total cohort risk score, where k = 0 to 200

$B_4(x_k*t)$ = time dependent interaction indicating rate of changes of the hazard ratio for the k^{th} total cohort risk score by time.

Age at onset of JD. The association of the control program with the age at detection of JD was evaluated using 4 different linear mixed models, where the ages of cattle in months at the first *MAP* bacterial culture positive result, first serum ELISA positive result, first *MAP* heavy fecal shedding result, and at detection of clinical JD were

used as dependent variables. This analysis used all the cows that tested positive or were JD clinical during the study period. All models included birth cohorts as explanatory variable and herd as random effect variable. Because the interest of this analysis was to evaluate the overall trend of the changes in age at first test positivity or clinical JD over successive birth cohorts, birth cohorts were treated as continuous variables instead of categorical variables, assuming linear association between birth cohorts and the outcomes. Model assumptions were evaluated using residual plots, and models were fitted using the Mixed procedure of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

2.4. RESULTS

Herds were enrolled in this study between 2000 and 2004, and they remained in the study between 5 to 10 years (Table 1). The herd size of the farms enrolled in the study varied from 42 to 341 cows. The vast majority of study cows were Holstein breed with an average ME305 milk production that varied by herd from approximately 9,700 to 15,600 kg per lactation at the year of enrollment. The annual apparent prevalence of *MAP* bacterial culture positive and JD serum ELISA positive cows varied from 3.6 to 21.8% and from 3.2 to 28.5%, respectively, at the year of enrollment, depending on the herd (Table 1).

The study population included 6,080 cows; however, 511 cows (8.4%) were not included in any analysis because no test was performed on those cows, samples were mislabeled, or bacterial culture was contaminated with fungal overgrowth. During the study period, 12,787 bacterial cultures and 12,735 serum ELISAs were performed. At the end of the study period, 7.26% of the 4,818 culled cows were culled due to clinical JD.

Evaluation of program compliance. At the beginning of the study, the mean (SD) total ARAS was 48.5 (14.7) points, while the same value at the end of the study was 30.6 (11.0) points. The mean (SD) risk assessment scores for the different production sections at the beginning and the end of the study were 22.2 (8.7) and 15.9 (8.4) for calving area, 4.5 (6.1) and 2.0 (1.4) for pre-weaning heifers, 4.5 (4.6) and 3.5 (3.3) for post-weaned

heifers, 12.7 (3.6) and 6.6 (3.1) for bred heifers, and 2.8 (1.6) and 2.6 (2.0) for cows and bulls.

The mean (SD) CRS of the first and last birth cohort under study were 50.9 (12.4) and 26.5 (10.8) points. Though there were variations in the CRS by birth cohort and across herds, overall all herds showed a numerical decrease in the CRS. The results of the exponential regression showed a decrease of 9.7% (95% CI 5.3 to 13.8) in consecutive birth cohorts ($P < 0.01$, Figure 1). The median (Interquartile Range) time between the date of fecal sample collection and the date of removal from the herd (culling date) was 8.4 months (9.8) for bacterial culture-positive cows. The same values for light fecal shedders and heavy fecal shedders were 10.3 months (11.0) and 4.7 months (4.5).

Association of incidence and recommended management practices. The Kaplan-Meier curves and the time-dependent Cox regression analysis included a total of 3,760 and 3,758 cows followed using *MAP* bacterial culture, and JD serum ELISA, respectively, in the 8 birth cohorts and 8 herds. A total of 3,911 cows were also observed for clinical signs of JD and included in these analyses. During the follow-up period 432, 350, 101 and 182 cows were positive to MAP bacterial culture, JD serum ELISA, *MAP* heavy fecal shedders and clinical JD, respectively (Table 2).

The Wilcoxon and the Log-rank tests of equality of Kaplan Meier survival curves indicated differences in the incidence of *MAP* test positive and clinical JD across birth cohorts for time to first *MAP* bacterial culture positive results ($P < 0.01$ and $P < 0.05$, respectively), time to first JD serum ELISA positive result ($P < 0.01$ and $P < 0.01$, respectively), and time to clinical JD ($P < 0.01$ and $P < 0.05$, respectively). The null hypothesis of no differences across birth cohort survival curves was not rejected for the time to first *MAP* heavy fecal shedding result (Figure 2).

In general, there was an increase of the percent of survivals or a decrease in the incidence of test positivity or clinical JD in successive birth cohorts. The proportion (95% confidence interval) of cows that remained negative to bacterial culture at 36 and 48 months of age were 0.93 (0.91 to 0.95) and 0.86 (0.83 to 0.90), respectively, in the birth cohort "-2", 0.94 (0.92 to 0.96) and 0.86 (0.83 to 0.90), respectively, in the birth cohort

"1", and 0.95 (0.93 to 0.98) and 0.85 (0.77 to 0.92), respectively in birth cohort "5". Similar results were observed for serum ELISA positivity, *MAP* heavy fecal shedders and clinical JD (Figure 2).

The variable home raised or purchased cattle and possible 2-way interactions were not significant in any of the survival models, and therefore were not included in the final models. The final time-dependent Cox regressions for the 4 outcomes, time to first *MAP* bacterial culture positive result, time to first JD serum ELISA positive result, time to first *MAP* heavy fecal shedding result, and time to clinical JD, included the same explanatory variables: herd, cohort, CRS and the interaction between CRS and time.

Time to first *MAP* bacterial culture positive result was associated with birth cohort ($P < 0.01$). Compared to the reference birth cohort ("-2"), the instantaneous hazard of testing bacterial culture positive for birth cohorts -1, 0 and 1 were similar to the reference birth cohort ("-2"); however, that instantaneous hazard of testing bacterial culture positive decreased approximately 40% in birth cohort 2, and approximately 60% in birth cohort 3, 4 and 5 compared to the reference birth cohort ("-2", Table 3). An increase of the CRS was also associated with an increase in the instantaneous hazard of testing *MAP* bacterial culture positive ($P < 0.01$). The instantaneous hazard of testing *MAP* culture positive increased 1.21 fold (95% CI = 1.19 to 1.23) for every point increase in the CRS; however, the strength of this association decreased by time ($P < 0.01$) by approximately 0.009 fold (95% CI = 0.008 to 0.01) per month of age.

Changes in the instantaneous hazard of *MAP* heavy shedding in feces were also associated with birth cohort ($P < 0.05$), CRS ($P < 0.01$) and the interaction between CRS and time ($P < 0.01$). No statistical differences in hazard ratios were found in birth cohorts -2 (reference birth cohort) and -1, 0, 1, and 2; however, the hazard ratios of birth cohort 3, 4 and 5 were lower than the same reference level (Table 3). The estimated increase in the instantaneous hazard of testing *MAP* heavy fecal shedding was 1.27 fold (1.22 to 1.33) per unit of increase in the CRS ($P < 0.01$); however the magnitude of this association decreased 0.01 fold (0.008 to 0.01) by month of age ($P < 0.01$).

A similar time dependent Cox regression to evaluate changes in the instantaneous hazard of testing *MAP* serum ELISA positive explained by birth cohorts, also showed a

relative constant decrease of the instantaneous hazard from birth cohorts 0 compared to previous birth cohorts ($P < 0.01$, Table 3). Cohort risk score was also associated ($P < 0.01$) with the instantaneous hazard of ELISA positive and this association was modified by time in the program ($P < 0.01$). The instantaneous hazard of testing serum ELISA positive increased 1.22 fold (95% CI = 1.20 to 1.25) for each point of increase in the CRS; however, the strength of this association was decreased 0.01 fold (0.009 to 0.01) per month of age.

Finally, the instantaneous hazard of clinical JD was associated with birth cohort ($P < 0.01$), CRS ($P < 0.01$), and the interaction between CRS and time ($P < 0.01$). A reduction in the instantaneous hazard of clinical JD was observed from birth cohort 1 and latter birth cohorts compared to the reference birth cohort ("-2", Table 3). The instantaneous hazard of clinical JD increased 1.33 fold (1.28 to 1.40) per unit of increase on the CRS; however the magnitude of this association decreased 0.013 fold (0.011 to 0.015) per month of age.

Age at onset of JD. The overall mean age (SD) of first *MAP* bacterial culture positive result was 46.8 (15.2) months across all herds and cohorts. The mean age of first serum ELISA positive result, *MAP* heavy fecal shedding, and clinical JD was 45.7 (14.8), 49.5 (16.0), and 50.7 (14.1) months, respectively. No overall tendency on the age at the first *MAP* bacterial culture positive, serum ELISA positive result, *MAP* heavy fecal shedding, and clinical JD was observed for successive birth cohorts.

2.5. DISCUSSION

Several management practices have been recommended to reduce transmission of JD in dairy cattle herds. While there are few studies that have shown the effectiveness of some of these management practices on reducing the prevalence of JD (Collins et al., 2010, Nielsen and Toft, 2011, Jubb and Galvin, 2004), this study is the first to evaluate herd management practice changes in JD incidence in multiple herds, adjusting for recommended management practice compliance. The Minnesota Johne's Disease Demonstration Herd project was in operation for up to 10 years, starting with herds

enrolled in 2000, longer than other herds in the JDDHP. A partial evaluation of this program with 6 herds and 6 years of follow-up was performed previously (Ferrouillet et al., 2009); however, the project included data from 2 additional herds and continued monitoring until 2009. Now that data collection for this project concluded, a complete evaluation involving the 8 herds enrolled for the whole study period was needed.

This study was a prospective longitudinal field study, in which changes in herd management practices were recommended in order to reduce the transmission of JD. The compliance on the implementation of the recommended management practices was annually evaluated using the ARAS. Based on the ARAS, the level of disease exposure of each birth cohort was estimated and quantified using the CRS, as explained above. We observed an overall decrease of the CRS throughout years in the study and birth cohorts. Herds reduced the CRS by almost 50%, when the first and the last cohort under study were compared. Cohort risk score represented a subjective evaluation of the disease exposure of each birth cohort, and a reduction in the CRS by birth cohorts indicated a decrease in the disease exposure in successive birth cohorts.

Even though herd managers and owners were willing to participate in the control program and were enthusiastic about the program, none of them were able to implement all the recommended management practices to reduce the ARAS or CRS score to 0. In fact, the compliance with the recommended practices, evaluated using the ARAS, was similar to values observed in other Minnesota dairy herds enrolled in the VBJDCP, when the same period of time is compared (Wells et al., 2008). Analyses of the factors that affect the level of compliance of the recommended management practices were beyond the scope of this study; however, we expected a positive association between the level of compliance and the ease and feasibility of implementation of recommended practices in the herds and between the level of compliance and the within herd prevalence of JD . All herds enrolled in this study showed a numerical reduction in the ARAS during the study period, and the observed decrease of the CRS across birth cohorts was a consequence of the reduction on the ARAS. Similarly, in Michigan, a study that used a similar risk assessment survey found that 6 out 7 dairy herds were able to reduce their risk score after 5 years in a control program (Pillars et al., 2011). Another study of 54 herds in Australia

that focused on calf rearing related management practices to control JD found that only 18.5% of the herds followed all the main recommendations on calf management practices recommended to control JD (Ridge et al., 2005).

It is hypothesized that a reduction on the CRS would decrease the *MAP* exposure and effective transmission of JD to susceptible cattle, and as a consequence, a decrease in the JD incidence on successive birth cohorts should be expected. The study design, however, did not include cows not subject to disease control measures, or herds that did not implement any changes in management practices in order to reduce disease transmission during the same study period. Since *MAP* exposure levels were quantified using the CRS for each birth cohort; within each herd, previous birth cohorts served as controls for the following birth cohorts, since they were exposed to different levels of *MAP*.

The selected study herds were willing to participate in the JD control program and they perceived JD as an important disease for the dairy production. To illustrate the perception of JD to Minnesota dairy producers, by 2006, Minnesota was the US state with the highest proportion of dairy herds (30.8%) enrolled in the VBJDCP at a national level (Wells et al., 2008). Even though, herd managers showed interest in reducing the incidence of JD, the reduction was only observed 1 or 2 years after the program formally started.

The unadjusted and non-parametric survival analysis using Kaplan Meier curves indicated differences in the survival curves of bacterial culture and serum ELISA test positive or clinical JD across birth cohorts. In general, those birth cohorts that were born after the beginning of the control program showed a lower risk of testing bacterial culture or serum ELISA positive or being a clinical JD.

The hazard ratios of testing bacterial culture positive showed an important reduction (approximately 60%) between birth cohorts 3 and 5 compared to previous birth cohorts. The main effect of changes on the management practices on the reduction of the disease transmission were not observed in cows that were born within the first 2 years of the program (birth cohorts 0 and 1). During this period, most of the practical and economically feasible management changes recommended were likely implemented by

the herds. The hazard ratios of testing bacterial culture positive for the birth cohort 3, 4, and 5 were almost constant, what may suggest that the disease transmission reached an equilibrium point with the host and environment of the farms and (or) the presence of cows that passively fecal shed *MAP* to a level of detection by the bacterial culture.

A reduction of the instantaneous hazard of testing bacterial culture positive was not observed until birth cohort 2, cows that were born within the third year after the beginning of the program. In contrary with what was observed by the hazard ratios for bacterial culture positive cows along birth cohorts, the hazard ratios of serum ELISA positive cows showed an overall reduction from birth cohorts 1 to 5. The reduction on the hazard ratios of serum ELISA positive along birth cohort was more constant (almost linear) in those cows that were born after the formal control program started, birth cohort 0. Although hazard ratios of bacterial culture positive and serum ELISA positive cows showed a decrease along birth cohorts, the decrease on the instantaneous hazard of serum ELISA positive cows was early compared to the decrease of bacterial culture positive hazard ratios. One explanation is that cows exposed to *MAP* contaminated environments and passively fecal shedding *MAP* may lead to positive bacterial culture of feces (false positive), but without humoral immune reaction detected by serum ELISA.

Unfortunately, it was not possible to directly compare these ratios between tests, because they are on a relative scale with respect to an unknown reference baseline, and the baseline for each test may be different.

Hazard ratios of *MAP* fecal heavy shedders showed a reduction in cows that were born within the fourth year of the control program (birth cohort 2); and later, compared to the reference birth cohort (-2). Study herd managers were asked to cull those cows that shed large amounts *MAP* into the environment (*MAP* heavy fecal shedders) as soon as they were detected. The observed reduction in new cases of *MAP* heavy fecal shedders could be expected as a consequence of a combination of several reasons: 1) decrease on JD transmission, 2) culling test positive cows before they became *MAP* heavy fecal shedders, and 3) premature culling of these cows because of decreased lactation performance.

A reduction in JD transmission in the herd as a consequence of the disease control program could have a direct impact reducing the incidence of not only *MAP* fecal shedders, and also by reducing the proportion of *MAP* fecal shedders that became *MAP* heavy fecal shedders later on. The reduction in the incidence of *MAP* heavy fecal shedders may also be explained as an indirect consequence of early detection and culling test (bacterial culture and serum ELISA) positive cows before these cows became *MAP* heavy fecal shedders. Finally, *MAP* heavy fecal shedding is associated with reduced milk production of approximately 1500 kg per lactation (Raizman et al., 2007), therefore *MAP* heavy fecal shedders could have been culled for low milk production before they were diagnosed as *MAP* heavy fecal shedders.

Herd managers were aware of the importance of culling cows with clinical JD, because the negative effect of the disease on the production performance (Raizman et al., 2007) and because these cows are likely shedding large amount of *MAP* into the environment and transmitting the disease to susceptible cattle. Hazard ratios of clinical JD showed a constant decrease of the instant hazard after the third year of the program and, in fact, the hazard ratio of clinical JD at birth cohort 5 was zero with no observed cases for that birth cohort.

All Cox regression models in this study had a significant time dependent interaction with the CRS, indicating that the association of CRS with the instantaneous hazard was not constant along the life of the cows. Cohort risk score was positively associated with the 4 different outcomes, indicating that an increase in the CRS was associated with an increasing instantaneous hazard of testing bacterial culture positive, serum ELISA positive, heavy shedding or clinical JD. The estimated values of the time dependent interaction indicated that the magnitude of the association between CRS and the 4 outcomes decreased with the age of the cows.

A decrease in the strength of the association between the level of implementation of the recommended management practices and the outcomes of the study during the life of the cows may be explained by the fact that the susceptibility against JD is age dependent (Windsor and Whittington, 2010). After birth, calves are considered to be the most susceptible and this susceptibility decreases when cattle become older. The risk

assessment score of the herds takes this into consideration by placing 40% of total possible score (80 out of 200) in the calving area. However, though adult cattle are less susceptible to JD, transmission of the disease among this age group may still occur, and, in fact, may play an important role in maintaining disease in herds with low frequency of the disease and in which disease transmission in calves has been minimized (Mitchell et al., 2008). Other factors that may contribute to maintaining JD in the herd may be the role that uncontrolled and/or unknown risk factors play in the dynamics of the disease. The importance of recommended management practices in controlling disease transmission may vary depending on the frequency of the disease. The management practices were recommended to minimize the risk of disease transmission based on the best current knowledge at that moment; however, at low disease frequency, uncontrolled and unknown risk factors may become proportionally more important, and may explain some levels of disease transmission in these herds after 5 to 10 years in the control program.

In this study, not all birth cohorts were followed during the same period of time. Older birth cohorts were followed for a longer period of time, which increased the probability of finding new test positive cows in older birth cohorts compared to younger birth cohorts. In order to control for the bias generated by differential follow up time among birth cohorts, the analysis of the data considered that all cows were censored at 60 months of age (approximately 3 lactations), or at the end of the study.

We also considered that the control program may not reduce transmission and incidence of the disease along birth cohorts, but instead may only delay the age of positive testing or culling for clinical JD. Censoring all cows at 60 months of age controlled the differential follow-up time bias for the birth cohorts; however, this approach did not allow us to evaluate whether the control program reduced or delayed the disease transmission. The objective of the analysis of age at first positive tests or culling for clinical JD was to evaluate the existence of differential age of testing positive and culled for JD among birth cohorts.

All birth cohorts showed similar mean age at first positive test or clinical JD. The mean age at first fecal culture positive was similar to the mean age at first serum ELISA test positive; however, as expected, the mean age of *MAP* heavy fecal shedding and

clinical JD was approximately 4 to 5 months older. Even though, based on these results, it is not possible to make inferences about the progression of the disease at the individual cow level and study how test results change along with JD progression over time, it is possible to make inferences at a population level about the expected age when cows become test positive, or clinically ill if infected. In a herd where JD is prevalent and under a control program, the likelihood that a cow tests positive for *MAP* or becomes clinical JD during the first 3 lactations is higher than a cow in the same herd in the fourth or fifth lactation.

The annual testing scheme of the cows in this study resulted in an overestimation of the time to test positive, because positive cows could have tested positive earlier if they were tested more frequently. Similarly, age at clinical JD may be overestimated because cows with clinical JD were recorded the day that they were culled, and cows could have shown signs of clinical JD before the culling event. Even though the age at positive test or clinical JD was likely over-estimated, this bias would not represent a differential misclassification, because hazard ratios are a relative measure that compare the instantaneous hazard of testing positive of each birth cohort with an unknown baseline hazard of the reference birth cohort (-2). Therefore, if the lag time between becoming positive or clinical JD and test detection as positive or culling is similar in all the birth cohorts, the estimated hazard ratios should be valid.

Although this observational study did not allow the authors to conclude causal associations between the implementation of the recommended management practices and the reduction of incidence of test-positive cattle and clinical JD, it is possible to conclude that herds enrolled in the study were able to effectively reduce the incidence of test-positivity and clinical JD in successive birth cohorts and the magnitude of this reduction was associated with a higher level of implementation of the recommended management practices suggested by the VBJDCP. The longitudinal nature of the study demonstrated not only a temporal association between changes in management practices and the incidence of test-positive cattle and clinical JD, but also estimated the strength of these associations. The results of this study reinforce the hypothesis that the implementation of

the recommended management practices reduces JD transmission leading to a reduction in the incidence of disease.

Table 1. Characteristics of the 8 dairy herds enrolled on the Minnesota Johne's Disease Demonstration Program at the beginning of the program

Herd	Housing system[1,2]	Herd size[1]	Fecal culture prevalence [1]	Serum ELISA prevalence[1]	Herd Average ME305[1](kg)	Follow-up period
1	FS	269	14.7%	28.5%	11,104	2000-2009
2	FS	42	38.3%	8.3%	9,698	2000-2008
3	FS	198	3.6%	3.2%	10,494	2004-2009
4	FS	181	11.5%	8.8%	15,592	2000-2008
5	TS	44	15.9%	18.2%	10,561	2001-2008
6	FS	246	15.9%	6.5%	11,595	2004-2008
7	FS	341	21.8%	9.2%	11,608	2000-2008
8	FS	225	13.8%	11.4%	9,271	2000-2005

[1] at the year of enrollment.

[2]FS = Free Stall, TS = Tie Stall.

Table 2. Number of cows followed and percentage of cattle censored by birth cohort and diagnostic test in 8 dairy herds under Johne's disease control program in Minnesota

Birth	Bacterial Culture[2]		Serum ELISA[3]		Heavy Fecal Shedders[4]		Clinical[5]	
Cohort[1]	N	Censor	N	Censor	N	Censor	N	Censor
-2	611	85.11	612	84.31	611	95.42	649	91.06
-1	582	88.49	584	87.50	582	97.25	588	93.71
0	537	90.13	535	90.47	537	97.95	542	94.65
1	519	86.51	521	90.98	519	97.11	538	95.35
2	462	88.96	469	92.11	462	96.97	474	96.62
3	329	88.15	335	91.94	329	97.87	343	96.79
4	342	89.47	353	96.03	342	97.66	357	98.32
5	378	93.39	349	98.57	378	99.47	420	100.00

[1]Birth cohorts represent annual birth cohorts before and after the implementation of the disease control program (before: -2 and -1; after: 0, 1, 2, 3, 4 and 5).

[2] *Mycobacterium avium* sp. *paratuberculosis* bacterial culture in Herrold's egg yolk media.

[3] Johne's disease serum ELISA (IDEXX).

[4] *Mycobacterium avium* sp. *paratuberculosis* bacterial culture in Herrold's egg yolk media with diagnostic test results > 50 colonies forming units/slant .

[5] Clinical Johne's disease as recorded by herd managers.

Table 3. Hazard Ratios (HR) and 95% Confidence Interval (95%CI) of test positive *Mycobacterium avium* subsp. *paratuberculosis* bacterial culture of feces, Johne's disease serum ELISA and bacterial culture heavy fecal shedder, and clinical Johne's disease, across birth cohorts in 8 dairy farms under a disease control program in Minnesota

Birth	Bacterial Culture[2]		Serum ELISA[3]		Heavy Fecal Shedders[4]		Clinical[5]	
Cohort[1]	HR	95%CI	HR	95%CI	HR	95%CI	HR	95%CI
-2	1.0	Ref.	1.0	Ref.	1.0	Ref.	1.0	Ref.
-1	0.97	0.70-1.35	0.91	0.66-1.24	0.70	0.37-1.32	0.79	0.47-1.33
0	0.81	0.57-1.15	0.71	0.50-1.00	0.51	0.25-1.05	0.58	0.33-1.01
1	1.11	0.80-1.53	0.65	0.45-0.93	0.84	0.43-1.62	0.66	0.38-1.14
2	0.64	0.42-0.97	0.42	0.27-0.66	0.60	0.28-1.26	0.08	0.03-0.23
3	0.47	0.30-0.75	0.24	0.15-0.40	0.28	0.11-0.74	0.07	0.02-0.19
4	0.44	0.28-0.69	0.11	0.06-0.20	0.32	0.13-0.80	0.03	0.01-0.9
5	0.36	0.22-0.60	0.04	0.02-0.09	0.09	0.02-0.39	0.0	0.0-0.0

[1]Birth cohorts represent annual birth cohorts before and after the implementation of the disease control program (before: -2 and -1; after: 0, 1, 2, 3, 4 and 5). Birth cohort -2 represents the reference level.

[2] *Mycobacterium avium* sp. *paratuberculosis* bacterial culture in Herrold's egg yolk media.

[3] Johne's disease serum ELISA (IDEXX).

[4] *Mycobacterium avium* sp. *paratuberculosis* bacterial culture in Herrold's egg yolk media with diagnostic test results > 50 colonies forming units/slant .

[5] Clinical Johne's Disease as recorded by herd managers.

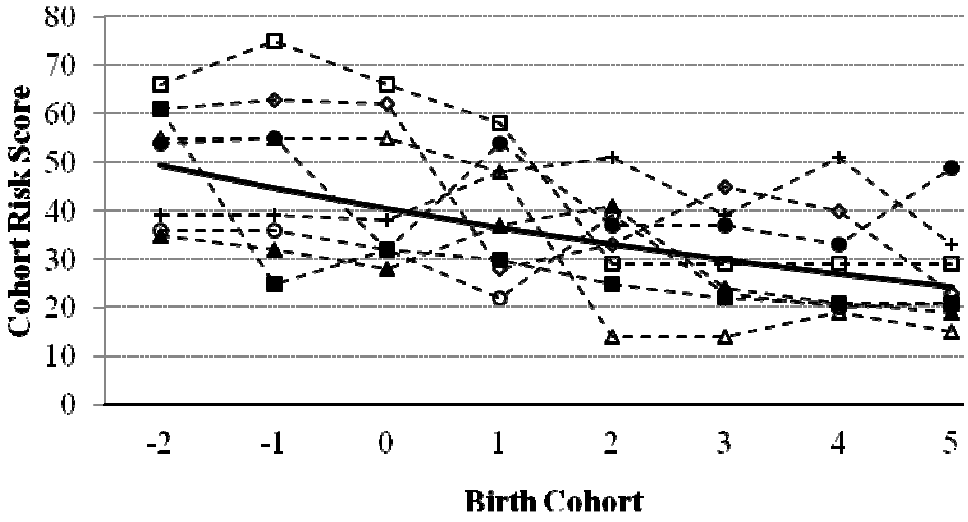

Figure 1. Cohort risk score of Johne's disease by birth cohort in 8 dairy herds under Johne's disease control program in Minnesota. Dashed lines with different shape symbols indicate herd CRSs and solid line is the exponential regression across herds

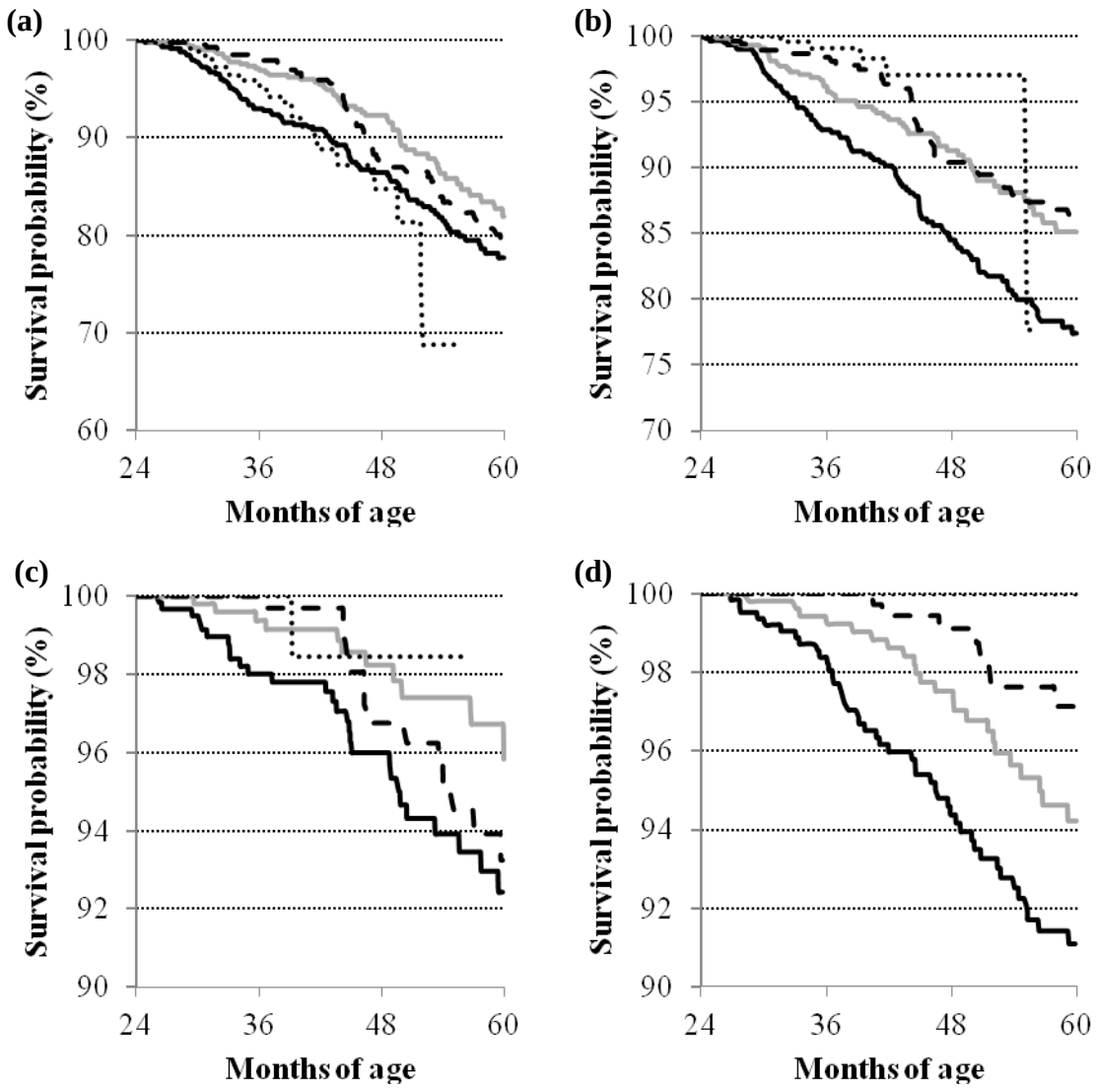

Figure 2. Kaplan Meier survival curves for the time to first *Mycobacterium avium* subsp. *paratuberculosis* positive bacterial culture (a), time to first positive Johne's disease serum ELISA (b), time to first *Mycobacterium avium* subsp. *paratuberculosis* heavy fecal shedder (c), and time to clinical Johne's disease (d) by annual birth cohorts in 8 dairy herds under Johne's disease control program in Minnesota. Annual birth cohorts represent birth cohort 2 year before (solid black line), at the year (solid grey line) and 2 (dotted line) and 5 (dashed line) years after the implementation of a Johne's disease control program

CHAPTER 3

EVALUATION OF THE ANALYTICAL SENSITIVITY OF BACTERIAL CULTURE AND FECAL PCR TO DETECT *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN BOVINE FECAL SAMPLES

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Evaluation of the analytical sensitivity of bacterial culture and fecal PCR to detect *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples

The objectives of this study were to estimate the analytical sensitivity of bacterial culture of feces and direct fecal PCR to detect *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) and the association between the concentration of *MAP* in feces and categorical bacterial culture results. We conducted two experiments in which three different strains of *MAP* were used to spike *MAP*-negative autoclaved and non-autoclaved bovine fecal samples at six different concentrations. The final concentrations of *MAP* in fecal samples were 10^8, 10^7, 10^5, 10^3, 10^1 and 0 *MAP*/g of feces in experiment 1 and 10^6, 10^5, 10^4, 10^3, 10^2, and 0 *MAP*/g in experiment 2. *MAP* spiked fecal samples were sent blind to two different laboratories to perform bacterial culture and direct PCR assays. Bacterial culture-positive results were observed in two of the *MAP* strains in experiment 1. Results of bacterial culture and fecal PCR indicated no differences between *MAP* strains, laboratories or autoclaved and non-autoclaved samples. The analytical sensitivity of the bacterial culture was 10^5 *MAP*/g of feces. The analytical sensitivities of the fecal PCR in experiments 1 and 2 using different approaches were 10^7 and 10^2 *MAP*/g of feces, respectively. The probability of a higher bacterial culture result increased with the concentration of *MAP* in the fecal sample.

3.2. INTRODUCTION

Johne's disease (**JD**) or paratuberculosis is a chronic enteritis in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (***MAP***, Gardner et al., 2011). Johne's disease causes economic losses directly associated with premature culling, poor reproductive performance as well as reduced milk production (Raizman et al., 2007; Smith et al. 2010). Infected animals can shed variable amounts of *MAP* into the environment (Whitlock et al., 2000) and susceptible cattle can become infected through the consumption of water or feed contaminated with feces infected with *MAP*. Infection

through placenta, milk and colostrum has also been described (Streeter et al., 1995; Sweeney, 1996; Whittington and Windsor, 2009).

Isolation of *MAP* using bacterial culture from fecal samples is considered a definitive diagnosis of *MAP* infection and classical analysis of sensitivity and specificity of other diagnostic tests has used bacterial culture of feces as the gold standard (Whitlock et al., 2000; Wells et al., 2006). Bacterial culture has also been used to classify *MAP* infected cattle into heavy, light or non-fecal shedders. This classification system has also been used to subjectively quantify the risk of transmission (vertical and horizontal transmission) of *MAP* among cows (Wells et al. 2006).

The bacterial culture procedure for detection of *MAP* involves three important steps: concentration and separation of the bacteria from feces, selective decontamination to eliminate other microorganisms while retaining *MAP*, and culture on selective media (Herrold's egg yolk media) slants (Collins, 1996; Clark et al., 2008). Since the procedure is not specific for *MAP*, viability of *MAP* can be compromised during the harsh decontamination procedures and an unknown number of *MAP* cells is expected to be lost.

Changes to the decontamination procedure (Whitlock et al., 1988; Whipple et al., 1992; Eamens et al., 1999), type of culture media (Whittington et al., 1998; Eamens et al., 1999), and predominant *MAP* strains in the sample (Whittington et al., 1998; Cernicchiaro et al., 2008) affect the performance of the bacterial culture. Moreover, there is limited information in the literature about the minimum detection levels of bacterial culture of *MAP*. The minimum detection level for *MAP* has been estimated at approximately 10^2 Colony Forming Units (**CFU**/g) on Lowenstein-Jensen media (Halldorsdottir et al., 2002) or liquid media (Reddacliff et al., 2003) using spiked fecal samples with *MAP* ATCC 19698 and *MAP* S strain, respectively. Visser (1999) and Collins et al. (2006) suggested that cows that shed low amounts of *MAP* in early stages of JD could not be detected by bacterial culture because *MAP* is typically contained in small clusters rather than homogenously distributed in the fecal sample or because of the lack of sensitivity of the current tests.

There is a need to quantify the precise number of *MAP* cells lost due to the bacterial culture procedure, and therefore estimate the concentration of *MAP* that cows actually shed into the environment. This will help to better understand the culture test results and quantify the amount of *MAP* that shedding cows release into the environment.

This is particularly important when interpreting the current in-field classification of moderate and light fecal shedders, as current management practices to control JD allow for maintaining these animals in herds despite their positive status while prioritizing the culling decision on heavy fecal shedding cows.

Important disadvantages of bacterial culture of feces for detection of *MAP* are the time required for the test completion and the laborious nature of the test. Another diagnostic test used to isolated *MAP* from feces and confirm fecal shedding status of cattle is Polymerase Chain Reaction (PCR, Wells et al. 2006). Though several studies have compared PCR and bacterial culture performance of fecal samples (Wells et al. 2006; Clark et al., 2008), PCR methods have changed through time, and there is limited evidence describing the analytical sensitivity of specific PCR assays. The objectives of this study were: 1) to estimate *MAP* losses during the bacterial culture procedure, and 2) to determine and characterize the association between test results (fecal culture and fecal PCR) and the concentration of *MAP* in fecal samples.

3.3. MATERIALS AND METHODS

This study was divided into two experiments (Figure 1). Both experiments included the following steps: 1) Collection of *MAP* negative fecal samples, 2) Growth of *MAP* in pure cultures, 3) Preparation of *MAP* dilutions, 4) Preparation of negative fecal samples, 5) Spiking of fecal samples and 6) Laboratory analysis of spiked fecal samples. Differences between experiments were the *MAP* strains used to spike fecal samples, the methodology to quantify the *MAP* concentration in pure cultures, the final concentration of *MAP* in the spiked fecal samples, and the direct PCR assay used. Experiment 1 was performed during the first semester of 2009, and the experiment 2 was performed during the first semester of 2010.

3.3.1. Collection of *MAP* negative fecal samples

Bovine feces and blood were obtained for each experiment on the same dairy herd (cows in tie-stall housing) on level 4 of the US Johne's Disease Test Negative Program

from Minnesota. This level of the program provided a 99 percent confidence of having a non-infected herd (USDA-APHIS, 2005). Samples were collected from five adult cows in their second and third lactation at the time of sampling. Approximately 1-2 kg of feces were collected from each cow directly from the rectum using individual plastic sleeves. Feces were stored at -20C in individual plastic bags. An aliquot of the feces from each cow was also collected in a 50 ml sterile specimen container for laboratory testing. Blood samples were obtained from the caudal (tail) vein using a sterile needle and 7 or 10 cc vacuum tube. Blood samples were allowed to clot and serum was collected for laboratory testing. Individual serum and fecal samples were refrigerated (4 C) and sent to the Minnesota Veterinary Diagnostic Laboratory (MNDVL) within 24 hours after collection to confirm that cows were negative to *MAP* using i) serum ELISA (IDEXX laboratories, Inc, Westbrook, ME), ii) bacterial culture of feces on Herrold's egg yolk media (HEYM, Wells et al., 2002) and iii) direct fecal PCR. In experiment 1, fecal samples were confirmed negative using the MAV2 TaqMan PCR described by Wells et al. (2006) and in experiment 2 using the TaqMan real time PCR using Ambion® reagents (Ambion®, Applied Biosystems, Carlsbad, CA). All fecal and serum samples were negative according to these tests which provided confirmation the cows were *MAP* negative.

3.3.2. Growth of *MAP* in pure cultures

All *MAP* strains were obtained from the Johne's Disease Integrated Program *MAP* strains repository. In experiment 1, pure cultures of *MAP* 1018, 7565, and 9179 strains were grown in Middlebrook 7H9 liquid media with 10% oleic acid-Catalase-Dextrose and Mycobactin J (2mg/L, Allied Monitor) for approximately 3-4 months. Before spiking, the concentrations of *MAP* in the pure cultures were estimated at $1x10^9$/ml, $2.5x10^9$/ml and $1x10^9$/ml for strains 1018, 7565, and 9179, respectively using spectrophotometry.

In experiment 2, pure cultures of *MAP* 1018, K-10, and 7565 strains were grown in Middlebrook 7H9 liquid media with 10% oleic acid-Catalase-Dextrose and Mycobactin J (2mg/L, Allied Monitor) for a period of approximately 2 months. The

concentration of *MAP* in the pure cultures (percentage of live bacteria) was estimated using Fluorescence Activated Cell Sorting (FACS) at $9.7x10^7$/ml (93.2%), $9.1x10^6$/ml (86.6%) and $9.9x10^6$/ml (89.2%) for strains 7565, K-10 and 1018, respectively.

3.3.3. Preparation of *MAP* dilutions

Pure cultures of the different *MAP* strains were diluted in series using the same Middlebrook 7H9 liquid media with 10% oleic acid-Catalase-Dextrose and Mycobactin J (2mg/L, Allied Monitor) in 50 ml sterile centrifuge tubes to reach the desired concentration for spiking the fecal samples. In experiment 1, pure cultures were diluted to reach a concentration of the spiking solution of 10^9, 10^8, 10^6, 10^4, 10^2 *MAP*/ml. In experiment 2, pure cultures were diluted to reach a concentration of the spiking solution of 10^6, 10^5, 10^4, 10^3, and 10^2*MAP*/ml. In both experiments, a spiking solution with only culture media was used as negative control. Therefore each experiment included five levels of *MAP* concentration and a negative control for each of the *MAP* strains.

3.3.4. Preparation of negative fecal samples

In experiments 1 and 2, frozen fecal samples from individual cows were thawed and mixed together. Thirty-six subsamples of 27± 0.05g were collected from this mixture in 50-ml sterile centrifuge tubes. Half of these tubes (18) were weighted and autoclaved at 120°C for 20 minutes in a steam autoclave. An aerobic culture of the autoclaved fecal samples was used to confirm the effectiveness of the autoclaving process and all samples were negative to aerobic bacteria. Autoclaved tubes were allowed to cool down to room temperature and reweighted. Sterile water was added to each tube to reach the original weight before the autoclaving process. Autoclaved and non-autoclaved samples were kept at 4C until spiking.

3.3.5. Spiking of fecal samples

The dilutions of the *MAP* strains and the negative controls were used to spike the fecal samples. An aliquot of 3 ml from each of the *MAP* dilutions and *MAP* strain was used to spike autoclaved and non-autoclaved fecal samples to complete a total of

approximately 30 g of *MAP* spiked feces per *MAP* dilution and *MAP* strain. The final concentrations of *MAP* in the autoclaved and non-autoclaved fecal samples were 10^8, 10^7, 10^5, 10^3, 10^1, and 0 *MAP*/g of feces in experiment 1, and 10^6, 10^5, 10^4, 10^3, 10^2, and 0 *MAP*/g in experiment 2 (Table 1). Fecal samples were vortexed for 5 minutes and triplicates were generated of approximately 10 g each in 15-ml centrifuge tubes. A total of 108 spike fecal samples were generated using triplicates of 3 strains of *MAP*, at 6 different concentrations spiking 2 types of bovine fecal samples (autoclaved and non-autoclaved). Samples were frozen at -70 C until laboratory tests.

3.3.6. Laboratory analysis of spiked fecal samples

In both experiments, all 108 *MAP* spiked fecal samples were sent blinded to the MNVDL and to the USDA National Veterinary Services Laboratory (NVSL) to perform a bacterial culture of feces using HEYM. The NVSL performed the centrifugation procedure to concentrate *MAP* before culture. The MNVDL performed the sedimentation procedure to concentrate *MAP*. After the *MAP* concentration step, both laboratories cultured the sample of 5 HEYM slants. One of the 5 HEYM slants did not contain mycobactin to differentiate *MAP*, which is mycobactin dependent, from other *Mycobacterium* able to produce mycobactin.

Additionally, in experiment 1, a single replicate of each the *MAP* spiked fecal samples (total 36 samples), was sent blinded to the MNDVL to perform the MAV2 TaqMan PCR on feces (Wells et al., 2006). In experiment 2, triplicates (total 108 samples) of the *MAP* spiked fecal samples were sent to the MNVDL to perform the TaqMan real time PCR using Ambion® reagents (Ambion®, Applied Biosystems, Carlsbad, CA).

1-ml samples of the *MAP* pure cultures of each *MAP* strain used to spike the fecal samples were also sent to the MNDVL to perform MAV2 TaqMan PCR (Wells et al. 2006) in experiment 1. A sample of 1-ml of the *MAP* dilutions used to spike the fecal samples were sent to the MNVDL to perform a TaqMan real time PCR using Ambion® reagents (Ambion®, Applied Biosystems, Carlsbad, CA) in experiment 2.

Results of the bacterial culture of feces were recorded using the 5-level ordered qualitative score, based on mean number of colonies per slant as follows: 0 = no *MAP* colonies; 1 = >0 and <10 *MAP* colonies; 2 = between ≥10 and <50 *MAP* colonies; 3 = ≥50 and <100 *MAP* colonies; and 4 = ≥ 100 *MAP* colonies. Similarly, results of the MAV2 TaqMan PCR were recorded as positive or negative as described by Wells et al. (2006). Results of the TaqMan real time PCR using Ambion® reagents (Ambion®, Applied Biosystems, Carlsbad, CA) were recorded using Cycles to threshold (Ct) values as positive (Ct <37) or negative (Ct≥ 37).

In experiment 2, the viability of *MAP* in the dilutions used to spike fecal samples and the spiked fecal samples with the higher and lower concentration of *MAP*/g (10^6 and 0 *MAP*/g, respectively) was evaluated using reverse transcriptase PCR. RNA from *MAP* spiking dilutions and positive controls (pure cultures of *MAP* K10, 1018 and 7565) were extracted using TriZol (Invitrogen, Carlsbad, CA) per manufacturer's instructions and the addition of a bead beating step. All RNA samples were treated with Dnase I (Ambion®, Austin, TX). RNA samples were stored at -80^{o}C until cDNA conversion. Total RNA was converted to cDNA using random hexamers from the SuperScript^{TM} III first-strand synthesis system (Invitrogen, Carlsbad, CA) for reverse transcriptase PCR as stipulated by the manufacturer. Polymerase chain reaction for IS900 (L1/L9 locus) was performed on all cDNA samples and analyzed on a 1.5 percent agarose gel. Positive controls included recently extracted RNA from *MAP* K-10, 1018 and 7565 as well as frozen samples, which were matched for the same storage time as fecal RNA.

3.3.7. Analysis

Two statistical approaches were used for the data analysis. The first approach was used for the bacterial culture and fecal PCR when test results were classified as positive or negative (binary). The second approach was used for the bacterial culture of feces when test results were classified using the 5-level score (0, 1, 2, 3, and 4).

The first approach used three different unconditional logistic regressions to explain the probability of a positive test result for bacterial culture and for PCR in

experiments 1 and 2. The logistic regression used to model the probability of a positive bacterial culture result included the results of all slants used to culture the fecal samples. These results were explained by the laboratory that performed the bacterial culture (NVSL or MNVDL), *MAP* strain, type of sample (autoclaved or non-autoclaved) and concentration of *MAP* in the fecal samples. All variables were categorical with the exception of the concentration of *MAP* in the fecal samples which was treated as a continuous variable in the logarithmic base 10 scale. The correlation within slants and replicates for bacterial culture and the correlation within replicates for PCR in experiment 2 were modeled assuming compound symmetry. This working correlation structure was chosen using the quasi-likelihood information criterion (Pan, 2001). The goodness of fit was evaluated using Pearson chi-squares test. All models included the logit link function. Predicted probabilities (95% confidence intervals) of positive bacterial culture and positive PCR results were obtained from these models at each concentration of *MAP* in the fecal samples. Models were fitted using Proc Genmod of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

The second approach evaluated the association of the *MAP* concentration in the spiked fecal samples and the 5-level score of the bacterial culture results. A nominal logistic regression was used to model the odds of observing a bacterial culture score (1, 2, 3 and 4) compared to the reference level (0). This model assumed there was no correlation within replicates or slants because this model had convergence problems. The odds of a bacterial culture score (1, 2, 3 or 4) compared to negative bacterial culture (score 0) were explained by the laboratory that performed the bacterial culture (NVSL or MNVDL), *MAP* strain, type of sample (autoclaved or non-autoclaved) and concentration of *MAP* in the fecal samples. All variables were categorical with the exception of the concentration of *MAP* in the fecal samples that was treated as a continuous variable in the logarithmic base 10 scale. Predicted probabilities (95% confidence intervals) of bacterial culture scores were obtained from the model at each concentration of *MAP* in the fecal samples. Goodness of fit of the model was evaluated using Pearson score. This model was fitted using Proc Logistic of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

3.4. RESULTS

3.4.1. Experiment 1

Pure cultures of the three *MAP* strains used to make the dilutions and spike the fecal samples were PCR positive. None of the fecal samples spiked with *MAP* strain 9179 were positive to bacterial culture of feces, which prevented the logistic regression from reaching maximum likelihood estimates (complete separation) and therefore were not included in the statistical analysis. All fecal samples spiked with MAP strain 1018 and 7565 with concentration of *MAP* 10^8 and 10^7 *MAP*/g were positive to bacterial culture; however only 33.3% and 50% of the spiked fecal samples with *MAP* strain 1018 and 7565, respectively, were positive to bacterial culture at 10^5 *MAP*/g. *MAP* colonies were also observed on the mycobactin negative in all the *MAP* strains, and therefore were included in the analysis. All the spiked fecal samples with *MAP* concentration of 10^3, 10^1 and 0 *MAP*/g were negative to bacterial culture.

Positive PCR results were only observed in all fecal samples spiked with the 3 *MAP* strains (1018, 7565 and 9179) at concentration of *MAP* in the fecal sample of 10^8 and 10^7 *MAP*/g. Lower concentrations of *MAP* were PCR-negative.

The logistic regression included bacterial culture results (positive or negative) from 720 slants. The probability of a positive bacterial culture was associated with the concentration of *MAP* in the fecal samples ($P<0.01$, Table 2). The odds of positive bacterial culture results increased 21.0 (95% CI= 7.2 to 61.2) times for every increase of one unit on the logarithmic base 10 scale. No associations between the probability of a positive bacterial culture result and the laboratory that performed the test, *MAP* strains or type of samples were found.

The nominal logistic regression with the 5-level bacterial culture results score as outcome also included 720 observations from the individual slants. The results of this model indicated that concentration of *MAP* in the fecal sample and the laboratory that performed the bacterial culture were associated with an increase of the bacterial culture score ($P<0.01$). This analysis also showed a trend towards the association between increasing the bacterial culture score and type of sample ($P=0.07$) or *MAP* Strain used to spike the fecal sample ($P=0.06$). Compared to bacterial culture 0 (negative), the NVLS

had higher odds of bacterial culture score 2, 3 and 4 than the MNVDL (Table 3). Similarly, an increase of 10 times the *MAP* concentration in fecal samples was associated with increased odds of higher bacterial culture score compared to a negative bacterial culture score. For example, bacterial culture score "2" was 143.9 times more likely to be observed than negative bacterial culture when the concentration of *MAP* increased 10 times in the fecal sample. Bacterial culture scores "2", "3" and "4" tended to be more likely in strain 1018 than in strain 7565. Similarly, bacterial culture tended to have higher scores at NVSL than at MVDL (Table 3). This model also predicted the probabilities of a bacterial culture score given the concentration of *MAP* in the fecal samples (Figure 2). The model predicted that almost 100% of the *MAP* spiked fecal samples with *MAP* concentrations $\leq 10^3$ *MAP*/g had a negative bacterial culture (score 0). Similarly, the predicted probabilities (95% CI) of a bacterial culture score 0 and 1 on *MAP* spiked fecal sample with *MAP* concentrations of 10^5 *MAP*/g was 74.6% (59.5 to 89.2) and 24.3% (9.6 to 39), respectively. In contrast, the predicted probability of a bacterial culture score 4 in on *MAP* spiked fecal sample with *MAP* concentrations of 10^8 *MAP*/g was 82.5% (71.6 to, 93.4).

Finally the logistic regression used to model the probability of a positive PCR results included 36 observations. The probability of a PCR positive result was also associated with the concentration of *MAP* in the fecal samples ($P<0.01$, Table 2). The odds of a positive PCR increased 4.9 (95% CI= 1.5 to 16.5) times for every increase of one unit on the logarithmic base 10 scale. No associations between the probability of a positive fecal PCR result and the *MAP* strains and type of samples were found.

3.4.2. Experiment 2

All the pure cultures were positive for real time PCR. The results of the PCR using the *MAP* dilutions used to spike the fecal samples with the three *MAP* strains (1018, K-10 and 7565) showed a decrease on the Ct-values along with an increase on the *MAP* concentration (Figure 3). None of the spiked fecal samples were positive for bacterial cultures at any of the concentrations used in this study; however real time RT-

PCR showed positive results at a *MAP* concentration as low as 10^2 *MAP*/g of feces in the three *MAP* strains.

The logistic regression included 108 positive and negative PCR results. The model results indicated that the probability of a positive real time PCR result increased with the concentration of *MAP* in the fecal sample (P<0.01, Table 2). No associations between real time PCR results and *MAP* strain, or type of sample (autoclaved or non-autoclaved) were found. The odds of a positive real time PCR result increased 4.4 (95%CI= 2.1 to 8.9) times when the concentration of *MAP* in the fecal sample increased 10 times.

Mycobacterium avium subspecies *paratuberculosis* dilutions used to spike fecal samples were positive for IS900 in contrast to spiked fecal samples. Dilutions positive for IS900 matched the corresponding bands for *MAP* K-10, 1018 and 7565 (both fresh and frozen).

3.5. DISCUSSION

In this study, bovine fecal samples were spiked with three different *MAP* strains found in naturally infected dairy herds in the United States and maintained in the JDIP *MAP* strain repository. *MAP* spiked fecal samples were sent to two different diagnostic laboratories to perform two of the most important diagnostic tests used to determine the fecal shedding status of the cows: bacterial culture and fecal PCR.

The concentrations of *MAP* in the spiked fecal samples in experiment 1 were selected to cover a wide range of possible levels of detection of the bacterial culture and fecal PCR (from 10^8 to 10^1 *MAP*/g of feces). In experiment 2, the range of *MAP* concentrations in the spiked fecal samples was reduced based on the test results of experiment 1 (from 10^6 to 10^2 *MAP*/g of feces) and to provide enhanced precision of estimation of shedding levels. The K10 *MAP* strain was included in experiment 2 because it represented a well known *MAP* strain (Lingling et al., 2005). We also used flow-cytometry and FACS in experiment 2, which allowed for the more precise estimation of the *MAP* concentration as well as the proportion of viable (live) *MAP* in the pure cultures. The proportion of viable *MAP* in experiment 2 varied from 86.6% to 93.2% of the total

MAP, depending on the *MAP* strain. In both experiments, the concentration of *MAP* in the spiking solutions was calculated based on the concentration of total *MAP* in the pure cultures, although the concentration of viable *MAP* in the pure cultures was available only in experiment 2. Even though the concentration of viable *MAP* (instead of total *MAP*) may help interpreting and understanding the results from bacterial culture, it may also represent a problem interpreting PCR results, because PCR can detect *MAP* DNA regardless its viability.

No differences in bacterial culture results were observed between different laboratories, even though differences in the performance were expected because these two laboratories implemented different *MAP* concentration procedures (sedimentation at MNVDL compared to centrifugation at NVSL), before culturing on HEYM. Previous studies suggest that the concentration of *MAP* in fecal samples using the centrifugation procedure may have higher epidemiological and analytical sensitivity than the sedimentation procedure (Whitlock et al., 1988; Whipple et al., 1992).

Autoclaved bovine fecal samples were considered in the study design in both experiments to eliminate any possibility that the fecal samples were positive for *MAP* due to the use of imperfect tests such as bacterial culture of feces and fecal PCR, and to prevent interaction of *MAP* and test results with other species of *Mycobacterium* and other organisms in feces. The use of autoclaved fecal samples before spiking represented a controlled laboratory condition; however, non-autoclaved fecal samples were also included as a more accurate representation of the field conditions used in testing fecal samples. No differences in the test results of the bacterial culture and fecal PCR were observed between autoclaved and non-autoclaved samples.

In other studies, different strains of *MAP* have led to diverse growth patterns in different culture media (Cernicchiaro et al., 2008). Similarly, Whittington et al. (1998) suggested that *MAP* strains from sheep and goats were more difficult to grow on solid than on liquid media compared to *MAP* strains from cattle. In this study, both experiments included *MAP* strains from cattle (1018, K10 and 9179) and sheep (7565); however, all statistical models failed to find differences on the bacterial culture and PCR

performance of attributable to *MAP* strains, when tests results were binary (positive or negative).

Based on the findings of experiment 1, *MAP* spiked fecal samples with the highest concentrations of *MAP* in experiment 2 were expected to be positive for bacterial culture; however, no positive bacterial culture was observed in experiment 2. The results of the reverse transcriptase PCR for IS900 suggested that post spiking processing of fecal samples drastically eliminates *MAP* cell numbers (CFU), resulting in the absence of growth in bacterial culture. The reasons of the decrease of viability of *MAP* in the spiked fecal samples to non-detectable levels by the bacterial culture are unknown; however, changes on store temperatures may be associated. After spiking process, samples were stored at -70°C until they were sent the laboratories; however, both laboratories store the samples at -20°C until bacterial culture was performed. A dormant stage of *MAP* used to spike the fecal samples may also explain the results of the bacterial culture in the experiment 2. Although the factors that trigger the dormancy stage on MAP are not well understood, *MAP* organisms could have entered to a dormancy phase that led to viable but non-cultivable using HEY media.

Even though the viability of *MAP* was compromised in experiment 2 and bacterial culture results were not available, PCR results were used to estimate the limit of detection, because the fecal PCR assay is often used to establish infection status of the cattle regardless of the *MAP* viability in the fecal sample.

The RT-PCR test in experiment 2 appeared to have much higher analytical sensitivity than that used in experiment 1. Positive PCR results were observed at a concentration of 10^2 *MAP*/g in experiment 2 and at concentration $\leq 10^5$ *MAP*/g in experiment 1. Even though the performance of the PCR for detection of *MAP* in fecal samples depends on the PCR technique (DNA extraction, targeted gene, and DNA amplification) and the PCR technique used in each experiment was different; performance of the PCR between experiments cannot be directly compared, because *MAP* spike fecal samples were different.

Unlike other *Mycobacterium*, *MAP* is unable to produce mycobactin to transport iron, therefore mycobactin must to be added to the culture media to allow *MAP* growth.

Mycobactin dependency is used during bacterial culture to differentiate *MAP* from other *Mycobacterium* by adding one slant without mycobactin to the culture procedure. In this study, *MAP* colonies were observed in both, mycobactin positive and negative slants. Although, mycobactin added to the culture media (Middlebrook 7H9 liquid media) used in the pure cultures was diluted in the spiking solutions, one possible explanation is that a low concentration of mycobactin was carried over to the slants allowing the growth of *MAP* on mycobactin negative slants.

The conditional probability of higher bacterial culture score increased with the concentration of *MAP* in the fecal samples. The estimated probability of a *MAP* concentration in the fecal samples given the bacterial culture score can be calculated using Bayes' theorem and the predicted conditional probability of a bacterial culture score given a concentration of *MAP* from this study. The application of the Bayes' theorem requires prior estimations of the concentration of *MAP* in the fecal sample. In general, the concentration of *MAP* in an environmental fecal sample should be low due to the dilution of *MAP* from fecal shedding cows with non-fecal shedding cows. In contrast, prior estimation of the concentration of *MAP* in an individual cow fecal sample may be difficult, especially if the cow is in a subclinical stage of JD.

Findings from this study provide information about the association between the *MAP* concentration in feces and the two most important diagnostic tests used to directly identify *MAP* in fecal samples: bacterial culture and PCR. Even though no *MAP* growth was observed on the bacterial cultures in experiments 1 (1 strain) and 2 (3 strains), the results of the bacterial cultures in experiment 1 and the results of the direct PCR assay in both experiments provided estimations of the magnitude and direction of the association between diagnostic test and *MAP* concentration. The results of this study also provide an estimation of the limits of detection (analytical sensitivity) of the bacterial culture and two different PCR assay techniques.

There are several published studies focused on the epidemiological sensitivity and specificity of bacterial culture and PCR (Whitlock et al., 2000; Collins et al., 2006; Wells et al., 2006). There is also published information about the analytical sensitivity of PCR to detect *MAP* in different types of samples (Halldorsdottir et al., 2002; Reddacliff et al.,

2003, Wells et al. 2006). To our knowledge, this is the first study focusing on not only the analytical sensitivity of these diagnostic tests, but also on the association between test results and *MAP* concentration in the fecal sample. Knowing the epidemiological and analytical sensitivity and specificity of these diagnostic tests and the association between test results and *MAP* concentration in the fecal samples is valuable information for the identification of infected cows and control of the disease in the management of infectious cattle.

Table 1. Final concentration and strains of *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) in autoclaved and non-autoclaved bovine fecal samples in two experiments.

	Concentration of *MAP* in autoclaved and non-autoclaved fecal samples (*MAP*/g)					
MAP strain	1	2	3	4	5	6
Experiment 1						
1018	$1x10^8$	$1x10^7$	$1x10^5$	$1x10^3$	$1x10^1$	0
7565	$0.4x10^8$	$2.5x10^7$	$2.5x10^5$	$2.5x10^3$	$2.5x10^1$	0
9179	$1x10^8$	$1x10^7$	$1x10^5$	$1x10^3$	$1x10^1$	0
Experiment 2						
1018	$1.0x10^6$	$1.0x10^5$	$1.0x10^4$	$1.0x10^3$	$1.0x10^2$	0
K-10	$1.0x10^6$	$1.0x10^5$	$1.0x10^4$	$1.0x10^3$	$1.0x10^2$	0
7565	$0.9x10^6$	$0.9x10^5$	$0.9x10^4$	$0.9x10^3$	$0.9x10^2$	0

Table 2. Predicted conditional probabilities of positive bacterial culture and fecal PCR results by concentration of *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) and experiment in spiked bovine fecal samples

MAP concentration (*MAP*/g)	Bacterial Culture[1] Probability (%)	Bacterial Culture[1] 95% Confidence Interval	Fecal PCR[2] Probability (%)	Fecal PCR[2] 95% Confidence Interval
Experiment 1				
$1x10^8$	99.9	99.3 to >99.9	98.6	68.3 to 99.7
$1x10^7$	99.30	95.1 to 99.9	93.6	55.8 to 99.4
$1x10^5$	25.66	15.5 to 39.8	37.4	12.3 to 71.7
$1x10^3$	0.08	0.01 to 0.9	2.4	0.1 to 35.9
$1x10^1$	<0.01	<0.01 to 0.02	0.1	<0.01 to 18.1
0	<0.01	<0.01 to <0.01	0.02	<0.01 to 12.7
Experiment 2				
$1x10^6$	-	-	99.8	95.4 to >99.9
$1x10^5$	-	-	99.0	89.7 to 99.9
$1x10^4$	-	-	96.0	78.2 to 99.5
$1x10^3$	-	-	85.8	59.8 to 96.9
$1x10^2$	-	-	63.1	35.6 to 87.2
0	-	-	11.9	2.0 to 46.5

1 Bacterial culture of MAP int Herrold's egg yolk media

2 Experiment 1= PCR method described by Wells et al. (2006), experiment 2 = Real time PCR using Ambion® reagents (Ambion®, Applied Biosystems, Carlsbad, CA)

Table 3. Odds ratios (95% confidence interval) of *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) bacterial culture score 1, 2, 3 and 4, compared to 0 in *MAP* spiked bovine fecal samples from a nominal logistic regression[1,2]

		Bacterial Culture Score Comparison			
Variable	Comparison[3]	1 vs. 0	2 vs. 0	3 vs. 0	4 vs. 0
MAP Strain	1018 vs. 7565	0.7 (0.3 to 1.5)	1.8 (0.5 to 6.2)	4.1 (0.8 to 21.3)	3.0 (0. 8 to 11.2)
Laboratory	MNVDL vs. NVSL	0.7 (0.3 to 1.5)	0.1 (0.02 to 0.3)	0.1 (0.03 to 0.7)	0.2 (0.05 to 0.9)
MAP Concentration (10^X*MAP*/g)	Increase one unit	11.5 (4.2 to 31.1)	143.9 (36.2 to 571.9)	>999.9 (165.5 to >999.9)	>999.9 (760.5 to >999.9)
Type of sample	Autoclaved vs. Non-Autoclaved	1.1 (0.5 to 2.4)	0.4 (0.1 to 1.3)	0.6 (0.1 to 3.0)	0.2 (0.06 to 0.9)

[1] Estimated baseline logits (intercepts (SE)) for bacterial culture score 1, 2, 3 and 4 were -13.3 (2.6), -29.9 (4.2), -46.2 (6.3) and -53.6 (5.1), respectively.

[2]Bacterial culture scores (Experiment 1): 0 = no *MAP* colonies; 1 = >0 and <10 *MAP* colonies; 2 = between ≥10 and <50 *MAP* colonies; 3 = ≥50 and <100 *MAP* colonies; and 4 = ≥ 100 *MAP* colonies.

[3] MNVDL= Minnesota Veterinary Diagnostic Laboratory, NVSL= USDA National veterinary Services Laboratories. Autoclaved=fecal samples autoclaved before spiking, Non-autoclaved=non-autoclaved fecal samples before spiking

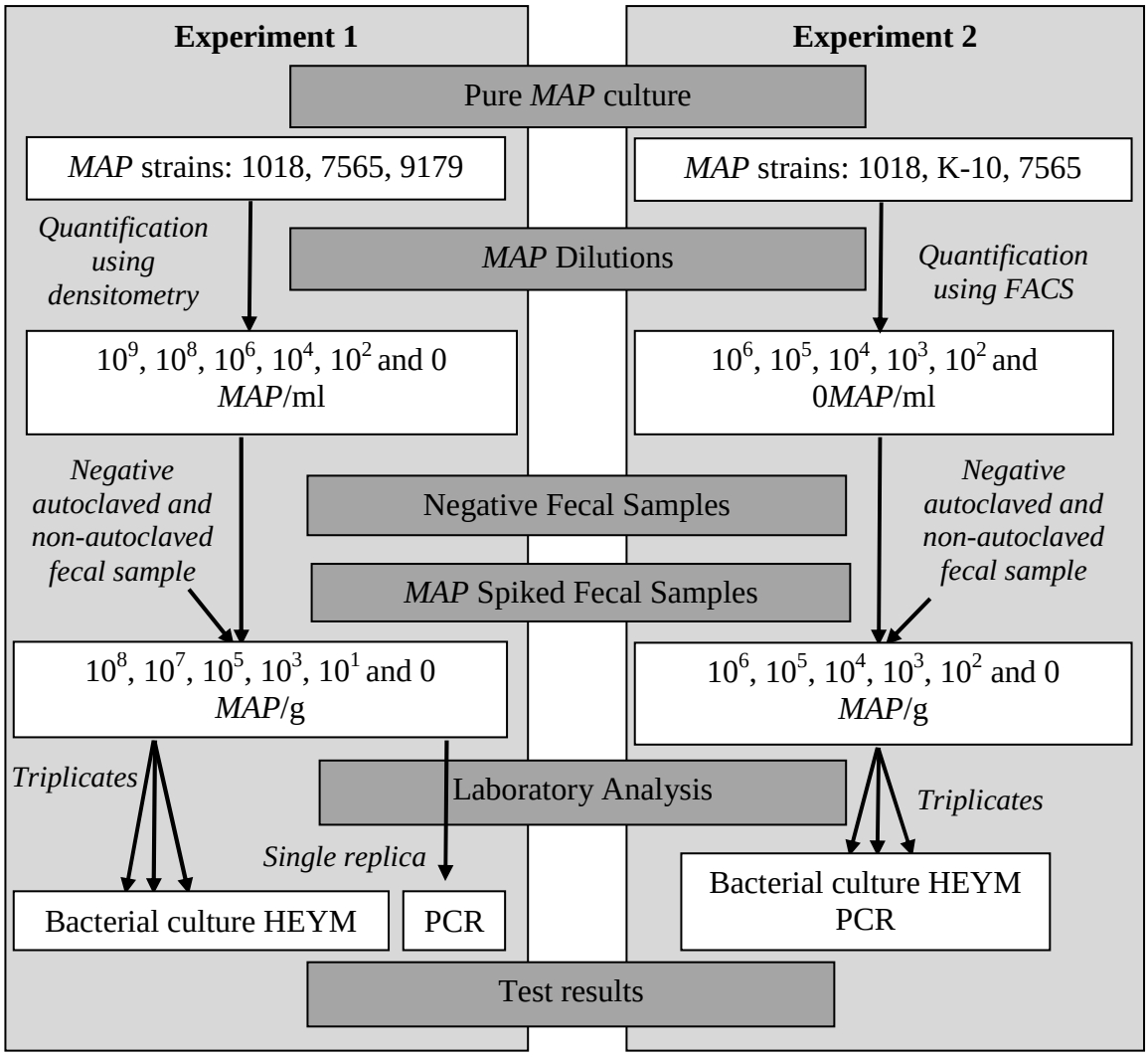

Figure 1. Diagram of two experiments of *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) spiked bovine fecal samples

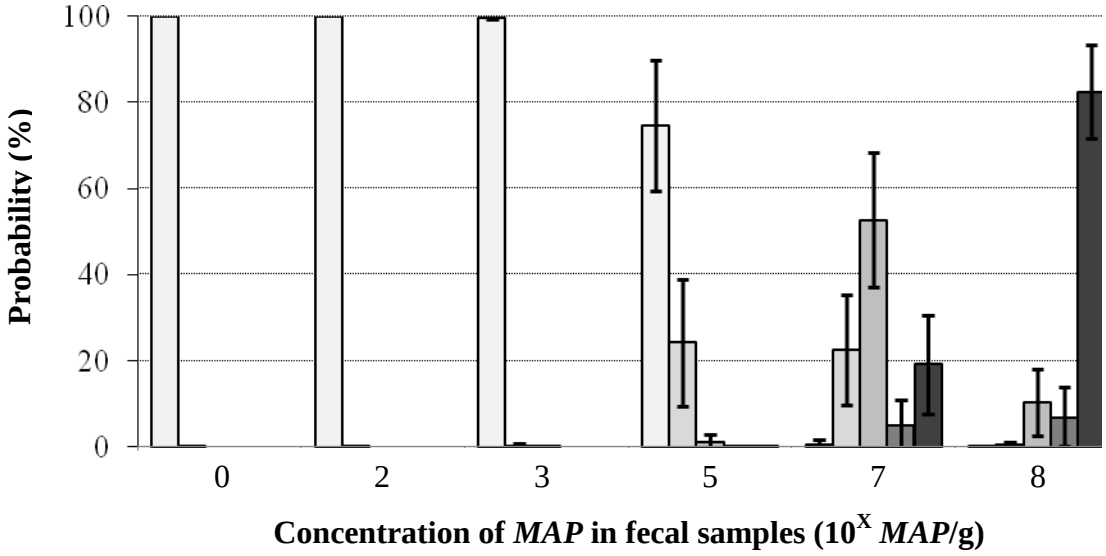

Figure 2. Predicted probability and 95% confidence interval of bacterial culture score (score 0, score 1, score 2, score 3 and ■ score 4) by *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) concentration in bovine fecal samples (Experiment 1)

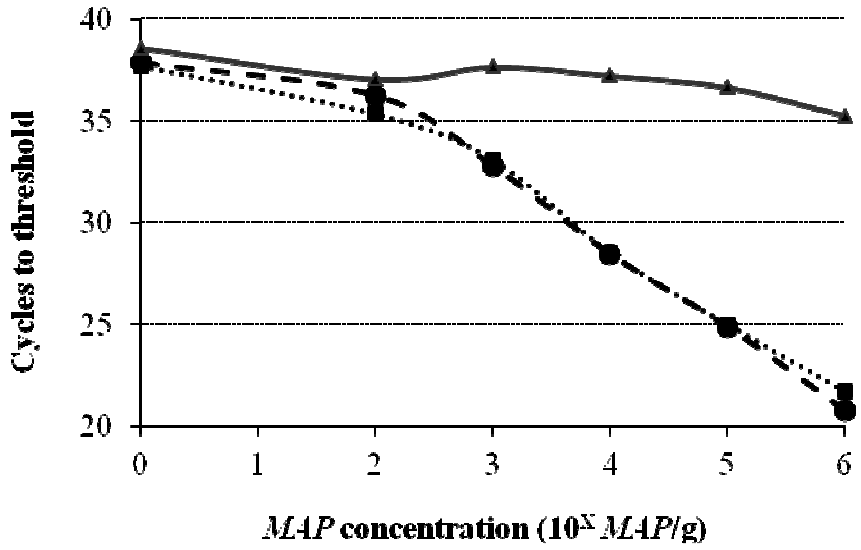

Figure 3. Cycles to threshold results of the real time PCR at different concentrations of *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) in spiked bovine fecal samples with 3 different *MAP* strains: 7565 (solid line and triangles), 1018 (dashed line and circles), and K10 (dotted line and squares), in experiment 2.

CHAPTER 4

EVALUATION OF PERFORMANCE OF BACTERIAL CULTURE OF FECES AND SERUM ELISA ACROSS STAGES OF JOHNE'S DISEASE IN CATTLE USING A BAYESIAN APPROACH

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Evaluation of test performance characteristics of bacterial culture of feces and serum ELISA across stages of Johne's disease in cattle using a Bayesian approach

The objective of this study was to evaluate the test performance characteristics of the bacterial culture of feces and serum ELISA to correctly identify *Mycobacterium avium* subsp. *paratuberculosis* heavy, light and non-fecal shedding cows. A total of 12,970, 15,680, and 1,962 parallel results from bacterial culture of feces and serum ELISA were collected from 17 dairy herds in Minnesota, Pennsylvania and Colorado, respectively. Samples were obtained from adult cows from dairy herds enrolled for up to 10 years in the National Johne's Disease Demonstration Herd Project. A latent class model using a Bayesian approach was fitted to estimate the posterior conditional probabilities that the diagnostic tests results correctly identified cows as high positive, low positive or negative that were heavy, light and non-fecal shedders, respectively. The model assumed that no gold standard test was available and conditional independency existed between diagnostic tests. The estimated conditional probabilities that bacterial culture of feces correctly identified heavy, light and non-fecal shedders were 70.8, 32.2 and 98.5%, respectively. The same values for the serum ELISA were 60.5, 18.8 and 99.5, respectively. Differences in the performance of diagnostic tests performance were observed among states. The agreement between diagnostic tests was moderate (Kappa = 0.44). These posterior conditional distributions improve the understanding of the bacterial culture of feces and serum ELISA, and could be used to model the transmission of the disease in dairy farms, taking into account the uncertainly of these tests.

4.2. INTRODUCTION

Johne's disease (JD) is a chronic infectious disease in cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). The disease has a slowly progressing detrimental effect on cow health and production. The progression of the disease can be categorized into multiple stages (Collins, 2002, Whitlock et al., 2000;

Nielsen and Toft, 2008); however, from the epidemiological point of view, a population of cattle can be divided into 3 different groups.

1) Non-fecal shedding cows. While this group may not be infected with JD, a proportion of this group is infected and in the latent stage of the disease. Latent infected cows generally do not shed detectable levels of *MAP* into the environment and there is not currently a practical way to differentiate latent infected cows from non-infected cows (Whitlock et al., 2000).

2) Light fecal shedding cows. Nearly all of the light fecal shedders are infected with JD and in general shed a low and intermittent concentration of *MAP* into the environment that can be occasionally detected using diagnostic tests. A small proportion of these cows may be *MAP* passive fecal shedders as a consequence of an environment highly contaminated with *MAP* that passes through the digestive system of the cows (Sweeney et al., 1992). This group has a lower risk of disease transmission to susceptible cattle compared to heavy fecal shedders.

3) Heavy shedding cows. This group represents infected cows in advanced stages of the infection. They shed large amounts of *MAP* into the environment and are likely to be detected using diagnostic tests. Heavy fecal shedders are associated with decreased milk production and clinical illness (Raizman et al., 2007). Because of the large concentrations of *MAP* that they shed into the environment, heavy fecal shedders represent the highest risk of disease transmission and their identification and culling is important for disease control (Lu et al., 2008).

In 2007, USDA-APHIS estimated that at least 68% of US dairy farms are infected with *MAP*, based on testing of environmental fecal samples (USDA-APHIS, 2008). Johne's disease causes economic losses directly associated with premature culling as well as reduced milk production (Raizman et al., 2007). Management practices are focused not only on reducing the transmission of *MAP* from infected shedding cows to uninfected young calves, but also on culling heavy fecal shedding cows as soon as they are detected (Raizman et al., 2007).

The most commonly used diagnostic tests for JD are bacterial culture of feces of feces and serum ELISA. Several studies have estimated the sensitivity and specificity of

these tests by using either multiple bacterial cultures of feces to confirm the infection status of the cattle or by using samples from confirmed negative herds, respectively (Whitlock et al. 2000; Collins et al. 2005; Wells et al., 2006); however, only the study from Wells et al. (2006) estimated the serum ELISA performance by fecal shedding level using 3 bacterial cultures of feces in parallel to confirm the fecal shedding status (non, light or heavy). Moreover, while a Bayesian approach was previously used to estimate the diagnostic tests performance for JD (Wells et al., 2006; Norton et al., 2010), this approach was used to estimate the performance of binary (positive or negative) test interpretation.

At the individual cow level, understanding how to interpret results of diagnostic tests for JD in the context of disease progression using multiple stages could be an important diagnostic tool for producers and veterinarians to elaborate breeding lists and prioritize culling decisions on the most infectious cattle. At the group or herd level, estimating the proportion of cows that shed no, low and large amounts of *MAP* into the environment in the herd may help producers and veterinarians choose and implement specific management practices in order to avoid the transmission of JD within the herd. Categorizing cattle populations by disease stage using accurate estimations of the JD diagnostic test performance in each disease stage could assist understanding the dynamics of disease transmission, implementation of accurate disease transmission models and evaluation of the impact and effectiveness of JD disease control or eradication programs (Lu et al. 2008).

The objective of this study was to estimate the accuracy of the bacterial culture of feces and serum ELISA to identify *MAP* heavy, light, and non-fecal shedding cattle into the environment.

4.3. MATERIALS AND METHODS

Johne's disease diagnostic test results from the National Johne's Disease Demonstration Herd Program (JDDHP) were obtained from the USDA-APHIS-VS. Herds were selected using the following criteria: 1) Whole adult herd test results were available, 2) Diagnostic tests used for *MAP* detection included both bacterial culture of

feces on Herrold's egg yolk media (HEYM) and serum ELISA (IDEXX laboratories, Inc, Westbrook, ME), 3) Fecal samples for bacterial culture of feces and blood samples for serum ELISA were collected the same day from cows in each herd, and 4) Herds did not implement vaccination against Johne's disease during the study period.

Diagnostic test results from 16 states and 66 herds were obtained; a total of 8 herds from Minnesota (MN), 7 herds from Pennsylvania (PA) and 2 herds from Colorado (CO) fulfilled the inclusion criteria. In those herds, samples were annually collected from 2000 to 2009 in MN, from 2002 to 2010 in PA and from 2003 to 2004 in CO, depending on the date of herd enrollment in the JDDHP. In almost all herds, samples were collected annually in approximately the same month every year to allow 12 months between sample collections within herds; however, in 2 herds in Minnesota, the sample collection was based on stage of the lactation of the cows from 2000 to 2004, and changed to annual testing in approximately the same month every year for the subsequent years of the study. Adult cow herd sizes varied from approximately 50 to 400, 50 to 800, and 300 to 400 adult cows in MN, PA and CO, respectively. The *MAP* apparent within-herd prevalence by bacterial culture of feces and serum ELISA at the first year of the study varied from 3.6 to 38.3% and 1.0 to 28.5%, respectively (Table 1).

After collection, serum and fecal samples from MN were sent to the Minnesota Veterinary Diagnostic Laboratory for testing. Similarly, serum and fecal samples from PA were sent to the Pennsylvania Animal Diagnostic Laboratory System. Serum samples from CO were sent to the Colorado State University Veterinary Diagnostic Laboratories, while fecal samples from CO were sent to Wisconsin Veterinary Diagnostic Laboratory. Fecal samples were submitted to the respective laboratories to perform bacterial culture of feces of feces using Herrold's egg yolk media (HEYM). Fecal samples from MN and CO were processed using 72h sedimentation procedure before culture and fecal samples from PA were processed using 30 min centrifugation procedure (Eamens et al., 2000). Serum samples were tested using serum ELISA (IDEXX Laboratories, Inc, Westbrook, ME) in the respective laboratories. During the study period, a total of 30,612 paired test results from bacterial culture of feces and serum ELISA were collected from MN (n=12,970), PA (n=15,680) and CO (n= 1,962, Table 2). All these laboratories passed the

annual USDA-APHIS-VS proficiency test administered by the National Veterinary Services Laboratories.

To differentiate test results from the true fecal shedding status of the cattle, this study used the following terminology: test results were classified as high, low or negative, and true status of the cattle were classified as heavy, light, or non-fecal shedder. A diagnostic test correctly identified heavy, light and non-fecal shedders if the diagnostic test result was high, low and negative, respectively. A diagnostic test incorrectly identified heavy, light and non-fecal shedders if the diagnostic test result was low or negative, high or negative, and high or low, respectively.

Serum ELISA results were classified using different cutoff points for the Sample to Positive Ratio (S/P), where negative samples had an S/P < 0.25, and positive samples had an S/P ≥ 0.25. Positive samples were also classified as low positive when 0.25 ≤ S/P < 1.0, and high positive when the S/P was ≥ 1.0 (Collins, 2002). Similarly, test results from bacterial culture of feces were classified as negative when no *MAP* was detected and as positive when more than 0 colonies of *MAP* were observed. Positive samples were also divided into low positive when the bacterial culture of feces results indicated an average of > 0 and < 50 *MAP* colonies per tube and as high positive when the bacterial culture of feces result was an average of ≥ 50 *MAP* colonies per tube.

4.3.1. Data Analysis

A Bayesian latent-class model was developed using the Markov Chain Monte Carlo (MCMC) method to estimate the conditional probability that diagnostic test results (high, low or negative), correctly or incorrectly identified a true heavy, low or non-*MAP* fecal shedding cow, according to the model initially described by Hui and Walter (1980) and Johnson et al. (2001) for one test with binomial result and one population.

Given the 3 possible test results: high (T_H), low (T_L) and negative (T_N), and the 3 possible true fecal shedding status: heavy (S_H), light (S_L) and non-fecal shedder (S_N), the conditional probabilities of the bacterial culture of feces (P_C) test result given the true fecal shedding status of the cows were: $P_C(T_H|S_H)$, $P_C(T_H|S_L)$, $P_C(T_H|S_N)$, $P_C(T_L|S_H)$, $P_C(T_L|S_L)$, $P_C(T_L|S_N)$, $P_C(T_N|S_H)$, $P_C(T_N|S_L)$, and $P_C(T_N|S_N)$. Similarly, the conditional

probabilities of the serum ELISA (P_E) test result given the true fecal shedding status of the cows were: $P_E(T_H|S_H)$, $P_E(T_H|S_L)$, $P_E(T_H|S_N)$, $P_E(T_L|S_H)$, $P_E(T_L|S_L)$, $P_E(T_L|S_N)$, $P_E(T_N|S_H)$, $P_E(T_N|S_L)$, and $P_E(T_N|S_N)$.

The latent-class model was built assuming conditional independency or non-conditional correlation between diagnostic tests. Cows in the *k* herd, where $k = 1$ to 17 herds in this study, had different bacterial culture of feces and serum ELISA diagnostic test results, given by the vector $y_k = (y_{HHk}, y_{HLk}, y_{HNk}, y_{LHk}, y_{LLk}, y_{LNk}, y_{NHk}, y_{NLk}, y_{NNk})$. Therefore the vector y_k represented the number of cows for all the 9 possible combinations of bacterial culture of feces and serum ELISA test results in the herd *k*, and is given by the number of cows with bacterial culture of feces high and a serum ELISA high (y_{HHk}), serum ELISA low (y_{HLk}) and serum ELISA negative (y_{HNk}); bacterial culture of feces low with serum ELISA high (y_{LHk}), serum ELISA low (y_{LLk}) and serum ELISA negative (y_{LNk}); and bacterial culture of feces negative with serum ELISA high (y_{NHk}), serum ELISA low (y_{NLk}) and serum ELISA negative (y_{NNk}). The sum of all the elements of the vector y_k will be equal to the size of the population in the herd *k* (n_k). Similarly, the vector p_k represented the proportion of cows in the herd *k* in either of the 9 possible combinations of bacterial culture of feces and serum ELISA test results and is described as $p_k = (p_{HHk}, p_{HLk}, p_{HNk}, p_{LHk}, p_{LLk}, p_{LNk}, p_{NHk}, p_{NLk}, p_{NNk})$. The sum of all the elements of the vector p_k added to 1. The true prevalence of heavy, light and non-fecal shedders in the *k* herd were given by π_{Hk}, π_{Lk} and $\pi_{Nk,}$ respectively.

Therefore, assuming that each cow represented an independent observation in each herd, the probability of a joint diagnostic test result y_k had a multinomial distribution:

$$y_k \sim \text{Multinomial}\ (n_k, (p_{HHk}, p_{HLk}, p_{HNk}, p_{LHk}, p_{LLk}, p_{LNk}, p_{NHk}, p_{NLk}, p_{NNk}))$$

And the probability of a combined bacterial culture and serum ELISA test results in the *k* herd was given by:

$$p_{HHk} = \pi_{Hk}\, P_C(T_H|S_H)\, P_E(T_H|S_H) + \pi_{Lk}\, P_C(T_H|S_L)\, P_E(T_H|S_L) + \pi_{Nk}\, P_C(T_H|S_N)\, P_E(T_H|S_N)$$

$$p_{HLk} = \pi_{Hk} P_C(T_H|S_H) P_E(T_L|S_H) + \pi_{Lk} P_C(T_H|S_L) P_E(T_L|S_L) + \pi_{Nk} P_C(T_H|S_N) P_E(T_L|S_N)$$

$$p_{HNk} = \pi_{Hk} P_C(T_H|S_H) P_E(T_N|S_H) + \pi_{Lk} P_C(T_H|S_L) P_E(T_N|S_L) + \pi_{Nk} P_C(T_H|S_N) P_E(T_N|S_N)$$

$$p_{LHk} = \pi_{Hk} P_C(T_L|S_H) P_E(T_H|S_H) + \pi_{Lk} P_C(T_L|S_L) P_E(T_H|S_L) + \pi_{Nk} P_C(T_L|S_N) P_E(T_H|S_N)$$

$$p_{LLk} = \pi_{Hk} P_C(T_L|S_H) P_E(T_L|S_H) + \pi_{Lk} P_C(T_L|S_L) P_E(T_L|S_L) + \pi_{Nk} P_C(T_L|S_N) P_E(T_L|S_N)$$

$$p_{LNk} = \pi_{Hk} P_C(T_L|S_H) P_E(T_N|S_H) + \pi_{Lk} P_C(T_L|S_L) P_E(T_N|S_L) + \pi_{Nk} P_C(T_L|S_N) P_E(T_L|S_N)$$

$$p_{NHk} = \pi_{Hk} P_C(T_N|S_H) P_E(T_H|S_H) + \pi_{Lk} P_C(T_N|S_L) P_E(T_H|S_L) + \pi_{Nk} P_C(T_N|S_N) P_E(T_H|S_N)$$

$$p_{NLk} = \pi_{Hk} P_C(T_N|S_H) P_E(T_L|S_H) + \pi_{Lk} P_C(T_N|S_L) P_E(T_L|S_L) + \pi_{Nk} P_C(T_N|S_N) P_E(T_L|S_N)$$

$$p_{NNk} = \pi_{Hk} P_C(T_N|S_H) P_E(T_N|S_H) + \pi_{Lk} P_C(T_N|S_L) P_E(T_N|S_L) + \pi_{Nk} P_C(T_N|S_N) P_E(T_N|S_N)$$

This model also assumed that there was no available gold standard test and the test performances were independent of herds and cows. Model outcomes were the conditional probability of test results given the true fecal shedding status of the cattle for bacterial culture of feces and serum ELISA. Model outcomes also included the true prevalence of heavy, light and non-fecal shedders in the *k* herd, π_{Hk}, π_{Lk} and π_{Nk}, respectively. The analysis was stratified by state to evaluate changes on the test performance by state.

Using the Bayes' theorem, estimated predictive values of heavy, light and non-fecal shedders for the bacterial culture of feces and serum ELISA were computed for each iteration of the MCMC in each *k* herd, therefore a posterior distribution of the predicted values was obtained for each test. The predicted values of the heavy fecal shedders corresponded to the conditional probability of heavy fecal shedder given that the bacterial culture of feces or the serum ELISA result was high, ($P_C(S_H|T_H)$ and $P_E(S_H|T_H)$, respectively. Similarly the predicted value of the light fecal shedder corresponded to the conditional probability of light fecal shedder given that the bacterial culture of feces or the serum ELISA result was low, ($P_C(S_L|T_L)$ and $P_E(S_H|T_L)$, respectively. Finally, the predicted value of the non-fecal shedder corresponded to the conditional probability of non-fecal shedder given that the bacterial culture of feces or the serum ELISA result was negative, ($P_C(S_N|T_N)$ and $P_E(S_N|T_N)$, respectively.

Informative prior distributions for the conditional probabilities of diagnostic test results (T) high, low or negative ($T_H, _L, _N$) given the true fecal shedding status (S) of

heavy, light or non-fecal shedding cows ($S_{H,\ L,\ N}$), were modeled using a Dirichlet distribution parametrized by the vector α_C for bacterial culture of feces (α_{CH}, $\alpha_{CL,}$ and α_{CN}) and the vectors α_E for serum ELISA (α_{EH}, $\alpha_{EL,}$ and α_{EN}). The values of the prior conditional probabilities of diagnostic test results for both diagnostic tests were assigned by consensus between three experts (Scott Wells, Ian Gardner and Elizabeth Patton , Table 3 and 4).

$P_C(T_{H,\ L,\ N}|S_H) \sim$ Dirichlet (α_{CH}), where $\alpha_{CH} = (P_C(T_H|S_H), P_C(T_L|S_H), P_C(T_N|S_H))$

$P_C(T_{H,\ L,\ N}|S_L) \sim$ Dirichlet (α_{CL}), where $\alpha_{CL} = (P_C(T_H|S_L), P_C(T_L|S_L), P_C(T_N|S_L))$

$P_C(T_{H,\ L,\ N}|S_N) \sim$ Dirichlet (α_{CN}), where $\alpha_{CN} = (P_C(T_H|S_N), P_C(T_L|S_N), P_C(T_N|S_N))$

$P_E(T_{H,\ L,\ N}|S_H) \sim$ Dirichlet (α_{EH}), where $\alpha_{EH} = (P_E(T_H|S_H), P_E(T_L|S_H), P_E(T_N|S_H))$

$P_E(T_{H,\ L,\ N}|S_L) \sim$ Dirichlet (α_{EL}), where $\alpha_{EL} = (P_E(T_H|S_L), P_E(T_L|S_L), P_E(T_N|S_L))$

$P_E(T_{H,\ L,\ N}|S_N) \sim$ Dirichlet (α_{EN}), where $\alpha_{EN} = (P_E(T_H|S_N), P_E(T_L|S_N), P_E(T_N|S_N))$

Since prior information of the herds true prevalences (π_k) of heavy (π_{Hk}), light (π_{Lk}) and non-fecal shedders (π_{Nk}) were not available for all *k* herds, they were included in the model using non-informative Dirichlet prior distributions, given by the vector α_π

$\pi_k \sim$ Dirichlet ($\alpha\pi$), where $\alpha_\pi = (\pi_{Hk}, \pi_{Lk}, \pi_{Nk}) = (1,1,1)$

Models were run using 3 parallel chains in the MCMC with sparse initial values and 10,000 iterations per chain. The first 500 iterations were discarded in each chain as a burning-in period. Models were implemented using OpenBugs software (Thomas et al., 2006). Posterior distributions of the diagnostic test conditional probabilities were described using the mean and the 95% credible interval. Convergence of the model was visually evaluated using trace plots and Gelman-Rubin diagnostic plots (Brooks and Gelman, 1998).

The sum of the conditional probabilities that the bacterial culture of feces (*C*) or serum ELISA (*E*) correctly identified a heavy, light or non-fecal shedder as high, low and negative, respectively; was used to obtain a index that summarized the performance of

each test (I_C and I_E). Percentage of changes of these indexes ((I_C and I_E) was used to evaluate the influential effect of each of the *k* herds on the estimated parameters for test performance, as an indicator of the sensitivity of the estimated parameter for the diagnostic test performance to the herds under study.

$$I_C = P_C(T_H|S_H) + P_C(T_L|S_L) + P_C(T_N|S_N)$$
$$I_E = P_E(T_H|S_H) + P_E(T_L|S_L) + P_E(T_H|S_N)$$

Agreement between diagnostic tests was evaluated using weighted Cohen's Kappa statistics. Similarly, to evaluate differences between diagnostic test agreement across states, data were stratified by state and the weighted Cohen's kappa statistics were calculated. Finally, differences among the kappa coefficients by state were evaluated using a chi-square test using the Freq procedure of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

4.4. RESULTS

4.4.1. Heavy fecal shedders

The estimated probabilities that bacterial culture of feces and serum ELISA will correctly identify a heavy fecal shedding cow as high were 70.8% (63.1 to 80.0) and 60.5% (54.6 to 66.1), respectively (Table 3). The proportion of heavy fecal shedding cows that were incorrectly classified as low was similar between bacterial culture of feces and serum ELISA; however, the proportion of heavy fecal shedders that were classified as negative was almost 6 times higher for serum ELISA than for bacterial culture of feces.

The stratified analysis indicated differences in the bacterial culture of feces of feces performance by state (Table 4). Overall bacterial culture of feces and serum ELISA performed similar in PA and CO; however, differences were observed when comparing the performance of these tests in these 2 states with the performance of the test in MN. The conditional probability of correctly identifying heavy fecal shedders as high using bacterial culture of feces was approximately 23 percent units lower in MN compare to

these values in CO and PA. In MN, most of the heavy fecal shedding cows that were not correctly classified as high by the bacterial culture of feces were instead classified as low fecal shedders. The false negative rate of bacterial culture of feces on heavy fecal shedding cows was low and similar among the 3 states (Table 3).

Contrary to what was observed on bacterial culture of feces in the stratified analysis, the serum ELISA in MN showed a higher probability of correctly identifying heavy fecal shedders as high compared to test performance in PA and CO. The probabilities of the serum ELISA of incorrectly classifying heavy fecal shedders as low or negative was similar among states (Table 4).

4.4.2. Light fecal shedders

The estimated probabilities that light fecal shedders would be correctly identified as low fecal shedders by the bacterial culture of feces and serum ELISA were 32.2% (26.1 to 39.1) and 18.8% (15.5 to 22.3), respectively. Most of the light fecal shedders were false negative to the bacterial culture of feces and serum ELISA and a small proportion of the light fecal shedders were identified as high when using these diagnostic tests (Table 3).

The stratified analysis by state showed that the conditional probability of bacterial culture of feces to correctly identify light fecal shedders as low was slightly lower in MN, compared to the values showed in PA and CO (Table 4); however, light fecal shedders were less likely to have bacterial culture of feces results as high in MN, compared to PA and CO. While the false negative probability was similar to the probability of correctly identifying a light fecal shedder using bacterial culture of feces in CO and PA, the probability of a false negative bacterial culture result was more than twice the probability of a low bacterial culture of feces in the population of light fecal shedders in MN (Table 4).

Similarly, the stratified analysis for the performance of the serum ELISA in the light fecal shedder population showed the serum ELISA was approximately twice as likely to correctly identify a light fecal shedder in MN compared to CO and PA; however

the probability of misclassifying light fecal shedder as negative and as high was similar across states (Table 4).

4.4.3. Non-fecal shedders

The estimated probability that bacterial culture of feces and serum ELISA correctly identified a non fecal shedder as negative (Specificity) was 98.5% (98.1 to 98.9) and 99.5% (99.3 to 99.7), respectively (Table 3). Most of the false positive test results were low positive when using bacterial culture of feces of serum ELISA; and only a small proportion of the false positive test results were high positive in either diagnostic tests.

The specificity of bacterial culture of feces was higher in PA than in MN. The probability of the bacterial culture of feces to incorrectly classify a non-fecal shedding cows as high was similar across states; however the probability of incorrectly classifying a non-fecal shedder as low in MN was approximately twice the values in CO and 3 times the value in PA (Table 5).

The specificity of the serum ELISA was similar across states. The conditional probability of the serum ELISA having a high or low result given that the cows were non-fecal shedders was also similar across states (Table 4).

4.4.4. True prevalence and predictive values

In general, considering the entire study period, the estimated true prevalence of fecal shedding cows was higher in MN compared to PA and CO (Table 1). The estimated within-herd true prevalence (95% credible interval) of heavy fecal shedders for the study period varied from 0.4% (0.1 to 1.1) to 5.4% (2.9 to 8.5). Likewise, the estimated within-herd true prevalence of light fecal shedders for the study period varied from 0.9% (0.1 to 2.1) to 25.2% (20.5 to 30.6). The ratio of the true prevalence of fecal shedders divided by the true prevalence of heavy fecal shedder varied from 1.9 to 16.0 and the Pearson's correlation between the true prevalence of light and heavy fecal shedders was 0.69 ($P<0.01$, Table 1).

As expected, positive predictive values of bacterial culture of feces and serum ELISA increased with the true prevalence of heavy, light and non-fecal shedders within the ranges of true prevalences observed in the population of herds and period under study. The bacterial culture of feces estimated predicted value for heavy fecal shedding, which is the conditional probability of heavy fecal shedding given that the bacterial culture of feces was high, varied from 56.2% (6.1 to 88.4) to 92.1% (83.5 to 97.1). The predicted value for heavy fecal shedding given that the serum ELISA was high varied by herd from 37.3% (2.1 to 78.0) to 89.8% (74.6 to 99.0). Similarly, the estimated predictive values for light fecal shedding varied by herd from 14.0% (0.7 to 36.6) to 81.2% (74.7 to 87.1) for bacterial culture of feces, and from 28.5% (2.9 to 58.3) to 78.2% (72.0 to 83.7) for serum ELISA. The negative predictive values for non-fecal shedding varied by herd from 78.4% (72.0 to 83.6) to 99.6% (98.7 to 100) for negative bacterial culture of feces, and from 76.1% (69.9 to 81.2) to 99.4% (98.5 to 99.9) for negative serum ELISA (Figure 1).

4.4.5. Test results agreement

The weighted Cohen's kappa coefficient for agreement between bacterial culture of feces and serum ELISA as 3-level test results was 0.44 (95% CI = 0.42 to 0.46). The coefficients (95% CI) for MN, PA and CO were 0.44 (0.41 to 0.47), 0.43 (0.40 to 0.47) and 0.38 (0.29, 0.46), respectively.

4.4.6. Sensitivity analysis of test performance

The sensitivity analysis of the effect of herd on test performance was performed using the percentage of change of the indices represented by the sum of the probabilities that either bacterial culture of feces or serum ELISA correctly identified a heavy fecal shedder as high, light fecal shedder as low, and non-fecal shedder as negative. The absolute value of the performance change of bacterial culture of feces varied from 0.2 to 15.3%. Similarly the absolute value of the performance change of the serum ELISA varied from 0 to 6.0%.

One herd from MN showed to be the most influential on the estimated bacterial culture of feces and serum ELISA performance (Figure 2). When this herd was excluded from the analysis, the probabilities of bacterial culture of feces correctly identifying a heavy fecal shedders as high, light fecal shedders as low and non-fecal shedders as negative were 84.4% (76.7 to 90.7), 50.4% (44.0 to 56.9), and 99.2% (98.8 to 99.6), respectively; while the same probabilities for serum ELISA were 55.3% (49.7 to 61.1), 13.5% (11.2 to 16.1), and 99.2% (99.0 to 99.4), respectively.

4.5. DISCUSSION

This is the first study to evaluate the accuracy of bacterial culture of feces and serum ELISA using a 3-level ordinal scale test result: negative, low and high, to correctly classify non, light and heavy fecal shedders, respectively. This study used a latent-class analysis with a Bayesian approach that allowed obtaining posterior distributions of the diagnostic test performance given the test results and the prior information of the diagnostic test performances and the prevalence of the fecal shedders. Unlike other published information about diagnostic test performance this approach does not need to assume a gold standard or a reference test.

Although, threshold values used to transform the quantitative diagnostic test results into a ordinal categorical scale (negative, low and high) could be modified in order to maximize the conditional probabilities of correctly identifying heavy, light and non-fecal shedders, this study used the threshold values recommended in the literature and commonly used to classified cattle based on the test results (Crossley et al, 2005; Collins, 2002).

This study used data from 17 herds dairy herds from 3 different states and more than 30,000 bacterial culture of feces and serum ELISA test results from fecal and serum samples taken at the same time. The 17 herds included in this study were under Johne's disease control programs with a high variation of the within-herd apparent prevalence of *MAP*. This program included changes in herd management practices in order to reduce the transmission of *MAP*, annual testing and culling of test positive cattle. These 17 herds were included in this study because they had available diagnostic test results from

bacterial culture of feces using HEYM and serum ELISA (IDEXX) from fecal and serum samples taken the same day in each adult cow. These herds were not vaccinated against Johne's disease during the study period; therefore diagnostic test performance was not influenced by vaccination.

Throughout the course of the *MAP* infection, cattle will develop different biological responses. Fecal shedding is a consequence of the colonization of *MAP* along the intestinal epithelia. Cellular and humoral immune response is observed as a consequence of exposure of *MAP* to the immune system (Stabel, 2000). When infection progresses and cow homeostasis is compromised, decreased production and clinical JD is often observed (Nielsen and Toft, 2008). Diagnostic tests have been developed to detect one or more biological processes as an indication of *MAP* infection. Bacterial culture of feces and serum ELISA are diagnostic tests that measures different biological phenomena in the cows. Since these two diagnostic tests measure different biological processes, this study assumed that both tests were conditionally independent. In other words, the tests results from serum ELISA are assumed to be independent from the test results of the bacterial culture of feces. However, the test results of both diagnostic tests are conditioned to the true fecal shedding status of the cows (Gardner et al., 2000).

Establishing the *MAP* infection status of cows based on their fecal shedding status may be difficult. Heavy and light fecal shedding cows primarily represent infected cattle that actively shed *MAP* in feces as a consequence of the colonization of *MAP* into the intestinal tissue; however, a small proportion of light fecal shedders could be attributed to passive fecal shedding of *MAP* as a consequence of an environment highly contaminated with *MAP* that passes through the digestive system of uninfected cows (Sweeney et al., 1992). These uninfected passive fecal shedders may test positive to bacterial culture of feces (likely to be low positive, Fecteau and Whilock, 2010). For disease control purposes, establishing the fecal shedding status of cattle is more important than identifying the actual infection status of the cattle, since fecal shedders are the most important source of *MAP* in the environment of susceptible cattle and actively transmit infection to their herdmates.

While results from bacterial culture of feces are associated with the concentration of *MAP* excreted in feces, serum ELISA results are associated with the intensity of the immune reaction against *MAP* antigens. Even though these diagnostic tests indicated the presence of different biological processes during the course of the infection, in practice, both diagnostic tests have been used to classify cattle at different stages of the disease (Collins, 2002; Collins et al., 2005), and serum ELISA may be used to estimate fecal shedding status of the cattle, because of the association between the development of *MAP* fecal shedding and the stage of the disease. The performance of the bacterial culture of feces and serum ELISA to identify the fecal shedding status of the cows was reflected by the moderate agreement between tests results observed on the Cohen's kappa coefficient.

Data collected in this study were treated as cross-sectional data, therefore test results from samples coming from the same cow at different years and test results from the same herd at different years were treated as independent observations. Similarly, estimated prevalences of heavy, light and non-fecal shedders were computed for the whole study period in each herd; however, these herds were under a disease control program and a decrease of the prevalence of fecal shedders throughout the time was expected. The progression of *MAP* infection while infected cows age may imply increasing concentrations of *MAP* fecal shedding resulting in changes in diagnostic test accuracy; however, Nielsen and Toft (2006) found no changes in diagnostic test performance to identify infectious cattle associated with age of cows using similar diagnostic tests (bacterial culture of feces and milk ELISA).

Bacterial culture of feces correctly identified as positive (high or low) approximately 98% of the heavy fecal shedding cows; however, only approximately 34% of the light fecal shedders were identified as positive (high or low) using bacterial culture of feces. Light fecal shedders are likely shedding low levels of *MAP* sometimes non-detectable when using bacterial culture of feces. Even though serum ELISA correctly identified most of the heavy fecal shedders as positive (approximately 86%), it was less accurate than bacterial culture of feces in this group of cows. Similar to bacterial culture of feces, most light fecal shedders were identified as negative by serum ELISA (approximately 74%). Despite the lower sensitivity of serum ELISA compared to

bacterial culture of feces to identify *MAP* positive cattle, as well as to identify their fecal shedding status (light or heavy), serum ELISA is a valuable diagnostic test to evaluate the infectiousness of cattle, as suggested by Nielsen and Toft (2006), especially when considering that serum ELISA is a faster and less expensive test than bacterial culture of feces.

While most heavy fecal shedders were correctly identified as high by both diagnostic tests, the probability of a negative (false negative) test results for a heavy fecal shedder was approximately 6 times higher for serum ELISA than bacterial culture. In contrast, most light fecal shedders had negative (false negative) bacterial culture and serum ELISA tests results, indicating the poor sensitivity of both diagnostic tests in this group of cows. The estimated conditional probabilities of a positive (low or high) serum ELISA given a light or a heavy fecal shedder were previously estimated by Wells et al. (2006) as 12.4 and 72.7%, respectively, using 3 bacterial cultures of feces in parallel as the positive reference test. These values were lower than the ones estimated in this study (25.8 and 86.1%, respectively) for the same diagnostic test.

Even though both diagnostic tests showed a high specificity (i.e. >98%), the specificity of the serum ELISA was approximately 1 percent unit higher than the bacterial culture of feces. This may be explained by the fact that approximately 2% of the test results were from serum ELISA positive (low or high) and bacterial culture of feces negative cows, and approximately 3.7% of the test results corresponded to bacterial culture of feces positive (low or high) and serum ELISA negative cows. Another explanation of a higher estimated specificity of serum ELISA may be associated to the prior estimations for these diagnostic tests. Experts set a prior specificity for the bacterial culture of feces (95%) lower than the same value for serum ELISA (98%), due to the expected passive fecal shedding phenomenon that some cows may experience in highly *MAP* contaminated environments (Sweeney et al. 1992). It was expected that a small proportion of cows with positive bacterial culture of feces results would be false-positive. Even with this passive shedding phenomena, specificity of the bacterial culture of feces has been expected to be close to 100%, especially when the growing bacteria colonies are confirmed to be *MAP* by using staining (acid-fast) or molecular techniques (E.g. IS900

PCR). Using another Bayesian approach, the specificity of the bacterial culture of feces was previously estimated at 99.8% (Wells et al., 2006) and 98.0% (Alinovi et al., 2009) using the same culture technique.

The specificity of the serum ELISA in this study (99.5%, 95%CI= 99.3 to 99.7), was higher than some of the values previously reported in the literature (Whitlock et al. 2000; Collins et al., 2005; Wells et al., 2006). Wells et at. (2006) previously reported a specificity of 94.9% using a similar Bayesian approach, and 95.3% using samples from JD negative herds. Further, Collins et al. (2005) reported a serum ELISA specificity of 97.0% using samples from JD negative herds, and Whitlock et al. (2000) reported 98.9% using 3 serial bacterial cultures of feces to confirm negativity of the cows. The higher specificity for the serum ELISA compared to previous published information may be explained by the absence of a gold standard based on bacterial culture of feces. It is possible that some light fecal shedding cows in this study shed non-detectable levels of *MAP* in feces, and therefore were negative (false-negative) to the bacterial culture of feces. These cows may have developed a detectable humoral immune response, but were assumed to be serum ELISA false-positive by the model.

Bacterial culture of feces performed better in correctly identifying light and heavy fecal shedders in PA and CO compared to MN. While this test was able to identify as positive (high or low) approximately half of the light fecal shedders in CO and PA, in MN this test only identified as positive approximately one third of light fecal shedders. Light fecal shedding cows were almost as likely to test positive (high or low) or negative in PA and CO and were more likely to test negative in MN. Differences of performance of the bacterial culture of feces among states may be explained by differences in the *MAP* concentration procedure before culture in the respective laboratories. While fecal samples sent to the MN Veterinary Diagnostic Laboratory were cultured using the sedimentation for 72h procedure of *MAP* concentration, fecal samples from PA sent to the Pennsylvania Animal Diagnostic Laboratory System were cultured using the centrifugation procedure. In contrast, the estimated performance of the bacterial culture of feces using sedimentation procedure at the University of Wisconsin Johne's Testing Center (samples from CO) seemed to be similar to the performance of the PA test using centrifugation

procedure for *MAP* concentration. Even though all 3 laboratories passed the USDA-APHIS-VS proficiency test, the culture procedures are not standard among laboratories. Whitlock et al. (2000) suggested that small changes on the culture procedure could lead to different performance of the bacterial culture of feces. Another factor that may explain different diagnostic test performance among states and herds may be associated with the predominant strain of *MAP* in the herd by state. Different subtypes of *MAP* may lead to diverse growth patterns in solid culture media like HEYM (Cernicchiaro et al., 2008).

The indices that represented the sum of the diagnostic test conditional probabilities of correctly identifying a heavy, light or non-fecal shedder as high, low or negative cow, respectively, showed that bacterial culture performance was influenced by the results of one of the herds. Inclusion of this herd in the analysis increased this index approximately 15% (Figure 2). This herd had the highest true prevalence of fecal shedders during the study period; however, this level of true prevalence is feasible in heavily infected herds. This herd was also characterized by the high apparent prevalence of serum ELISA positive cows compared to the apparent prevalence of bacterial culture positive cows during the study period (Table 1, herd 1). Although this herd may be considered as an influential outlier for the estimation of bacterial culture of feces performance, it was included because this herd may represent other herds with similar characteristics.

As suggested above, the performance of the diagnostic test results is sensitive to small changes in the techniques used in the diagnostic tests, therefore the results of this study may be applied with caution and only to bacterial culture of feces using HEY media and to serum ELISA using the IDEXX test kit. It is also important to take into account that there is a continuous development to improve the accuracy of the diagnostic test through time; however the Bayesian approach used in this study could be applied to evaluate the accuracy of further developments of these or other diagnostic tests (E.g: direct *MAP* PCR assay of fecal samples).

For veterinary practice and for decision making on the farm, knowing the probability of the diagnostic test result given the true fecal shedding status of the cattle is not really of interest. The probabilities of true fecal shedding status given the diagnostic

test results (predictive values) are the parameters of interest in a practical context. In this case, predicted values of heavy, light and non-fecal shedders could be estimated using the Bayes' theorem and the true prevalence of fecal shedding cows.

In this study, bacterial culture of feces and serum ELISA were found to be accurate diagnostic tools to find heavy and non-fecal shedders; however the performance of these diagnostic tests in the population of light fecal shedders was poor and most light fecal shedders were categorized as negative by both tests. The estimated posterior conditional distributions from this study improve understanding of the bacterial culture of feces and serum ELISA, and not only provide a estimation of test performance that can be used for veterinarians to detect fecal shedding of cattle using evidence-based medicine, but also provide a posterior distribution of these parameters that can also be used to model the transmission of Johne's disease in dairy farms taking into account the uncertainly of diagnostic test accuracy .

Table 1. Dairy herd characteristics, apparent prevalence of *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture of feces and Johne's disease serum ELISA, and true prevalence of *Mycobacterium avium* subspecies *paratuberculosis* fecal shedding cows in 17 commercial dairy herds, 8 in Minnesota, 2 in Colorado, and 7 in Pennsylvania

Herd	State	Cows tested, mean[1]	Prevalence Bacterial Culture of feces (%)[2]	Prevalence Serum ELISA (%)[2]	True Prevalence Fecal shedders (%)[3]	
					All	Heavy
1	MN	230	14.7	28.5	30.0	4.8
2	MN	41	38.3	8.3	20.4	4.4
3	MN	246	3.6	3.2	3.1	1.6
4	MN	316	6.7	8.8	6.6	0.9
5	MN	37	15.9	18.2	12.1	5.4
6	MN	285	15.9	6.5	10.1	3.0
7	MN	356	21.8	9.2	16.0	2.8
8	MN	228	13.8	11.4	13.3	2.5
9	PA	596	4.7	2.6	10.9	1.6
10	PA	199	7.9	4.5	6.9	1.9
11	PA	338	6.3	2.7	6.0	1.2
12	PA	422	5.8	8.3	7.7	2.4
13	PA	131	1.9	1.0	1.9	0.4
14	PA	66	9.8	6.6	8.5	1.5
15	PA	375	3.4	3.8	1.6	0.7
16	CO	408	3.9	2.9	6.1	0.4
17	CO	355	5.3	3.9	6.4	0.9

[1]Mean adult cows (age > 24 months) tested per year

[2]Annual apparent prevalence at the beginning of the study

[3] Bayesian estimation of the true prevalence of *Mycobacterium avium* subspecies *paratuberculosis* fecal shedding (All) and heavy fecal shedding (Heavy) cows for the study period.

Table 2. Distribution of the combined *Mycobacterium avium* subspecies *paratuberculosis* test results from bacterial culture of feces and serum ELISA in 17 commercial dairy herds (8 in Minnesota, 2 in Colorado, and 7 in Pennsylvania)

Bacterial Culture[1]	Serum ELISA[2]	N
Negative	Negative	28,121
Low	Negative	1,017
High	Negative	121
Negative	Low	501
Low	Low	179
High	Low	109
Negative	High	116
Low	High	197
High	High	251
TOTAL		30,612

[1] Negative: bacterial culture of feces =0 colonies/slant, Low: bacterial culture of feces > 0 and < 50 colonies/slant, High: bacterial culture of feces ≥ 50 colonies/slant.

[2] Negative: sample to positive ratio < 0.25, Low: sample to positive ratio ≥ 0.25 and < 1.0, High: sample to positive ratio ≥ 1.0

Table 3. Mean and 95% Credible Interval (95% CI) of the posterior conditional probability of *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture of feces and Serum ELISA test results given the true status of the cattle

Test	Test results[1]	Fecal shedding True status	Prior Probability (%)	Conditional Probability (%)	
				Mean	95% CI
Bacterial culture of feces					
	Negative	Non	95	98.5	98.1, 98.9
	Low	Non	4	1.4	0.9, 1.8
	High	Non	1	0.1	0.03, 0.3
	Negative	Light	45	66.2	59.1, 72.6
	Low	Light	45	32.2	26.1, 39.1
	High	Light	10	1.6	0.8, 2.6
	Negative	Heavy	3	2.3	0.5, 5.5
	Low	Heavy	12	26.9	18.0, 33.9
	High	Heavy	85	70.8	63.1, 80.0
Serum ELISA					
	Negative	Non	98	99.5	99.3, 99.7
	Low	Non	1.5	0.5	0.3, 0.7
	High	Non	0.5	0.01	<0.001, 0.1
	Negative	Light	80	74.2	68.5, 79.6
	Low	Light	15	18.8	15.5, 22.3
	High	Light	5	7.0	4.48, 10.08
	Negative	Heavy	15	13.9	8.3, 20.2
	Low	Heavy	35	25.6	21.7, 29.7
	High	Heavy	50	60.5	54.6, 66.1

[1] Bacterial culture of feces negative =0 colonies/slant, Low > 0 and < 50 colonies/slant, High: ≥ 50 colonies/slant. Serum ELISA negative: sample to positive ratio < 0.25, Low: sample to positive ratio ≥ 0.25 and < 1.0, High: sample to positive ratio ≥ 1.0

Table 4. Mean and 95% credible interval (95% CI) of the distribution of the posterior conditional probability of *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture of feces and Johne's disease serum ELISA test results given the true status of the cattle, in 8 dairy herds in Minnesota (MN), 7 in Pennsylvania (PA) and 2 in Colorado (CO)

Diagnostic Test	Test results[1]	Fecal shedding true status	MN Mean	MN 95% CI	CO Mean	CO 95% CI	PA Mean	PA 95% CI
Bacterial culture of feces								
	Negative	Non	97.7	96.8-98.5	98.6	97.4-99.5	99.2	98.7-99.6
	Low	Non	2.3	1.5-3.2	1.3	0.4-2.4	0.7	0.3-1.1
	High	Non	0.01	<0.01-0.02	0.1	<0.01-0.4	0.1	<0.01-0.3
	Negative	Light	68.2	61.9-73.9	46.3	36.8-56.0	49.8	40.7-58.6
	Low	Light	29.7	24.3-35.7	46.2	37.1-55.5	43.4	35.4-51.8
	High	Light	2.1	1.1-3.3	7.5	3.8-12.5	6.8	4.0-10.2
	Negative	Heavy	2.7	0.6- 6.2	3.0	0.7-7.2	4.6	1.0-10.1
	Low	Heavy	37.1	30.6-43.2	13.6	7.3-21.6	11.7	6.8-17.3
	High	Heavy	60.2	53.6-67.3	83.4	75.0-90.3	83.7	76.1-90.2
Serum ELISA								
	Negative	Non	99.4	98.9-99.8	98.9	97.9-99.6	99.3	99.1-99.5
	Low	Non	0.5	0.1-0.9	0.8	0.2-1.6	0.7	0.4-0.8
	High	Non	0.1	<0.01-0.2	0.3	<0.01-0.9	0.01	<0.01-0.1
	Negative	Light	69.4	62.3-75.8	74.4	66.2-82.1	83.3	78.7-87.5
	Low	Light	24.2	19.2-29.8	14.5	8.9-21.3	11.4	8.2-15.0
	High	Light	6.4	4.0-9.4	11.1	5.6-17.3	5.3	3.0-7.8
	Negative	Heavy	9.6	5.8-13.8	14.6	8.9-21.3	14.4	8.7-20.9
	Low	Heavy	22.0	17.5-26.9	35.7	26.8-45.1	32.3	26.7-38.3
	High	Heavy	68.4	62.9-73.9	49.7	40.0-59.3	53.3	46.6-59.9

[1] Bacterial culture of feces negative =0 colonies/slant, Low > 0 and < 50 colonies/slant, High: ≥ 50 colonies/slant. Serum ELISA negative: sample to positive ratio < 0.25, Low: sample to positive ratio ≥ 0.25 and < 1.0, High: sample to positive ratio ≥ 1.0

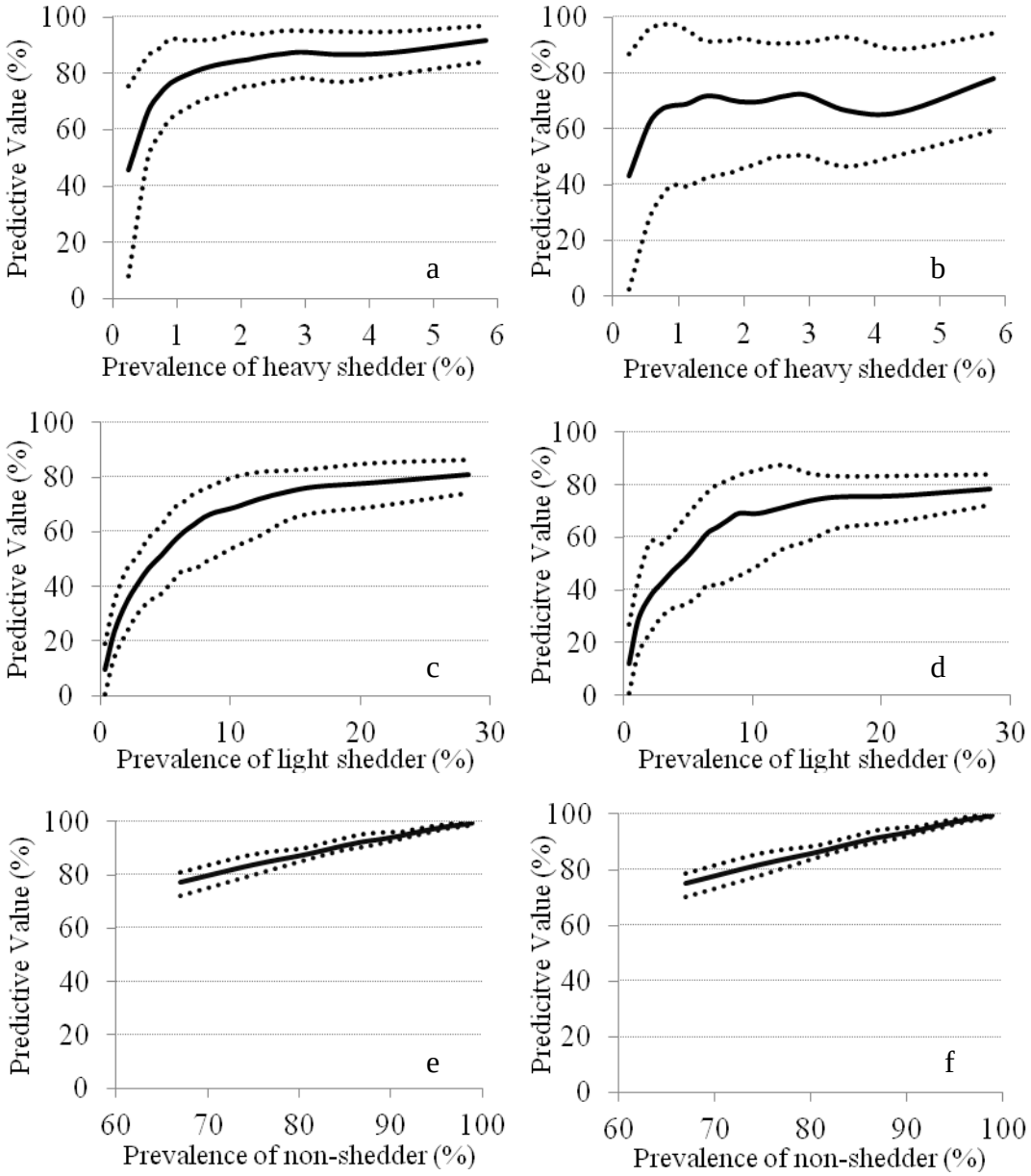
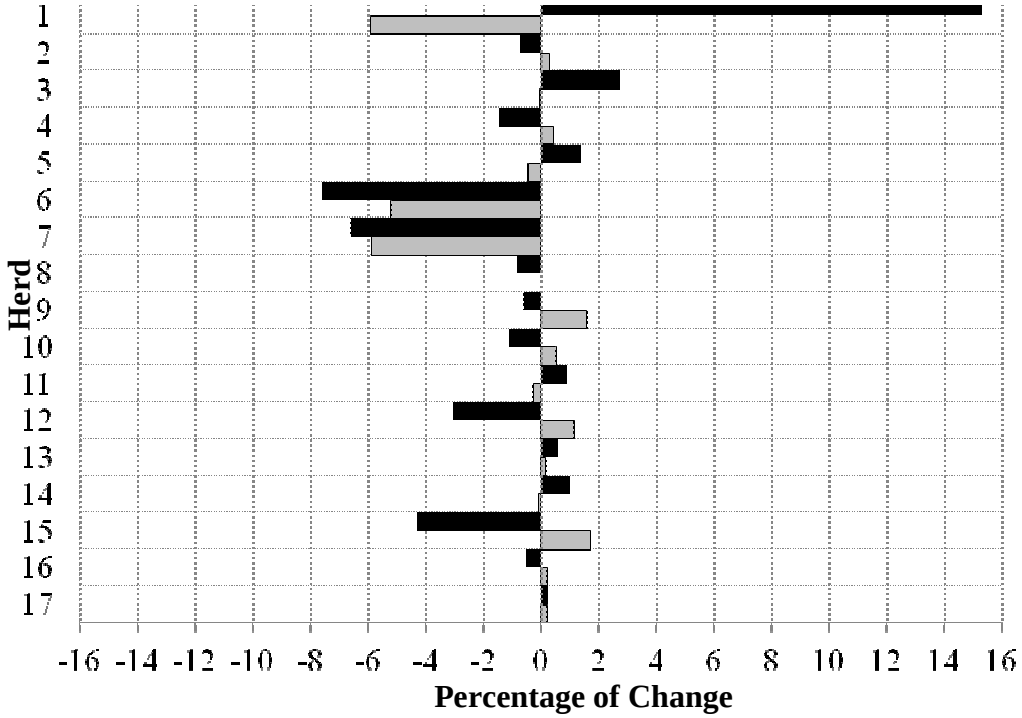


Figure 1. Mean (solid line) and 95% credible interval (dotted lines) of *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture of feces (a,c,e) and Johne's disease serum ELISA (b,d,f) predictive values of high (a,b), low (c,d) and negative (e,f) test results by true prevalence of heavy, light and non-fecal shedding, in cattle in 8 dairy herds in Minnesota, 7 in Pennsylvania and 2 in Colorado

Figure 2. Sensitivity analysis of the herd effect on the percentage of change of the sum of the conditional probabilities that *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture of feces (black) and Johne's disease serum ELISA (grey) correctly identify a heavy, light or non fecal shedder as high, low and negative, respectively; in 17 Johne's disease demonstration herds.



CHAPTER 5

ASSOCIATION BETWEEN COW-LEVEL AND POOLED ENVIRONMENTAL FECAL SAMPLE RESULTS FOR DETECTION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN DAIRY HERDS

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Association between cow-level and pooled environmental fecal sample results for detection of *mycobacterium avium* subspecies *paratuberculosis* in dairy herds

The objective of this cross sectional study was to evaluate the association between *Mycobacterium avium* subspecies *paratuberculosis* (***MAP***) bacterial culture results from pooled environmental fecal samples and from pooled individual fecal samples with the apparent prevalence of *MAP* bacterial culture and Johne's disease serum ELISA positive cows in dairy cattle herds under a Johne's disease control program. Seventy-nine annual herd visits on 17 commercial dairy herds were performed between 2003 and 2010 to collect pooled environmental samples of manure from alleys, manure storage areas, groups of cows, and individual cows from all adult cows in the herd at the time of the visit. From each herd and visit, *MAP* bacterial culture on pooled environmental fecal samples and fecal samples from individual cows, and Johne's disease serum ELISA from serum samples of individual cows were performed. Results were categorized as negative, low positive and high positive. The mean within-herd apparent prevalence of bacterial culture low and high positive cows was 4.3% and 1.2%, respectively, for an overall apparent prevalence of 5.5%. The mean within-herd apparent prevalence of serum ELISA low and high positive cows were 5.6% and 3.4%, respectively, for an apparent test-positive prevalence of 9.0%. The sensitivity and specificity of the parallel interpretation of bacterial cultures on pooled environmental samples from the herd to detect at least one individual cow with high positive bacterial culture result in the herd was 98.2% and 43.5%, respectively. The sensitivity and specificity of the bacterial culture on pooled individual samples to detect at least one heavy fecal shedding cow in the pool were 100% and 91%, respectively, and these values did not change when pool size increased from 5 to 10 cows per pool. The magnitude of the association between the probabilities of low or high positive bacterial culture of feces using pooled environmental samples and within-herd apparent prevalence of *MAP* heavy fecal shedders increased when the herd size increased. Similarly, the probability of a low or high positive bacterial culture using

pooled group samples was associated to the interaction between group size and within-groups apparent prevalence of *MAP* heavy fecal shedders.

5.2. INTRODUCTION

Johne's disease (**JD**) or paratuberculosis is a chronic infectious disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (***MAP***, Harris and Barletta, 2001). It is a multistage disease characterized by chronic diarrhea, weight loss, and weakness during its clinical stage (Whitlock and Buergelt, 1996). The infection is mainly transmitted via fecal-oral routes, although *MAP* shedding through placenta, milk and colostrum has also been described (Streeter et al., 1995; Sweeney, 1996; Whittington and Windsor, 2009). In the United States, the herd-level apparent prevalence of infection on dairy farms was estimated at 68%, based on testing of environmental fecal samples. This apparent prevalence estimate is higher in large herds with >500 cows (95%, USDA-APHIS, 2008).

The control of JD in cattle herds is primarily focused on reducing transmission from infectious to susceptible animals. To accomplish this objective, changes in herd management practices should focus on avoidance of exposure of young susceptible replacement cattle to infectious adult cattle and their feces. One important method of avoiding this exposure is to identify and cull cows shedding a high concentration of *MAP* in feces (heavy fecal shedders) as these cattle represent the highest risk of disease transmission. Besides the implication of heavy fecal shedders in the transmission of the infection, several studies have shown an association between heavy fecal shedding and high risk of clinical JD (Whitlock et al., 2000, Raizman et al., 2007), lower milk production and reduced reproductive performance (Raizman et al., 2007, Smith et al., 2009). Early identification and culling of heavy fecal shedders would help dairy and beef cattle producers not only reduce transmission of *MAP* from infectious to susceptible animals, but also reduce the negative impact of heavy fecal shedding on production and reproduction.

Due to the costs involved in sample collection and diagnostic testing, pooling individual and environmental fecal samples may represent a lower cost alternative to

identify infected cattle or infected herds. However, there is limited information in the scientific literature about the performance of *MAP* diagnostic tests performed on pooled fecal samples from the environment of cattle or pooled fecal samples from individual cows to detect fecal shedding cows in the pool or herd. The few studies available using pooled samples from individual dairy cows suggest that an increase in the concentration of *MAP* in the samples and reducing the number of cows represented in the pool leads to an increase in the sensitivity of the pooled individual fecal culture (Wells et al., 2002; van Schaik et al., 2003b; Wells et al., 2003). Environmental fecal pooled samples have been used to evaluate JD dairy herd status in prevalence studies, and as an alternative to individual serological tests to classify dairy herds in the Test-Negative component (now the JD herd classification levels) of the US Voluntary JD Control Program (USDA-APHIS, 2010). Information has been lacking to date to evaluate use of pooled fecal samples in beef cattle herds, or for additional purposes in dairy herds, such as to evaluate use of pooled environmental samples to estimate the proportion of fecal shedding cows in herds. Testing of pooled environmental samples to estimate the proportion of cows shedding high or low concentrations of *MAP* into the environment could help veterinarians and producers to establish a strategic sampling program to reduce the cost of detection of fecal shedders. The objective of this study was to evaluate the association between *MAP* bacterial culture results from pooled environmental fecal samples and from pooled individual fecal samples with the apparent prevalence of *MAP* bacterial culture and Johne's disease serum ELISA positive cows in dairy cattle herds under a Johne's disease control program.

5.3. MATERIALS AND METHODS

5.3.1. Herds, samples and test results

This cross sectional study included 17 commercial dairy herds in Pennsylvania (PA, 10) and Minnesota (MN, 7) enrolled in the National Johne's Disease Demonstration Herd Project between 2003 and 2010. Herds were annually visited for a total of 2 to 8 times, depending on the herd. Herd sizes ranged approximately from 51 to 711 cows in PA and from 41 to 380 cows in MN. The mean (SD) group sizes in these herds was 54

(42) cows, ranging from 2 to 230 cows per group. Herds were visited annually to obtain pooled environmental samples of feces and individual serum and fecal samples from all adult cows in the herd (≥24 month of age). All pooled environmental fecal samples and individual cow fecal and serum samples were collected at the time of the herd visit. Samples were sent to Veterinary Diagnostic Laboratories within each state. Each diagnostic laboratory was approved by USDA-APHIS-VS National Veterinary Laboratories Services to perform bacterial culture of feces using Herrold's egg yolk media (HEYM, Wells et al., 2002) and serum ELISA (IDEXX laboratories, Inc, Westbrook, ME). During the study period, a total of 20,564 bacterial cultures and 20,515 serum ELISA results were recorded from individual cows in 79 herd visits to the 17 herds under study. Similarly, a total of 654 bacterial culture test results from pooled environmental manure samples were obtained from the 79 herd visits to the 17 herds during the study period (Table 1).

The pooled environmental fecal samples included in this study were categorized as pooled environmental samples from the "Whole Herd Environment" (HERD) that represented all or a high proportion of the cows in the herd (high cow traffic alleys, manure storage areas, holding pens, manure spreaders, etc), or pooled environmental samples from a "Group of Cows Environment" (GROUP) that represented a known group of cows with common production characteristics (cow pens). While pooled environmental HERD samples were collected on 63 herd visits in PA, pooled environmental samples in MN did not include HERD, only GROUP. In general, pooled environmental samples from HERD and GROUP were collected following the recommendation of the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program (USDA-APHIS, 2010).

In order to associate the individual bacterial culture and serum ELISA test results from individual cows with the test results of the pooled environmental fecal samples at the time of the visit, information about the group location of each cow was recorded at each herd and visit, and pooled environmental samples from the GROUP were collected. Evaluation of this association was not possible for 17 out of 79 herd visits during the study period, either because information on the group location of the cows was not

available or pooled environmental samples from GROUP were not collected. Therefore, during the study period, including the 17 herds and 62 herd visits, test results from 277 bacterial cultures on pooled environmental samples from GROUP were evaluated with the individual bacterial culture and serum ELISA test results from individual cows on those groups.

5.3.2. Diagnostic tests and interpretation of test results

Serum ELISA test results from individual cows had a binary classification as positive or negative based on the Sample to Positive ratio (S/P). Positive serum ELISA test results had S/P $\geq$ 0.25, while negative serum ELISA had S/P < 0.25. In order to differentiate high and low positive serum ELISA cows, another 3-level classification method based on S/P of the serum ELISA was used as follows: negative cows had S/P < 0.25, low positive cows had S/P $\geq$ 0.25 and <1.0 and high positive cows had S/P $\geq$ 1.0. Similarly, the test results of the bacterial culture of feces on fecal samples from individual cows and from pooled environmental fecal samples were classified in a binary scale as positive or negative based on the presence of *MAP* colony forming units (**CFU**) on the 4 slants. Positive bacterial cultures had at least one CFU in any of the 4 slants, while negative bacterial culture results had no CFU on the 4 slants. Bacterial culture of feces test results were also classified as low positive bacterial cultures based on mean CFU > 0 and < 50 on the slants and high positive bacterial cultures based on mean CFU $\geq$ 50 on the slants. Individual cows with high positive bacterial culture test results were designated as heavy fecal shedders, and individual cows with low positive bacterial culture test results were considered light fecal shedders. Real time PCR results were interpreted as positive or negative based on the Cycles to threshold (Ct) values. Negative test result had Ct $\geq$ 37 and positive test results had Ct < 37.

5.3.3. Within-herd and within-group apparent prevalence

The within-herd proportions of serum ELISA or bacterial culture positive cows was calculated by dividing the number of test positive cows by the total of number of cows tested with the respective tests at the herd visit. Similarly, the proportions of cows

with high and low positive serum ELISA and bacterial culture were calculated by dividing the number of cows with high or low test results by the total number of cows tested at the time of the visit.

The within-group apparent prevalence of positive (low or high) serum ELISA or bacterial culture cows was calculated by dividing the number of test positive (low and high) cows by the total number of cows in the group tested with the respective test at the herd visit.

5.3.4. Individual pooled samples

Individual fecal samples from the herds in Minnesota were pooled in groups of 5 and 10 cows per pool. The pooling procedure was performed at the Minnesota Veterinary Diagnostic Laboratory from individual fecal samples. Individual pooled samples from 5 cows (**IP5**) were obtained by pooling together 2 grams of feces from 5 different individual fecal samples in a 15 ml centrifuge tube. Tubes with IP5 were vortexed for 30 seconds to obtain the IP5. Similarly, samples of 3 grams each were obtained from 2 different IP5s, mixed together in a 15ml centrifuge tube and vortexed for 30 seconds to obtain individual pooled samples from 10 cows (**IP10**). Individual fecal samples from cows included in the IP5 and IP10 were obtained from cows in the same group and herd and the number of IP5 and IP10 samples per group depended on the group size. Bacterial culture of feces and *MAP* Real-time PCR using Ambion® reagents (Ambion®, Applied Biosystems, Carlsbad, CA) were performed on the IP5 and IP10 and results were interpreted based on the CPU as positive or negative, and as negative, low positive and high positive as described above. The results of the Real-time PCR were interpreted based on the number of cycle to threshold (Ct-values). A total of 284 IP5 and 140 IP10 were tested using bacterial culture.

5.3.5. MAP status of the group of cows

The *MAP* status of a group of cows in a specific herd and visit was established using the bacterial culture test result from the pooled environmental GROUP samples of that group. Positive or negative bacterial culture test result on Pooled environmental

GROUP samples represented positive or negative *MAP* status of the group at the time of the visit, respectively. Similarly, low or high positive bacterial culture test results from pooled environmental GROUP samples of a group of cows represented a low or high *MAP* status of the group at the time of the visit, respectively.

5.3.6. MAP status of the herds

Since several pooled environmental HERD and GROUP samples were collected from each herd and visit, the *MAP* status of the herds at the time of the visit was established using 3 different scenarios: 1) *MAP* status of the herd was given by bacterial culture test results from a combination of available pooled environmental samples from HERD and GROUP, 2) *MAP* status of the herd was given by bacterial culture test results only from pooled environmental GROUP samples, and 3) *MAP* status of the herd was given by bacterial culture test results only from pooled environmental HERD samples.

Several pooled environmental HERD and GROUP samples were collected from each herd and visit; therefore, in each scenario a parallel interpretation of the bacterial culture test results was used to declare the *MAP*-positive status of the herd and a serial interpretation was used to declare a negative *MAP* status of the herd. In other words, at the time of the visit a herd was considered as positive if at least one of the bacterial cultures from pooled environmental combining HERD and GROUP (scenario 1), only GROUP (scenario 2), or only HERD (scenario 3) samples was positive. Alternatively, a herd was considered as *MAP* negative if all the bacterial cultures test results from pooled environmental fecal samples for each scenario were negative at the time of the visit (Figure 1).

Likewise a herd was classified as high positive *MAP* status if at least one of the bacterial culture on HERD or GROUP samples results were high positive, low positive *MAP* status if at least one of the positive bacterial culture on HERD or GROUP samples results was low positive and none were high positive, and negative *MAP* status if all the positive bacterial culture on HERD or GROUP samples results were negative at the time of the visit.

5.3.7. Sensitivity and specificity for fecal shedders and heavy fecal shedders

The herd-level sensitivity of fecal shedding (HSeS) was described as the probability of a positive *MAP* status of the herd based on bacterial culture of feces on environmental fecal samples for each of the 3 proposed scenarios (scenario 1 a combination of HERD and GHE samples, scenario 2 only GROUP samples and scenario 3 only HERD samples) given that there was at least one fecal shedding cow in the herd at the time of the visit. Herd-level specificity for non-fecal shedders was not calculated, since all herds were considered infected.

The herd-level sensitivity of heavy fecal shedding (HSeHS) was described as the probability of a positive *MAP* status of the herd based on bacterial culture of feces on environmental fecal samples for each of the 3 proposed scenarios, given that there was at least one heavy fecal shedding cow in the herd at the time of the visit. All these herds were considered infected during the study period, and therefore herd-level specificity for fecal shedders was not calculated.

The herd-level specificity of heavy fecal shedding (HSpHS) was represented by the probability of a negative *MAP* status of the herd based on the bacterial culture of feces on environmental fecal samples for each of the 3 proposed scenarios, given that there was no heavy fecal shedding cow in the herd at the time of the visit. The fecal shedding status of the cows in the herds was based on the interpretation of the bacterial culture of feces results on individual fecal samples explained above.

The group-level sensitivity of fecal shedding (GSeS) for bacterial culture from group pooled environmental GROUP samples represented the probability of a positive bacterial culture result given that there was at least one fecal shedding cow in the group.

The group-level specificity of fecal shedding (GSpS) for bacterial culture from pooled environmental GROUP samples represented the probability of a negative bacterial culture result given no fecal shedding cows were present in the group at the time of the visit.

The group-level sensitivity of heavy fecal shedding (GSeHS) for bacterial culture from group pooled environmental GROUP samples represented the probability of a

positive bacterial culture result given that there was at least one heavy fecal shedding cow in the group.

The group-level specificity of heavy fecal shedders (GSpHS) for bacterial culture from pooled environmental GROUP samples represented the probability of a negative bacterial culture result given no heavy fecal shedding cows were present in the group at the time of the visit.

Therefore, using a parallel interpretation of different bacterial culture results from pooled environmental samples; at herd-level the relative sensitivities for fecal shedders and for heavy fecal shedders (HSeH and HSeSH, respectively), and the relative specificity for heavy fecal shedders (HSpHS) were estimated. Similarly, using the results of the bacterial culture from pooled environmental (GROUP) samples, at group-level the relative sensitivities for fecal shedders and heavy fecal shedders (GSeS and GSeHS, respectively), and the relative specificities of fecal shedder and heavy fecal shedders (GSpS and GSpHS, respectively), were estimated.

Results of bacterial culture and direct fecal PCR assay on pooled individual fecal samples and individual fecal samples were used to estimate the relative sensitivity and specificity for fecal shedders and heavy fecal shedders. The conditional probability of a positive bacterial culture or positive PCR from IP5 or IP10 samples given that there was at least one fecal shedding (P5SeS and P10SeS, respectively) cow and at least one heavy fecal shedder (P5SeHS and P10SeHS, respectively) in the pool was described as the sensitivity for fecal shedding and sensitivity for heavy fecal shedding, respectively. Similarly, the specificity of the bacterial culture of PCR on IP5 and IP10 samples for fecal shedding (P5SpS and P10SpS, respectively) and for heavy fecal shedding (P5SpHS and P10SpHS, respectively) were described as the conditional probability of a negative bacterial culture given that there was no fecal shedder and no heavy fecal shedding cow in the pool, respectively. Fecal shedding and heavy fecal shedding status of the individual cows was established based on bacterial culture of the individual cow fecal samples.

The 95% confidence interval for estimated sensitivities and specificities were calculated using the Wilson score method for confidence interval (Newcombe, 1998).

5.3.8. Analysis

Statistical analysis was performed at both the group and herd-level. At both levels, the analysis included the 2-level test results (negative or positive) and 3-level test results (negative, light or heavy) bacterial culture test results from pooled environmental samples. Therefore, four models were built to accomplish the objectives of the study.

Herd-level. The probability of positive *MAP* status of the herd at the time (experimental unit) was modeled using a logistic regression with repeated measurements and a logit link function. The *MAP* disease status at the time of the visit was established based on the test results of the bacterial culture from pooled environmental HERD and GROUP (scenario 1) samples taken at the time of the visit. The explanatory variables were the within-herd apparent prevalences of high and low bacterial culture and serum ELISA, and herd size at the time of the visit. The model assumed compound symmetry of the working correlation for different visits within-herd. The working correlation was chosen using quasi-likelihood information criterion (Pan, 2001) and model parameters were estimated using GEE. All the explanatory variables were included in the model as continuous variables; however, within-herd apparent prevalences of high positive and low positive bacterial culture and serum ELISA were expressed per thousand cases, because overall apparent prevalences were low. Similarly, herd sizes at the time of the visit were divided by 10 when included in the model to avoid extremely small parameter estimates. Interaction terms between explanatory variables were initially included in the model, but removed when statistical significance (P-value <0.05) was not reached. Model was fitted using proc GENMOD in SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

An ordinal logistic regression with repeated measurements and a cumulative logit link function was used to model the probability of changing the *MAP* status of the herds from negative to low positive and from low positive to high positive. The *MAP* status (negative, low positive or high positive) of the herd at the visit was established based on bacterial culture test results from pooled environmental HERD and GROUP samples (scenario 1). The probability of the *MAP* status of the herd at the time of the visit was explained by the within-herd apparent prevalence of high and low bacterial culture and

serum ELISA, and herd size. All these explanatory variables were also included in the model as continuous variables. The assumption of proportionality of the odds was evaluated using score tests and no violation of this assumption was found. Model assumed independence of the working correlation structure of the observations within-herd. Model parameters were estimated using GGE and fitted using Proc GENMOD in SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

Group-level. Logistic regression with repeated measurements using generalized estimated equations (GEE) and a logit link function was used to model the probability of positive *MAP* status of the group of cows (experimental unit). The *MAP* status of each group of cows in a herd was obtained from the bacterial culture test results from pooled environmental GROUP sample in all herds and visits. This probability was explained by the four within-group apparent prevalences of high and low positive bacterial culture and serum ELISA and group size. After checking for linear associations between the explanatory variables and the logit (Natural logarithmic transformation of the odds) of positive *MAP* group of cows, within-group apparent prevalences were included as continuous variables in the model; however, within-group apparent prevalences were also included as proportion per thousand, and group sizes were also divided by 10 as explained above. The correlation among groups by herd and visit was modeled using compound symmetry. This working correlation structure was chosen using quasi-likelihood information criterion (Pan, 2001). Interaction terms between explanatory variables were initially included in the model, but removed when statistical significance (P-value <0.05) was not reached. The model was fitted using Proc GENMOD in SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

An ordinal logistic regression with repeated measures using GEE was used to model the probability of changing the *MAP* status of a group of cows from negative to low positive or from low positive to high positive. The *MAP* status of the groups of cows by herd and visit was established by using the bacterial culture test results from pooled environmental GROUP sample. The probability of increasing the *MAP* status of the groups was explained by the four within-group apparent prevalences of high and low

positive bacterial culture and serum ELISA and group size. Within-group apparent prevalences were included as continuous variables in the model and expressed per thousand cases and group sizes were divided by 10. The original model violated the assumption of the proportionality of the odds (parallel slopes among the levels of the outcome) when evaluated using the score test; therefore a partial proportional odds approach for ordinal logistic regression (Peterson and Harrell, 1990) was used instead. In the partial proportional odds approach, the score statistics for type 3 GEE analysis were used to identify explanatory variables that may violate the model assumption of proportionally of the odds. Then, these variables were allowed to have different estimations for the probability of increasing the *MAP* status of the group from negative to low positive and from low to high positive. The correlation among groups by herd and visit was modeled using compound symmetry. This working correlation structure was chosen using quasi-likelihood information criterion (Pan, 2001). Interaction between explanatory variables were initially included in the model, but removed when statistical significance (P-value <0.05) was not reached. Model was fitted using Proc GENMOD in SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

5.4. RESULTS

Even though some herds had no bacterial culture and/or serum ELISA positive cows at some herd visits, all herds showed at least one test positive cow during the study period. The within-herd apparent prevalence of bacterial culture and serum ELISA positive cows at the time of the visit had a mean (range) of 5.5% (0 to 24.4) and 9.0% (0 and 61.5) respectively. Similarly, the mean (range) within-herd apparent prevalence of low positive bacterial culture and serum ELISA test results was 4.3% (0 to 22.2) and 5.6% (0 to 40.4), respectively. The mean (range) within-herd apparent prevalence for high positive bacterial culture and serum ELISA was 1.2% (0 to 13.5) and 3.4% (0 to 44.3), respectively.

From the 277 groups of cows sampled during the study period, 127 groups (45.8%) were found to be *MAP*-positive using bacterial culture based on testing of pooled environmental GROUP samples. Thirteen out of these 127 (10.2%) *MAP*-positive groups

of cows were high positive *MAP* status and 35.7% of the 277 groups of cows in the study had at least one heavy fecal shedding cow in the group.

The proportion of herd visits with positive *MAP* status based on bacterial culture on pooled environmental samples varied depending on the scenario. For scenario 1, where test results of HERD and GROUP samples were combined, 86.1% of the herds were classified as positive JD at the time of the visit. The same value for scenario 2, where only the test results from GROUP samples was used, was 77.2%. Finally, the proportion of *MAP* positive herds under the scenario 3, where only test results from HERD samples were used, was 69.8%. These values represented the apparent prevalence of *MAP* positive herds at the time of the visit, based on the results of the bacterial cultures on different types of pooled environmental samples.

5.4.1. Herd-level

The HSeS of bacterial culture using a combination of HERD and GROUP samples showed to be higher than the same HSeS using GROUP samples and HSeS using HERD samples (Table 2).

The accuracy of the bacterial culture based on environmental samples for detection of at least one heavy fecal shedder varied depending on the type of pooled environmental samples used. The HSeHS showed to be higher in scenario 1, where the *MAP* status of the herd was given using a combination of pooled environmental samples from HERD and GROUP, followed by the HSeHS on scenario 2 where the *MAP* status of the herd was given using pooled environmental samples only from GROUP samples, and the lowest HSeHS was observed in the scenario 3, where the *MAP* status of the herd was defined only using the test results of the bacterial culture on HERD samples. The higher HSpHS was observed on the scenario 2, followed by scenario 3 and 1 (Table 3)

The logistic regression model to explain the probability of positive outcome showed no association between the probability of a *MAP* positive herd and the within-herd apparent prevalence of high and low positive bacterial culture and serum ELISA and herd size.

The results of the ordinal logistic regression, when the herd *MAP* status was divided into 3 ordinal categorical levels (negative, low positive and high positive), showed an increase in the probability of higher *MAP* status of the herds (i.e. from negative to low positive, or from low to high positive) associated with the within-herd apparent prevalence of heavy fecal shedders ($P<0.01$) and herd size ($P<0.01$). The apparent prevalences of low and high positive serum ELISA were not associated with probability of higher *MAP* status of the herds and therefore eliminated from the model. Under ceteris paribus conditions, or keeping the other variable constant, a 0.1 percent units increase in the within-herd apparent prevalence of heavy fecal shedders was associated with a 12.06% (96% CI = 7.6 to 16.7) increase in the probability of a higher JD status of the herd. Similarly, under the same conditions, an increase of 5.6% (96% CI =3.4 to 7.8) in the probability of higher JD status of the herds was expected for every 10 adult cows increase in herd size.

5.4.2. Group-level

A total of 277 groups of cows from the 17 herds and 79 herd visits were included in the analysis. Out of the 277 groups of cows tested using bacterial culture on GROUP samples, 127 were positive and 150 negative. Out of the same 277 groups of cows tested, 191 had at least one fecal shedder and 99 groups had at least one heavy fecal shedder.

The GSeS and GSeHS (95% confidence interval) of bacterial culture performed on GROUP samples were 61.3% (54.2 to 67.9) and 82.8% (74.2 to 89.0) to detect at least one fecal shedding cow and at least one heavy fecal shedding cow in the group, respectively. Similarly, the GSpS and GSpHS of bacterial culture was 88.4% (79.9 to 93.6) and 74.7% (67.9 to 80.5) to correctly detect non-fecal shedding cows and non-heavy fecal shedding cows in the group, respectively.

From logistic regression, the probability of positive *MAP* status of the groups was associated with the within-group apparent prevalence of light fecal shedders, group size and the interaction between the within-group apparent prevalence of heavy fecal shedders and group size. In contrast, the probability of positive *MAP* status of the groups was not associated with the within-group apparent prevalence of low and high positive serum

ELISA. Under ceteris paribus conditions, the probability (95% confidence interval) of positive JD status of the group increased 0.9% (0.4 to 1.4) for each increase of 0.1 percent units the within-group apparent prevalence of low positive bacterial culture cows. Similarly, the probability of positive JD status of the group increased 7.9% (0.4 to 14.8) when the group size increased by 10 cows; however, this association was modified by the within-group apparent prevalence of high positive bacterial culture cows (heavy fecal shedders). An increase of 0.1 percent units in the apparent prevalence of heavy fecal shedders increased the magnitude of the association of group size and JD status of the group by approximately 2.3% (1.6 to 2.8).

The score test in the partial proportional odds model identified within-group apparent prevalence of light and heavy fecal shedders, and group size as the variables that violated the proportional odds assumption. Therefore, the model allowed estimating different parameters for changing the JD status of the groups from negative to low positive and from low to high positive in these variables. Within-group apparent prevalence of light fecal shedders and the interaction between within-group apparent prevalence of heavy fecal shedders and group size were associated with the probability of increasing the JD status of the group of cows. The probability (95% confidence interval) of increasing the JD status of the group from negative to low positive increased 1.2% (0.6 to 1.7) for every 0.1 percent units increase in the within-group apparent prevalence of light fecal shedders; however, the probability of increasing the JD status of the group from low to high positive was not associated with the within-group apparent prevalence of low shedders. The association between group size and the probability of increasing the JD status of the group from negative to low positive increased 2.4% (1.7 to 3.1) for every 0.1 percent units increase in the within-group apparent prevalence of heavy fecal shedders. Likewise, the association between group size and the probability of increasing the JD status of the group from low to high positive increased 1.6% (0.7 to 2.6) for every 0.1 percent units of increase in the within-group apparent prevalence of heavy fecal shedders (Table 4). The probability of increasing the *MAP* status of the group of cows was not associated to with the within-group apparent prevalence of low and high positive serum ELISA.

5.4.3. Individual pooled samples

The estimated relative sensitivity and specificity of bacterial culture for detection of at least one fecal shedder or at least one heavy fecal shedder in a pool of individual cows was similar when using 5 or 10 individual cows per pool. The estimated P5SeS and P10SeS of the bacterial culture were low; however, the estimated P5SpS and P5SpS were high (Table 5). In this study, bacterial culture showed a perfect (100%) P5SeSH and P10SeSH; however, it was observed that approximately 9% of the pools were classified as bacterial culture false positive, when the objective is the detection of at least one heavy fecal shedder in the pool (Table 5).

The estimated relative sensitivity and specificity of direct fecal PCR assay for detection of at least one fecal shedder or at least one heavy fecal to in a pool of individual cows were also similar when using 5 or 10 individual cows per pool. The P5SeS and P10SeS of PCR were higher than the corresponding sensitivities for bacterial culture. In contrast, the P5SpS and P10SpS were lower that the corresponding specificities of the bacterial culture.The performance P5SeSH and P10SeSH using direct fecal PCR assay were equivalent to the corresponding values of the bacterial culture; however, P5SpS and P10SpHS were lower than the corresponding values of bacterial culture.

5.5. DISCUSSION

Herds in this study were enrolled in the National Johne's Disease Demonstration Herd Program. The objective of this project was to control the transmission of JD over several years through changes in herd management practices, as well as practices related to testing and culling of test-positive cattle; therefore, variable JD prevalences among herds and visits were expected. Although a few herds had no adult cows either serum ELISA or bacterial culture positive on a specific herd visit, all herds had JD test positive cows and pooled environmental JD positive samples on at least one visit; therefore all study herds were considered endemically infected with JD during the study period.

There was a wide variation on the group size that could be explained by the variability of herd sizes observed in the study herds (41 to 711 adult cows) and the

variation of the grouping strategies observed among herds. The description of the groups of cows in the herds included the whole production cycle of a typical dairy operation (i.e. groups with different lactating cows, dry-cows, and special needs cows).

The apparent prevalence of JD and low and high JD positive cattle showed wide variation depending on the herd, visit and diagnostic test used. Even though bacterial culture of feces is considered the optimal test, because it is the most sensitive and specific for diagnosis of fecal shedders, its sensitivity is still only 30-60% with 99% specificity (Whitlock et al., 2000, Wells et al., 2006, Collins et al., 2006). Considering that diagnostic tests are imperfect and the sensitivity of the bacterial culture and serum ELISA are rather low for detecting true JD infected cattle, a higher true prevalence of JD is expected compared to apparent prevalence.

The use of bacterial culture of environmental fecal samples to identify herds infected with *MAP* has been previously evaluated (Raizman et al., 2004, Lombard et al., 2006, Smith et al., 2011) and used as part of the Voluntary Bovine Johne's Disease Control Program (USDA-APHIS, 2010). Even though bacterial culture of pooled environmental samples has been previously used to determine the *MAP* infection status of the herd, to our knowledge, this is the first study that evaluated the used of pooled environmental and pooled individual fecal samples to identify the presence and location of heavy shedding cows in the herd.

At herd-level, the sensitivity or the conditional probability of at least one positive bacterial culture based on pooled environmental samples given that the herd had at least one fecal shedding cow varied between approximately 70 to 87% depending on the type of pooled environmental sample used (HERD, GROUP or both). In contrast, at the group-level, the sensitivity of the bacterial culture to classify a group of cows as positive using GROUP samples given that there was at least one fecal shedding cow in the group was approximately 60%.

Even though the sensitivity of the bacterial culture seemed to be lower for detecting a fecal shedder in a group of cows than in the whole herd, the JD status of the herd was confirmed using the results the several bacterial cultures from pooled environmental samples taken at different locations, while the JD status of the group of

cows was based on a single bacterial culture from a pooled environmental sample from the group (GROUP). The test results from bacterial culture used to establish the JD status of the herd was performed using a parallel interpretation of the different results, which increased the sensitivity of this strategy to detect fecal shedding cattle.

These results were similar to the 76% probability of correctly identifying at least one fecal shedding cow in the herd using bacterial culture based on HERD sample reported by Lombard et al (2006). Smith et al. (2011) reported 40% sensitivity when using bacterial culture based on HERD samples; however, the within-herd apparent prevalence of fecal shedding cows was lower than that observed in this study.

While the probability of false negative bacterial culture on pooled environmental samples to identify at least one fecal shedders in the herd varied, depending on the type of pooled environmental samples used (HERD, GROUP, or both), from approximately 13% to 30%, the same probability to identify at least one heavy fecal shedder varied from 2 to 16%. These results may be explained by the exponential level of environmental *MAP* contamination caused by a heavy fecal shedder compared to a light fecal shedder.

The estimated herd-level sensitivity of detection of at least one heavy fecal shedder using pooled environmental samples (scenario 1) was higher than the estimated group-level sensitivity of detection of at least one heavy fecal shedder (HSeHS:98.2% vs. GSeHS:82.8%). In contrast, the group-level specificity of detection of heavy fecal shedders on pooled environmental samples appeared higher than herd-level specificity (GSpHS: 74.7% vs. HSpHS:43.5%). Differences in performance between group and herd-levels may also be explained by the number of samples used to classify a herd or a group as positive or negative status and the parallel interpretation of the test results.

As mentioned above, bacterial culture is an imperfect diagnostic test when used on individual fecal samples (Whitlock et al., 2000, Wells et al., 2006, Collins et al., 2006). The accuracy of this diagnostic test on pooled environmental samples showed to be even lower when using bacterial culture on individual samples as reference test. In this study, the estimated values of sensitivity and specificity of the bacterial culture on pooled environmental samples to detect at least one fecal shedder or heavy fecal shedder assumed the bacterial culture on individual fecal samples as gold standard test.

Beyond the factors that affect the accuracy of the bacterial culture on individual fecal samples, the application of this test on pooled environmental samples may also increase the false positive and negative rates of the test. False negative bacterial culture results when detecting heavy fecal shedding cows in the groups of cows or herds may be explained by borderline heavy fecal shedding. The amount of *MAP* shed in feces in heavy fecal shedding cows may vary from thousands, millions or even billions of *MAP* per gram of feces. A proportion of these heavy fecal shedding cows shed enough *MAP* to be categorized as heavy fecal shedding cow using bacterial culture on the individual fecal sample; however they do not shed high enough *MAP* to overcome the dilution effect when mixing with feces from non-fecal shedding cows in the environment leading to negative bacterial culture test results for the environmental fecal sample. This dilution effect may be accentuated in groups of cattle with low apparent prevalence of light and heavy fecal shedders or/and a large group or herd sizes.

False positive bacterial culture results when detecting heavy fecal shedders may be explained by the presence of light fecal shedders that shed enough amounts of *MAP* into the environment that may lead to a positive bacterial culture results when using environmental samples of these cows. Therefore, if at least one light fecal shedding cow was present in the group of cows or in the herd, there was a probability that at least one positive bacterial culture resulted from testing of pooled environmental samples.

The use of bacterial culture on pooled individual samples for detection of fecal shedders has also been previously evaluated as alternative to reduce the cost of sampling and testing cattle (Wells et al., 2002). In this study, the sensitivity of bacterial culture and direct fecal PCR assay on pooled individual samples to detect a fecal shedding cow in the pool increased when the pooled sized decreased from 10 to 5 cows per pool; however, the sensitivity of the bacterial culture on pooled individual samples to detect at least one heavy fecal shedding cow in the pool did not change and remained at 100% when increasing the pool size from 5 to 10 cows per pool. Almost no differences in the sensitivity of direct fecal PCR assay on IP5 or IP10 were observed when detecting heavy fecal shedders. This study showed that bacterial culture and direct fecal PCR on larger pool sizes (10 individual per pool) may be used when detecting heavy fecal shedders.

Wells et al. (2002) also concluded that the sensitivity of the bacterial culture using pooled individual samples increased when the pool size decreased and when the pool sample contained fecal samples from heavy fecal shedders. Beside pool size, other factors also need to be considered when evaluating the application of pooled individual samples, such as herd size, prevalence of fecal shedders or heavy fecal shedders, false positive rate and costs and time to results of the tests results (Wells et al., 2003; van Schaik et al., 2003b, Tavorpanich et al., 2004).

Direct fecal PCR assay showed a higher sensitivity to detect fecal shedders and heavy fecal shedders for both IP5 and IP10; however, direct fecal PCR assay had a slightly lower specificity compared to bacterial culture. An important advantage of the direct fecal PCR assay was that the test results were available within a week, compared to the 16 weeks incubation period needed for the bacterial culture before test result at similar costs.

At the herd-level, increasing the JD status of the herd from negative to low or from low to high was associated with an increase in herd size. With larger herd size, herds were more likely to have a higher JD status based on the results of the pooled environmental samples. Larger herds had a higher probability of high positive *MAP* status, and smaller herds had a higher probability of negative *MAP* status. The reason for this association is not clear, but it is possible that, smaller herds tend to be close herds and less likely to introduce infected cattle to the herd, compared to larger herds. It is also possible that fecal contamination of feed and therefore *MAP* contamination is more frequent in larger herds with free-stalls housing than in smaller with tie-stalls housing. Finally it is also possible that compared to larger herd, herd managers in smaller herds paid more attention to individual cows and culled or isolated fecal shedders from the rest of the herd reducing the environmental contamination with *MAP* and the probability of a positive bacterial culture on pooled environmental sample. It is also possible, that

At a herd level, the ordinal logistic regression showed that an increase in the within-herd apparent prevalence of heavy fecal shedders was associated with an increase in the MAP status of the herd (from negative to low or from low to high). Therefore, the higher the prevalence of heavy fecal shedders, the higher the concentration of *MAP* in the

environment, and the higher the probability of a high positive bacterial culture result from pooled environmental samples.

At the group-level, the logistic regression showed that larger groups of cows were more likely to be *MAP* positive than smaller sized groups. The probability of a *MAP*-positive group was also associated with the interaction between group size and within-group apparent prevalence of heavy fecal shedders, indicating that at the same within-group apparent prevalence of heavy fecal shedders, larger groups were more likely to be *MAP* positive, or in other words, at the same group size, groups with higher within-group apparent prevalence of heavy fecal shedders were more likely to be *MAP* positive.

Similar results were found in the partial proportional odds model, when bacterial culture results from pooled environmental GROUP samples were divided into negative, low positive and high positive results. The analysis showed that increasing the within-group apparent prevalence of light fecal shedders was associated with a higher probability of going from negative to low positive *MAP* status; however, as expected, the within-group apparent prevalence of light fecal shedders was not associated with the probability of a high positive *MAP* status. It is likely that *MAP* from light shedding cows was diluted with the feces of non-fecal shedding cows, therefore the higher the within-group apparent prevalence of light fecal shedders the less likely *MAP* was diluted, and the more likely to be detected by bacterial culture. The environmental concentration of *MAP* from light fecal shedders would not reach the threshold limit of detection of the bacterial culture to be categorized as high positive.

As previously described for the probability of JD group positivity, the association between group size and the probability of low or high JD group positivity was modified by the within-group apparent prevalence of heavy fecal shedders. At the same within-group apparent prevalence of heavy fecal shedders, larger sized groups were more likely to be JD low or high positive than smaller sized groups, and at the same group size, groups with higher within-group apparent prevalence of heavy fecal shedders were more likely to be a JD low or high positive; however, the magnitude of the interaction between these explanatory variables decreased approximately 66% (from 2.4% to 1.6%) for JD low positive groups compared to high JD positive groups. This indicated that the

probability of high JD group positivity was more similar among group sizes at the same within-group apparent prevalence of heavy fecal shedders and the probability of low JD group positivity was less similar among group sizes at the same within-group apparent prevalence of heavy fecal shedders.

The apparent prevalences of low and high positive serum ELISA were not associated with *MAP* environmental contamination of the group of cows or the herd. Collins (2002) suggested an association between the serum ELISA results and the progression of the disease. Higher S/P ratio on the serum ELISA is associated with more advanced stages of JD and higher levels of fecal shedding. Even though an association between *MAP* fecal shedding level and humoral immune response at individual cow-level is expected, this association was not observed at the group-level or herd level in this study.

The JD status of the herd based on pooled environmental samples was associated with the within-herd apparent prevalence of heavy fecal shedders and herd size. The performance of the JD surveillance system that uses this type of sample to determine the disease status of the herd is sensitive to the within-herd apparent prevalence of heavy fecal shedders and herd size; therefore small herds with a low apparent prevalence of heavy fecal shedders may be classified as negative status (false negative), affecting the sensitivity of the surveillance system. Positive bacterial cultures from pooled environmental samples from the whole herd or from a group of cows not only indicates the presence of fecal shedding cows, but also the presence of heavy fecal shedding cows in the group or in the herd. As long as special attention is taken to collect representative samples from the group of cows, pooled environmental sampling may be especially useful to detect the presence of a small number of heavy fecal shedders (low apparent prevalence) in large groups of cows. Targeted surveillance in these bacterial culture positive groups can then identify heavy fecal shedding cows. The use of bacterial culture from a combination of pooled environmental samples from the whole herd and from specific group of cows maximized the sensitivity of the surveillance system to detect the presence of MAP fecal shedding cows in the herd (infected herd); however, the best combination of sensitivity and specificity to detect heavy fecal shedders in the herd was

obtained when only bacterial culture results from pooled environmental samples from the group of cows was used.

Use of bacterial culture on pooled HERD samples alone showed the lowest sensitivity to identify the presence of fecal shedding (69.8%) or heavy fecal shedding cows in the herd (83.7). A higher sensitivity was obtained when using bacterial culture on pooled GROUP samples to identify the presence of fecal shedding cows (78.2%) and heavy fecal shedding cows (96.4%). This is an important difference not only in a surveillance system when the objective is identifying *MAP* infected herds, but also is important in a control program when the objective is reducing the incidence of the disease in *MAP* infected herds. Moreover, bacterial culture on pooled GROUP samples also provides indications about the location of the fecal shedders or heavy fecal shedders in the herd that could be used to target the sampling collection and testing on bacterial culture positive groups.

Once the presence of fecal shedders or heavy fecal shedders is suspected of specific groups of cows based on the bacterial culture test results, it may be possible to pool individual samples in groups of 5 or 10 cows per group in order to reduce the costs of testing. Both bacterial culture and direct fecal PCR assay showed high sensitivity to detect heavy fecal shedders in pooled individual samples, and therefore is an alternative that needs to be further evaluated taking into account sampling and testing costs.

We also concluded that within-group or within-herd apparent prevalence of fecal shedding or heavy fecal shedding cows and herd or group size are important factors associated with the probability of a *MAP* positive group or herd. Though this study provides estimations of the accuracy of the bacterial culture performance on pooled environmental samples and pooled individual samples, further cost-effectiveness analysis using these type of samples under different herd sizes and prevalences conditions is needed in order to evaluate its implementation in JD control programs in dairy herds.

One primary finding of this study was the estimation of sensitivity and specificity of bacterial culture using pooled environmental fecal samples. These estimates showed that bacterial culture of pooled environmental samples can be useful to detect the presence of heavy fecal shedding cows in the herd.

Table 1. *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture and Johne's disease serum ELISA test results from pooled environmental manure samples and individual cow fecal and serum samples on 7 dairy herds in Minnesota (MN) and 10 dairy herd in Pennsylvania (PA) enrolled on the Johne's disease demonstration herd project.

		Bacterial Culture[1]		Serum ELISA[2]	
Samples	State	Positive (%)	Total	Positive (%)	Total
Pooled Environmental[3]					
Whole herd	MN	0 (0)	0	-	-
	PA	91 (54.5)	167	-	-
	Total	91 (54.5)	167		
Group of cows	MN	40 (54.8)	73		
	PA	174 (42.0)	414	-	-
	Total	214 (43.9)	487		
Individual Cow	MN	244 (6.8)	3,611	108 (3.0)	3,566
	PA	795 (4.7)	16,953	991 (5.8)	16,949
	Total	1,039 (5.1)	20,564	1,099 (5.4)	20,515

[1] Herrold's egg yolk media. Negative = 0 colonies/slant, positive >0 colonies/slant.

[2] IDEXX. Negative: S/P <0.25, positive: S/P ≥ 0.25.

[3] Pooled environmental whole herd samples represented samples of manure collected from high cow traffic areas (alleys) or manure storages (manure pit). Polled environmental group of cows samples represented samples of manure collected from cow housing areas (groups).

Table 2. Relative sensitivity of the *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture using different sources of pooled environmental manure samples to detect at least one *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture positive cows in the herd in 7 dairy herds in Minnesota (MN) and 10 dairy herd in Pennsylvania (PA) enrolled on the Johne's disease demonstration herd project[1]

Source [2,3]	**Sensitivity (%)**	**95 % Confidence Interval**
Group of cows	78.2	67.8 to 85.9
Whole herd	69.8	57.7 to 79.8
Whole herd and Group of cows[4]	87.2	78.0 to 92.9

[1] Herrold's egg yolk media. Negative = 0 colonies/slant, positive >0 colonies/slant.

[2] Source of the pooled environmental sample

[3] Pooled environmental whole herd samples represented samples of manure collected from high cow traffic areas (alleys) or manure storages (manure pit). Polled environmental group of cows samples represented samples of manure collected from cow housing areas (groups).

[4] Using both types of samples, pooled environmental fecal samples from the whole herd and pooled environmental fecal samples from groups of cows to detect at least one *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture positive cow in the herd.

Table 3. Relative sensitivity and specificity (95% confidence interval) of the *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture using different sources of pooled environmental manure samples to detect at least one *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture high positive cow (heavy fecal shedder) in the herd in 7 dairy herds in Minnesota (MN) and 10 dairy herd in Pennsylvania (PA) enrolled on the Johne's disease demonstration herd project

Source [2]	**Sensitivity (%)**	**Specificity (%)**
Whole herd and Group of cows	98.2 (90.5 to 99.7)	43.5 (25.6 to 63.2)
Group of cows	96.4 (87.9 to 99.0)	69.6 (49.1 to 84.4)
Whole herd	83.7 (70.0 to 91.9)	60.0 (38.7 to 78.1)

[1] Herrold's egg yolk media. Negative = 0 colonies/slant, positive >0 colonies/slant.

[2] Source of the pooled environmental sample

[3] Pooled environmental whole herd samples represented samples of manure collected from high cow traffic areas (alleys) or manure storages (manure pit). Polled environmental group of cows samples represented samples of manure collected from cow housing areas (groups)

Table 4. Odd ratios (95% Confidence interval) from a partial proportional odds model for 3-level *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture results on pooled environmental samples from groups of cows in 7 dairy herds in Minnesota (MN) and 10 dairy herd in Pennsylvania (PA) enrolled on the Johne's disease demonstration herd project

		MAP status of the group[1,2]	
Variable	Comparison	Negative vs. Low	Low vs. High
Prevalence of heavy[3]	Increase 0.1 Percent units	0.99 (0.97 to 1.0)	0.99 (0.97 to 1.0)
Prevalence of light[3]	Increase 0.1 Percent units	1.01 (1.00 to 1.02)	1.00 (0.99 to 1.01)
Group Size	Increase 10 cows	1.10 (1.03 to 1.19)	0.61 (0.44 to 0.85)
Prevalence of high[3] and group size interaction	Increase in 0.1 Percent, keeping group size constant	1.024 (1.017 to 1.031)	1.016 (1.007 to 1.026)

[1] Herrold's egg yolk media. Negative = 0 colonies/slant, low >0 and <50 colonies/slant, high ≥50 colonies/slant

[2] From the pooled environmental group of cows samples

[3] Apparent prevalence of light and heavy given by bacterial culture of feces based on individual samples

Table 5. Relative sensitivity and specificity (95% Confidence Interval) of *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture and RT-PCR on pooled individual samples in groups of 5 and 10 cows per pool to detect at least one *Mycobacterium avium* subspecies *paratuberculosis* fecal shedder and at least one heavy fecal shedders in the pool, in 7 herds in Minnesota enrolled on the Johne's disease demonstration herd project[1]

Test for pools	**Pools**	**Detection[2]**	**Sensitivity (%)**	**Specificity (%)**
Bacterial Culture	Pool of 5 cows	Fecal Shedder	47.9 (36.9 to 59.2)	97.5 (95.2 to 99.3)
		Heavy fecal shedder	100.0 (79.6 to 100)	91.1 (87.1 to 93.9)
	Pool of 10 cows	Fecal Shedder	36.1 (25.2 to 48.6)	97.5 (91.2 to 99.3)
		Heavy fecal shedder	100.0 (77.2 to 100)	91.3 (85.2 to 95.1)
RT-PCR	Pool of 5 cows	Fecal Shedder	68.9 (60.7 to 76.1)	86.1 (81.5 to 89.6)
		Heavy fecal shedder	97.0 (84.7 to 99.5)	73.8 (69.2 to 78.0)
	Pool of 10 cows	Fecal Shedder	57.1 (47.6 to 66.2)	89.5 (81.2 to 94.2)
		Heavy fecal shedder	100.0 (88.3 to 100)	76.0 (69.1 to 81.8)

[1] Reference test was Herrold's egg yolk media bacterial culture on individual cows fecal sample.

[2] Fecal shedder = bacterial culture result >0 colonies/slant, heavy fecal shedder = bacterial culture result ≥50 colonies/slant.

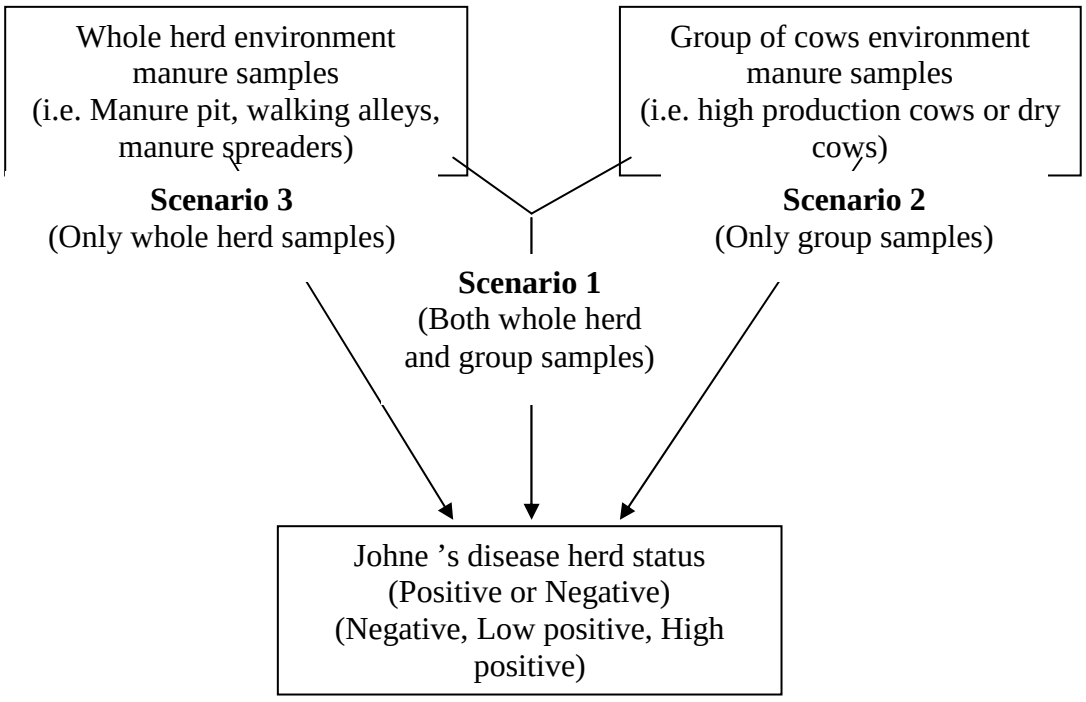

Figure 1. Types of pooled environmental manure samples used to establish the Johne's disease status of the herd in 17 commercial dairy herds in Pennsylvania and Minnesota.

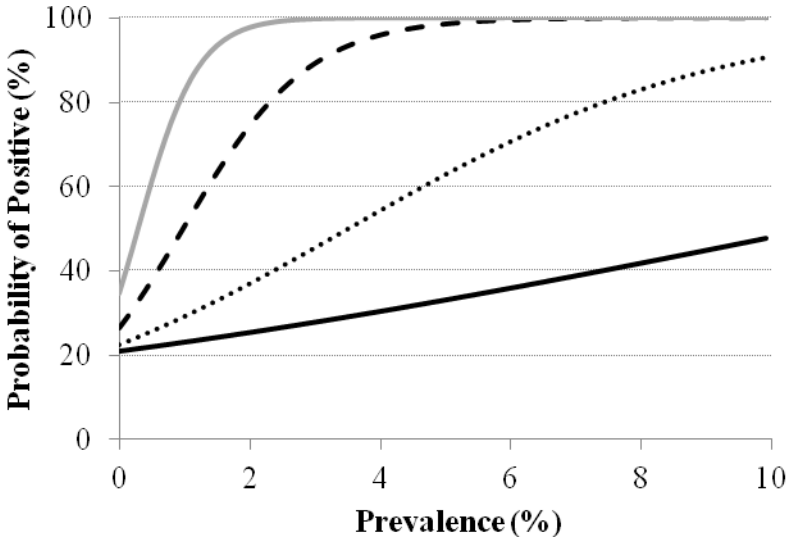

Figure 2. Probability of group of cows positive to *Mycobacterium avium* subspecies *paratuberculosis* bacterial culture by within-group apparent prevalence of heavy fecal shedders at different group sizes: 10 cows (solid black), 20 cows (dotted), 50 cows (dashed), and 100 cows (solid grey) in 17 commercial dairy herds in Pennsylvania and Minnesota under Johne's disease control program.

SUMMARY, GENERAL CONCLUSIONS AND FURTHER RESEARCH

Summary, general conclusions and further research

Although the chapters of this thesis had different objectives, all of them were designed to expand the knowledge about the epidemiology of Johne's Disease (JD). The overall aim of this project was to provide practical answers to common challenges faced by field veterinarians and dairy herd managers when implementing Johne's disease control programs in dairy herds.

The literature review presented in Chapter 1 summarizes the current state of knowledge of the epidemiology and management of JD in an effort to identify the gaps in research that may be important for the control of JD.

Chapter 2 described a study of 8 dairy herds enrolled in the Minnesota Johne's Disease Demonstration Herd Control Program. This program hypothesized that implementation of recommended management practices (including annual testing and culling of JD infected cattle) would reduce *MAP* transmission leading to reduction of the incidence of JD in the herd. We demonstrated that the dairy herds were able to implement recommended management practices; the level of implementation increased in every birth cohort. We also showed how the level of implementation of the recommended management practices was associated with a reduction of the incidence of test positivity and clinical JD along birth cohorts. This reduction was not explained by a delay in the onset of test positivity or clinical JD through time in study herds related to implementation of recommended control practices.

Since the levels of MAP exposure of susceptible cattle play an important role in the probability of infection transmission, Chapter 3 was focused on improving the understanding of two of the most important diagnostic tests used to identify MAP in fecal samples: bacterial culture of feces and the direct fecal PCR assay. We evaluated the analytical sensitivity of these diagnostic tests and the association of bacterial 5-level score with the concentration of MAP in fecal samples. The minimum detection levels (analytical sensitivity) of these diagnostic tests were obtained and we estimated the probability of a negative or positive test result, given the concentration of MAP in the fecal sample, by using probabilistic models. We found a positive association of bacterial

culture score with the concentration of MAP in the fecal sample and estimated the probability of a bacterial culture score given the concentration of MAP in the sample. This information could be used in the field to estimate the concentration of MAP in feces based on the diagnostic test results. As an example of the usefulness of this information, a dairy with a positive fecal culture result from a pooled environmental fecal sample of 4 provides evidence of at least 10^7 *MAP* colonies per gram of feces in this location.

Johne's disease is a multistage disease and the accuracy of the diagnostic tests varies depending on the stage of the disease in the cow. Diagnostic test results can be used not only to identify infected cattle but also evaluate the stage of infection of the cows. Chapter 4 evaluated the performance of the bacterial culture and serum ELISA to identify cattle at three different stages of infectiousness (heavy, light and non fecal shedding). We implemented a methodology that did not require or assume gold standard tests by estimating the conditional probabilities of correctly and incorrectly identifying cattle as heavy, light, or non fecal shedders, given the diagnostic test results. These conditional probabilities and the results of the diagnostic test can be used in the field to identify the cows that shed high concentrations of *MAP* into the environment (heavy fecal shedder) and, therefore, represent an important risk of transmission of the infection to susceptible cattle in the herd, including replacement heifers.

In the last chapter (Chapter 5), we focused on the evaluation of bacterial culture and direct fecal PCR in pooled environmental fecal samples to identify heavy fecal shedders as an alternative to identifying and culling all infected cattle in the herd, which can be expensive and impractical. We found that bacterial culture performed well identifying the presence of heavy fecal shedders in herds or groups of cows using pooled environmental fecal samples. Bacterial culture of feces and PCR also performed well identifying heavy fecal shedders in pooled individual samples in both groups of 5 or 10 cows per pool. Bacterial culture and PCR of pooled environmental fecal samples and pooled individual fecal samples can be used in the field to identify the presence of heavy fecal shedders and this practice may reduce the cost and time of sampling and testing.

In the epidemiology of JD, avoiding contact between susceptible cattle and infective doses of *MAP* is one key component to controlling Johne's disease. Reducing

the infection pressure or concentration of *MAP* in the environment of susceptible cattle should reduce the transmission and thus the incidence of disease. We found that JD can be controlled in dairy herds by implementing management practices that reduce the incidence of infection. We also provided the tools to estimate the concentration of *MAP* in fecal samples based on the diagnostic test results. Future studies can evaluate the effectiveness and cost-benefit of implementing single specific management practices for reduction of the concentration of *MAP* in the environment (infection pressure) and transmission of *MAP* to susceptible cattle.

A surveillance system is also an important element to control Johne's disease. Fecal shedding cows need to be identified and managed so their contribution to the environmental *MAP* contamination is minimized. Based on diagnostic test results, cattle can be classified by their contribution to the environmental *MAP* contamination and, therefore, by the risk they represent to disease transmission. A targeted sampling and testing focused on identifying heavy fecal shedders can also be implemented using diagnostic tests on pooled environmental fecal samples and pooled individual fecal samples. These studies provide the elements to interpret the diagnostic tests results using individual, pooled individual and pooled environmental samples.

Future research should focus on the cost-effectiveness of a sampling and testing strategy using different diagnostic tests and different types of samples to identifying heavy fecal shedders under different herd sizes and the prevalence levels of heavy fecal shedders. A potential objective could be to evaluate the use of different test strategies using bacterial culture of feces, fecal PCR, and serum and milk ELISA on individual, individual pooled and pooled environmental samples to identify heavy shedding cows under different JD prevalences and herd size scenarios. In this future study, the effectiveness and cost-effectiveness of identifying *MAP* heavy fecal shedders using different testing strategies, diagnostic tests, types of samples, prevalence levels and herd sizes could be evaluated using a probabilistic (Monte-Carlo) model. The different testing strategies could be evaluated based on the overall sensitivity and specificity of the strategy to correctly identify true *MAP* heavy fecal shedders and non-heavy fecal shedders in the herd.

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