APPLICATION OF MOLECULAR TECHNIQUES FOR THE DECTECTION OF MYCOBACTERIUM BOVIS IN THE ENVIRONMENT

By

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A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Comparative Medicine and Integrative Biology

2012

ABSTRACT

APPLICATION OF MOLECULAR TECHNIQUES FOR THE DETECTION OF MYCOBACTERIUM BOVIS IN THE ENVIRONMENT

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This Master's thesis project was designed to compare molecular techniques with traditional methods of culture processing in detecting *Mycobacterium bovis* in environmental substrates. A polymerase chain reaction (PCR) assay was applied to environmental substrates previously inoculated with varying concentrations of *M. bovis* and stored for various times under a range of temperatures. PCR was statistically equivalent to bacterial culture for detection of *M. bovis* in samples of water and hay but overall more effective than bacterial culture for detection of *M. bovis* in soil samples. In a second experiment, *M. bovis* was detected by PCR for up to 11 months in water samples exposed to natural weather conditions. In contrast, *M. bovis* was not detected by culture longer than 2 months post-inoculation in any substrate. PCR techniques were applied to a final set of environmental substrates that were sampled from cattle farms with previous occurrences of *M. bovis* infection. However, none of the analyzed samples tested positive for *M. bovis* DNA.

In epidemiologic investigations of TB farms and wildlife sites, PCR-based assays may be useful for parallel testing with bacterial culture to enhance detection of *M. bovis* in the environment. The data presented supports the fact that *M. bovis* can persist in the environment long enough to pose a potential infection risk to susceptible animals. Molecular techniques can be a useful and efficient tool in investigations concerning the role of indirect transmission in the epidemiology of bovine tuberculosis.

ACKNOWLEDGEMENTS

The completion of this project has come with many obstacles and challenges, but I would like to acknowledge and thank all of those who helped me through this journey, both academically and personally. I would like to acknowledge my major advisor, Dr. John B.Kaneene for his guidance and leadership, as well as the members of my graduate committee, Dr. Steve Bolin, Dr. Carole Bolin and Dr. Amanda Fine for all of their academic direction and feedback throughout this project. I would like to recognize my graduate program director, Dr. Vilma Yuzbasiyan-Gurkan, for introducing me to this opportunity and for all of her encouragement and support. I would also like to thank RoseAnn Miller for her help with data analysis and the staff at MSU's Diagnostic Center for Population and Animal Health (DCPAH) for their patience and laboratory assistance. The academic team listed above has been in invaluable asset to the completion of this project and I am thankful for the opportunity to have worked with each individual.

To my parents, thank you for teaching me the significance and importance of education. To my sister, Kania, thank you for showing me the value of hard work and persistence, and for providing me with a lifetime of inspiration. I am forever indebted to my family and close friends for all of their love, support, thoughts, prayers and cheers along the way.

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INTRODUCTION

PROBLEM STATEMENT

Mycobacterium bovis (*M. bovis*), the causative agent of bovine tuberculosis, has been of major concern in Michigan since the late 1990s, when the first case of bovine TB in cattle was confirmed in the northeast lower peninsula of Michigan since the state was declared TB-free in 1979 (O'Brien et al., 2006; Miller and Kaneene, 2006). It is suspected that for a majority of the cattle farms identified as TB positive in this same region of Michigan, *M. bovis* infection was caused by a spillover from white-tailed deer (*Odocoileus virginianus*) to cattle. Bovine TB likely became endemic in deer when there were large numbers of *M. bovis* infected cattle in the state during the late 1950s (Miller and Kaneene, 2006). Indirect transmission of *M. bovis* through contaminated environmental substrates, such as hay, water and soil may play a critical role in the transmission of *M. bovis* between wildlife and cattle (O'Brien et al., 2002; DeLiberto et al., 2004; Kaneene et al., 2002; Miller et al., 2003).

Bacterial culture, the standard method for detecting *M. bovis*, has many drawbacks and demonstrates the need for better techniques to improve our ability to detect *M. bovis* as well as characterize its persistence in the environment.

STUDY RATIONALE

The use of mycobacterial culture for the detection of *M. bovis* in the environment is challenged on a number of fronts. First, environmental samples (soil, feed, fecal material, pond/stream water, etc.) contain large numbers of saprophytic bacteria, molds and other infectious organisms. These other organisms interfere with mycobacteria

isolation by overgrowing and out-competing the mycobacteria during the bacterial culture process. Therefore, a decontamination step is essential. The decontamination process not only eliminates saprophytic organisms but also reduces the viability of mycobacteria in the specimen and; therefore, interferes with the sensitivity of detection of mycobacteria by bacterial culture methods (Kent and Kubica, 1985; Yajko et al., 1995). Secondly, the inherent properties of *M. bovis* make it particularly difficult to process and culture. *M. bovis*, like other mycobacteria, has the tendency to clump and form cords so it is often not evenly distributed in a processed sample. Its thick, waxy cell wall makes it buoyant and reduces the success of centrifugation methods aimed at concentrating the organism in the sample. In addition, *M. bovis* requires 6-8 weeks for growth on solid media, prolonging the time the specimen must be maintained at optimal conditions (37ºC, moist and free of other microbial contamination).

HYPOTHESIS AND OBJECTIVES

The hypothesis being tested is that molecular detection techniques will improve our ability to detect *Mycobacterium bovis* in environmental substrates, thereby offering an accurate characterization of the persistence and distribution of *M. bovis* in farm environments.

This Master's project was designed to compare molecular techniques with traditional methods of culture processing in detecting *Mycobacterium bovis* in environmental substrates. All samples used during this project were obtained from original experiments where samples were collected or experimentally inoculated with *M. bovis* and processed using traditional mycobacterial culture (Fine, 2006; Fine et al., 2011a,b). The specific objectives of this project included: 1) Determining a technique for

processing environmental samples for *M. bovis* detection by polymerase chain reaction (PCR); 2) Applying the molecular technique to an extensive set of experimentally inoculated environmental substrates to characterize the persistence of *M. bovis* DNA in the environment under conditions typical of the bovine TB endemic region within the state of Michigan; and 3) Testing environmental samples collected from sites of natural bovine TB transmission and previously processed for mycobacterial culture.

OVERVIEW

Chapter 1 is a literature review briefly addressing the epidemiology, pathology and diagnosis of bovine tuberculosis and presenting studies examining the evidence of *Mycobacterium bovis* persistence in the environment and the methods of detection used. **Chapter 2** compares molecular techniques with traditional bacterial culture for the detection of *M. bovis* in experimentally inoculated samples of hay, soil and water as well as the differences in detection rates between substrate types. **Chapter 3** presents an experimental study designed to determine the length of survival of *M. bovis* in experimentally inoculated substrates exposed to natural environmental conditions in Michigan and compares results obtained by molecular techniques with those obtained previously (Fine, et. al.) through bacterial culture. **Chapter 4** presents the results of a study designed to collect evidence of *M. bovis* in environmental samples obtained from TB positive cattle farms and wildlife areas in Michigan with high prevalence rates of *M. bovis* in free-ranging white-tailed deer.

The outcomes and overall comparisons of bacterial culture versus molecular techniques for the detection of *M. bovis* presented throughout this thesis are discussed in the final section: Overall Summary and Conclusions.

Chapter 1

LITERATURE REVIEW OF THE PERSISTENCE AND DETECTION OF *MYCOBACTERIUM BOVIS* IN THE ENVIRONMENT AND THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MICHIGAN

1.0 INTRODUCTION

By the early 20th century, bovine tuberculosis had become pervasive throughout the United States and presented a great threat to the livestock industry. The USDA began efforts to eradicate bovine tuberculosis from the country in 1917. The eradication program consisted of an extensive test and slaughter campaign that resulted in the destruction of nearly 4 million cattle – almost 6% of the cattle population during the time period ranging from 1917 through 1940 (Olmstead and Rhode, 2004). The program was such a success that by 1941, all counties in the United States were reporting infection rates of 0.5% or lower in their livestock.

Michigan was declared a TB-free state in 1979 after livestock surveillance found no new cases of tuberculosis for the preceding 5 years (Miller and Kaneene, 2006). In the late 1990's, bovine TB re-emerged in the northeast lower peninsula of Michigan as the result of contact of cattle with white-tailed deer (*Odocoileus virginianus*), which had become a reservoir for *M. bovis* (Schmitt et al., 1997). This caused Michigan to lose its USDA status as "accredited-free of TB" (O'Brien et al., 2006). A historical evaluation showed that high numbers of deer and a severe shortage of feed during the winter due to natural habitat destruction led to the practice of feeding deer to maintain population

numbers above the natural carrying capacity of the Michigan northeast lower peninsula. Deer became dependent on livestock feed for survival during winter months which led to increased contact with domestic cattle. This practice facilitated cattle-to-deer transmission of bovine TB during the mid-20th century (Miller and Kaneene, 2006).

In 1996, the Michigan Department of Natural Resources (MDNR) created Deer Management Unit 452 (DMU452) to facilitate deer surveillance within the core tuberculosis-affected areas during the deer hunting season. This unit encompassed a 4-county area within the northern lower peninsula of approximately 1500 km^2. Management strategies were implemented to control the deer population and reduce the prevalence of the disease in deer. In an effort to reduce the deer population to carrying capacity, more liberal hunting permits were issued and additional harvest seasons were instated for antlerless deer in an attempt to reduce reproductive capacity. In addition, restrictions were applied to the supplemental feeding and baiting of deer (Hickling, 2002).

Retrospective studies have shown initial decreasing trends in disease prevalence among deer that were concurrent with the implementation of the MDNR's management strategies (O'brien et al, 2006; Miller et al., 2007). Even with these advances, apparent disease prevalence has shown no significant downward trend since 2002 (O'Brien et al., 2011) and cases of bovine tuberculosis are still appearing within Michigan's cattle herds, suggesting continued spillover from white-tailed deer. Studies were initiated to further determine the modes of transmission between and within these two species.

1.1 PATHOLOGY AND DIAGNOSIS OF BOVINE TUBERCULOSIS

Effective TB control in cattle is dependent upon the accurate detection and removal of infected animals from the herd. Cattle infected with *Mycobacterium bovis* are often asymptomatic and may become infectious long before displaying signs of infection, which may include emaciation, lethargy, weakness, anorexia, low grade fever, and pneumonia with a chronic, moist cough (USDA, 2008). Consequently, effective ante mortem testing for bovine TB must focus on detecting infection during early stages of the disease with the use of immunodiagnostic tests.

The original test-and-slaughter program during the early to mid-20th century used the caudal-fold tuberculin test (CFT) to identify positive cattle within a herd. The CFT is the primary diagnostic tool used today to identify potential infected herds. The test involves an intradermal injection of Purified Protein Derivative (PPD) tuberculin into the caudal tail fold. The CFT is merely a screening tool that measures an animal's immune response to *Mycobacterium bovis*. The test is meant only to identify suspect animals within a herd, which would then undergo further testing to determine true infection status. Positive responders, identified by swelling or discoloration at the site of injection, are retested using the comparative cervical tuberculin (CCT) test within one week of the CFT test reading (Grooms and Molesworth, 2000a).

The CCT test involves two separate injections into the neck region of the animal; bovine PPD at one location and avian PPD at the other. Skin thickness at the site of injection is measured before injection and again at 72-hrs post-injection. This test is designed to distinguish positive reactors due to *M. bovis* versus those that are caused by *Mycobacterium avium*, a closely related bacterium that causes false positive results to the

CFT test (Grooms and Molesworth, 2000b). Animals that are determined to be true reactors are submitted to an animal diagnostic laboratory for necropsy and further diagnostic testing, including histopathology, culture and molecular methods of diagnosis.

1.2 TRANSMISSION OF BOVINE TUBERCULOSIS

Lesion location in an infected animal is often used to determine the route of transmission, whether oral or intranasal. Tubercle lesions in cattle primarily occur in the respiratory system lymph nodes, suggesting intranasal infection through the inhalation of infected aerosol droplets from another infected animal (Collins, 1996). Aerosol spread and active secretion of M. bovis are thought to be facilitated by the development of tubercle lesions in the respiratory system which invade the airways (Francis, 1958; Steele and Ranney, 1958). Lesions in the abdominal cavity are rare and it is suggested that they may occur as a result of infected cattle swallowing their own *M. bovis*-contaminated sputum (Neill et al., 1988). Looking primarily at an intranasal infection route, bovine TB can be spread either directly through nose-to-nose contact between an infected and susceptible animal, or indirectly through contaminated aerosolized droplets or through the contamination of feed, water and other environmental substrates.

1.2.1 Direct

Experimental studies have demonstrated direct transmission of TB from cattle to cattle using infected calves. Costello et al. designed an experiment that housed infection-free calves in close contact with calves naturally infected with *M. bovis* (Costello et al., 1998). Transmission of the infection occurred in 4 out of the 10 infection-free calves used in the study. Cassidy et al. designed a similar experiment using calves experimentally inoculated intranasally with *M. bovis* (Cassidy et al., 1999). The in-

contact calves developed lesions that were similar in distribution and appearance to those found in cattle naturally infected with the disease. Direct transmission between deer and cattle is less likely to occur as the two species are seldom observed in close proximity with each other (Palmer et al., 2012).

1.2.2 Indirect

Indirect transmission of M. bovis through contaminated substrates likely has a major role in the transmission of M. bovis between wildlife and cattle (Palmer et al., 2012; DeLiberto et al., 2004; Miller et al., 2003; Kaneene et al., 2002; O'Brien, et al., 2002). Indirect transmission from deer to deer was shown through the daily transferring of unconsumed feed from the pens of experimentally infected deer to the pens of uninfected (naïve) deer (Palmer et al., 2001). Direct contact between the two groups of deer was avoided, along with aerosol transmission and transmission through personnel. Upon necropsy after 150 days, all of the naïve deer had lesions consistent with tuberculosis and *M. bovis* was isolated from various tissues, mainly the lungs, tracheobronchial lymph nodes, and mediastinal lymph nodes.

In a similar study, the occurrence of indirect transmission between experimentally infected white-tailed deer and susceptible calves was investigated (Palmer et al., 2004). Calves testing negative to tuberculin skin testing were transferred to soiled pens where experimentally infected deer were previously held and were allowed to eat from the deer's leftover feed daily. No direct contact between the two species occurred and 100% (nine out of nine) of the calves developed *M. bovis* infection. The study also showed that keeping calves in their original pens and only transferring leftover feed from infected deer pens also led to infection in 4 out of 9 calves.

Observation of indirect cattle-to-cattle transmission was attempted by grazing calves on pasture that had been contaminated with *M. bovis* from experimentally infected calves (Maddock, 1936). None of the calves that were introduced on pasture post-contamination developed tuberculosis.

1.3 DETECTING MYCOBACTERIUM BOVIS IN THE ENVIRONMENT

1.3.1 Detecting *Mycobacterium bovis* using culture methods

Attempts to isolate *M. bovis* from presumed naturally infected environmental samples by bacterial culture have failed (Cooney et al., 1997; Livanainen, 1995; Livanainen et al., 1999; Palmer and Whipple, 2000; Fine et al., 2011b). However, numerous papers have focused on the persistence of *Mycobacterium bovis* in the environment using experimental study designs. Fine et. al. conducted a study concerning the persistence of *M. bovis* in experimentally inoculated substrates exposed to natural weather conditions (Fine et. al., 2011a). Survival of *M. bovis* was detected for up to 88 days in soil, 58 days in water and hay and 43 days on corn using conventional mycobacterial culture techniques. Williams and Hoy conducted early studies concerning the persistence of *M. bovis* in feces shed by an infected cow (Williams and Hoy, 1930). Survival of *M. bovis* survival was detected for up to four months when contaminated feces were spread on pasture and monitored. The recovery of *M. bovis* from naturally contaminated feces after 12 months of storage in cool, dark conditions also was reported and *M. bovis* was recovered after 2 years from experimentally inoculated feces stored under similar conditions. Other studies have shown *M. bovis* can persist in the environment for periods ranging from four weeks to six months (Duffield and Young, 1985; Maddock, 1933; Maddock, 1934; Whipple and Palmer, 2000).

Those studies have shown that under experimental conditions, *M. bovis* survival times are longer in controlled storage conditions versus the survival times of experimentally inoculated substrates stored outdoors under natural environmental conditions. Survival times for *M. bovis* appear to be shortened with exposure to seasonal environmental factors including high ambient temperatures, intensity of solar radiation and loss of moisture through evapotranspiration (Duffield and Young, 1985; Fine et al., 2011). In contrast, other experimental studies report minimal persistence in the environment (Little et al., 1982) and conclude that environmental contamination does not play a major role in the epidemiology of bovine tuberculosis in cattle and wildlife in New Zealand (Jackson et al., 1995).

1.3.2. Detecting *Mycobacterium bovis* using molecular methods

Using molecular detection techniques, Young et al. detected *M. bovis* DNA in soil from a farm environment up to 12 months after possible contamination (Young et al., 2005). Young et al. also showed that DNA did not persist in dead cells in soil based on DNA decay rates and it can be assumed that detection of DNA signifies viable organism. Sweeney et al. used an immunomagnetic capture technique to detect *Mycobacterium bovis* in naturally infected environmental samples of soil, feces and urine (Sweeney et al., 2006). In addition, molecular detection techniques have been used to effectively detect the presence of other organisms, including Escherichia coli O157:H7 in soil and water (Campbell et al., 2001), and Salmonella typhimurium (Marsh et al., 1998) and Mycoplasma species (Marois et al., 2002) in soil, thus avoiding the need to selectively cultivate these organisms.

1.4 CONCLUSIONS

The re-emergence of bovine TB in the state of Michigan has been a major concern since the late 1990s. White-tailed deer have become a reservoir of *M. bovis* and the most likely source of infection for Michigan's cattle herds. Transmission of *M. bovis* through direct nose-to-nose contact is thought to be the most effective form of transmission between the species, although this type of contact has been rarely noted in studies. Indirect transmission of the disease through contaminated environmental elements is thought to play a potential role in the spread of the disease between white-tailed deer and cattle.

Although bacterial culture is the standard for identifying *M. bovis* in environmental samples, the process comes with many limitations that affect detectability of the organism. Environmental substrates are often contaminated with other organisms that may out-compete and overgrow *M. bovis* during the culture process, potentially leading to false negative results. Decontamination methods used to prevent contamination have also been shown to reduce viability of mycobacterium species and increase the minimum incubation time required to detect positive cultures (Palomino and Portaels, 1998; Corner et al., 2012).

Molecular detection techniques may provide a more beneficial tool for quantifying and characterizing the persistence of *M. bovis* within the environment. Marois et al. compared detection results of *Mycoplasma synoviae* in poultry environmental samples by culture and PCR (Marois et al., 1999). Results show a significant increase in the number of positive samples obtained by PCR over those obtained by culture. Thacker et al. concluded that the use of a PCR assay may provide a

more rapid method than culture for providing diagnostic test results for the detection of *M. bovis* in tissue samples (Thacker et al., 2011).

Determining the best technique for the detection of *M. bovis* in the environment is a vital step to understanding the persistence and transmission of the disease. Proper and effective detection techniques will enhance epidemiological investigations of TB farms and wildlife sites allowing for a better understanding of indirect transmission of bovine TB and its role in the epidemiology of the disease.

Chapter 2

COMPARISON OF MOLECULAR TECHNIQUES WITH TRADITIONAL BACTERIAL CULTURE FOR THE DETECTION OF *MYCOBACTERIUM BOVIS* IN EXPERIMENTALLY INOCULATED SUBSTRATES

2.0 Abstract

Objective: To test the utility of a polymerase chain reaction (PCR) assay for detection of M. bovis in samples of soil, hay and water and to compare results with the traditional diagnostic procedure of bacterial culture.

Design: Experimental inoculation

Sample Population: *M. bovis* inoculated substrates of hay, soil and water previously processed for isolation of *M. bovis* by mycobacterial culture.

Procedure: A PCR assay was chosen and applied to environmental substrates previously experimentally inoculated with varying concentrations of *M. bovis* and stored under various times and temperatures. *M. bovis* recovery by PCR was compared to recovery obtained by mycobacterial culture.

Results: PCR was statistically equivalent to bacterial culture for detection of *M. bovis* in samples of water and hay. The PCR assay was most effective for detection of *M. bovis* in soil samples regardless of concentration of the inoculum, storage time, or storage temperature.

Conclusion: Contaminating organisms often overgrow and out-compete mycobacteria during the culture process, reducing overall detection rates of certain environmental

substrates. However, the PCR assay is consistent for detecting *M. bovis* across all substrate types.

2.1 INTRODUCTION

M. bovis has been of major concern in the state of Michigan since the late 1990s, when a case of bovine TB in cattle was confirmed in the northeast lower peninsula of Michigan (O'Brien et al., 2006). This was the first case of bovine TB in the state since Michigan was declared TB-free in 1979 (Miller and Kaneene, 2006). It is suspected that for a majority of the cattle farms identified as bovine TB positive in this same region of Michigan, *M. bovis* infection was caused by a spillover from white-tailed deer (*Odocoileus virginianus*) to cattle. It is thought that indirect transmission of *M. bovis* through contaminated substrates plays a role in the ongoing transmission of *M. bovis* between wildlife and cattle in Michigan (DeLiberto et al., 2004; Kaneene et al., 2002; Miller et al., 2003; O'Brien, et al., 2002).

Attempts to isolate *M. bovis* from presumed naturally infected environmental samples by bacterial culture have failed (Cooney et al., 1997; Livanainen, 1995; Livanainen et al., 1999; Palmer and Whipple, 2000; Fine et al., 2011b). However, using molecular detection techniques, Young et al. detected *M. bovis* DNA in soil from a farm environment up to 12 months after possible contamination (Young et al., 2005).

With the use of conventional bacterial culture as the method of detection, numerous papers have focused on the persistence of *Mycobacterium bovis* in the environment using experimental study designs. Fine et. al. conducted a study concerning the persistence of *M. bovis* in experimentally inoculated substrates exposed to natural weather conditions (Fine et. al., 2011a). Survival of *M. bovis* was detected for up to 88

days in soil, 58 days in water and hay and 43 days on corn using conventional mycobacterial culture techniues. Williams and Hoy conducted early studies concerning the persistence of *M. bovis* in feces shed by an infected cow (Williams and Hoy, 1930). Survival of *M. bovis* was detected for up to four months when contaminated feces were spread on pasture and periodically monitored, using bacterial culture. That same group also reported the recovery of *M. bovis* from naturally contaminated feces after 12 months of storage in cool, dark conditions and *M. bovis* was recovered after 2 years from experimentally inoculated feces stored under similar conditions. Other studies have shown *M. bovis* can persist in the environment for periods ranging from four weeks to six months (Duffield and Young, 1985; Maddock, 1933; Maddock, 1934; Whipple and Palmer, 2000).

These experimental studies have shown that under experimental conditions, *M. bovis* survival times are longer in controlled storage conditions versus the survival times of experimentally inoculated substrates stored outdoors under natural environmental conditions. Survival times for *M. bovis* appear to be shortened with exposure to seasonal environmental factors including higher ambient temperatures, increased intensity of solar radiation and higher loss of moisture through evapotranspiration (Duffield and Young, 1985; Fine et al., 2011). In contrast, other experimental studies report minimal persistence in the environment (Little et al., 1982) and conclude that environmental contamination does not play a major role in the epidemiology of bovine tuberculosis in cattle and wildlife in New Zealand (Jackson et al., 1995).

The current study used molecular detection techniques on a set of experimentally inoculated environmental substrates exposed to varying controlled storage conditions and previously tested with bacterial culture (Fine, 2006). The purpose of the study was to

optimize a PCR procedure for *M. bovis* isolation from environmental samples and compare the results to those obtained previously using bacterial culture methods for *M. bovis* detection. We hypothesized that molecular detection techniques would improve detection of *M. bovis* in soil, hay, water and similar substrates found on a farm enabling a more accurate characterization of the distribution of *M. bovis* in farm environments.

2.2 MATERIALS AND METHODS

2.2.1 Samples used for Molecular Testing

A total of 202 inoculated samples of water, soil and hay were processed for *M. bovis* detection by PCR. The samples were obtained from a previous study designed to compare two techniques for processing environmental samples for *M.* bovis isolation by bacterial culture (Fine et al, 2006).

2.2.2 Sample Processing

The Michigan straing of *M. bovis* was obtained from a frozen culture originally isolated in 2001 from a 5-year old cow from northeast Michigan. Samples were either inoculated as is or sterilized prior to inoculation. Each group of samples was inoculated for varying concentrations of *M. bovis* ranging from <10CFU to 10,000CFU. Samples were then stored at controlled temperatures [-20ºC, 4ºC, and room temperature (20-25ºC)] for up to 19 days. Samples were processed for bacterial culture using one of two chemical decontamination methods (CB-18 or NaOH) as described by Fine (Fine, 2006). Prior to further processing, a 0.5 mL sample was transferred to a 2.0 mL labeled cryogenic vial and frozen at -80 ºC. This 0.5mL sample was stored for later DNA-based

PCR processing and testing. The remainder of each sample was further processed for bacterial culture (Fine et al, 2006).

2.2.3 *DNA Extraction*

Samples previously frozen at -80 ºC were thawed for DNA extraction. To extract bacterial DNA, a 150-µL aliquot was removed from each thawed sample and transferred to a 1mL microcentrifuge tube. The tubes were then sonicated for 15-minutes, boiled for 10-minutes, flash frozen in an ethanol dry ice bath, boiled for another 5-minutes, then centrifuged for 10-minutes at 10,000 g. The supernatant was removed from each tube and stored frozen at -20ºC until used in polymerase chain reaction (PCR) assays.

2.2.4 *PCR Procedure*

Preliminary experiments were done using 6 published sets of PCR primers. This included 1 primer set each targeting mycobacterial genes encoding Hsp65, mpb64, and mpb70; as well as insertion sequence 1810, and 2 primer sets targeting insertion sequence (IS) 6110 (Young et al., 2005; Zumárraga et al., 2005). Our preliminary experiments indicated that the 2 primer sets targeting the IS 6110 insertion sequence were more efficient at detecting *M. bovis* DNA in known positive samples than the primer sets targeting the Hsp65, mpb64 and mpb70 genes or the IS 1810 insertion sequence. Young et al. also indicated this point within their study (Young et al., 2005). PCR primers may produce false positive results from the contamination of negative samples with positive DNA or from a non-optimized amplification program, which can cause non-specific binding and/or the formation of primer dimers (Noordhoek, 1994). After evaluating these previously published procedures and in an attempt to increase our assay sensitivity, we

created a nested PCR procedure using a mix of the two PCR primer sets targeting IS6110. In our hands, this nested PCR assay proved to be the most sensitive at detecting *M. bovis* DNA when working with processed environmental samples.

The nested PCR amplification was done using an initial 25-µL reaction mixture containing 12.5µL Promega GoTaq® Green 2X Master Mix, 7.1µL water, and 5µL DNA with 0.2 µL each of forward outer primer CGTGAGGGCATCGAGGTGGC and reverse outer primer CCTGCGAGCGTAGGCGTCGG . This primer set targeted the IS6110 insertion sequence found in the *M. bovis* genome and amplified a product of 252-bp. The initial denaturation and enzyme activation step of 94ºC for 4 minutes was followed by 20 cycles of 94ºC for 30 seconds, 67ºC for 30 seconds, 72ºC for 30 seconds; and a final extension step of 72ºC for 5 minutes. The second PCR reaction used a 1µL aliquot from the completed first PCR reaction added to a reaction mixture containing 12.5µL Promega GoTaq® Green 2X Master Mix and 10.7µL water with 0.4µL each of inner forward primer CTCGTCCAGCGCCGCTTCGG and inner reverse primer GCGTAGGCGTCGGTGACAAA that produced a product of 116-bp (figure 2.1). The reaction conditions for the second PCR were 94ºC for 4 minutes; 40 cycles of 94ºC for 30 seconds, 65ºC for 30 seconds, and 72ºC for 30 seconds; and a final extension step of 72ºC for 5 minutes. Gel electrophoresis of the product from the second PCR was done using a 1.5% agarose gel and ethidium bromide for detection of DNA (figure 2.2). The PCR amplicons were verified as being from *Mycobacterium tuberculosis* complex by nucleic acid sequencing followed by *in silico* analysis using the Basic Local Alignment Search Tool available from the National Center for Biotechnology Information.

2.3 RESULTS

A total of 202 samples (67 water samples, 67 hay samples and 68 soil samples) from the CB-18/NaOH comparison study were analyzed for detection of *M. bovis* DNA using a nested PCR assay. *M. bovis* was detected in 48 out of 67 water samples, 50 out of 67 hay samples and 49 out of 68 soil samples. Figure 2.3 compares *M. bovis* detection by PCR with the detection rates obtained by culture within the same set of samples. Table 2.1 compares sensitivity and specificity estimates of PCR to the standard culture method. Results are displayed by substrate type.

Figure 2.1 Nested PCR targeting the IS6110 gene in *M. bovis* DNA, producing a final product amplicon size of 116bp

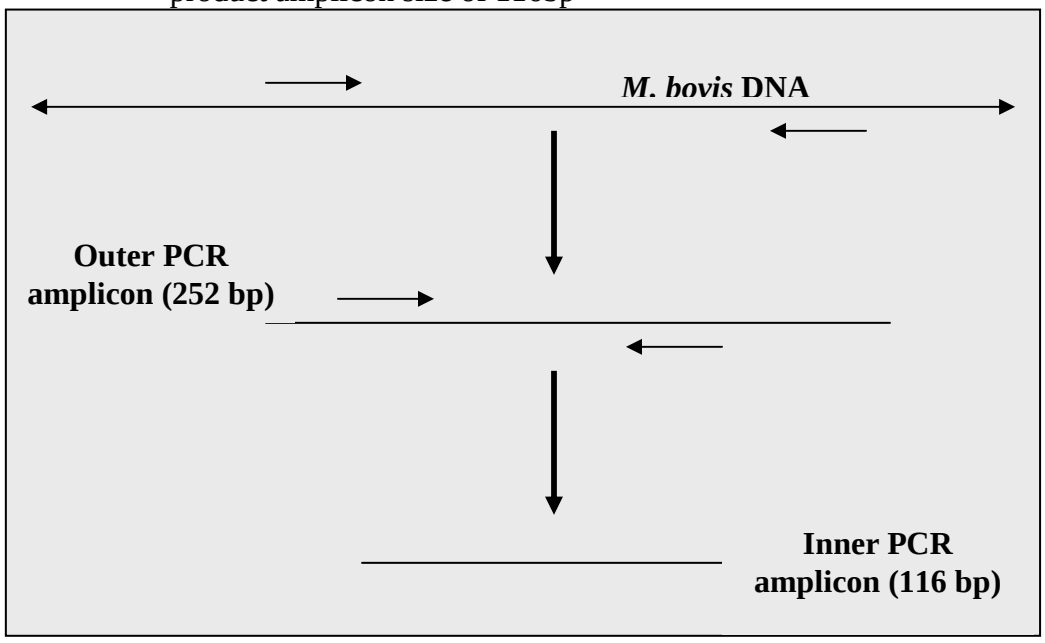


Figure 2.2 Gel electrophoresis performed on 1.5% agarose gel with ethidium bromide

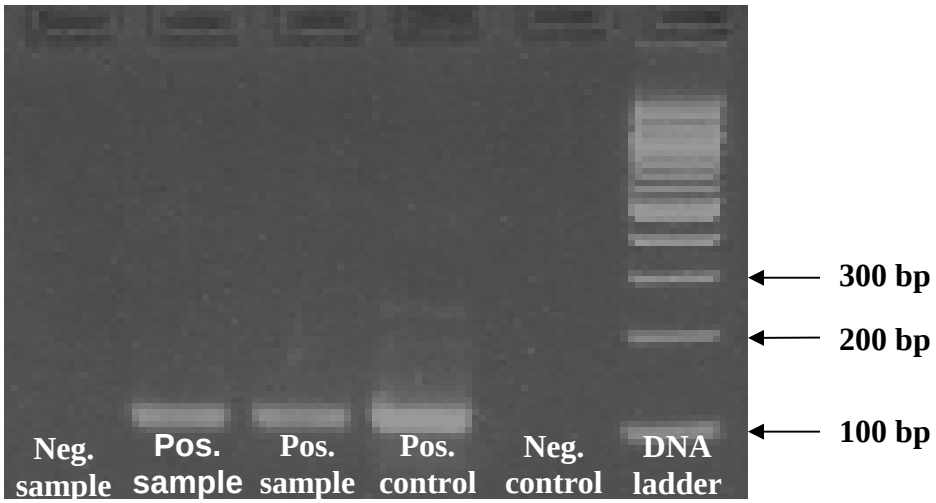
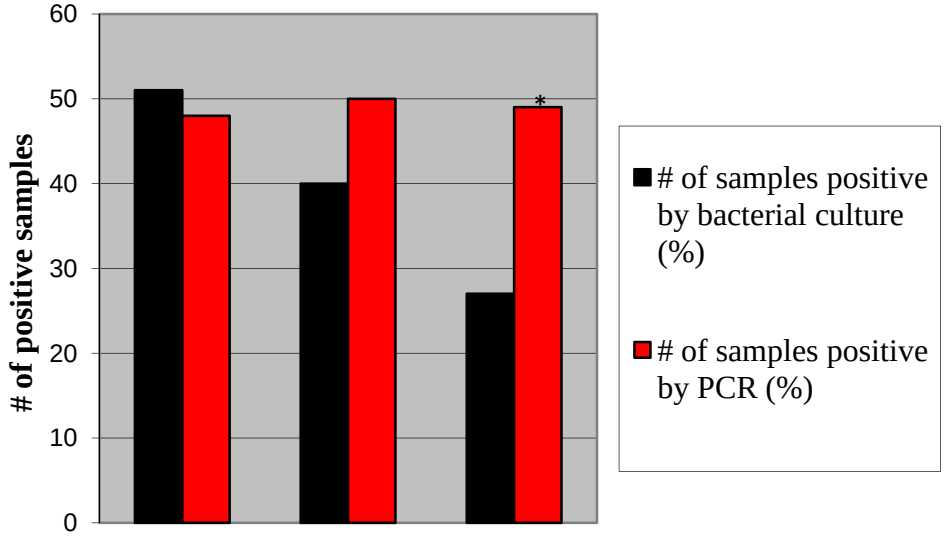


Figure 2.3 - Number of samples positive for the detection of *M. bovis* (*For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.)*



*denotes statistically significant difference (p-value <0.05)

Table 2.1 - PCR sensitivity and specificity estimates for water, hay and soil based on culture results.

	Water (N=67)		
	Culture Positive	**Culture Negative**	**Totals**
PCR Positive	45	3	48
PCR Negative	6	13	19
Totals	51	16	67
	sensitivity = 88.24%	specificity = 81.25%	

	Hay (N=67)		
	Culture Positive	**Culture Negative**	**Totals**
PCR Positive	37	13	50
PCR Negative	3	14	17
Totals	40	27	67
	sensitivity = 92.5%	specificity = 51.85%	

	Soil (N=68)		
	Culture Positive	**Culture Negative**	**Totals**
PCR Positive	24	25	49
PCR Negative	3	16	19
Totals	27	41	68
	sensitivity = 88.89%	specificity = 39.02%	

2.4 DISCUSSION

While working with our initial PCR primer sets, we found that two PCR primer pairs, each targeting the 6110 insertion sequence found within the *M. bovis* genome, could be combined and mixed to create a nested PCR assay. In our preliminary experiments, this newly created nested PCR primer set proved to be more sensitive than the other primer pairs we originally tested that targeted other areas within the *M. bovis* genome. Nested PCR increases test sensitivity by using a second reaction to re-amplify the product from the first reaction. This procedure also increases test specificity because the inner primers only amplify if the first reaction yields a specific PCR amplicon.

Our nested PCR assay produced an initial reaction product of 252 base pairs and a final amplicon of 116 base pairs. As DNA degrades over time, targeting a smaller-sized amplicon may prove to be beneficial when looking for evidence of *M. bovis,* which might have been present in the environment for months before testing.

PCR was essentially equivalent to bacterial culture for detection of *M. bovis* in samples of water and hay. PCR was most effective for detection of *M. bovis* in soil samples regardless of concentration of inoculum, storage time, or storage temperature. Soil was also the substrate that was most likely to produce contamination during the culture process, regardless of sample decontamination method.

Contamination plays a major role in the culture process of *Mycobacterium bovis* and samples collected from the environment are likely to be contaminated with non-mycobacteria species. Contaminating organisms often overgrow and out-compete mycobacteria during the culture process, reducing overall detection rates of certain environmental substrates leading to false negatives. However, the optimized nested PCR assay we developed was consistent for detecting *M. bovis* across all substrate types,

regardless of processing method or storage variables. Since our nested PCR procedure targets a DNA sequence specific to the mycobacterium tuberculosis complex (MTBC), contamination from other organisms is of little concern to the PCR process. Another strength of PCR is its ability to detect lower concentrations of *M. bovis* from environmental samples as compared to culture. Optimizing the PCR amplification process can improve the sensitivity of a PCR assay, reducing the occurrence of false negative results. Optimized molecular detection techniques provide a valuable tool in epidemiologic investigations involving samples collected from the outside environment.

Chapter 3

COMPARISON OF MOLECULAR DETECTION TECHNIQUES AND BACTERIAL CULTURE: A STUDY OF THE DURATION OF DETECTION OF *MYOCOBACTERIUM BOVIS* IN THE ENVIRONMENT

3.0 ABSTRACT

Objective: To characterize the persistence of the Michigan strain of *Mycobacterium bovis* in the environment under natural weather conditions and compare results using molecular detection techniques and bacterial culture.

Design: Experimental Study

Sample Population: Environmental substrates previously inoculated with *Mycobacterium bovis*, exposed to natural weather conditions and examined over varying lengths of time up to 12 months.

Procedure: PCR was compared with bacterial culture for the detection of *M. bovis* from experimentally inoculated environmental substrates in an attempt to characterize the persistence of *M. bovis* in the environment.

Results: *M. bovis* was detected by PCR in experimentally inoculated samples up to 10 months after exposure to natural weather conditions in hay, 11 months after exposure to natural weather conditions in water, 8 months after exposure to natural weather conditions in soil and 9 months after exposure to natural weather conditions in corn. In contrast, *M. bovis* was not detected by culture longer than 2 months post-inoculation in any water, hay or corn and no longer than 88 days in soil.

Conclusions and Clinical Relevance: PCR was able to detect *M. bovis* in experimentally inoculated substrates exposed to natural weather conditions up to 9 months longer than culture. PCR provides quicker results than culture and is not hindered by contaminating organisms. PCR also gives information about the presence and distribution of *M. bovis* in the environment much longer after the initial contamination than culture. In epidemiologic investigations of TB farms and wildlife sites, PCR-based assays may be useful for parallel testing with bacterial culture to enhance detection of *M. bovis* in the environment.

3.1 INTRODUCTION

Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, has the largest host range among pathogenic mycobacteria and is capable of infecting both domestic and wild mammals, as well as humans (Ojo et al., 2008). Bovine TB is transmitted to humans orally through ingestion of raw milk as well as nasally through inhalation of infectious droplet nuclei (Thoen and Barletta, 2005; Thoen et al., 2009). The oral route of infection in humans has become of less concern with the growing practice of milk pasteurization among developed countries. However, airborne infection is still of major concern among slaughterhouse and other meat industry workers in countries where *M. bovis* remains prevalent in its cattle herds (Thoen et al., 2006; Thoen et al., 2009). Bovine tuberculosis results in severe economic losses in the livestock industry in many countries worldwide due to decreased production and increased mortality, as well as condemnation of infected carcasses. It also puts restrictions on the international trade of animals and animal products (Suazo et al., 2003).

M. bovis has been of concern in Michigan since the late 1990s, when a case of bovine TB in cattle was confirmed in the northeast lower peninsula of Michigan (O'Brien et al., 2006). This was the first case of bovine TB in the state since Michigan was declared TB-free in 1979 (Miller and Kaneene, 2006).

It is suspected that for a majority of the cattle farms identified as bovine TB positive in this same region of Michigan, *M. bovis* infection was caused by a spillover from white-tailed deer (*Odocoileus virginianus*) to cattle. Bovine TB likely became endemic in deer when there were large numbers of *M. bovis* infected cattle in the state during the late 1950s (Miller and Kaneene, 2006). It is thought that indirect transmission of *M. bovis* through contaminated substrates plays a role in the ongoing transmission of *M. bovis* between wildlife and cattle in Michigan (DeLiberto et al., 2004; Kaneene et al., 2002; Miller et al., 2003; O'Brien, et al., 2002).

Using molecular detection techniques, Young et al. detected *M. bovis* DNA in soil from a farm environment up to 12 months after possible contamination (Young et al., 2005). Marois et al. compared detection results of *Mycoplasma synoviae* in poultry environmental samples by culture and PCR (Marois et al., 2000). Results show a significant increase in the number of positive samples obtained by PCR over those obtained by culture. Thacker et al. concluded that the use of a PCR assay may provide a more rapid method than culture for providing diagnostic test results for the detection of *M. bovis* in tissue samples (Thacker et al., 2011). Although these studies have shown the benefits of molecular techniques over traditional culture methods, studies have yet to compare results of *M. bovis* detection in experimentally inoculated environmental substrates.

A study designed to identify and collect samples from Michigan TB positive farms failed to isolate *M. bovis* from collected samples using bacterial culture (Fine et al., 2011b). Other attempts to isolate *M. bovis* from presumed naturally infected environmental samples by bacterial culture have also failed (Cooney et al., 1997; Livanainen, 1995; Livanainen et al., 1999; Palmer and Whipple, 2000; Witmer et al., 2010). Numerous papers have focused on the persistence of *Mycobacterium bovis* in the environment using experimental study designs. In a study conducted by Fine et al., environmental substrates were experimentally inoculated with *M. bovis* and exposed to natural weather conditions in Michigan (Fine et al., 2011a). *M. bovis* persisted up to 88 days in soil, 58 days in water and hay, and 43 days on corn as detected by bacterial culture. Williams and Hoy conducted early studies concerning the persistence of *M. bovis* in feces shed by an infected cow (Williams and Hoy, 1930). Survival of *M. bovis* was detected for up to four months when contaminated feces were spread on pasture and monitored. The recovery of *M. bovis* from naturally contaminated feces after 12 months of storage in cool, dark conditions also was reported and *M. bovis* was recovered after 2 years from experimentally inoculated feces stored under similar conditions. Other studies have shown *M. bovis* can persist in the environment for periods ranging from four weeks to six months (Duffield and Young, 1985; Maddock, 1933; Maddock, 1934; Whipple and Palmer, 2000). Survival times for *M. bovis* appear to be shortened with exposure to seasonal environmental factors including higher ambient temperatures, increased intensity of solar radiation and higher loss of moisture through evapotranspiration (Duffield and Young, 1985; Fine et al., 2011a). Other experimental studies have reported minimal persistence in the environment (Little et al., 1982) and conclude that

environmental contamination does not play a major role in the epidemiology of bovine tuberculosis in cattle and wildlife in New Zealand (Jackson et al., 1995).

The current study used molecular detection techniques on a set of experimentally inoculated environmental substrates exposed to natural, varying weather conditions in Michigan. That set of experimentally inoculated environmental samples were previously tested using bacterial culture (Fine et al., 2011a). The purpose of the study was to compare the results of using molecular techniques (detection of the presence of DNA from *M. bovis*) with those previously obtained through bacterial culture. We hypothesized that molecular detection techniques would improve detection of *M. bovis* in soil, hay, water and similar substrates found on a farm enabling a more accurate characterization of the distribution of *M. bovis* in farm environments.

3.2 MATERIALS AND METHODS

3.2.1 Samples used for Molecular Testing

A total of 687 inoculated samples of hay, soil, corn and water were processed for *M. bovis* detection by PCR. The samples were obtained from a previous study designed to evaluate the persistence of *M. bovis* in environmental substrates under natural weather conditions in Michigan (Fine et al, 2011a). Half of the samples in each group of environmental substrates were autoclaved for sterilization. Thus, each group of environmental substrate contained 2 sterile and 2 non-sterile samples of grass hay, soil, water, and shelled corn. Each environmental substrate was inoculated with 50,000 colony forming units (CFUs) of a strain of *M. bovis* isolated from a cow in Michigan. The groups of inoculated environmental substrates were transported to an outdoor experimental enclosure and placed within plastic containers on 2 stainless steel tables.

One steel table was shaded and the other table was exposed to direct sunlight (Table 3.1). Four time periods were used in the study. The first sampling period spanned 12 months from November 2004 through November 2005. During this sampling period, samples of each environmental substrate were processed monthly for isolation of *M. bovis*. The remaining three sampling periods covered up to 12 weeks each and began on November 8, 2004, February 4, 2005 or May 20, 2005. During each sampling period, samples were processed at the time of inoculation and then at 11 additional time points during the sampling period.

Table 3.1 Sample layout for placement of the inoculated environmental substrates within the secured outdoor enclosure.

Stainless Steel Table A	
Shade	Shade
Sterile samples of grass hay, shelled corn, soil, and water inoculated with *M. bovis*	Non-sterile samples of grass hay, shelled corn, soil, and water inoculated with *M. bovis*

Stainless Steel Table B	
Sunlight	Sunlight
Sterile samples of grass hay, shelled corn, soil, and water inoculated with *M. bovis*	Non-sterile samples of grass hay, shelled corn, soil, and water inoculated with *M. bovis*

3.2.2 *Sample Processing*

After samples were collected from the experimental enclosure, they were processed within their original container using a standardized protocol by Fine et al. for processing environmental samples for mycobacterial culture (Fine, 2006). Samples were pulverized and homogenized for 30-seconds on the high setting of a household blender. Samples were placed upright and allowed to settle for 30-minutes. The top 5 mL of fluid from each sample was removed and transferred to a 50-mL conical tube containing 10-mL of Decontamination Solution. Samples were mixed by vortex and incubated at 37 ºC for 75 minutes. Sterile water was added to the 50-mL mark on each tube, mixed and centrifuged at 3,000 g for 20-minutes. Pellet-containing tubes were completely decanted and a pipette was used to remove all but 1-3 mL of liquid from samples without a visible pellet. The pellet was suspended in the supernatant backwash. One mL of sterile water was added and mixed. A 0.5 mL sample was transferred to a 2.0 mL labeled cryogenic vial and frozen at -80 ºC. This 0.5mL sample was stored separately for later DNA-based PCR processing and testing. The remainder of each sample was further processed for bacterial culture (Fine et al, 2011a).

3.2.3 *DNA Extraction*

Samples previously frozen at -80 ºC were thawed for DNA extraction. To extract bacterial DNA, a 150-µL aliquot was removed from each thawed sample and transferred to a 1mL microcentrifuge tube. The tubes were then sonicated for 15-minutes, boiled for 10-minutes, flash frozen in an ethanol dry ice bath, boiled for another 5-minutes, then centrifuged for 10-minutes at 10,000 g. The supernatant was removed from each tube and stored frozen at -20ºC until used in polymerase chain reaction (PCR) assays.

3.2.4 *PCR Procedure*

Nested PCR amplification was done using an initial 25-µL reaction mixture containing 12.5µL Promega GoTaq® Green 2X Master Mix, 7.1µL water, and 5µL DNA with 0.2 µL each of forward outer primer CGTGAGGGCATCGAGGTGGC and reverse outer primer CCTGCGAGCGTAGGCGTCGG . This primer set targeted the IS6110 insertion sequence found in the *M. bovis* genome and amplified a product of 252-bp. The initial denaturation and enzyme activation step of 94ºC for 4 minutes was followed by 20 cycles of 94ºC for 30 seconds, 67ºC for 30 seconds, 72ºC for 30 seconds; and a final extension step of 72ºC for 5 minutes. The second PCR reaction used a 1-µL aliquot from the completed first PCR reaction added to a reaction mixture containing 12.5µL Promega GoTaq® Green 2X Master Mix and 10.7µL water with 0.4µL each of inner forward primer CTCGTCCAGCGCCGCTTCGG and inner reverse primer GCGTAGGCGTCGGTGACAAA that produced a product of 116-bp. The reaction conditions for the second PCR were 94ºC for 4 mins; 40 cycles of 94ºC for 30 sec, 65ºC for 30 sec, and 72ºC for 30 sec; and a final extension step of 72ºC for 5 minutes. Gel electrophoresis of the product from the second PCR was done using a 1.5% agarose gel and ethidium bromide for detection of DNA. The PCR amplicons were verified as being from *Mycobacterium tuberculosis* complex by nucleic acid sequencing followed by *in silico* analysis using the Basic Local Alignment Search Tool available from the National Center for Biotechnology Information.

3.2.5 *Data Analysis*

Results from bacterial culture (Fine et al, 2011a) were compared with the results of PCR assays done in the current study by season and substrate type using Fisher's Exact Test. Differences were considered significant if the Fisher's Exact Test calculates a p-value of ≤ 0.05. The survival functions of *M. bovis* DNA detected by PCR were plotted using SAS statistical software (SAS PROC LIFETEST; SAS version 10.0, Cary N.C.: SAS Institute, Inc.).

3.3 RESULTS

A total of 128 samples from the 12-month study (lasting from November 2004 through October 2005) were analyzed for detection of *M. bovis* DNA using a nested PCR assay. A total of 176 samples (44 samples each of water, hay, corn and soil) from the Winter Sampling period "A", beginning November 8, 2004 and ending January 6, 2005, were analyzed for detection of *M. bovis* DNA using a nested PCR assay. 191 samples (48 samples each of water, hay, and soil and 47 samples of corn) were analyzed from the Winter/Spring Sampling period "B" beginning February 4, 2005 and ending May 3, 2005 and 192 samples (48 samples each of water, hay, corn and soil) were analyzed from the Summer Sampling period "C" beginning May 20, 2005 and ending August 2, 2005.

The DNA from *M. bovis* was detected by PCR in 42% (73/176) of the samples from Winter sampling period "A", 38% (73/191) of the samples from Winter/Spring sampling period "B" and 22% (43/192) of the samples from Summer sampling period "C" (Table 2). Sampling period "A" was, on average, the coldest of the three sampling periods and Sampling Period "C" was the warmest.

For water samples (n=140) across all sampling periods, *M. bovis* was detected in 35% (n=49) of the samples by PCR and in 37.86% (n=53) by bacterial culture. In hay samples (n=140) across all sampling periods, *M. bovis* was detected in 56.43% (n=79) of the samples by PCR and in 45% (n=63) by bacterial culture. Across all soil samples (n=140), *M. bovis* was detected in 26.43% (n=37) by PCR and in 47.86% (n=67) by bacterial culture. Across all corn samples (n=139 PCR, n=140 bacterial culture) *M. bovis* was detected in 18.71% (n=26) by PCR and in 30% (n=42) by bacterial culture (see Table 2). *M. bovis* DNA was detected by PCR up to 88 days for all sample types within the Winter/Spring Sampling period. *M. bovis* was detected by bacterial culture up to 88 days within the same sampling period, but only in soil samples (Figure 3.1). Figure 3.2 shows the survival curve of each substrate type combining PCR results from sampling periods "A" (November 8 – January 5), "B" (February 4 – May 3) and "C" (May 20 – August 2).

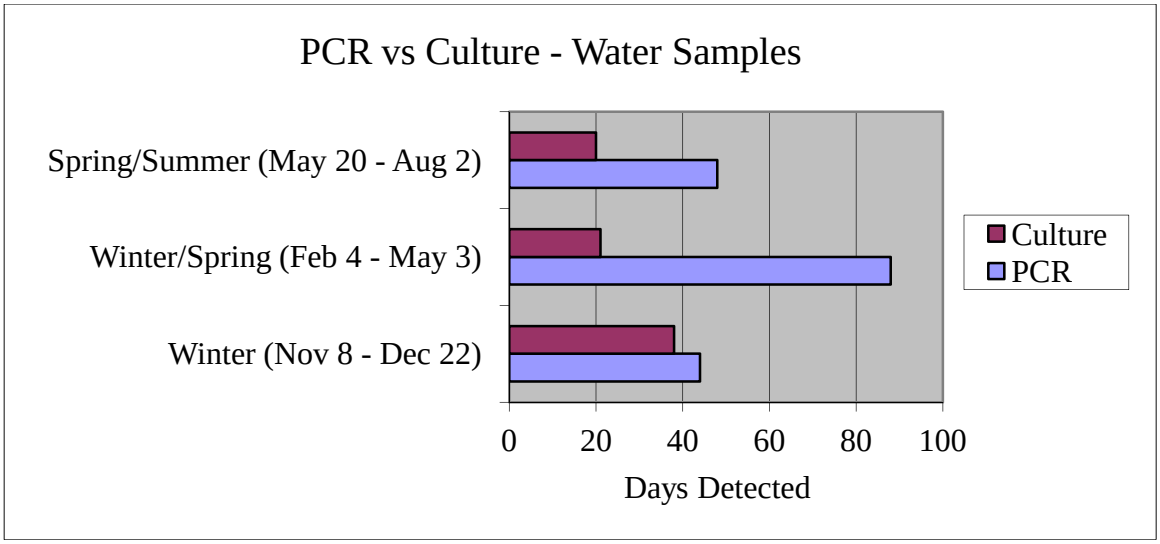
Within the 12-month study, *M. bovis* was not detectable by culture in any sample type after month 2 (see Figure 3.3). In contrast, *M. bovis* DNA was detectable by PCR up to 8 months within soil samples exposed to natural weather conditions, 9 months within corn samples exposed to natural weather conditions, 10 months within hay samples exposed to natural weather conditions, and up to 11 months within water samples exposed to natural weather conditions. Figure 3.5 shows the survival curve of each substrate type comparing PCR to culture results for samples processed from the 12-month study.

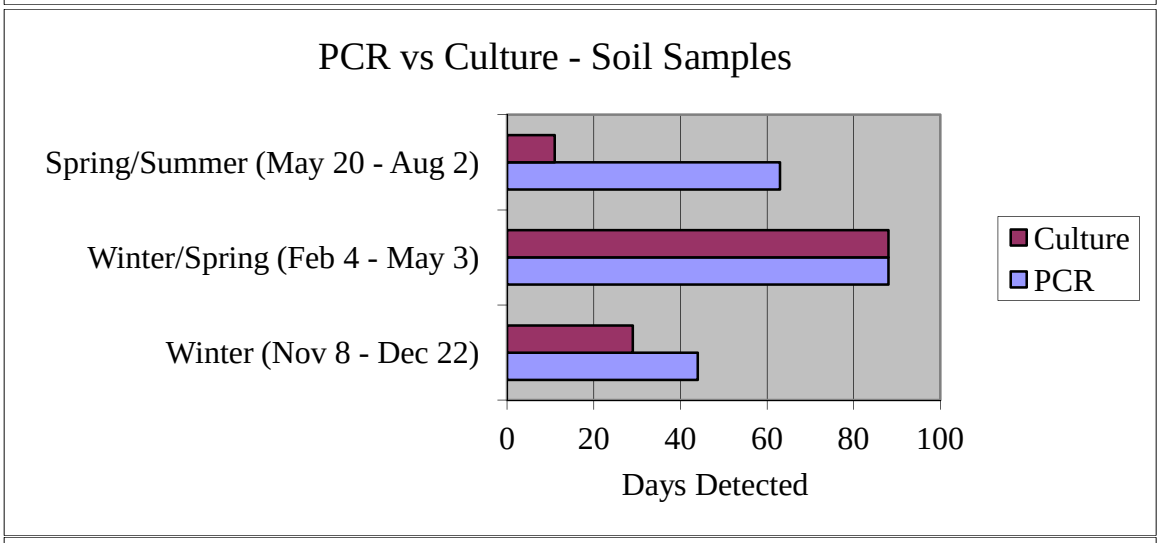
Table 3.2 Number of *M. bovis* positive samples by PCR and Culture within each season. P-values ≤0.05 obtained by the Fisher's Exact Test indicate a significant difference between PCR and culture results. Significant values are denoted by an asterisk (*).

Season	Substrate	PCR(+)	Culture(+)	P-value
Winter	Water	24	35	0.022*
Winter	Hay	25	40	<0.001*
Winter	Soil	17	30	0.009*
Winter	Corn	9	26	<0.001*
Winter/Spring	Water	17	8	0.061
Winter/Spring	Hay	36	19	<0.001*
Winter/Spring	Soil	10	28	<0.001*
Winter/Spring	Corn	10	10	>0.999
Spring/Summer	Water	8	10	0.794
Spring/Summer	Hay	18	4	0.001*
Spring/Summer	Soil	10	9	>0.999
Spring Summer	Corn	7	6	>0.999
Combined	Water	49	53	0.709
Combined	Hay	79	63	0.072
Combined	Soil	37	67	<0.001*
Combined	Corn	26	42	0.035*

Season Key:
Winter: …….. Season "A": November 4 – January 5
Winter/Spring:……Season "B": February 4 – May 3
Spring/Summer: …… Season "C": May 22 – August 2

Figure 3.1 Days *M. bovis* was detected by PCR and culture in water, soil, hay and corn samples within each sampling season. Seasonal sampling durations: Winter – 44 days; Winter/Spring – 88 days; Spring/Summer – 74 days.





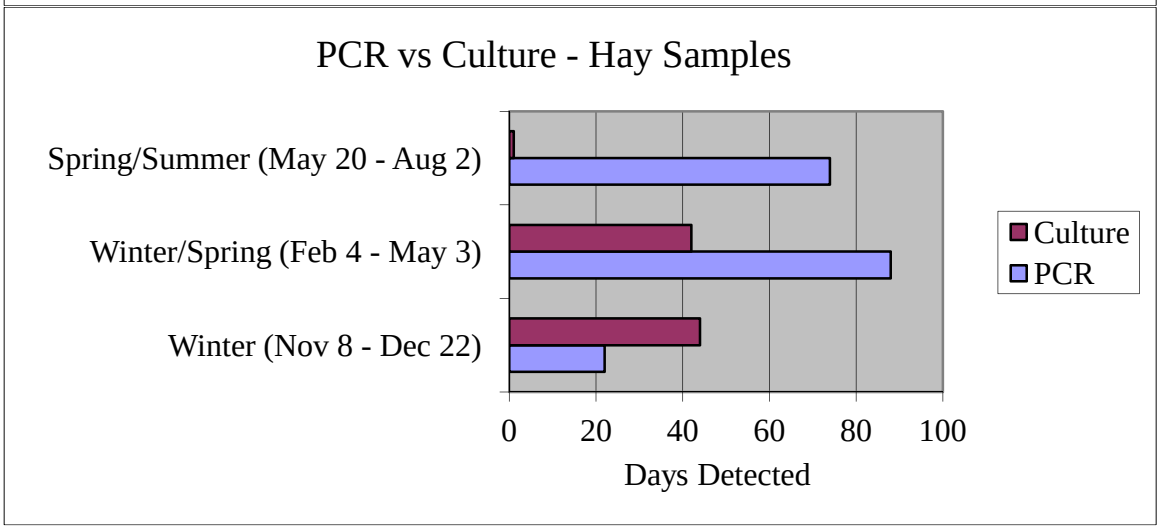


Figure 3.1(cont'd)

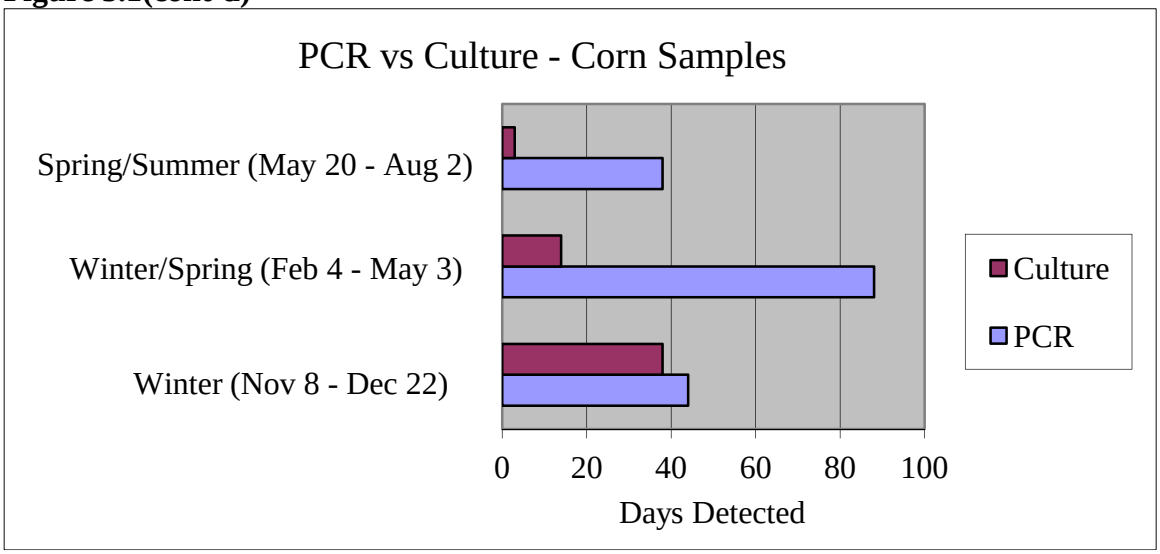


Figure 3.2 Survival Curve – Rate of detection, by PCR, of *M. bovis* DNA exposed to natural environmental conditions across all three 3-month sampling periods; N=48.

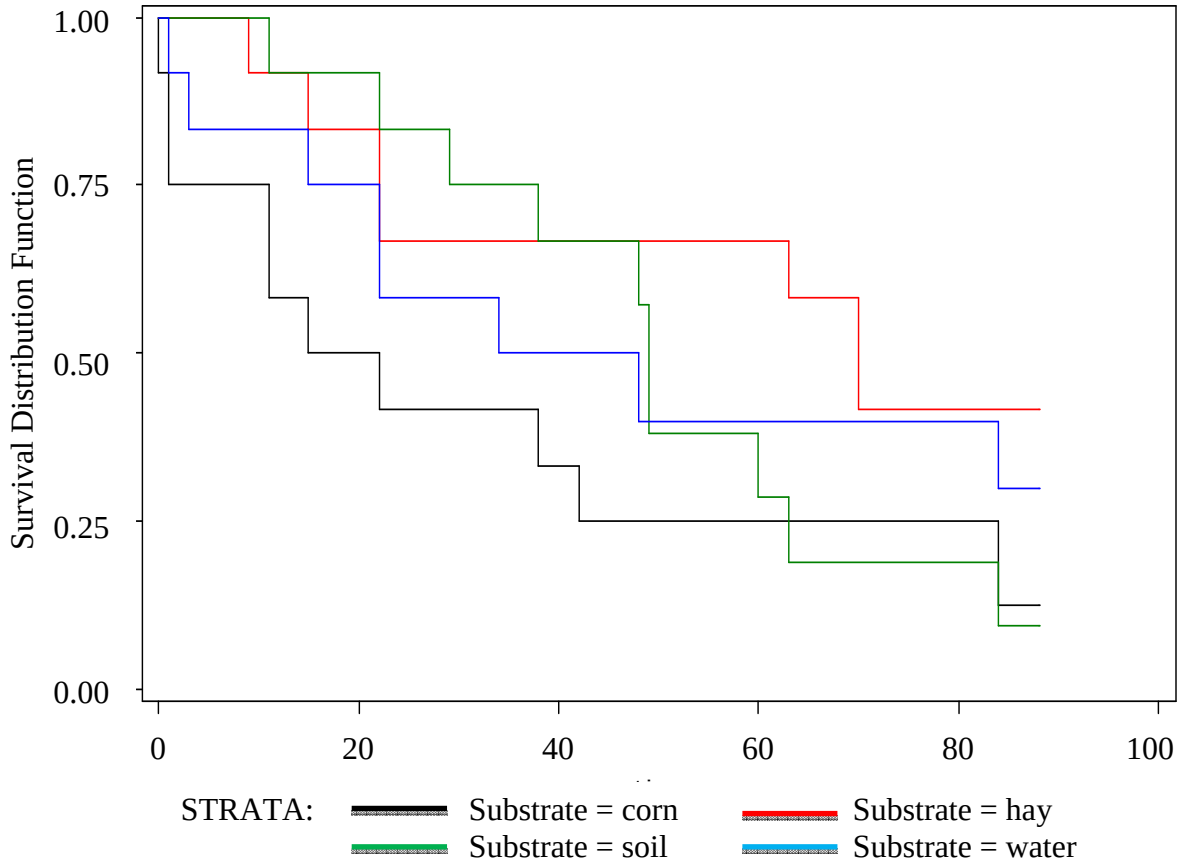


Figure 3.3 Months *M. bovis* was detected by PCR and culture in water, hay, soil and corn samples during the 12-month persistence study

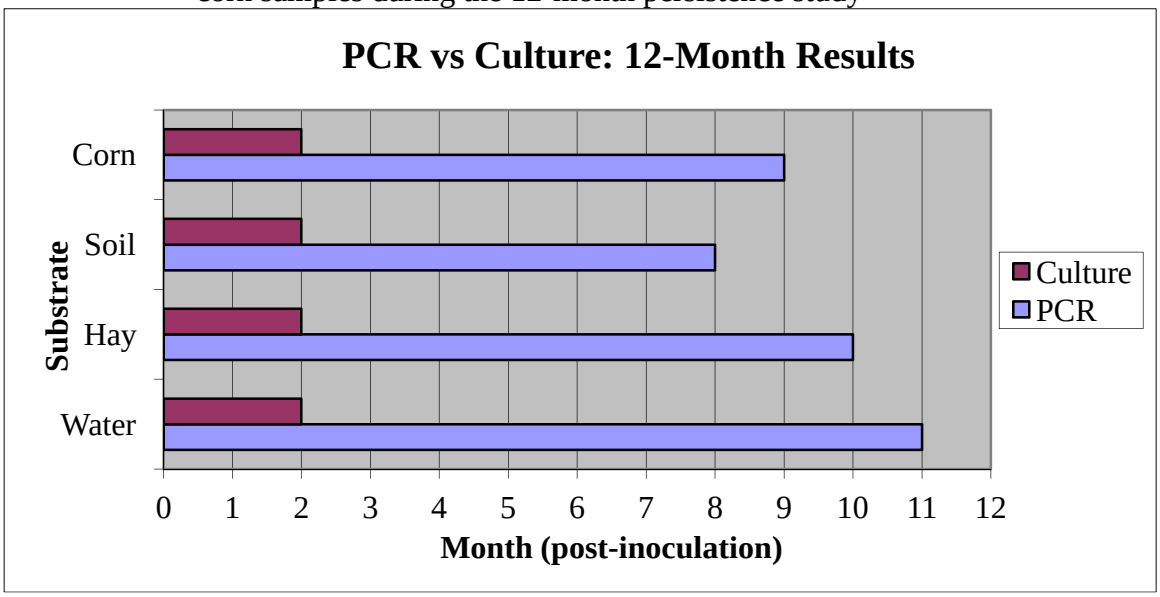


Figure 3.4 – Number of positive replicates obtained at each sampling point by substrate type

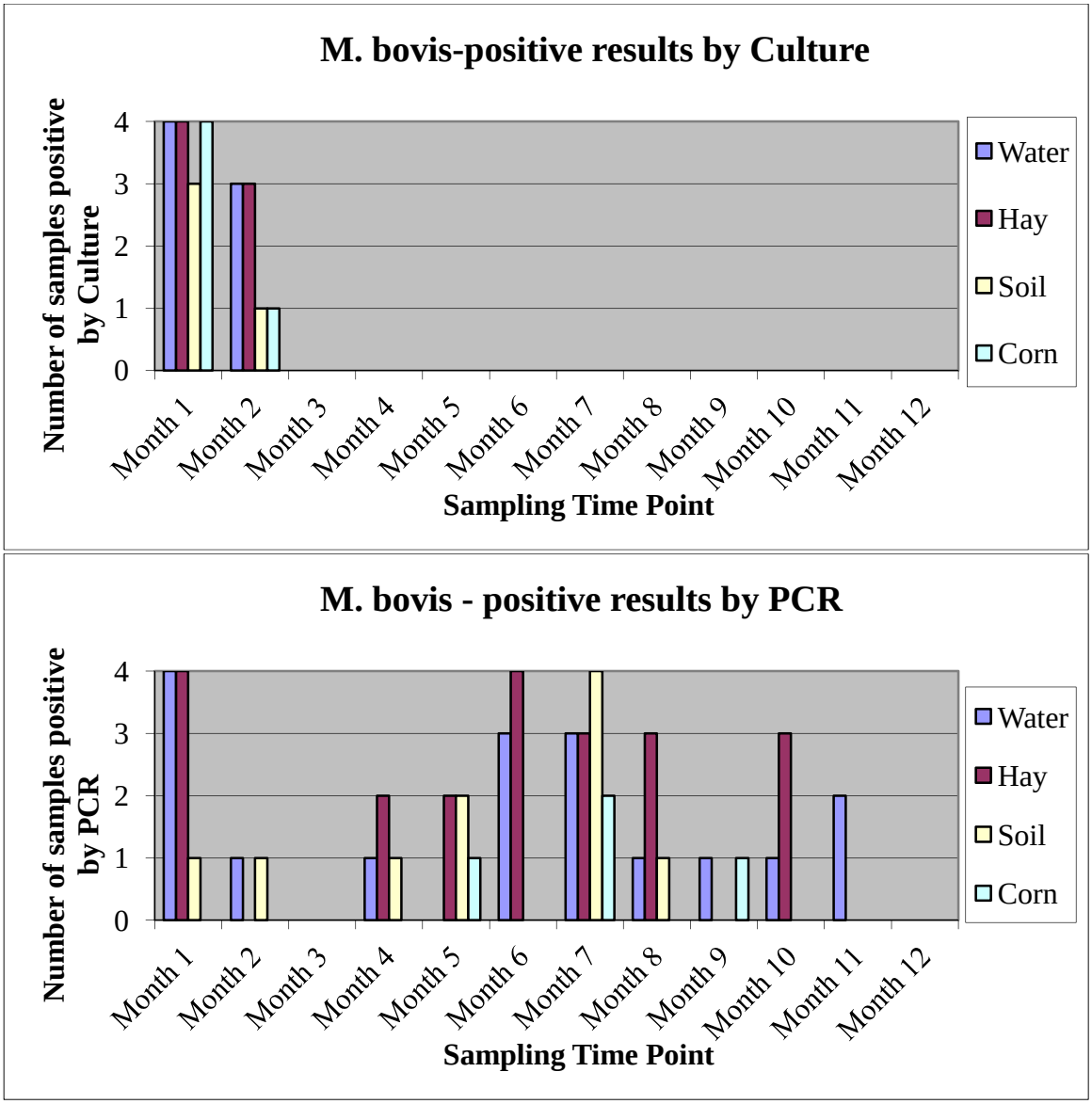
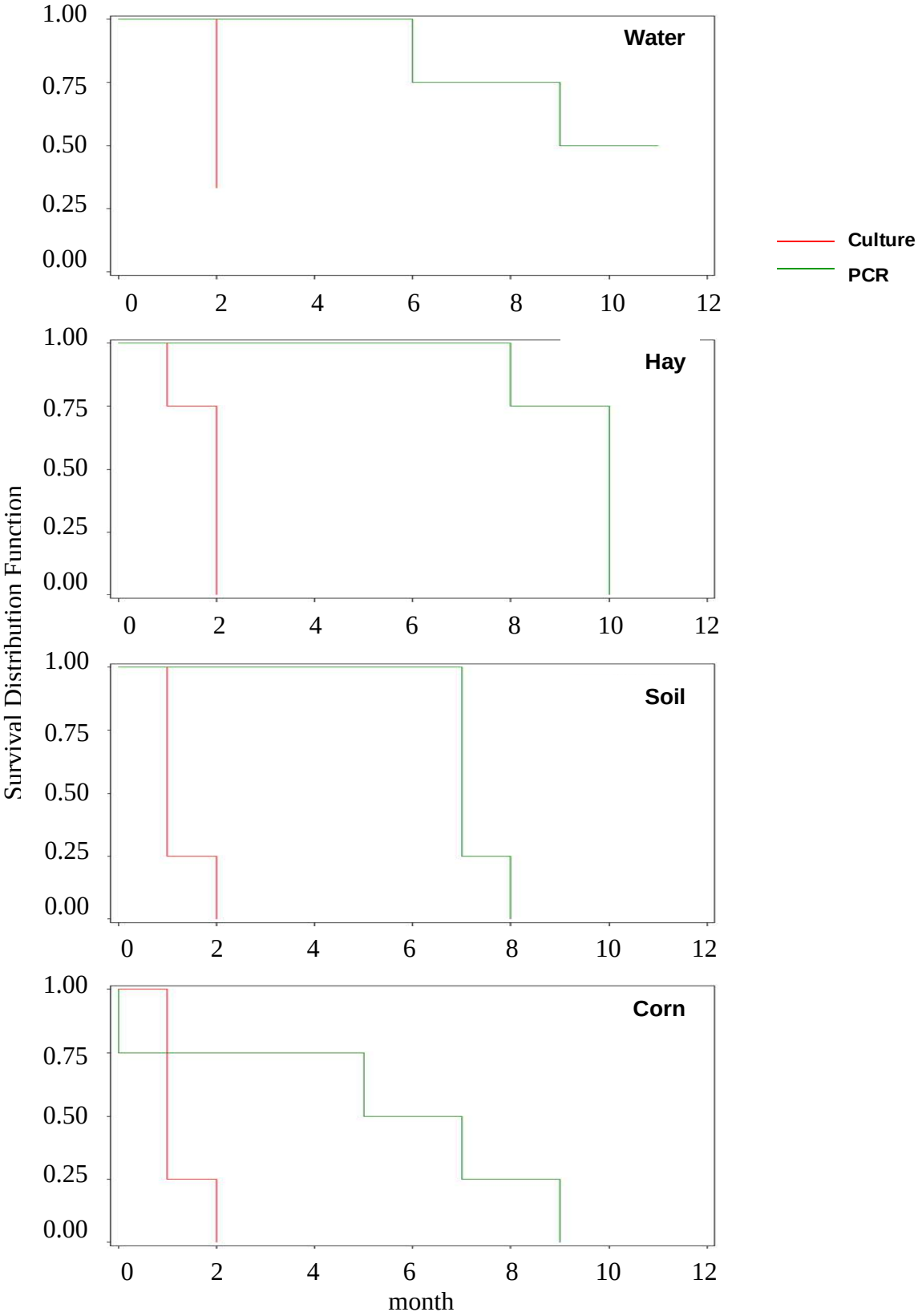


Figure 3.5 Survival curves for detection of *M. bovis* in samples exposed to natural environmental conditions over 12 months using PCR and culture, by substrate type



3.4 DISCUSSION

This study was designed to compare the effectiveness (determined by the number of detectable positive samples) as well as the duration of *M. bovis* detectability of molecular detection techniques (PCR) against the standard of bacterial culture in experimentally inoculated samples.

The persistence of *Mycobacterium bovis* in the environment was evaluated over varying time periods and bacterial culture results were compared with results obtained by PCR. We hypothesized that molecular techniques would improve our ability to detect *M. bovis* in the environment. A total of 559 inoculated samples of hay, soil, corn and water from the winter, winter/spring and spring/summer sampling seasons were analyzed for *M. bovis* detection along with an additional 128 samples from the 12-month study.

Within the 12-month study, PCR detected *M. bovis* in environmental substrates up to 9 months longer than culture (Figure 3.3). Across each of the three sampling seasons, the effectiveness of PCR was shown to vary depending on the substrate type. PCR was slightly more effective than bacterial culture for the detection of *M. bovis* in samples of hay, while PCR was essentially equivalent to bacterial culture for the detection of *M. bovis* in samples of water. Bacterial culture was more effective than PCR for the detection of *M. bovis* in samples of soil and corn. Bacterial culture detected the presence of *M. bovis* in more samples than PCR in the winter sampling period (November 8 – December 22) regardless of substrate type. Within the winter/spring sampling period (Feb 5 – May 4) PCR was more effective than bacterial culture for the detection of *M. bovis* in hay samples, less effective in soil samples and essentially the same as culture in

corn and water samples. In the spring/summer sampling period (May 20 – August 2), PCR was more effective than culture for the detection of *M. bovis* in samples of hay, but PCR and culture were essentially equivalent for the detection of *M. bovis* in water, soil and corn samples.

We hypothesized that molecular detection techniques would improve our ability to detect *M. bovis* in soil, hay, water and similar substrates. The effectiveness of PCR compared with bacterial culture, as shown by number of positive samples detected, varied across substrate type and sampling period; however, PCR was able to detect the presence of *M. bovis* over a longer period of time than bacterial culture independent of substrate type, as shown by the 12-month study.

Although the duration of detectable *M. bovis*-positive samples was longer for PCR than culture, the survival curves in figures 3.2 and 3.5 show that the detectability of *M. bovis* using PCR steadily decreases over time. During the spring/summer season, which was the warmest and driest of the three, we saw the lowest rate of detection of *M. bovis*-positive samples by both PCR and culture. As has been shown in previous studies, an increase in temperature and a loss of moisture were found to be associated with a decrease in the persistence of *M. bovis* in the environment (Fine et al., 2011a; Jackson et al., 1995; Tanner and Michel, 1999).

The bacterial load used to inoculate each substrate was 50,000 CFU of *M. bovis*. Although this is larger than the minimum infective oral dose of *M. bovis* for cattle (5,000 CFU) and white-tailed deer (300 CFU) (Palmer et al., 2002; Palmer et al., 2004), this amount of inoculum is thought to emulate the amount of *M. bovis* that could be shed by an infected animal (Fine et al., 2011a).

The use of molecular techniques and bacterial culture are both critical for the detection of *M. bovis* within environmental samples. Young et al. showed that DNA did not persist in dead bacterial cells in soil based on DNA decay rates. Therefore, DNA detected using PCR assays signifies the presence of viable organisms (Young, 2005). Both bacterial culture and PCR assays allow detection of viable bacteria in the environment. The strength of the PCR assay is that it provides quicker results (within hours) than detection of *M. bovis* by bacterial culture, which can take up to 12 weeks, and is not hindered by contaminating organisms that can overgrow a bacterial culture. PCR also gives information about the presence and distribution of *M. bovis* in the environment much longer after the initial contamination than culture. In epidemiologic investigations of TB farms and wildlife sites, PCR-based assays may be useful for parallel testing with bacterial culture to enhance detection of M. bovis in the environment.

Chapter 4

A STUDY OF THE DETECTION OF *MYCOBACTERIUM BOVIS* IN SAMPLES COLLECTED FROM BOVINE TUBERCULOSIS TRANSMISSION SITES IN MICHIGAN

4.0 ABSTRACT

Objective: To recover *M. bovis* DNA from environmental substrates collected from bovine tuberculosis transmission sites in an attempt to document the persistence of *M. bovis* in the environment.

Design: Cross-Sectional

Sample Population: Environmental substrates obtained from 3 cattle farms identified as bovine TB positive and previously processed for isolation of *M. bovis* by mycobacterial culture.

Procedure: Previously collected samples from bovine tuberculosis (TB) positive cattle farms were processed for *M. bovis* persistence using molecular detection techniques.

Results: None of the analyzed samples were positive for *M. bovis* DNA.

Conclusions and Clinical Relevance: Farm management practices likely play a role in the indirect transmission of bovine tuberculosis between deer and cattle in northeast lower Michigan.

4.1 INTRODUCTION

The emergence and persistence of bovine tuberculosis in white-tailed deer and subsequently in Michigan's cattle herds has raised concern over farm management practices that may aid in the indirect transmission of *M. bovis* infection from deer to cattle. Previous investigations of farms and wildlife areas have identified factors that may contribute to the spread of the disease, such as maintenance/housing of the animal and feeding/watering practices (Kaneene et al., 2002; Fine et al., 2011b). The current study used molecular detection techniques on a set of environmental substrates previously sampled from potential sites of bovine TB transmission (Fine, 2011b). The purpose of this study was to recover *M. bovis* DNA from environmental substrates collected from bovine tuberculosis transmission sites. We hypothesized that molecular techniques would detect the presence of *M. bovis* in naturally occurring environmental substrates enabling a more accurate characterization of the persistence of *M. bovis* in the environment and identifying farm management practices that may facilitate the indirect transmission of bovine tuberculosis.

4.2 MATERIALS AND METHODS

4.2.1 Sample Used for Molecular Testing

A total of 508 samples were previously collected from 13 cattle farms and 5 wildlife areas located within the TB "core area" in northern Michigan's lower peninsula (Fine et al., 2011b). The farms included were 11 out of the 12 Michigan cattle farms confirmed to have the presence of bovine TB positive cattle on site between June 2002 and September 2004. Samples from two additional cattle farms identified as bovine TB positive in 2000 and 2001 were also included within the study. Wildlife areas were

selected that had high probabilities of being a potential TB transmission site based on the prevalence of bovine TB positive white-tailed deer in the area.

Of the 508 samples collected and processed for *M. bovis* by mycobacterial culture, a total of 93 samples were selected for PCR processing based on the farms that were sampled closest to the date they were declared TB-positive (table 4.1). Collected sample substrates included manure/manure mix, hay/straw, water, bedding, grain/feed, soil and grass (table 4.2).

4.2.2 Sample Processing

Collected samples were processed using a standardized protocol by Fine et al. for processing environmental samples for mycobacterial culture (Fine, 2011b). Samples were pulverized and homogenized for 30-seconds on the high setting of a household blender. Samples were placed upright and allowed to settle for 30-minutes. The top 5 mL of fluid from each sample was removed and transferred to a 50-mL conical tube containing 10-mL of Decontamination Solution. Samples were mixed by vortex and incubated at 37 ºC for 75 minutes. Sterile water was added to the 50-mL mark on each tube, mixed and centrifuged at 3,000 g for 20-minutes. Pellet-containing tubes were completely decanted and a pipette was used to remove all but 1-3 mL of liquid from samples without a visible pellet. The pellet was suspended in the supernatant backwash. One mL of sterile water was added and mixed. A 0.5 mL sample was transferred to a 2.0 mL labeled cryogenic vial and frozen at -80 ºC. This 0.5mL sample was stored separately for later DNA-based PCR processing and testing. The remainder of each sample was further processed for bacterial culture (Fine et al, 2011b).

4.2.3 DNA Extraction

Samples previously frozen at -80 ºC were thawed for DNA extraction. To extract bacterial DNA, a 150-µL aliquot was removed from each thawed sample and transferred to a 1mL microcentrifuge tube. The tubes were then sonicated for 15-minutes, boiled for 10-minutes, flash frozen in an ethanol dry ice bath, boiled for another 5-minutes, then centrifuged for 10-minutes at 10,000 g. The supernatant was removed from each tube and stored frozen at -20ºC until used in polymerase chain reaction (PCR) assays.

4.2.4 PCR Procedure

A nested PCR amplification was performed using an initial 25-µL reaction mixture containing 12.5µL Promega GoTaq® Green 2X Master Mix, 7.1µL water, and 5µL DNA with 0.2 µL each of forward outer primer CGTGAGGGCATCGAGGTGGC and reverse outer primer CCTGCGAGCGTAGGCGTCGG. This primer set targeted the IS6110 insertion sequence found in the *M. bovis* genome and amplified a product of 252-bp. The initial denaturation and enzyme activation step of 94ºC for 4 minutes was followed by 20 cycles of 94ºC for 30 seconds, 67ºC for 30 seconds, 72ºC for 30 seconds; and a final extension step of 72ºC for 5 minutes. The second PCR reaction used a 1-µL aliquot from the completed first PCR reaction added to a reaction mixture containing 12.5µL Promega GoTaq® Green 2X Master Mix and 10.7µL water with 0.4µL each of inner forward primer CTCGTCCAGCGCCGCTTCGG and inner reverse primer GCGTAGGCGTCGGTGACAAA that produced a product of 116-bp. The reaction conditions for the second PCR were 94ºC for 4 minutes; 40 cycles of 94ºC for 30 seconds, 65ºC for 30 seconds, and 72ºC for 30 seconds; and a final extension step of 72ºC

for 5 minutes. Gel electrophoresis of the product from the second PCR was done using a 1.5% agarose gel and ethidium bromide for detection of DNA.

4.3 RESULTS

All samples collected were negative for *M. bovis* by both mycobacterial culture as well as PCR.

Table 4.1 Time between farm investigation (environmental sampling) and official TB positive date. Shaded farms were sampled closest to the date the farm was declared TB-positive and were thus selected for further testing by PCR

Farm #	Date TB+	Sampling Date	TB+ to Sampling (Days)
101	**09/20/02**	**09/10/02**	**-10**
102	**07/17/02**	**09/10/02**	**55**
103	**07/16/02**	**09/13/02**	**59**
104	**06/12/02**	**09/27/02**	**107**
105	**04/06/01**	**12/09/02**	**612**
106	**06/02/00**	**12/18/02**	**929**
107	**01/09/03**	**02/28/03**	**50**
108	**11/27/02**	**03/04/03**	**97**
109	**01/27/03**	**05/05/03**	**98**
110	**05/27/03**	**07/02/03**	**36**
111	**11/10/03**	**12/02/03**	**22**
112	**12/23/03**	**03/03/04**	**71**
113	**08/20/04**	**09/02/04**	**13**

Table 4.2 Number of samples (by substrate type) processed by PCR for *M. bovis* recovery.

	Substrate Type							
Farm	*Manure/ Manure mix*	*Hay/Straw*	*Water*	*Bedding*	*Grain/Feed*	*Soil*	*Grass*	**Totals**
101	8	6	1	0	1	1	3	**20**
111	2	6	6	2	2	2	1	**21**
113	0	3	12	0	0	20	17	**52**
Totals	**10**	**15**	**19**	**2**	**3**	**23**	**21**	**93**

4.4 DISCUSSION

Failure to isolate *M. bovis* from TB positive cattle farm samples does not necessarily indicate lack of persistence of the organism in the environment. Experimental studies have shown that *M. bovis* is capable of surviving outdoors under natural weather conditions for months after contamination has occurred (Williams and Hoy, 1930; Duffield and Young, 1985; Maddock, 1933; Maddock, 1934; Whipple and Palmer, 2000; Fine et al., 2011a). Although many attempts to isolate *M. bovis* from naturally infected environmental substrates have failed, Young et al. used molecular techniques to detect *M. bovis* DNA in soil from a farm environment up to 12 months after possible contamination (Young et al., 2005). The large spatial distribution of farm lands and wildlife areas makes it difficult to pinpoint exact sample locations that may be positive for *M. bovis*. However, experimental studies have identified indirect contact through contaminated feed and bedding as a viable and likely component of the transmission of the disease which should not be ignored (Palmer et al., 2001; Palmer et al., 2004).

OVERALL SUMMARY AND CONCLUSIONS

SUMMARY

This thesis has examined the persistence of *Mycobacterium bovis* in the environment through molecular detection techniques (specifically PCR) and has compared these results with those obtained in an earlier set of experimental and field-based studies (Fine, 2006) by traditional bacterial culture methods. Culture methods present many challenges concerning the isolation *Mycobacterium bovis.* Due to the organism's characteristics, it is easily out-competed and overgrown by contaminating organisms during the culture process. For this reason, decontamination methods are a necessary processing step in attempts to isolate the organism, but this process also reduces *M. bovis* viability and may lead to false negatives.

In contrast, PCR is not hindered by contaminating organisms as it targets DNA sequences that are specific to the organism of interest. It is able to detect low concentrations of *M. bovis* and it also provides results within hours, versus weeks by culture. However, PCR primers may produce false positive results from the contamination of negative samples with positive DNA or from a non-optimized amplification program, which can cause non-specific binding and/or the formation of primer dimers.

During the initial study, we were able to create a nested PCR procedure targeting the IS6110 insertion sequence of the *M. bovis* genome. The advantage of using nested PCR is that it increases the sensitivity of the amplification process, targeting the same DNA sequence twice and reducing the possibility of obtaining false positive results. Over 900

environmental samples previously processed by traditional culture were processed using this PCR procedure and results were compared.

CONCLUSIONS

This research has shown that a nested PCR amplification process can improve the sensitivity of a PCR assay, reducing the occurrence of falsenegative results. PCR is an invaluable tool to have during epidemiologic investigations. Itis a great complement to traditional culture methods as it can quickly detect or confirm positive results and is able to be utilized with samples that may fail detection by culture due to contamination.

Under controlled conditions, PCR consistently detects *M. bovis* across all samples regardless of substrate type. PCR was also able to detect *M. bovis* in experimentally inoculated substrates exposed to natural weather conditions for up to 11 months. This data supports the idea that indirect transmission plays a role in the epidemiology of bovine tuberculosis. As long as the state of Michigan remains TB-positive, procedures and practices should remain in place that reduce or eliminate the potential for indirect transmission between white-tailed deer and cattle herds.

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