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THE EFFECT OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* ON THE CAUDAL FOLD TUBERCULIN (CFT) AND GAMMA INTERFERON (γ-IFN) TESTS FOR BOVINE TUBERCULOSIS

presented by

JOHN RICHARD DUNN

has been accepted towards fulfillment
of the requirements for the

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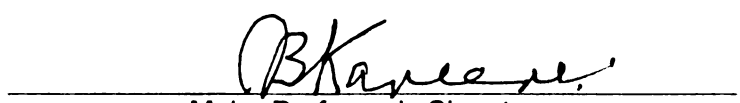

Major Professor's Signature

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Date

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By

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ABSTRACT

THE EFFECT OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* ON THE CAUDAL FOLD TUBERCULIN (CFT) AND GAMMA INTERFERON (γ-IFN) TESTS FOR BOVINE TUBERCULOSIS

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The studies described were performed to determine whether cattle testing positive for *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) were more likely to have false positive results on the caudal fold tuberculin (CFT) test and the γ-IFN assay for bovine tuberculosis (TB) than negative cattle. *M. paratuberculosis* fecal culture and antibody ELISA were performed for 1,043 Holstein cattle from 10 Michigan herds on the day the CFT test was read. Blood samples were also used to test for γ-IFN response following overnight stimulation with phosphate buffered saline (control) and purified protein derivative (PPD) from *M. bovis* and *M. avium*.

The total number of CFT suspects from all herds was 180 (17.3%), and 8 cattle (0.8%) were positive for γ-IFN stimulated by *M. bovis*. Cattle testing positive for *M. paratuberculosis*, as measured by a positive *M. paratuberculosis* fecal culture or antibody ELISA test, appeared to have an increased likelihood of false positive results on the CFT test, although the association was not statistically significant. Further studies involving a larger sample size need to be conducted to confirm these findings. No significant association was found between cattle testing positive by *M. paratuberculosis* fecal culture or antibody ELISA, and positive results of the γ-IFN assay for TB. Cattle positive for γ-IFN stimulated by *M. avium* were more likely to be CFT suspects than negative cattle, which may be related to early stages of Johne's disease.

In loving memory of my father,
Richard J. "Dick" Dunn
1930-2003

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KEY TO ABBREVIATIONS

CCT test	Comparative Cervical Tuberculin test
CFT test	Caudal Fold Tuberculin test
ELISA	Enzyme-Linked Immunosorbent Assay
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. paratuberculosis	*Mycobacterium avium* subsp. *paratuberculosis*
MDA	Michigan Department of Agriculture
OD	Optical Density
PPD	Purified Protein Derivative
TB	Bovine tuberculosis
Th1 cells	Type 1 Helper T lymphocyte
USDA	United States Department of Agriculture
γ-IFN	Gamma-interferon

INTRODUCTION

Purpose

Johne's disease and bovine tuberculosis (TB) are both important diseases in cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) and *Mycobacterium bovis*, respectively. The caudal fold tuberculin (CFT) test and gamma-interferon (γ-IFN) assay are screening tests for detection of TB in cattle. Since Johne's disease and TB are both caused by mycobacteria, it is speculated that cross-reactivity may occur on the CFT test and γ-IFN assay causing false positives in animals infected with Johne's disease. The effect of infection with *M. paratuberculosis* on the CFT test and γ-IFN assay is important for determining confidence of positive results among herds infected with Johne's disease.

Objectives

1. Compare CFT test results for TB between cattle testing positive and negative for *M. paratuberculosis*.
2. Compare γ-IFN assay results for TB between cattle testing positive and negative for *M. paratuberculosis*.
3. Evaluate the association between γ-IFN assay results stimulated by *M. avium* PPD with test results for *M. paratuberculosis*.
4. Compare CFT test results for TB between cattle with a positive response for γ-IFN stimulated by *M. avium* PPD and cattle with a negative response.

Hypotheses Tested

1. Cattle testing positive for *M. paratuberculosis* will have a higher likelihood of false positive results on the CFT test compared to cattle testing negative for *M. paratuberculosis*.
2. Cattle testing positive for *M. paratuberculosis* will have a higher likelihood of false positive results on the γ-IFN assay for TB compared to cattle testing negative for *M. paratuberculosis*.
3. Cattle testing positive for *M. paratuberculosis* will have a higher likelihood of positive results for γ-IFN stimulated by *M. avium* compared to cattle testing negative for *M. paratuberculosis*.
4. Cattle with a positive response of γ-IFN stimulated with *M. avium* will have a higher likelihood of false positive results on the CFT test compared to cattle with a negative response.

Overview

Chapter 1 is a literature review of Johne's disease and TB with primary emphasis on ante-mortem diagnostic tests for each disease. Chapter 2 details a cross-sectional study performed in Michigan evaluating the effect of infection with *M. paratuberculosis* on the results of the CFT test in cattle. Chapter 3 is from the same study performed in Michigan, but this chapter focuses on the effect of infection with *M. paratuberculosis* on the results of the γ-IFN assay for TB in cattle. Chapter 4 evaluates the γ-IFN assay, specifically the response from *M. avium* PPD stimulation and the association with *M. paratuberculosis* infection. The conclusion summarizes the findings as a whole and presents the relevance of these findings and potential recommendations.

CHAPTER 1

LITERATURE REVIEW

Johne's disease

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative agent for Johne's disease in cattle. Johne's disease is a chronic disease characterized by gradual weight loss despite a normal appetite. Decrease in production is common among infected cattle, as well as a decrease in reproductive performance. Cattle positive for *M. paratuberculosis* antibody ELISA have been to shown to have a 28-day increase in days open compared to negative cows (Johnson-Ifearulundu et al., 2000). Calves are the most susceptible to infection during their first year of life, but clinical signs generally do not develop until the animal is greater than two years old.

The terminal part of the ileum is the most frequent site of lesions associated with Johne's disease. Once ingested, *M. paratuberculosis* is taken up in the ileum by microfold cells (M cells), which are specialized epithelial cells associated with Peyer's patches and lymphoid follicles. M cells are efficient antigen-presenting cells that present the bacteria directly to lymphocytes within the Peyer's patch. The immune system responds by recruiting macrophages and developing giant cells (Buergelt et al., 1978). The intestinal wall develops a corrugated appearance from increasingly thickened villi as the organism continues to multiply, and gradually the intestinal wall loses its ability to absorb nutrients. Eventually the intestinal wall begins to leak protein from blood into the intestine and further lowers the animals absorbed nutrients, despite having a normal appetite. The malabsorption and protein-losing enteropathy result in the hypoproteinemia, which in advanced disease commonly results in cachexia and

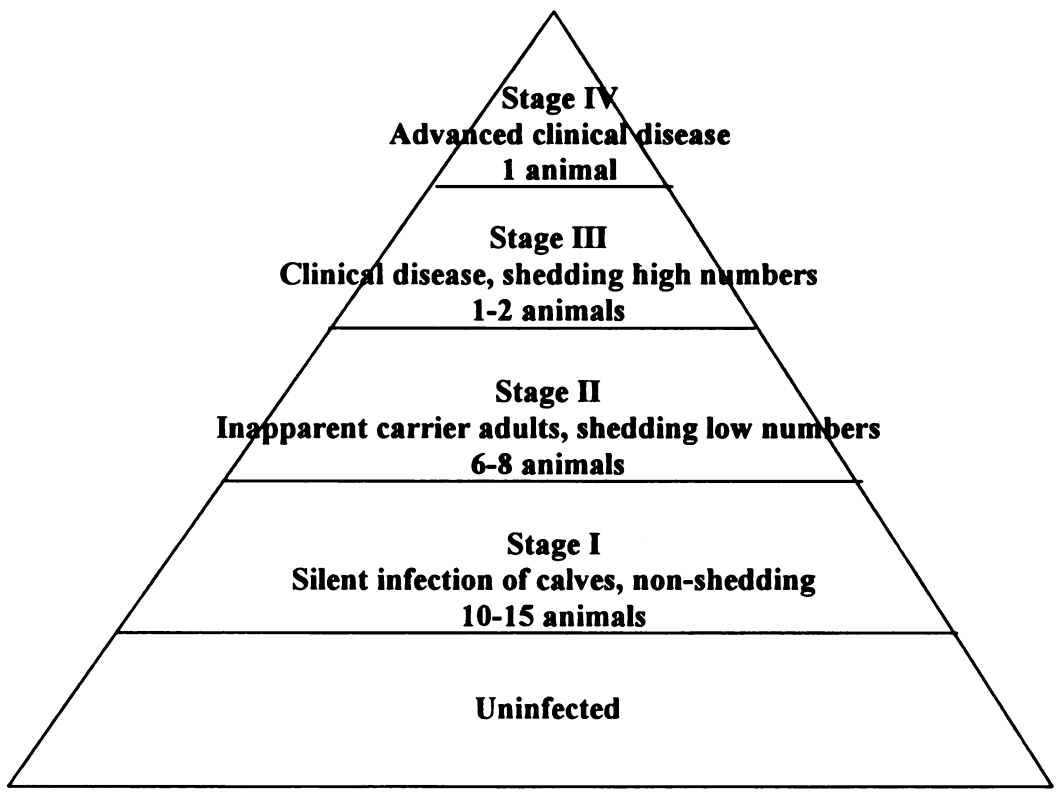
pipestream diarrhea. Intermandibular edema also develops in rare cases. In addition to the intestine, lesions have been observed in the liver, spleen, lungs, kidneys, uterus, placenta, and nonmesenteric lymph nodes.

The primary mode of transmission for *M. paratuberculosis* is fecal-oral, most typically from manure or fecal contaminated feed or water (Sweeney, 1996). Neonates may ingest contaminated manure by suckling from a manure-contaminated teat or by other manure-contaminated sources in their birth environment. Contaminated colostrum or milk from the infected dam or originating from other infected cattle are other potential sources of ingestion (Streeter et al., 1995). In utero infection may also occur in 25% of calves born to cows with clinical signs, and 18% from asymptomatic infected cows (Sweeney et al., 1992). Difficulty in detecting subclinical Johne's disease leads to the propagation of the disease within a herd, despite common disease preventative procedures.

Johne's disease is commonly divided into four stages of infection (Figure 1-1). Stage I is described as the silent infection stage (Whitlock and Buergelt, 1996). This stage primarily involves young calves, as calves less than 30 days of age are most susceptible with the majority of cattle becoming infected before four months of age (Hagan, 1938). This first stage generally lasts greater than two years and may surpass ten years. Laboratory tests rarely detect cattle in stage I of infection. Stage II is referred to as the inapparent carrier adult stage. Cattle with subclinical disease can have an increased γ-IFN response by sensitized T cells or an increased antibody response to *M. paratuberculosis* (Stabel, 1996). Infected animals may also potentially be positive on fecal culture in Stage II. Animals in Stage II do not have diarrhea or weight loss but are

now shedding organisms in their manure and infecting other animals on the farm. Stage III is defined as clinical disease. Animals in this stage have a normal appetite and vital signs but are beginning chronic diarrhea and gradual weight loss. Infected animals may test positive for *M. paratuberculosis* on fecal culture or antibody ELISA and agar gel immunodiffusion (AGID). The final progression of infection, Stage IV, is advanced clinical disease. Infected animals are normally lethargic, emaciated, and have pipestream diarrhea and intermandibular edema. The animal's condition typically deteriorates within one or two days. Death can result from extreme dehydration and cachexia.

Figure 1-1: Johne's disease progression based on clinical presentation and shedding of *M. paratuberculosis*, including an approximation of the number of cattle in stages I-III based on one animal in stage IV (Whitlock and Buergelt, 1996).



Early stages of *M. paratuberculosis* infection are generally characterized by a cell-mediated immune response followed by a predominately humoral response in later stages of the disease (Bendixen, 1978; Stabel, 2000). In the final stages of infection, anergy may develop in which there will be no immune response of any type to specific antigens (Stabel, 1996). The easiest stage in which to diagnose infection with *M. paratuberculosis* is during the clinical phase when overt clinical signs exist and antibodies can be detected using a variety of methods. The greatest challenge exists with detecting *M. paratuberculosis* during the subclinical stage, before a humoral response has begun.

Diagnostic tests for Johne's disease

Ante-mortem diagnostic tests for Johne's include those for direct bacteria detection, serological tests for the detection of antibody, and assays for cellular immunity. Methods for direct bacteria detection consist of standard fecal culture, radiometric fecal culture (BACTEC), and DNA probe (PCR). Direct detection of the bacteria is the definitive method for detecting *M. paratuberculosis* (specificity is close to 100%). Unfortunately, *M. paratuberculosis* bacteria are shed intermittently with shedding increasing as the animal progresses towards the clinical phase of disease. In addition, the standard fecal culture takes 12-16 weeks, costs more than the rapidly run ELISA, and has a relatively low sensitivity (see Table 1-1). Radiometric fecal culture is faster than standard culture, however the special technique is expensive, requires special equipment, and involves the handling of radioisotopes. The DNA probe is the fastest of

the three methods for directly detecting bacteria, however the test is considered less sensitive and more expensive compared to fecal culture.

Table 1-1: Sensitivity and specificity for standard *M. paratuberculosis* fecal culture

Author	Sample size	Sensitivity
Sockett et al., 1992	182 infected*	45.1%
	111 fecal shedders†	73.8%
Zimmer et al., 1999	75 clinically affected‡	84.0%
	57 subclinically infected§	87.7%
Collins et al., 1990	75 positive¶	60%

*Isolation of *M. paratuberculosis* from any fecal or tissue sample
† Positive on either radiometric fecal culture, conventional fecal culture, or commercial PCR/DNA probe
‡Cattle showing typical signs in herds known to be affected by Johne's disease
§Cattle positive on either Ziehl-Neelsen staining, fecal culture, or DNA-Probe test
¶ Positive on either radiometric fecal culture or conventional fecal culture

There are three serological tests used to detect antibodies for *M. paratuberculosis*. Enzyme-linked immunosorbent assay (ELISA) is the most sensitive, followed by agar-gel immunodiffusion (AGID), and complement fixation (CF). The sensitivity of these tests, however, has several problems related to the course of *M. paratuberculosis* infections. One limitation is the relative lateness of the humoral immune response during the course of Johne's disease infection (Kreeger, 1991). The introduction of absorbed ELISA's improved the second limitation, which was cross-reactivity with other environmental bacterial species that can potentially reduce specificity of serological tests (Ridge et al., 1991). The antibody ELISA test is fast and low-cost, however, reported sensitivities are even lower than those reported for *M. paratuberculosis* fecal culture (see Table 1-2).

Table 1-2: Sensitivity and specificity for Parachek* *M. paratuberculosis* antibody ELISA

Author	Sample size	Sensitivity	Specificity
Reichel et al., 1999	106 positive, 341 negative	31.1%	97.9%
Sockett et al., 1992	177 positive, 196 negative	43.4%	99.0%
Collins et al., 1991	150 positive, 196 negative	47.3%	99.0%
Ridge et al., 1991	150 positive, 1,000 negative	47.3%	99.8%
Ellis et al., 1998	1000 negative		99.7%

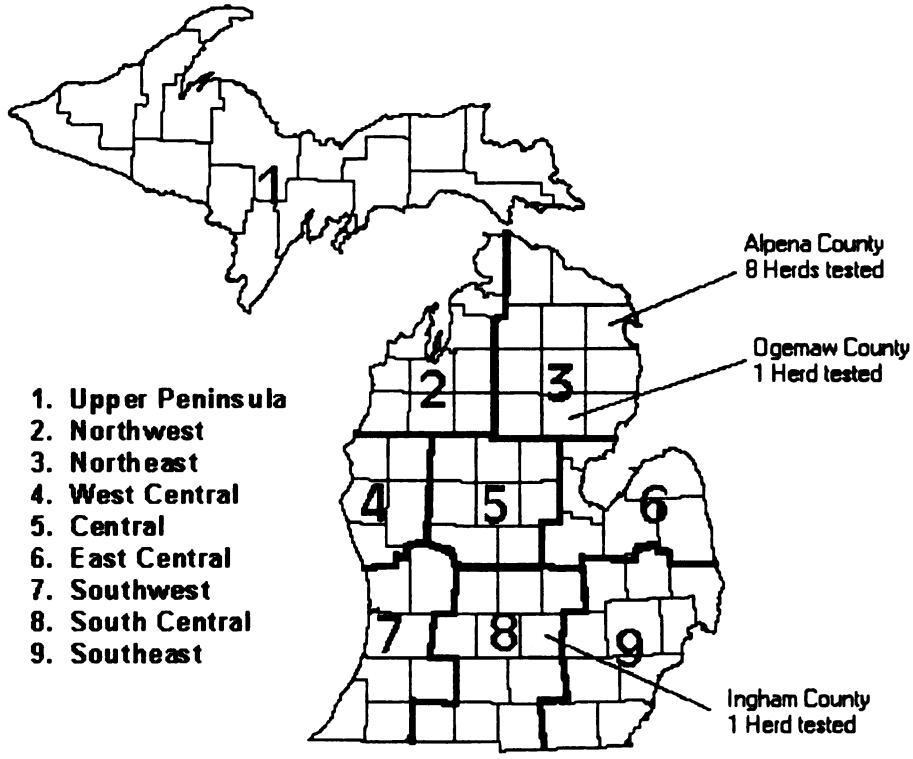
*Parachek™, BioCor Animal Health, Omaha NE

The third category of tests used to detect *M. paratuberculosis* infection includes those that measure a cell-mediated immune response. These tests include the Johnin skin test and γ-IFN assay. The γ-IFN assay is a measurement of γ-IFN released by sensitized T lymphocytes stimulated by johnin PPD. The γ-IFN assay has a high number of false positives with specificity in a recent study ranging from 66.1-93.6% depending on the algorithm used for the calculation (Kalis et al., 2003).

Prevalence of Johne's disease

M. paratuberculosis is prevalent in the United States with estimates of individual prevalence ranging from 1.6-20% in different parts of the country (Braun et al., 1990; Whipple et al., 1991; Thoen and Baum, 1988; Kreeger, 1991). A recent study in Michigan found that 55% of the state's herds had ≥2 cows that were positive for *M. paratuberculosis* with a total prevalence of 6.9%, using antibody ELISA (Johnson-Ifearulundu and Kaneene, 1999). The highest prevalence of positive herds coincided with the eastern portion of the Lower Peninsula in agricultural district 6 (94.1%), district 9 (87.5%), and district 3 (80%) (see Figure 1-2).

Figure 1-2: Agricultural districts of Michigan showing the counties with numbers of herds used for our study



Bovine tuberculosis

Tuberculosis in cattle, most commonly caused by *Mycobacterium bovis*, is the other most recognized mycobacterial disease in cattle. Bovine tuberculosis (TB) is an important health risk due to the potential spread of the disease to other livestock and humans. Infected cattle often do not show clinical signs, however, some cattle have chronic weight loss, anorexia, weakness, lethargy, low-grade fever, or a soft, moist, chronic cough. Inhalation is the primary mode of natural transmission for *M. bovis* in 80-90% of cattle (Morris et al., 1994). Ingestion is considered secondary to inhalation as a mode of transmission, which can occur directly from infected cattle or from contaminated pastures, water, or fomites (Menzies and Neill, 2000).

As a result of inhalation exposure, primary lesions for TB are typically found in the lungs. The majority of lung lesions are found in the caudal lobes of the left or right lungs, and are capable of being single, multiple, unilateral or bilateral (McIlroy et al., 1986). The initial lesion is normally comprised of the invading bacilli surrounded by a cluster of neutrophils. Macrophages soon replace the neutrophils and ingest the mycobacteria. During the cell-mediated immune response, sensitized T lymphocytes produce cytokines that activate the macrophages, increasing their ability to inhibit the growth of the phagocytosed bacteria or helping to kill them effectively. The activated macrophages can also change in shape or fuse together to form multinucleated giant cells. As more macrophages are recruited to the site of antigen insult, classic granulomas form which also contain multinucleated giant cells, epithelioid macrophages/histiocytes, lymphocytes, fibroblasts, and necrosis. Tuberculous granulomas are generally caseous, meaning that the cells and tissue in the center of the lesion are destroyed during the process caseous necrosis. Collagenous connective tissue eventually begins to surround the lesion, which may increase in size large enough to penetrate blood or airway vessels and allow dissemination to other organs. The most frequent gross and histological lesions have been found in the thoracic lymph nodes of cattle (Whipple et al., 1996). Only about 1% of infected cattle usually have lesions in the udder, and rarely infect other cattle by milk (Collins et al., 1987).

Screening tests for bovine tuberculosis

The type IV delayed hypersensitivity reaction responsible for the formation of granulomas is the basis for ante-mortem screening tests used for TB. Type IV

hypersensitivity is a reaction involving sensitized T lymphocytes that react with cell bound antigen causing the release of cytokines. These cytokines cause mononuclear cell accumulation, tissue damage, and inflammation usually observed at least 24 hours after exposure to the antigen. Following initial infection of mycobacteria in an animal, the bacteria are phagocytosed by macrophages and presented to cells involved in innate and acquired immune responses. Antigen-specific $CD4^+$, $CD8^+$, and γ/δ T cells become activated after exposure to the bacteria (Flynn and Chan, 2001; Hope et al., 2000; Liebana et al., 1999; Mustafa et al., 1986; Pollock et al., 1996). These sensitized T cells are recruited to the site of subsequent infections and produce cytokines such as γ-IFN and tumor necrosis factor alpha (Feng et al., 1999). The release of γ-IFN increases the ability of macrophages to kill the bacilli that they have phagocytosed (Flesch and Kaufmann, 1991). In addition to releasing γ-IFN, $CD4^+$, $CD8^+$, and γ/δ T cells have also been shown to be directly involved in cytotoxic activity (Tan et al., 1997; Skinner et al., 2003).

Purified protein derivative (PPD) tuberculin is a crude preparation of mycobacterial antigens used for the detection of TB in cattle. Bovine PPD tuberculin is produced from *M. bovis* (AN5 or Vallee strains), and each batch must be tested in animals to be compared to a reference standard (Monaghan et al., 1994). A recent study evaluating the genome of *M. bovis* AN5, for possible gene deletions during in vitro passage, determined that the PPD strains do not possess any dramatic differences between other strains of *M. bovis* and thus are suitable for detection of *M. bovis* infection (Inwald et al., 2003). When PPD tuberculin is injected intradermally, dendritic cells take up some of the antigen and migrate to the regional lymph node. In a previously sensitized animal, the PPD will attract sensitized T cells to the injection site, in addition

to neutrophils and macrophages. Circulating Th1 cells, such as $CD4^+$, $CD8^+$, and γ/δ T cells are activated by the antigen and secrete γ-IFN and other cytokines within a few hours of being stimulated by the antigen. These cytokines activate macrophages to increase their ability for cell killing, and also lead to the palpable swelling at the site of PPD injection due to inflammatory edema, fibrin deposition, and local thrombosis.

Intradermal tuberculin skin tests are the most widely used screening tests for TB in cattle. The two most common of these skin tests in cattle in the U.S. are the caudal fold tuberculin (CFT) test and the comparative cervical tuberculin (CCT) test. The CFT test is administered by injecting bovine PPD into the caudal fold at the base of the tail, whereas the CCT test compares the response from separate injections of bovine PPD and avian PPD in the cervical region. Diagnostic tests using PPD, however, are limited because most of the proteins in PPD are shared between different mycobacteria species (Aagaard et al, 2003). The reported sensitivity and specificity of the CFT test has varied with past studies, but the test is frequently used because it is simplest to perform and relatively inexpensive (see Table 1-3). A high sensitivity is desired to detect asymptomatic disease within the herd, whereas a high specificity minimizes the number of false positives. Sensitivity and specificity are important for determining the presence of disease in a herd, but predictive value is generally more useful at the level of the individual animal. Predictive value is an estimate of the likelihood that a test result is true. Positive predictive value estimates the likelihood that an animal testing positive is truly positive, a measure of the specificity in the context of disease prevalence. The predictive value is affected by prevalence, such that the lower the prevalence of disease in a population, the lower the positive predictive value. The CFT test has a low positive

predictive value due to a low prevalence of TB in the cattle population, resulting in the disadvantage of a higher likelihood of false positives for the test.

False positives on the CFT test have been attributed to the subjectivity of reading the CFT test, as well as exposure of cattle to *M. paratuberculosis*, *M. avium*, *M. tuberculosis*, environmental *Mycobacteria spp.*, or certain non-mycobacteria agents such as *Nocardia spp* (Karlson, 1962). A study in 1981 showed that cattle infected with *M. avium* complex serotypes 6, 14, and 18 produced positive reactions to the CFT test (Ketterer et al., 1981). The reclassification of *M. paratuberculosis* as a subspecies of *M. avium* would suggest the animal might respond similarly to *M. avium* on the CFT and CCT tests (McIntyre and Stanford, 1986).

Table 1-3: Sensitivity and specificity for the CFT test

Author	Sample size	Sensitivity	Specificity
Whipple et al., 1995	204 positive	80.4%	
Wood et al., 1991	125 positive; 6177 negative	65.6%	99.5%
Wood et al., 1992	22 positive; 1340 negative	68.2%	97.3%
Francis et al., 1978	135 positive; 3820 negative	72.0%	98.8%

The γ-IFN assay is also approved as a screening test for TB in cattle. This *in vitro* test measures γ-IFN that is released by lymphocytes during cell-mediated immune response to antigen stimulation. The assay compares the animal's γ-IFN response to bovine PPD and avian PPD. The γ-IFN assay has the benefits that animals do not have to be contained for 72 hours to wait and read the test, subjective differences in interpreting the results between veterinarians is eliminated, and the cattle don't have to be injected with PPD. Sensitivity and specificity estimates of the test have varied (see Table 1-4).

Table 1-4: Sensitivity and specificity for Bovigam* γ-IFN assay

Author	Sample size	Sensitivity	Specificity
Ryan et al., 2000	163 positive, 213 negative	85%	93%
Scacchia et al., 2000	36 positive, 70 negative	91.7%	84.3%
Lauzi et al., 2000	1557 negative		88.8%
Wood et al., 1992	22 positive, 1340 negative	81.8%	99.1%

*Bovigam™, BioCor Animal Health, Omaha NE

Prevalence of TB

M. bovis is currently endemic in free-ranging white-tailed deer (*Odocoileus virginianus*) in northeastern Michigan, and since 1998 it has been diagnosed within the same region in 25 beef cattle herds, 5 dairy cattle herds (Schmitt et al., 2002; Michigan Bovine TB Web site, 2003), and in one captive cervid farm (Kaneene et al., 2002). *M. bovis* has also been detected in a cat in the same endemic area (Kaneene et al., 2003), as well as other free-ranging carnivores including coyotes, raccoons, a red fox, black bear, and bobcat (Bruning-Fann et al., 2001). Since 2001, TB has also reemerged in areas of Texas, California, and New Mexico (TAHC, 2002; CDFA, 2002; APHIS, 2003). In deer, the disease is efficiently transmitted through nasal secretions, saliva, or contaminated feed (Palmer et al., 2001). Infected deer are considered to be a reservoir for the spread of TB to cattle in Michigan, with increased spread among deer likely as a result of unnaturally dense congregations around contaminated feed from supplemental feeding and baiting (Kaneene et al., 2002; Miller et al., 2003; Payeur et al., 2002).

Conclusion

Johne's disease and TB are important diseases in cattle, both caused by mycobacteria species. Limitations exist for ante-mortem tests used for each disease, especially with regards to sensitivity and specificity. Cross-reactivity between varying mycobacterial antigens is one of the limitations for ante-mortem testing and has led to speculation that *M. paratuberculosis* infection may significantly influence an animal's reaction to the CFT test (Morrison et al., 2000). Due to a high prevalence of *M. paratuberculosis* among cattle in Michigan and the United States, the studies described in the following chapters were designed to determine whether cattle testing positive for *M. paratuberculosis* are more likely to have false positive results on the CFT test and γ-IFN assay than cattle testing negative for *M. paratuberculosis* in Michigan. Evaluating the effect of *M. paratuberculosis* on the CFT test and γ-IFN assay under field and laboratory conditions is an important step for determining the amount of confidence given to positive results among herds infected with Johne's disease.

CHAPTER 2

THE EFFECT OF INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* ON THE CAUDAL FOLD TUBERCULIN (CFT) TEST FOR BOVINE TUBERCULOSIS IN CATTLE.

Abstract

Objective – To determine whether cattle testing positive for *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) were more likely to have false positive results on the caudal fold tuberculin (CFT) test than cattle testing negative.

Animals – 1,043 Holstein cattle from 10 herds in Michigan

Procedure –Fecal and whole blood samples were collected from all cattle ≥24 months of age on the day the CFT test was read. Fecal samples were submitted for mycobacterial culture, and samples of plasma were tested for antibody against *M. paratuberculosis*.

Results – The total number of CFT suspects from all herds was 180 (17.3%). Of the 1043 cattle tested in this study, 45 (4.3%) were positive for *M. paratuberculosis* by fecal culture and 115 (11.0%) were positive by antibody ELISA. Percentage of cattle positive for the CFT test ranged from 16.6%, in cattle negative for *M. paratuberculosis* fecal culture and antibody ELISA, to 25.0% in cattle positive for both tests. No variables were significantly associated with a positive CFT test.

Conclusions –Cattle testing positive for *M. paratuberculosis*, as measured by a positive *M. paratuberculosis* fecal culture or antibody ELISA test, appear to have an increased likelihood of false positive results on the CFT test, although the association is not statistically significant.

Introduction

M. avium subsp. *paratuberculosis* (*M. paratuberculosis)* has been speculated to influence an animal's reaction to the CFT test due to cross reactivity between varying mycobacterial antigens (Morrison et al., 2000). This study was designed to determine whether cattle testing positive for *M. paratuberculosis* are more likely to have false positive results on the CFT test when compared to cattle testing negative for *M. paratuberculosis* in Michigan. Evaluating the effect of *M. paratuberculosis* on the CFT test under field conditions is an important step for determining the amount of confidence given to positive results among herds infected with Johne's disease.

Methods

Animals

Samples of feces and whole blood were collected from 1043 Holstein cattle from 10 separate herds from three counties in Michigan. Eight of the herds sampled were within a TB high-risk area (Alpena county), one in a TB-free area (Ingham county), and one near the high-risk area (Ogemaw county) (Figure 1-1). Criteria for inclusion were a willingness to participate, having a total number of cattle less than 250 (for cost reasons), and being able to sample the herd on the day their required whole-herd TB tests were being read. All cattle greater than or equal to 24 months of age were sampled from each herd. None of the herds had been previously vaccinated for *M. paratuberculosis* or *M. bovis*.

Tuberculosis skin testing

The CFT tests were performed by either accredited private practice veterinarians or by veterinarians employed by the Michigan Department of Agriculture (MDA) using the methods described in the USDA Uniform Methods and Rules (USDA, 1999). The same MDA veterinarian tested seven of the 10 herds. All comparative cervical tuberculin (CCT) tests were performed by USDA or MDA veterinarians. Results of both skin tests were interpreted 72 ± 6 hours after injection of tuberculin.

Sampling

Blood and fecal samples were collected on the day the CFT test was read. A new plastic sleeve was used for each animal to collect fecal samples, which were placed in separate plastic whirl-pack bags after collection. Fecal samples were transported in coolers at ambient temperature to the Diagnostic Center for Population and Animal Health at Michigan State University and stored at -80°C until cultured. Blood was collected on the day the CFT test was read via the middle coccygeal vein using a 20-gauge, 1-in. needle. The blood was collected into a 10 ml Vacutainer tube containing sodium heparin (Corning Glass Works, Corning, NY). Blood samples were transported to the lab in plastic coolers, chilled with ice packs, and were processed within 24 hours of when the samples were drawn.

Laboratory methods – A detailed description of laboratory methods is located in the appendix.

M. paratuberculosis testing

M. paratuberculosis laboratory diagnostic tests consisted of fecal culture and testing for antibodies using a plasma ELISA. Fecal samples were cultured to detect the presence of *M. paratuberculosis* using standard procedures recommended by USDA-National Veterinary Service Laboratory and based on the procedures used by Whitlock et al. at the University of Pennsylvania (Fyock and Whitlock, 1999).

Plasma samples were tested for antibodies to *M. paratuberculosis* with a commercial antibody ELISA test kit.[1] The samples were tested in duplicate wells and the average optical density (OD) was calculated. The corrected OD was calculated by subtracting the average OD of two negative serum control wells from the average OD of duplicate sample wells. A corrected OD greater than 0.1 was considered positive.

Statistical methods

Prevalence of Johne's disease within each herd was computed for each type of Johne's test (fecal culture or antibody ELISA) by the number of positive cattle for each test divided by the total number of cattle tested within each herd.

Statistical analyses were conducted using a standard software package[2], testing associations between CFT test results and CFT testing veterinarian, animal age, and Johne's disease status. In the analyses, multivariable logistic regression models with random effects were developed to assess associations between animal CFT status and animal factors and Johne's disease status. Because the analysis was done on an individual animal level, the random effect function was used in all the models to adjust

[1] Parachek™, BioCor Animal Health, Omaha NE
[2] SAS V8, SAS Institue, Cary, NC

for the fact that animals from the same herd are more alike in terms of the exposure than animals in other herds. Model outcome was CFT test status (positive or negative), and risk factors including testing veterinarian, animal age, results of individual Johne's tests, and herd prevalence of Johne's disease tests (Table 2-1).

A backwards stepwise model development approach was used to create final models with risk factors significant at $p \leq 0.05$. In brief, a full model was generated, and possible interactions and confounding were assessed and corrected during the model development process. Odds ratios (OR) with 95% confidence intervals were computed for parameter estimates. With the exception of the potential confounders forced into the model, each risk factor was tested by examining the effects of removal of that factor from the model. If removal of the risk factor resulted in a change in the OR of the remaining variables of more than 10%, the risk factor and its interaction terms were retained in the model.

Results

Johne's disease results

Of the 1043 cattle tested in this study, 45 (4.3%) were fecal culture positive and 115 (11.0%) were positive by antibody ELISA (Table 2-2). The herd prevalence for each test ranged from 0 to 15.4% (mean 4.5%, median 2.8%) for the *M. paratuberculosis* fecal culture, and 1.2 to 30.8% (mean 9.7%, median 6.9%) for the *M. paratuberculosis* antibody ELISA.

TB results

All cattle that responded to the CFT test (any visible or palpable response) were classified as suspect and retested with the CCT test. Of the 1043 total cattle, 180 were CFT suspects (17.3%). Herd response ranged from 8.0% to 56.1% (mean 21.2%, median 20.1%) for the CFT test (Figure 2-1). Five of the 180 CFT suspects were also CCT suspects and a sixth animal was a CCT reactor. The CCT suspects and the CCT reactor were classified as negative for TB based on necropsy, histological examination of tissues and mycobacterial culture. Johne's disease test results were separated displayed, comparing those animals positive versus those negative to the CFT test (Figure 2-2). CFT test results were also displayed based on Johne's disease test results (Figure 2-3). The percentage of cattle positive for the CFT test ranged from 16.6%, in cattle negative for *M. paratuberculosis* fecal culture and antibody ELISA, to 25.0% in cattle positive for both tests (Figure 2-3).

Statistical results

In the multivariable regression model for the CFT test, the individual animal and herd level test results of *M. paratuberculosis* fecal culture and antibody ELISA did not significantly affect the CFT test results (Table 2-3). The veterinarian performing the CFT test and age of the animal also lacked a significant association with a positive CFT test. *M. paratuberculosis* fecal culture and antibody ELISA test results were poorly correlated with a spearman correlation coefficient of 0.1663 ($p<0.0001$).

Discussion

Cross-reactivity between different mycobacterial antigens have led to the suggestion that infection with *M. paratuberculosis* may lead to false positive responses to the CFT test (Morrison et al., 2000). It is important to determine the cause of false positives responses as they may lead to unnecessary culling, increased time and cost of animal handling, increased cost of follow-up testing, as well as psychological stress to producers and veterinarians.

False positive responses to the CFT test have been reported in a study following vaccination of calves against *M. paratuberculosis* (Köhler et al., 2001). Four calves were vaccinated at 28 days of age with a commercially available modified-live *M. paratuberculosis* vaccine, resulting in positive or suspicious skin reactions on the CFT test for as long as 2 years after vaccination (Köhler et al., 2001). Vaccination in their study was shown to create a cell-mediated immune reaction detectable by tuberculin skin tests over a long period of time. Our study included adult cattle that were not vaccinated for *M. paratuberculosis*, but rather attempted to evaluate the relationship between natural *M. paratuberculosis* infection and the CFT test.

When comparing cattle that were positive to the CFT test with cattle negative to the CFT test, a higher percentage of CFT positive cattle were positive for *M. paratuberculosis* fecal culture, antibody ELISA, and a combination of the two tests (Figure 2-2). Similarly, cattle testing positive for *M. paratuberculosis* fecal culture, antibody ELISA, and a combination of the two tests had a higher percentage of cattle testing positive for the CFT test compared to cattle negative by fecal culture and ELISA. Cattle positive for *M. paratuberculosis* were estimated to be four times as likely to be

positive on the CFT test than negative cattle, although the estimation was not statistically significant (Table 2-3).

Despite the minimal trend of a higher likelihood of a positive CFT test among cattle testing positive for *M. paratuberculosis*, the most surprising finding of our study was the lack of a statistically significant association. There was no significant association at either the individual animal or the herd level between test status for Johne's disease and risk of CFT response. The lack of significant association between positive Johne's disease tests and the CFT test could have the following possible explanations. First, both tests used to detect *M. paratuberculosis* in this study have relatively poor sensitivities (Table 1-1; Table 1-2). A low sensitivity would result in a high number of false negatives, resulting in an underestimation of the true number of cattle infected with *M. paratuberculosis*. This misclassification would reduce the power to detect a true positive association between cattle testing positive for *M. paratuberculosis* and a positive CFT test result. Second, the sample size may have been too small to detect a true association. The sample size used for this study was based on the ability to detect a 25% difference in the CFT test results between cattle testing positive and negative for *M. paratuberculosis*. In this study, the difference in CFT test results were only 7% between cattle testing positive and negative by *M. paratuberculosis* fecal culture, and 4% between cattle testing positive and negative by ELISA. With 80% power (the probability of detecting a true association), the true sample size needed to detect a significant association between *M. paratuberculosis* tests and the CFT test would have to be 6,304 based on culture and 7,736 based on ELISA, compared to the total sample size of 1,043 used for this study (Table 2-4). Third, there was a possible lack of the true prevalence of Johne's disease

between herds. All ten herds in this study had a Johne's disease prevalence of 15% or less, as measured by *M. paratuberculosis* fecal culture. There were no high prevalence herds that would have increased the number of total positive animals, helping to increase the test positive sample size, as well as increasing the power of detecting an association between herd level *M. paratuberculosis* results and positive CFT test results.

The results of this study show a minimal trend between positive *M. paratuberculosis* test results and a positive CFT test result for individual animals. A combination of factors may have led to the lack of statistical significance of this association. The most likely combination is a misclassification of true positives as Johne's disease test negative due to the low sensitivity of the current tests. This misclassification may have led to a difference of the percent of CFT positive cattle between cattle testing positive and negative for *M. paratuberculosis* that was smaller than the true difference. The smaller difference decreased the statistical power, which would make a larger sample size necessary to detect a significant association.

The CFT test is prone to false positives from cross-reactivity due to the fact that many of the proteins in PPD are shared between different mycobacteria species. Current research is underway to identify proteins that are specific to *M. bovis*, able to be used in a whole-blood assay. A recently published study concluded that a mix of the proteins ESAT-6, CFP-10, TB27.4, and TB10.4 may have a sensitivity as good as the CFT test with an improved specificity (Aagaard et al., 2003). It is important that further research continue with either larger sample sizes or more specific diagnostic tests in order to determine whether an association truly exists between cattle testing positive for *M. paratuberculosis* and positive CFT test results.

Table 2-1: Description and descriptive statistics for risk factors evaluated

Outcome	Values	Description	n	%*
CFT Test Result	1	Positive	180	17
	0	Negative	863	83
Risk Factor	**Values**	**Description**	**n**	**%**
CFT Testing Veterinarian	1	Veterinarian 1 (CFT tested 1 herd)	195	19
	2	Veterinarian 2 (CFT tested 1 herd)	94	9
	3	Veterinarian 3 (CFT tested 7 herds)	647	62
	4	Veterinarian 4 (CFT tested 1 herd)	107	10
M. paratuberculosis ELISA	1	Positive	115	11
	0	Negative	928	89
M. paratuberculosis Culture	1	Positive	45	4
	0	Negative	998	96
M. paratuberculosis ELISA and Culture	1	Positive for both	16	2
	0	Negative for one or both	1027	98
		Continuous variables		
Age (months)	24-144	Age of animal in months	1043	100
M. paratuberculosis Culture Herd Prevalence	0-15.4	% cattle positive for *M. paratuberculosis* fecal culture for each herd	1043	100
M. paratuberculosis ELISA Herd Prevalence	1.2-30.8	% cattle positive for *M. paratuberculosis* antibody ELISA for each herd	1043	100

*Percentage of cattle described by the respective value (= n/1043)

Table 2-2: Descriptive analysis of CFT test results based on positive Johne's disease tests

Johne's disease test (positive results)	All Cattle (n=1043)		CFT suspect (n=180)		CFT negative (n=863)	
	#	%	#	%	#	%
Fecal Culture	45	4.3	11	6.1	34	3.9
Antibody ELISA	115	11.0	24	13.3	91	10.5
Fecal Culture or Antibody ELISA	144	13.8	31	17.2	113	13.1
Fecal Culture and Antibody ELISA	16	1.5	4	2.2	12	1.4
Negative to both Johne's disease tests	899	86.2	149	82.8	750	86.9

Table 2-3: Results of a multivariable logistic regression analysis of the effect of individual and herd level *M. paratuberculosis* test results on the CFT test as the outcome (classified as suspect or negative).

		Full Model				
Risk Factor		**Estimate**	**S.E.**	**X^2 p**	**OR**	**95% C.I.**
Testing vet:	1	.34	1.61	0.83	1.41	.06 – 33.1
	2	2.37	3.33	0.48	10.67	.02 – 7288
	3	3.92	3.38	0.25	50.26	.07 – 3.8E4
	4	0	-	-	-	-
Age (months)		.01	.01	0.10	1.01	.998 – 1.02
Day 3 Para Result		-.37	.89	0.67	0.69	.12 – 3.93
Culture		1.46	1.01	0.15	4.29	.59 – 31.0
CultureANDELISA		.01	.84	0.99	1.01	.20 – 5.22
CultureHerdPrev		-1.23	7.04	0.86	0.29	3.0E-7 – 2.9E5
ParaHerdPrev		14.39	12.2	0.24	1.8E6	7.3E-5 – 4.3E16
Culture*Day3ParaResult		0	-	-	1	-
Agem*Day3ParaResult		.01	.02	0.41	1.01	.98-1.04
Agem*Culture		-.02	.02	0.29	0.98	.95-1.02
		Model –2 Res Log Likelihood = 5139.0				
		Reduced Model				
Risk Factor		**Estimate**	**S.E.**	**X^2 p**	**OR**	**95% C.I.**
Age (months)		0.01	.004	0.06	1.01	.999-1.02
		Model –2 Res Log Likelihood = 5088.9				

Table 2-4: Sample size required to detect a true association based on the actual percentage of CFT positive cattle and *M. paratuberculosis* test results, and power calculations based on the actual sample size

CFT results vs. ELISA	Confidence	Power	Ratio of ELISA negative: ELISA positive	% CFT positive of ELISA negative cattle	% CFT positive of ELISA positive cattle	Minimum sample size of ELISA negative cattle	Minimum sample size of ELISA positive cattle	Total required sample size
Sample size required to detect true association	95%	80%	928:115	17%	21%	6,883	853	**7,736**
Power based on actual sample size	95%	**17%**	928:115	17%	21%	928	115	1,043
CFT results vs. Culture	Confidence	Power	Ratio of Culture negative: Culture positive	% CFT positive of Culture negative cattle	% CFT positive of Culture positive cattle	Minimum sample size of Culture negative cattle	Minimum sample size of Culture positive cattle	Total required sample size
Sample size required to detect true association	95%	80%	998:45	17%	24%	6,032	272	**6,304**
Power based on actual sample size	95%	**20%**	998:45	17%	24%	998	45	1,043

Figure 2-1: Graph of the percentage of positive test results by herd

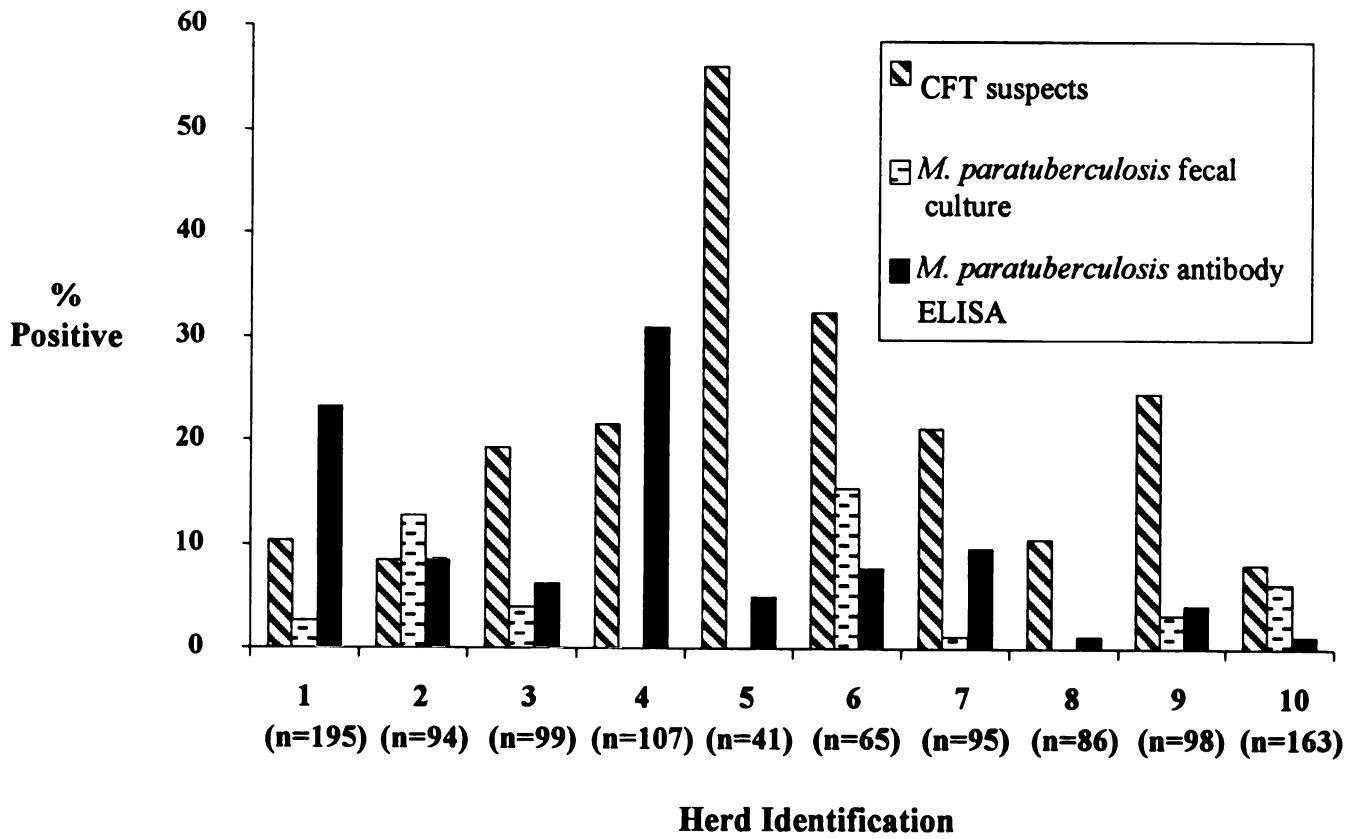


Figure 2-2: Percentage of cattle positive for *M. paratuberculosis* fecal culture and antibody ELISA tests separated by CFT status

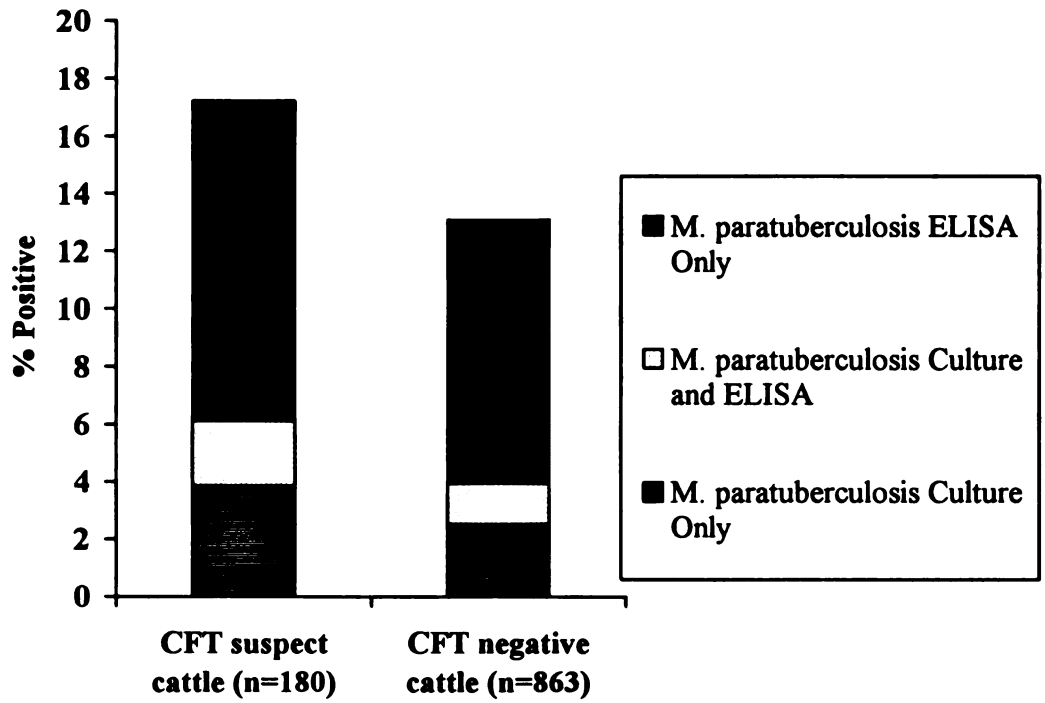
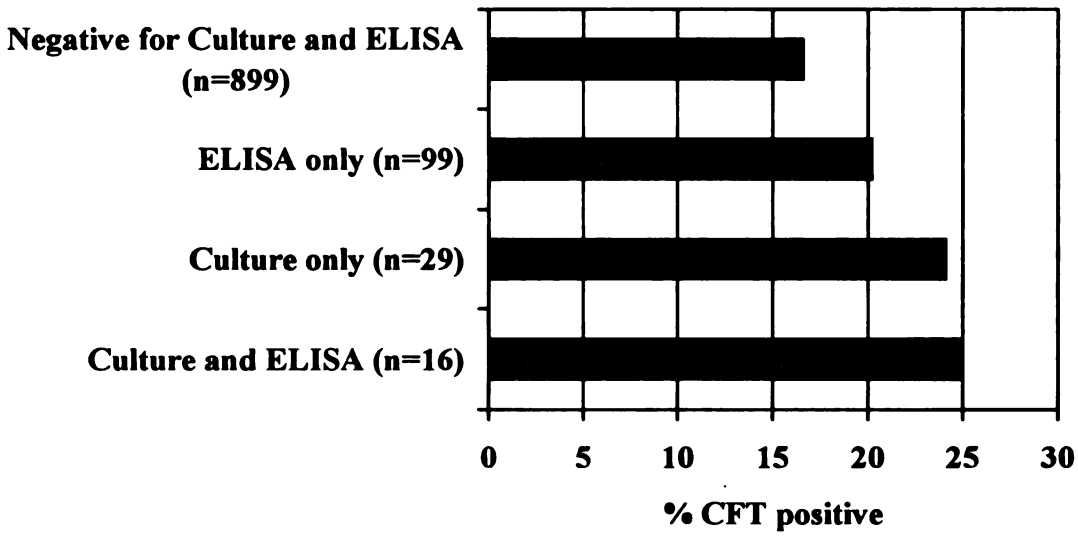


Figure 2-3: Percentage of cattle with positive CFT results based on positive *M. paratuberculosis* test results



CHAPTER 3

THE EFFECT OF INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* ON THE GAMMA INTERFERON (γ-IFN) ASSAY FOR BOVINE TUBERCULOSIS IN CATTLE.

Abstract

Objective – To determine whether cattle testing positive for *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) are more likely to have false positive results on the γ-IFN assay for bovine tuberculosis than cattle testing negative.

Animals – 1,043 Holstein cattle from 10 herds in Michigan

Procedure – Fecal and whole blood samples were collected from all cattle ≥24 months of age on the day each herd's annual caudal fold tuberculin (CFT) test was read. Fecal samples were submitted for mycobacterial culture. Samples of plasma were tested for antibody against *M. paratuberculosis*, as well as for γ-IFN after whole blood samples were stimulated with phosphate buffered saline solution or purified protein derivative (PPD) from either *M. bovis* or *M. avium*.

Results – Of the 1043 cattle tested in this study, 45 (4.3%) were positive for *M. paratuberculosis* by fecal culture and 115 (11.0%) were positive by antibody ELISA. A total of 8 cattle (0.8%) were positive in the γ-IFN assay after stimulation with PPD from *M. bovis*. None of the eight cattle were also positive for either *M. paratuberculosis* fecal culture or antibody ELISA.

Conclusions – No significant association was found between cattle testing positive by *M. paratuberculosis* fecal culture or antibody ELISA, and positive results of the γ-IFN assay for bovine tuberculosis.

Introduction

The γ-IFN assay for TB compares an animal's γ-IFN response to whole blood stimulated by bovine purified protein derivative (PPD) tuberculin with the γ-IFN response stimulated by avian PPD. Cross-reactivity is speculated to cause false positives on diagnostic tests that utilize PPD due to shared proteins among mycobacterial species (Aagaard et al., 2003). As a result, the high prevalence of Johne's disease in Michigan cattle may lead to cross-reactivity on the γ-IFN assay used to detect bovine tuberculosis (TB). A recent study in Denmark demonstrated a lower specificity of the γ-IFN assay for TB among *M. paratuberculosis*-infected herds compared to uninfected herds (Jungersen et al., 2002).

This study was designed to determine whether individual cattle testing positive for *M. paratuberculosis* are more likely to have false positive results on the γ-IFN assay for TB than cattle testing negative for *M. paratuberculosis*. Evaluating the effect of *M. paratuberculosis* on the γ-IFN assay is an important step for determining the amount of confidence given to positive results among herds infected with Johne's disease.

Methods

Animals

The study included 1,043 Holstein cattle from 10 separate herds located in three counties in Michigan. Eight of the herds sampled were within a TB high-risk area (Alpena county), one in a TB-free area (Ingham county), and one near the high-risk area (Ogemaw county) (Figure 1-1). Criteria for inclusion were a willingness to participate, having a total number of cattle in the herd less than 250 (for cost reasons), and being able

to sample the herd on the day their required whole-herd TB test was being read. All cattle greater than or equal to 24 months of age were sampled from each herd. None of the herds had been previously vaccinated for *M. paratuberculosis* or *M. bovis*.

Sampling

Blood and fecal samples were collected on the day the CFT test was read. A new plastic sleeve was used for each animal to collect fecal samples, which were placed in separate plastic whirl-pack bags after collection. Fecal samples were transported in coolers at ambient temperature to the Diagnostic Center for Population and Animal Health at Michigan State University and stored at -80°C within 24 hours of sampling until cultured. Blood was collected via the middle coccygeal vein using a 20-gauge, 1-in. needle. The blood was collected into a 10 ml Vacutainer tube containing sodium heparin (Corning Glass Works, Corning, NY). Blood samples were transported to the lab in plastic coolers, chilled with ice packs, and were processed within 24 hours of when the samples were drawn.

Laboratory methods – A detailed description of laboratory methods is located in the appendix.

***M. paratuberculosis* testing**

M. paratuberculosis laboratory diagnostic tests consisted of fecal culture and testing for antibodies using a plasma ELISA. Fecal samples were cultured to detect the presence of *M. paratuberculosis* using standard procedures recommended by USDA-

National Veterinary Service Laboratory and based on the procedures used by Whitlock et al. at the University of Pennsylvania (Fyock and Whitlock, 1999).

Plasma samples were tested for antibodies to *M. paratuberculosis* with a commercial antibody ELISA test kit.[3] The samples were tested in duplicate wells and the average absorbance values, or optical density (OD) was calculated. The corrected OD was calculated by subtracting the average OD of two negative serum control wells from the average OD of duplicate sample wells. A corrected OD greater than 0.1 was considered positive.

γ-IFN testing

Whole blood samples were tested for γ-IFN using a commercially available antigen capture ELISA, following the manufacturers recommended protocol.[4] Using the OD values, an animal was classified as positive for *M. bovis* γ-IFN if the difference between the mean OD value from the bovine PPD samples and the nil antigen samples were ≥ 0.1 and the difference between the mean OD value from the bovine PPD samples and avian PPD samples were also ≥ 0.1.

Statistical methods

Prevalence of Johne's disease within each herd was computed for each type of Johne's disease test (fecal culture or antibody ELISA) by the number of positive cattle for each test divided by the total number of cattle tested within each herd.

[3] Parachek™, BioCor Animal Health, Omaha NE
[4] Bovigam™, BioCor Animal Health, Omaha NE

Statistical analyses were conducted using a standard software package[5], testing associations between γ-IFN assay results and animal age and Johne's disease status. In the analyses, multiple logistic regression with random effects was performed with positive γ-IFN result for TB as the model outcome and risk factors including Johne's test results, Johne's herd prevalence for each test, and animal age (Table 3-1). Because the analysis was done on an individual animal level, the random effect function was used in all the models to adjust for the fact that animals from the same herd are more alike in terms of the exposure than animals in other herds.

A backwards stepwise model development was used to create final models with risk factors significant at $p \leq 0.05$. In brief, a full model was generated, and possible interactions and confounding were assessed and corrected during the model development process. Odds ratios (OR) with 95% confidence intervals were computed for parameter estimates. With the exception of the potential confounders forced into the model, each risk factor was tested by examining the effects of removal of that factor from the model. If removal of the risk factor resulted in a change in the OR of the remaining variables of more than 10%, the risk factor and its interaction terms were retained in the model.

Results

Johne's disease results

Of the 1043 cattle tested in this study, 45 (4.3%) were positive for *M. paratuberculosis* fecal culture and 115 (11.0%) were positive for antibody ELISA (Table 3-2). The herd prevalence for each test ranged from 0 to 15.4% (mean 4.5%, median

[5] SAS V8, SAS Institue, Cary, NC

2.8%) for *M. paratuberculosis* fecal culture, and 1.2 to 30.8% (mean 9.7%, median 6.9%) for *M. paratuberculosis* antibody ELISA (Figure 3-1).

TB results

Eight cattle (0.8%) were positive for γ-IFN after stimulation of blood samples with PPD from *M. bovis*. Herd response ranged from 0.0% to 3.5% (mean 0.9%, median 0.3%) (Figure 3-1). Two of the eight cattle positive for γ-IFN were CFT suspects, however, both were negative on the CCT test. This study was done before the γ-IFN assay became an official screening test for TB, so none of the eight cattle that were γ-IFN positive were removed from the herd for post-mortem examination. In addition, none of the eight cattle that were positive for γ-IFN were also positive for *M. paratuberculosis* fecal culture or antibody ELISA (Table 3-2). To date, *M. bovis* has not been diagnosed on any of the farms in this study.

Statistical results

The γ-IFN test results following stimulation with *M. bovis* PPD for each animal were analyzed with individual Johne's test results, Johne's herd prevalence for each test, and age (Table 3-3). None of the animals positive for γ-IFN were also positive for *M. paratuberculosis* antibody ELISA or fecal culture. No variables were significantly associated with positive γ-IFN test results.

Discussion

The γ-IFN assay has several advantages over tuberculin skin tests for diagnosis of bovine tuberculosis. First, blood samples are taken only once, so animals are not required to be held for 72 hours and handled twice, which is required for administration and reading of the CFT and CCT skin tests. Second, cattle can be retested at any time, unlike the skin tests, which require retesting within 10 days or after 60 days from the previous skin test (USDA, 1999). Third, unlike the skin tests, the immune response of the animal is not potentially compromised from the intradermal injection of tuberculin (Radunz and Lepper, 1985). Fourth, the assay eliminates the subjective variability present between veterinarians reading skin tests. The γ-IFN assay also has several disadvantages. Laboratory facilities are required with the ability to cultivate the blood cells and detect the γ-IFN production. The blood must also be processed quickly since cells must be viable in order to produce γ-IFN during stimulation with PPD. A study in 1992 determined that blood must be processed within 8 hours for optimal production of γ-IFN (Rothel et al., 1992).

The USDA's Center for Veterinary Biologics licensed the γ-IFN assay on December 10, 2001 as an official supplemental test for the bovine TB eradication program (USDA, 2002). The manufacturer recommends that the test be used as an ancillary test for serial (confirmation of positives) or parallel (confirmation of negatives) testing, three to thirty days after skin testing (Biocor, 2002). False positive responses could cause a problem if used as a confirmation of cattle with positive responses from tuberculin skin testing. These false positive responses could lead to unnecessary culling,

unnecessary time and cost of follow-up testing, as well as possible psychological stress to the producer and veterinarian.

There were only eight cattle positive for the γ-IFN assay for TB from 1,043 cattle tested in this study. Two of the eight cattle were positive on the CFT test, but were negative on the follow-up CCT test. None of the eight cattle positive for *M. bovis* γ-IFN were also positive for *M. paratuberculosis* antibody ELISA or fecal culture. Because there was no overlap between positive *M. paratuberculosis* test results and positive *M. bovis* γ-IFN results, the 95% confidence intervals for the odds ratios were extremely large. The lack of overlapping values resulted in a large standard error for the estimate of the independent variable, leading to the infinite range of the confidence interval.

Although no significant effect was found between the *M. paratuberculosis* test results and positive *M. bovis* γ-IFN results, the lack of effect was only based on eight γ-IFN positive animals. Due to the small number of false positives, a true association due to *M. paratuberculosis* would have been impossible to identify. The sensitivity of the γ-IFN assay used in this study has been reported to range from 81.8-91.7%, with a specificity ranging from 84.3-99.1% (Table 1-4). The low number of animals positive for γ-IFN in this study supports a high specificity for the assay. If *M. paratuberculosis* truly has no effect on the γ-IFN assay, the low number of false positives may make it a useful supplemental screening test in herds infected with Johne's disease.

Further studies involving a large sample size, however, need to be conducted to confirm these findings. In addition, research should focus on using proteins that are specific to *M. bovis*, rather than PPD that share proteins with other mycobacterial species. The recent mapping of the complete genome sequence for *M. bovis* allows specific

proteins to be identified that are unique to *M. bovis* (Garnier et al., 2003). A recently published study identified a mixture of the proteins ESAT-6, CFP-10, TB27.4, and TB10.4 as a potential novel cocktail for the development of new tests for *M. bovis* (Aagaard et al., 2003). It is important that genomic sequence be studied in depth to help generate more possible proteins that can be tested for potential highly specific diagnostic assays for TB.

Table 3-1: Description and descriptive statistics for risk factors evaluated

Outcome	Values	Description	n	%*
γ-IFN Assay Result	1	Positive	8	1
	0	Negative	1035	99
Risk Factor	**Values**	**Description**	**n**	**%**
M. paratuberculosis ELISA	1	Positive	115	11
	0	Negative	928	89
M. paratuberculosis Culture	1	Positive	45	4
	0	Negative	998	96
M. paratuberculosis ELISA and Culture	1	Positive for both	16	2
	0	Negative for one or both	1027	98
		Continuous variables		
Age (months)	24-144	Age of animal in months	1043	100
M. paratuberculosis Culture Herd Prevalence	0-15.4	% cattle positive for *M. paratuberculosis* fecal culture for each herd	1043	100
M. paratuberculosis ELISA Herd Prevalence	1.2-30.8	% cattle positive for *M. paratuberculosis* antibody ELISA for each herd	1043	100

*Percentage of animals described by the respective value (=n/1043)

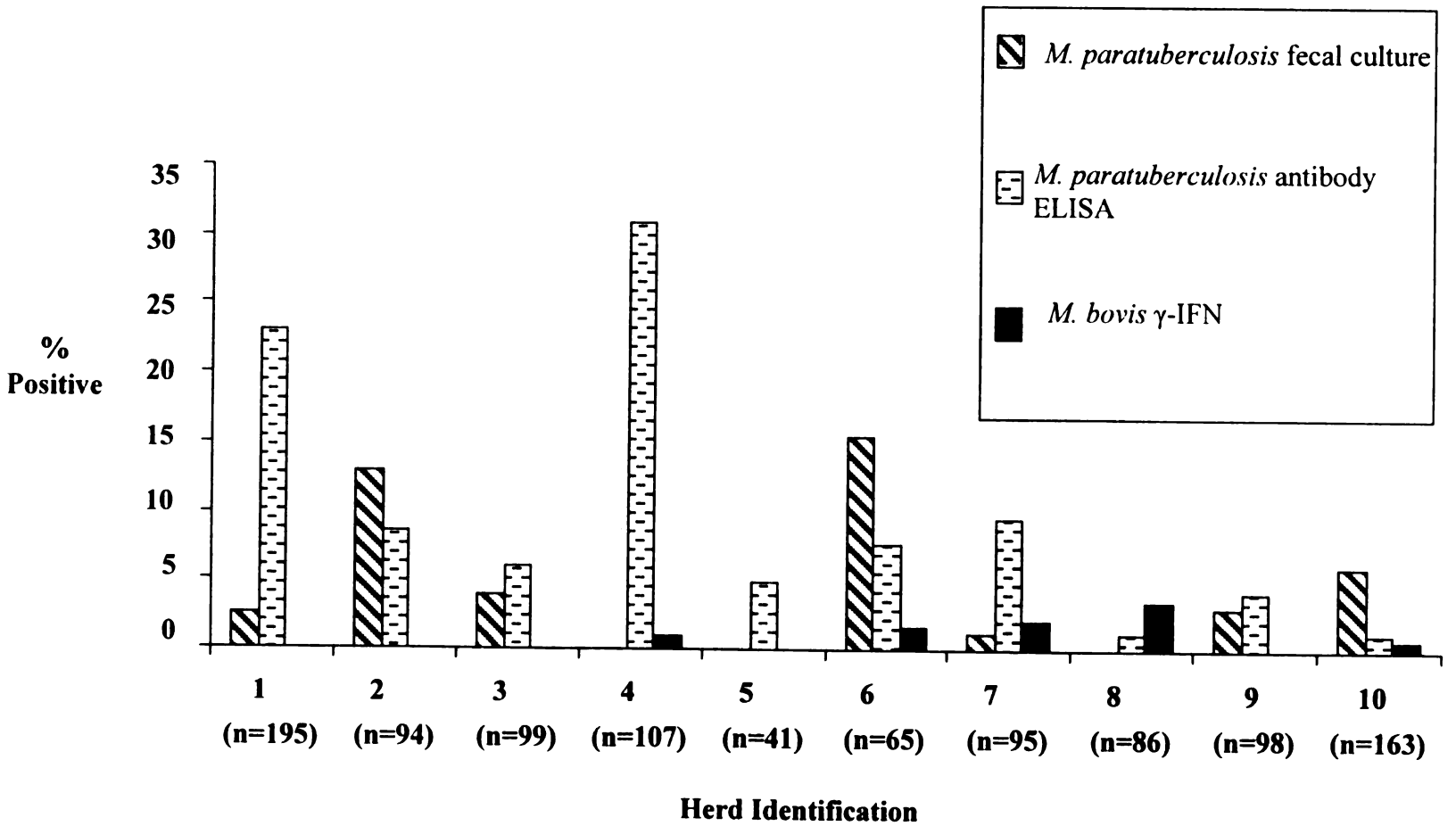
Table 3-2: Descriptive analysis of TB test results based on positive Johne's disease tests

Johne's disease test (positive results)	All Cattle (n=1043) #	%	γ-IFN positive (n=8) #	%
Fecal Culture	45	4.3	0	0
Antibody ELISA	115	11.0	0	0
Fecal Culture or Antibody ELISA	144	13.8	0	0
Fecal Culture and Antibody ELISA	16	1.5	0	0
Negative to both Johne's disease tests	899	86.2	8	100

Table 3-3: Results of a multivariable logistic regression analysis of the effect of individual Johne's Disease test results and herd levels on the γ-IFN test as the outcome (classified as positive or negative).

	Full Model				
Risk Factor	**Estimate**	**S.E.**	**X^2 p**	**OR**	**95% C.I.**
Age (months)	0.01	.01	0.49	1.01	.98 – 1.04
Day 3 Para Result	-16.67	10864	0.998	$5.7E^{-8}$	0-∞
Culture	-16.95	13297	0.999	$4.3E^{-8}$	0-∞
CultureANDELISA	17.83	11201	0.999	$5.5E^{7}$	0-∞
CultureHerdPrev	-3.08	11.1	0.78	.05	$1.6E^{-11}$ – $1.3E^{8}$
ParaHerdPrev	-1.79	6.00	0.77	.17	$1.3E^{-6}$ – $2.1E^{4}$
Culture*Day3ParaResult	0	-	-	1	-
Agem*Day3ParaResult	-0.01	232	1.00	.99	$3.3E^{-198}$-$3.0E^{197}$
Agem*Culture	-0.02	209	0.999	.98	$1.2E^{-178}$-$7.9E^{177}$
	Model –2 Res Log Likelihood = 9909.4				

Figure 3-1: Graph of the percentage of positive test results by herd



CHAPTER 4

EVALUATION OF THE ASSOCIATION BETWEEN THE GAMMA INTERFERON (γ-IFN) ASSAY, STIMULATED BY *MYCOBACTERIUM AVIUM*, AND THE CAUDAL FOLD TUBERCULIN (CFT) TEST AND JOHNE'S DISEASE IN CATTLE

Abstract

Objective – To determine whether cattle testing positive for *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) are more likely to be positive on the γ-IFN assay stimulated by *Mycobacterium avium* PPD than cattle testing negative. The association between the *M. avium* γ-IFN assay and the CFT test was also evaluated.

Animals – 1,043 Holstein cattle from 10 herds in Michigan

Procedure – Fecal and whole blood samples were collected from all cattle ≥24 months of age on the day the CFT test was read. Fecal samples were submitted for *M. paratuberculosis* culture. Samples of plasma were tested for antibody against *M. paratuberculosis*, as well as for γ-IFN after whole blood samples were stimulated overnight with phosphate buffered saline solution (control) and purified protein derivative (PPD) from *M. bovis* or *M. avium*.

Results –The multivariable model for *M. avium* γ-IFN test results identified positive *M. paratuberculosis* fecal culture and herd prevalence of *M. paratuberculosis* antibody ELISA as statistically significant risk factors, with odds ratios of 27.1 ($p=0.0066$) and 8.4 E^5 ($p=0.0461$), respectively. Cattle positive for *M. avium* γ-IFN were 5.4 times more likely to be CFT suspects than those negative for *M. avium* γ-IFN ($p<0.0001$).

Conclusions and Clinical Relevance – Cattle positive for *M. avium* γ-IFN were more likely to be CFT suspects than negative cattle, which may be related to early stages of Johne's disease.

Introduction

Determining whether an animal is infected with Johne's disease is a challenge, especially in the earlier stages of the disease, before a humoral response has begun. The beginning stage of Johne's disease is typically characterized by cell-mediated immunity to antigen stimulation, which progresses to a strong humoral immune response as the disease progresses from subclinical to clinical stages (Bendixen, 1977). Because γ-IFN relies on a cell-mediated response, we attempted to use the γ-IFN assay, comparing stimulation of blood with *M. avium* PPD to phosphate buffered saline (control), as an indicator for early cell-mediated stages of Johne's disease. *M. avium* PPD was used instead because *M. paratuberculosis* is a subspecies of *M. avium*, and Johnin PPD is not commercially available (McIntyre and Stanford, 1986). The γ-IFN response to *M. avium*, however, is not an approved diagnostic test for *M. paratuberculosis*.

A recent study measuring γ-IFN after stimulation with *M. avium* PPD and Johnin PPD concluded that the γ-IFN assay could be used to identify subclinical Johne's disease, although the test is highly susceptible to cross-reactivity between mycobacteria species (Jungerson et al., 2002). This first goal of our study was to measure this association between individual cattle with positive *M. paratuberculosis* test results and the γ-IFN response to *M. avium*. To measure this association, our study calculated the odds of a positive γ-IFN response based on positive results by *M. paratuberculosis* fecal culture

and antibody ELISA tests. This assumes that a cell-mediated immune response remains detectable after progression of Johne's disease to a humoral response, detected by ELISA.

The second goal of this study was to evaluate the effect of *M. paratuberculosis*, using a positive response of γ-IFN to *M. avium* as well as positive *M. paratuberculosis* fecal culture and antibody ELISA, with a positive response on the CFT test for bovine tuberculosis. This second goal is intended to supplement Chapter 2, by adding positive *M. avium* γ-IFN results as third diagnostic test for Johne's disease infection.

Methods

Animals

The study included 1,043 Holstein cattle from 10 separate herds located in three counties in Michigan. Eight of the herds sampled were within a TB high-risk area (Alpena county), one in a TB-free area (Ingham county), and one near the high-risk area (Ogemaw county) (Figure 1-1). Criteria for inclusion of a herd in this study were a willingness to participate, having a total number of cattle in the herd less than 250 (for cost reasons), and being able to sample the herd on the day when their required whole-herd TB test was read. All cattle greater than or equal to 24 months of age were sampled from each herd. None of the herds had been previously vaccinated for *M. paratuberculosis* or *M. bovis*.

Tuberculosis skin testing

The CFT tests were performed by either accredited private practice veterinarians or by veterinarians employed by the Michigan Department of Agriculture (MDA) using the methods described in the USDA Uniform Methods and Rules (USDA, 1999). Seven of the 10 herds were CFT tested by the same MDA veterinarian. All comparative cervical tuberculin (CCT) tests were performed by USDA or MDA veterinarians. Results of both skin tests were interpreted 72 ± 6 hours after injection of tuberculin.

Sampling

Blood and fecal samples were collected on the day the CFT test was read. A new plastic sleeve was used for each animal to collect fecal samples from the rectum. The fecal samples were placed in separate plastic whirl-pack bags after collection. Fecal samples were transported in coolers at ambient temperature to the Diagnostic Center for Population and Animal Health at Michigan State University and stored at -80°C within 24 hours of sampling until cultured. Blood was collected via the middle coccygeal vein using a 20-gauge, 1-in. needle. The blood was collected into a 10 ml Vacutainer tube containing sodium heparin (Corning Glass Works, Corning, NY). Blood samples were transported to the lab in plastic coolers, chilled with ice packs, and were processed within 24 hours of when the samples were drawn.

Laboratory methods – A detailed description of laboratory methods is located in the appendix.

M. paratuberculosis testing

M. paratuberculosis laboratory diagnostic tests consisted of fecal culture and testing for antibodies using a plasma ELISA. Fecal samples were cultured to detect the presence of *M. paratuberculosis* using standard procedures recommended by USDA-National Veterinary Service Laboratory and based on the procedures used by Whitlock et al. at the University of Pennsylvania (Fyock and Whitlock, 1999).

Plasma samples were tested for antibodies to *M. paratuberculosis* using a commercial ELISA kit.[6] The samples were tested in duplicate wells, as recommended by the manufacturer. The average optical density (OD) was calculated for each pair of wells. The corrected OD was calculated by subtracting the average OD of two negative serum control wells from the average OD of duplicate sample wells. A corrected OD greater than 0.1 was considered positive.

M. avium γ-IFN testing

Whole blood samples were tested for γ-IFN using a commercially available antigen capture ELISA, following the manufacturers recommended protocol.[7] Using the OD values, an animal was classified as positive for *M. avium* γ-IFN if the difference between the mean OD value from the avian PPD samples and the nil antigen samples were ≥ 0.1 and the difference between the mean OD value from the avian PPD samples and bovine PPD samples were also ≥ 0.1.

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[6] Parachek™, BioCor Animal Health, Omaha NE
[7] Bovigam™, BioCor Animal Health, Omaha NE

Statistical methods

Prevalence of Johne's disease within each herd was computed for each type of Johne's disease test (fecal culture, antibody ELISA, or *M. avium* γ-IFN) by the number of positive cattle for each test divided by the total number of cattle tested within each herd.

Statistical analyses were conducted in two parts, using a standard software package[8]: 1) testing associations between *M. avium* γ-IFN test results and animal age and Johne's disease status; and 2) testing associations between CFT test results and age, CFT testing veterinarian, and Johne's disease status. In the first analyses, multivariable logistic regression models with random effects were developed with the outcome of positive γ-IFN response from *M. avium* stimulation and risk factors included Johne's disease test results, Johne's disease herd prevalence for each test, and animal age. In the second analyses, model outcome was CFT test status (positive or negative), and risk factors included testing veterinarian, animal age, results of individual Johne's disease tests, and herd prevalence of Johne's disease (Table 4-1). Because the analysis was done on an individual animal level, the random effect function was used in all the models to adjust for the fact that animals from the same herd are more alike in terms of the exposure than animals in other herds.

A backwards stepwise model development was used to create final models with risk factors significant at $p \leq 0.05$. In brief, a full model was generated, and possible interactions and confounding were assessed and corrected during the model development process. Odds ratios (OR) with 95% confidence intervals were computed for parameter estimates. With the exception of the potential confounders forced into the model, each risk factor was tested by examining the effects of removal of that factor from the model.

[8] SAS V8, SAS Institue, Cary, NC

If removal of the risk factor resulted in a change in the OR of the remaining variables of more than 10%, the risk factor and its interaction terms were retained in the model.

Results

Johne's disease results

Of the 1043 cattle tested in this study, 45 (4.3%) were positive for *M. paratuberculosis* by fecal culture, 115 (11.0%) were positive by antibody ELISA, and 154 (14.8%) were positive for *M. avium* γ-IFN (Table 4-2). The herd prevalence for each test ranged from 0 to 15.4% (mean 4.5%, median 2.8%) for *M. paratuberculosis* fecal culture, 1.2 to 30.8% (mean 9.7%, median 6.9%) for *M. paratuberculosis* antibody ELISA, and 0 to 68.2% (mean 9.3%, median 1.7%) for *M. avium* γ-IFN (Figure 4-1).

TB results

All cattle that responded to the CFT test (any visible or palpable response) were classified as suspect and retested with the CCT test. Of the 1043 total cattle, 180 were CFT suspects (17.3%). Herd response ranged from 8.0% to 56.1% (mean 21.2%, median 20.1%) for the CFT test (Figure 4-1). Five of the 180 CFT suspects were also CCT suspects and a sixth animal was a CCT reactor. The CCT suspects and the CCT reactor were classified as negative for TB based on necropsy, histological examination of tissues and mycobacterial culture. Of the cattle positive for *M. paratuberculosis* by fecal culture, 24.4% were CFT suspects, while 20.9% of the antibody ELISA positive cattle were CFT suspects, and 19.5% of those positive for *M. avium* γ-IFN were CFT suspects.

Statistical results

The initial multivariable model for *M. avium* γ-IFN assay results identified positive individual animal *M. paratuberculosis* fecal culture and increasing herd prevalence of *M. paratuberculosis* antibody ELISA as statistically significant risk factors for positive result on the γ-IFN assay, with odds ratios of 27.1 (p=0.0066) and 8.4 E^5 (p=0.0461), respectively (Table 4-3).

In the multivariable regression model for the CFT test, γ-IFN released from the stimulation of blood with *M. avium* PPD was the only statistically significant risk factor identified (OR=5.4, p<0.0001) (Table 4-4). The individual animal and herd level of *M. paratuberculosis* fecal culture and antibody ELISA test results did not significantly affect the CFT test results. The veterinarian administering the CFT test was identified as a confounding variable, and therefore controlled for in the modeling process.

Discussion

Cross-reactivity between different mycobacteria have led to the assumption that infection with *M. paratuberculosis* may lead to false positive responses to the CFT test (Morrison et al., 2000). It is important to determine the cause of false positives responses as they may lead to unnecessary culling, increased time and cost of animal handling, increased cost of follow-up testing, and possible psychological stress to producers and veterinarians.

In the current study, Johne's disease infection was classified according to *M. paratuberculosis* fecal culture and antibody ELISA results, and γ-IFN response when stimulated by *M. avium*. As mentioned earlier, *M. avium* γ-IFN is not an approved

diagnostic test for Johne's disease but was used for this purpose in our study. We calculated the odds of a positive response of γ-IFN stimulated by *M. avium*, based on positive results from *M. paratuberculosis* fecal culture and antibody ELISA. After learning the relationship between *M. avium* γ-IFN and other approved Johne's disease diagnostic tests, we evaluated the effect these diagnostic tests had on the outcome of the CFT test, including the γ-IFN response to *M. avium* stimulation.

Because Johne's disease is thought to progress from cell-mediated immunity to a strong humoral immune response as the disease progresses from subclinical to clinical stages, we attempted to use *M. avium* γ-IFN as an indicator for early stages of Johne's disease where the immune response is predominately cell-mediated. For later stages of Johne's disease that have progressed to humoral immunity, we attempted to detect antibodies using antibody ELISA and to detect shedding bacteria with fecal culture.

First, we examined the effect of individual Johne's disease test results on the outcome of a positive *M. avium* γ-IFN result. Cattle positive for *M. paratuberculosis* fecal culture were 27.1 times more likely to be positive for *M. avium* γ-IFN than cattle with a negative culture (Table 4-3). Herds with a higher prevalence of cattle testing positive for *M. paratuberculosis* antibody ELISA were also more likely to be positive for *M. avium* γ-IFN than cattle from herds with lower prevalence. The 95% confidence interval for the odds ratio was large for ELISA herd prevalence, so it is difficult to comment on the true effect on a positive *M. avium* γ-IFN response. This relationship suggests that *M. avium* γ-IFN may be detecting a portion of cattle infected with *M paratuberculosis.*

Cattle positive for *M. avium* γ-IFN were 5.4 times more likely to be CFT suspects than cattle negative for *M. avium* γ-IFN (Table 4-4). A positive trend existed between positive *M. paratuberculosis* fecal culture and antibody ELISA tests and false positive reactions to the CFT test, however this association was not statistically significant (See Chapter 2).

Although positive *M. paratuberculosis* fecal culture results were associated with positive *M. avium* γ-IFN results, this study was not designed to determine whether *M. avium* γ-IFN indicates early or late stages of Johne's disease. The association was significant, however, which may indicate that cattle infected with *M. paratuberculosis* can successfully be detected with *M. avium* γ-IFN, or the association may also be due to an increased false positive cross-reactivity for the γ-IFN assay due to the genetic relationship between *M. avium* and M. *paratuberculosis*. Animals with positive *M. avium* γ-IFN results were more likely to be CFT suspects compared to negative animals. It is difficult to conclude whether this association was due to proteins in common with PPD since both tests are a measurement of cell-mediated immunity based on PPD, or if specific infection with Johne's disease causing positive *M. avium* γ-IFN results resulted in the false positive CFT results.

Although positive *M. paratuberculosis* fecal culture results were associated with positive *M. avium* γ-IFN results, cattle positive for *M. paratuberculosis* fecal culture were not more likely to be CFT suspects. Several possible explanations may account for this lack of significant association. First, the sensitivity of fecal culture is low, due to the fact that the bacteria are only shed intermittently and in low numbers for most stages of Johne's disease. A low sensitivity would result in a large number of false positives,

which would decrease the likelihood of detecting an association due to the misclassification. Second, the sample size may have been too small to detect a true association between positive fecal culture and a positive CFT test. In this study, the difference in CFT test results were only 7% between cattle testing positive and negative by *M. paratuberculosis* fecal culture. The sample size used for this study was based on the ability to detect a 25% difference in the CFT test results between cattle testing positive and negative for fecal culture, thus the power to detect a true association was not adequate. Third, all ten herds in this study had a prevalence of positive fecal culture of 15% or less, such that we did not have any high prevalence herds which would have increased the number of positive cattle, increasing the ability to detect individual and herd level associations with positive CFT results. Fourth, despite the mild trend between *M. paratuberculosis* positive cattle and positive CFT results, it is possible that *M. paratuberculosis* may not significantly affect the CFT test. Either way, further studies with a larger sample size are necessary to confirm whether an association truly exists or not. Determining that a true association exists would help people in TB eradication programs decide whether the CFT test could be most useful in Johne's disease-free herds or if a high number of false positives are inherent to the test despite Johne's disease status.

Future studies are important to determine whether γ-IFN is successful at detecting early stages of Johne's disease, undetectable by *M. paratuberculosis* fecal culture or antibody ELISA. If γ-IFN proves to be an accurate diagnostic assay for subclinical Johne's disease, the high number of CFT suspects in this study may indicate that the majority of subclinically infected cattle in these herds are predominately exhibiting cell-

mediated immunity and haven't progressed to humoral immunity. The result could be a large number of CFT false positives in regions that have a high prevalence of Johne's disease.

Finally, it is important that research continues for improving diagnostic tests for TB that successfully compares *M. bovis* to *M. avium* and *M. paratuberculosis* during the initial round of testing. The recent mapping of the complete genome sequence for *M. bovis* allows specific proteins to be identified that are unique to *M. bovis* (Garnier et al., 2003). A recently published study identified a mixture of the proteins ESAT-6, CFP-10, TB27.4, and TB10.4 as a potential novel cocktail for the development of new tests for *M. bovis* (Aagaard et al., 2003). Further work identifying new diagnostic tests that detect antigens specific to *M. bovis* will dramatically aid in the eradication of TB.

Table 4-1: Description and descriptive statistics for risk factors evaluated

Outcome	Values	Description	n	%*
CFT Test Result	1	Positive	180	17
	0	Negative	863	83
Risk Factor	**Values**	**Description**	**n**	**%**
CFT Vet	1	Veterinarian 1 (CFT tested 1 herd)	195	19
	2	Veterinarian 2 (CFT tested 1 herd)	94	9
	3	Veterinarian 3 (CFT tested 7 herds)	647	62
	4	Veterinarian 4 (CFT tested 1 herd)	107	10
M. avium γ-IFN	1	Positive	154	15
	0	Negative	889	85
M. paratuberculosis ELISA	1	Positive	115	11
	0	Negative	928	89
M. paratuberculosis Culture	1	Positive	45	4
	0	Negative	998	96
M. paratuberculosis ELISA and Culture	1	Positive for both	16	2
	0	Negative for one or both	1027	98
		Continuous variables		
Age (months)	24-144	Age of animal in months	1043	100
M. paratuberculosis Culture Herd Prevalence	0-15.4	% cattle positive for *M. paratuberculosis* fecal culture for each herd	1043	100
M. paratuberculosis ELISA Herd Prevalence	1.2-30.8	% cattle positive for *M. paratuberculosis* antibody ELISA for each herd	1043	100
M. avium γ-IFN Herd Prevalence	0-68.2	% cattle positive for *M. avium* γ-IFN for each herd	1043	100

*Percentage of animals described by the respective value (=n/1043)

Table 4-2: Descriptive analysis of *M. avium* γ-IFN results based on positive Johne's disease test results

Johne's test (positive results)	All Cattle (n=1043)		*M. avium* γ-IFN positive (n=154)		*M. avium* γ-IFN negative (n=889)	
	#	%	#	%	#	%
Fecal Culture	45	4.3	12	7.8	33	3.7
Antibody ELISA	115	11.0	35	22.7	80	9.0
ELISA + Culture	16	1.5	6	3.9	10	1.1
Negative for both	899	86.2	113	73.4	786	88.4

Table 4-3: Descriptive analysis of CFT test results based on positive Johne's disease test results

Johne's test (positive results)	All Cattle (n=1043)		CFT suspect (n=180)		CFT negative (n=863)	
	#	%	#	%	#	%
Fecal Culture	45	4.3	11	6.1	34	3.9
Antibody ELISA	115	11.0	24	13.3	91	10.5
M. avium γ-IFN	154	14.8	30	16.7	124	14.4
Johne's negative	749	71.8	121	67.2	628	72.8

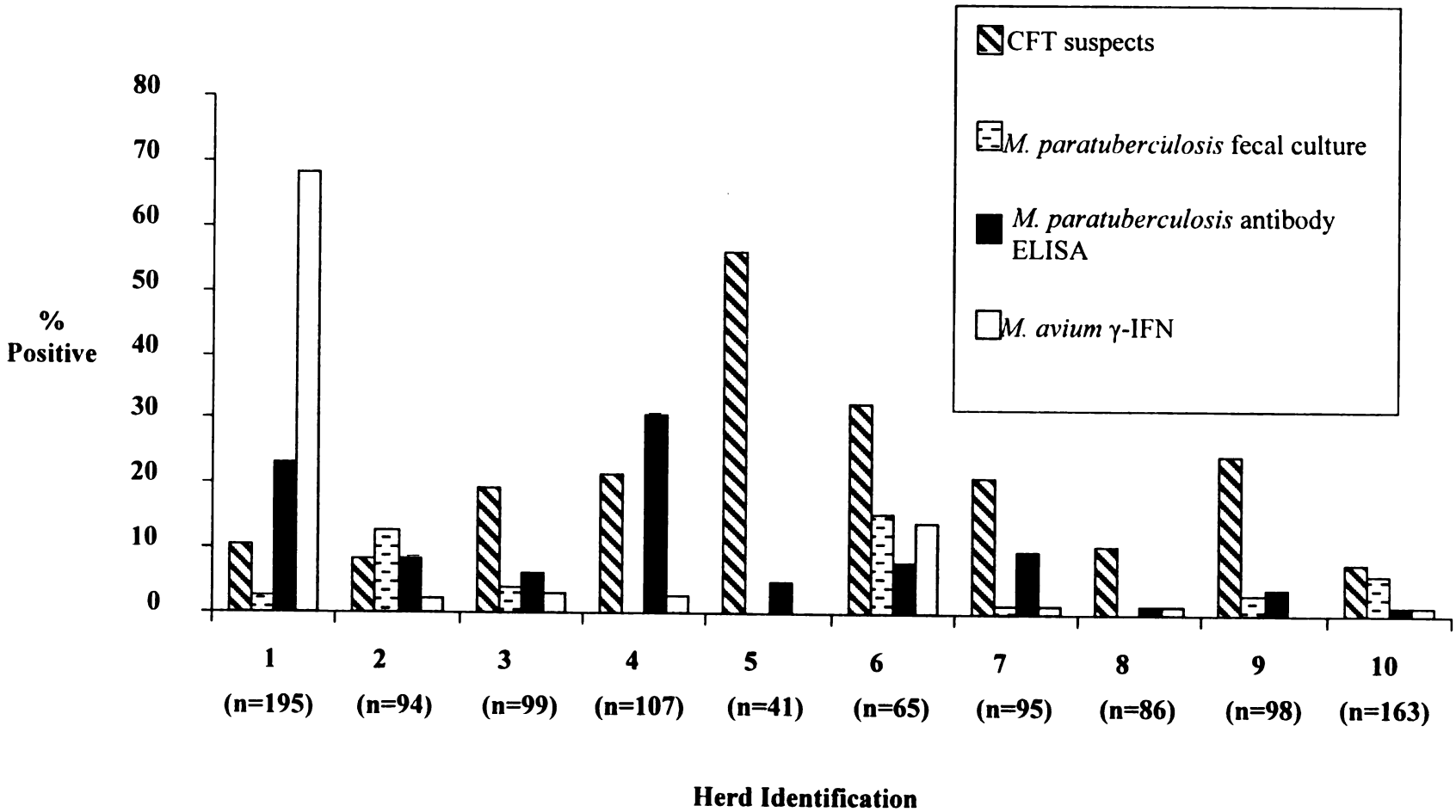
Table 4-4: Results of a multivariable logistic regression analysis of the effect of Johne's disease test results on the *M. avium* γ-IFN test as the outcome.

	Final Model				
Risk Factor	**Estimate**	**S.E.**	**X^2 p**	**OR**	**95% C.I.**
M. paratuberculosis Culture	3.30	1.21	.01	27.1	2.52-292
M. paratuberculosis ELISA Herd Prevalence	13.64	6.83	.05	$8.4E^{5}$	$1.28\text{-}5.5E^{11}$
M. paratuberculosis Culture Herd Prevalence	11.09	12.3	.37	$6.5E^{4}$	$2.0E^{-6}$-$2.1E^{15}$
Age (months)	-.003	.01	.66	1.00	.98-1.01
Age (months)* *M. paratuberculosis* Culture	-.02	.02	.33	.98	.93-1.02
	Model –2 Res Log Likelihood = 6796.4				

Table 4-5: Results of a multivariable logistic regression analysis of the effect of individual Johne's Disease test results and herd levels on the CFT test as the outcome (classified as suspect or negative).

		Final Model				
	Risk Factor	**Estimate**	**S.E.**	**X^2 p**	**OR**	**95% C.I.**
	M. avium γ-IFN	1.69	.40	< .0001	5.45	2.47-12.03
	Age (months)	.01	.01	.07	1.01	.999-1.02
	1	-2.07	1.22	.09	0.13	.01-1.38
CFT	2	-.95	1.21	.43	0.39	.04-4.13
Vet	3	.13	0.89	.89	1.13	.20-6.49
	4	0	-	-	1	-
		Model –2 Res Log Likelihood = 5154.9				

Figure 4-1: Graph of the percentage of positive results by herd



SUMMARY AND CONCLUSIONS

In the first chapter, an introduction to Johne's disease and bovine tuberculosis (TB) was presented along with a review of current ante-mortem diagnostic tests used for each disease. Johne's disease and TB both exist in Michigan, with spread of TB in cattle occurring from infected white-tailed deer. A major problem for TB screening tests is the relatively high number of false positive responses, thought to occur as a result of cross-reactivity between mycobacteria and other closely related species. Due to the high prevalence of Johne's disease among Michigan herds, information was needed about the association between infection with *M. paratuberculosis* and false positive responses to the CFT and γ-IFN screening tests for TB.

The second chapter described a cross-sectional study performed in Michigan evaluating the effect of *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) on the CFT test for TB in cattle. Cattle testing positive for *M. paratuberculosis*, as measured by a positive *M. paratuberculosis* fecal culture or antibody ELISA test, appear to have an increased likelihood of false positive results on the CFT test, although the association is not statistically significant. Further studies involving a larger sample size need to be conducted to confirm these findings.

The third chapter was an evaluation of the effect of *M. paratuberculosis* on the γ-IFN assay for TB in cattle, using the same animals and herd visits described in chapter 2. No significant association was found between cattle testing positive by *M. paratuberculosis* fecal culture or antibody ELISA, and positive results of the γ-IFN assay for bovine tuberculosis. It may not be valid to conclude whether an association truly exists because only eight cattle were positive for γ-IFN stimulated by *M. bovis*. None of

the eight cattle with a positive γ-IFN response were also positive for either *M. paratuberculosis* fecal culture or ELISA. Further studies involving a large sample size with more false positives need to be conducted to confirm these findings.

The fourth chapter looked at the possibility of γ-IFN, stimulated by *M. avium*, as a test for diagnosis of early stages of Johne's disease. Cattle positive for *M. paratuberculosis* fecal culture were 27.1 times more likely to be positive for *M. avium* γ-IFN than cattle with a negative culture. Herds with a higher prevalence of cattle testing positive for *M. paratuberculosis* antibody ELISA were also more likely to be positive for *M. avium* γ-IFN than cattle from herds with lower prevalence. Cattle positive for *M. avium* γ-IFN were 5.4 times more likely to be CFT suspects than cattle negative for *M. avium* γ-IFN. It is difficult to conclude whether this association was due to proteins in common with PPD since both tests are a measurement of cell-mediated immunity based on PPD, or if true infection detected by *M. avium* γ-IFN resulted in the false positive CFT results. Further work is necessary to determine whether *M. avium* γ-IFN is detecting early stages of Johne's disease and whether early stage Johne's disease is the reason for an increased likelihood of positive CFT test results, as measured by *M. avium* γ-IFN. Confirmation of the results in these studies will help to determine necessary improvements for screening tests currently used for detection or TB. As diagnostic tests are improved, further work identifying new diagnostic tests that detect antigens specific to *M. bovis* will dramatically aid in the eradication of TB.

APPENDIX

DETAILED LABORATORY PROTOCOL

Fecal Culture

Fecal culture was performed on all samples following a protocol used by Whitlock et al. at the University of Pennsylvania (Fyock and Whitlock, 1999). Samples were stored at -80°C until processed. Two grams of each fecal sample were diluted with sterile distilled water in a labeled tube, shaken and allowed to stand for 30 minutes before transferring 5mL from the center of the supernatant into a tube containing 1/2XBHI-HPC (Half strength Brain Heart Infusion [BHI] broth with 1-Hexadecylpyridinium Chloride). The tube was then incubated overnight at 35-37°C. After incubation, the samples were centrifuged at 900 x G for 30 minutes. Supernatant was then discarded and 1mL of antibiotic brew was added with a sterile disposable pipet, followed by incubation overnight at 35-37°C. Antibiotic brew consists of 1 Liter of half strength BHI broth mixed with 5mL Amphotericin B (10mg/mL), 10mL Vancomycin (10mg/mL), and 5mL Naladixic Acid (10mg/mL).

Inoculation of the media took place either the next day or the following two days. Five tubes of Herrold's Egg Yolk Agar, four containing Mycobactin J and one without Mycobactin J, were then inoculated with 0.25mL of well mixed suspension. Tubes were rolled to coat the entire service of media with inoculum. Tubes were incubated in a 35-37°C aerobic incubator in a slanted position with the media service horizontal until the inoculum dried. After drying, the tubes were returned to upright position and the caps tightened. Tubes were evaluated for growth and contamination every 2 weeks for 16 weeks. Colonies appearing after 6 weeks were evaluated for typical acid fastness and

morphological appearance of *M. paratuberculosis*. Each culture was then evaluated for mycobactin dependency before being reported as positive.

Antibody ELISA

Plasma samples were tested for antibodies to *M. paratuberculosis* with a commercial antibody ELISA test kit[b], using the manufacturer's recommended protocol. Briefly, 25μL of plasma and control samples were combined with serum diluent buffer, mixed and incubated for 1 hour at room temperature. Next, 100μL of test and control samples were added to microtiter plates containing bound *M. paratuberculosis* antigens and incubated at room temperature for 30 minutes. The microtiter wells were then washed 6 times with an appropriately diluted buffer supplied by the manufacturer. Next, 100μL of horseradish peroxidase labeled anti-bovine Ig was added to each well and incubated at room temperature for 30 minutes. Plates were washed six times with wash buffer, followed by the addition of 100μL enzyme substrate solution to each well. Finally, 50μL of enzyme stopping solution (0.5M H_2SO_4) was added to each well once positive control wells had absorbance between 0.35 and 0.40 using a 620nm filter. The absorbance was then read using a 450nm filter between 2 and 5 minutes after stopping the reaction.

γ-IFN testing

Whole blood samples were tested for γ-IFN using a commercially available antigen capture ELISA, following the manufacturers recommended protocol.[9] Heparinzed blood was separated into 1.5mL aliquots and placed into four wells of a 24

[9] Bovigam™, BioCor Animal Health, Omaha NE

well tissue culture plate for each animal. To the first well, 100μL phosphate buffered saline (0.01M, pH 7.2) was added as a negative control (nil antigen). In the second and third wells, 100μL bovine PPD[10] or avian PPD[11] were added to stimulate the release of γ-IFN, respectively. Pokeweed mitogen[12] was added to the fourth well to a final concentration of 10μg/mL of blood (Stabel, 1996). The pokeweed mitogen served as a control for cell function, as this mitogen stimulates production and release of γ-IFN from mononuclear leukocytes. Samples were incubated for 16-24 hours at 38°C in a humidified atmosphere. Samples were centrifuged at 1,200 x g for 15 minutes following incubation and 500μL of plasma were harvested from each well. For the test procedure, 50μL of test plasma and an appropriate number of positive and negative control samples provided by the manufacturer were combined with plasma diluent buffer, mixed and incubated for 60 minutes at room temperature. The microtiter wells were then washed out and filled with wash buffer six times, removing as much wash buffer as possible after the last wash. Next, 100μL of horseradish peroxidase labeled anti-bovine γ-IFN was added to each well and incubated at room temperature for 60 minutes. Plates were washed six times with wash buffer as earlier, followed by the addition of 100μL enzyme substrate solution to each well, and then incubated for 30 minutes. Finally, 50μL enzyme stopping solution (0.5M H2SO4) was added to each well at the end of the incubation and the absorbance was read using a 450nm filter within 5 minutes of stopping the reaction. Using the absorbance values measured by the spectrophotometer, or optical density (OD) values, an animal was classified as positive for *M. avium* γ-IFN if the difference between the mean OD value from the avian PPD samples and the nil antigen samples were ≥ 0.1

[10] CSL Limited, Victoria, Australia, Cat. No. 20901501
[11] CSL Limited, Victoria, Australia, Cat. No. 20911501
[12] *Phytolacca americana*, cell culture tested, Sigma, St. Louis, MO

and the difference between the mean OD value from the avian PPD samples and bovine PPD samples were also ≥ 0.1. An animal was classified as positive for *M. bovis* γ-IFN if the difference between the mean OD value from the bovine PPD samples and the nil antigen samples were ≥ 0.1 and the difference between the mean OD value from the bovine PPD samples and avian PPD samples were also ≥ 0.1.

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