This is to certify that the
dissertation entitled

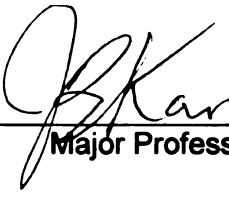
The Role of Indirect Transmission in the Epidemiology of Bovine Tuberculosis in Cattle and White-Tailed Deer in Michigan

presented by

Amanda Elizabeth Fine

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Large Animal Clinical Sciences (Epidemiology)



Major Professor's Signature

04/12/06

Date

MSU is an Affirmative Action/Equal Opportunity Institution

DATE DUE	DATE DUE	DATE DUE
[illegible]		

THE ROLE OF INDIRECT TRANSMISSION IN THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN CATTLE AND WHITE-TAILED DEER IN MICHIGAN

By

Amanda Elizabeth Fine

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Large Animal Clinical Sciences

(Epidemiology)

2006

ABSTRACT

THE ROLE OF INDIRECT TRANSMISSION IN THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN CATTLE AND WHITE-TAILED DEER IN MICHIGAN

By

Amanda Elizabeth Fine

Understanding the epidemiology of *Mycobacterium bovis* transmission in Michigan is an essential component of nationwide efforts to control and eradicate bovine tuberculosis (TB). Determining the role of indirect transmission in bovine TB dynamics is a key to the application of epidemiologically effective methods of disease control in both livestock and wildlife populations. The objective of this dissertation was to characterize the persistence of *M. bovis* in the environment and its potential role in the indirect transmission of disease among and between cattle (*Bos taurus*) and white-tailed deer (*Odocoileus virginianus*) in Michigan.

Optimized techniques for isolating *M. bovis* from environmental substrates were developed. These were applied to the testing of samples collected from cattle farms with a known history of *M. bovis* infection. Samples collected opportunistically from locations within areas with a high prevalence of *M. bovis* infection in white-tailed deer were also tested. Though mycobacterial isolation was successful, none of the isolates were identified as *M. bovis*. The inability to detect *M. bovis* from sites of known bovine TB transmission suggests that the pathogen is not distributed broadly across the landscape, making the identification of a specific site of contamination difficult.

To address the question of *M. bovis* persistence in the environment, given the limitations of detecting the bacilli under natural conditions, a 12-month long experiment

was designed. Environmental substrates were experimentally inoculated with *M. bovis* and exposed to natural weather conditions. The effects of environmental conditions on the survival of *M. bovis* over time were assessed.

Persistence of *M. bovis* in the Michigan environment under natural weather conditions was recorded for an average of 30 days in the cooler months of the year (November – May), and an average of 7 days in the warmer months (May - August). This recorded persistence strongly suggests an important potential role for indirect transmission in the epidemiology of bovine TB in the region. These data supplement those produced through experimental *M. bovis* disease transmission studies that have proven the feasibility of indirect transmission of *M. bovis* among and between cattle and white-tailed deer. They also support the analyses of observational data on *M. bovis* infection in cattle and white-tailed deer in Michigan that indicate the importance of indirect transmission in the interspecies transmission of *M. bovis*.

Local, State and Federal bovine TB control and eradication policy needs to consider indirect transmission of *M. bovis* through contaminated environmental substrates in the creation and implementation of epidemiologically appropriate disease management plans. In the bovine TB endemic region of Michigan, interspecies transmission of bovine TB should be considered by both wildlife and livestock health agencies. If this component of the epidemiology of *M. bovis* transmission in the region is ignored, efforts to control and eventually eradicate the disease will fail.

To the many individuals and institutions dedicated to the global control and eradication of bovine tuberculosis.

ACKNOWLEDGEMENTS

I have the deepest appreciation for all of the individuals who have helped to make this project possible. Like the approach to the control of bovine tuberculosis (TB) in Michigan, I have benefited greatly from the assistance and guidance of a multidisciplinary and team. I would like to thank my two major advisors, Dr. John Baker who created the opportunity for me to study TB in Michigan and pursue this PhD, and Dr. John Kaneene who supervised the development of this research project and provided expertise at every step of its progress toward completion. I would also like to thank the members of my graduate committee, Dr. Carole Bolin, Dr. Joseph Gardiner, Dr. Dan O'Brien, Dr. Scott Winterstein, and Dr. Gary Witmer for their guidance, support and the valuable contributions to this research.

I would like to acknowledge the assistance of Dr. Charles Thornton (Integrated Research Technology), Mr. Dale Berry (Michigan Department of Community Health) and the staff at the National Veterinary Laboratories in Ames, Iowa, who were willing to pick up the phone and give me advice on the development of the laboratory techniques used in this research. For assistance in the laboratory at Michigan State University (MSU) Diagnostic Center for Population and Animal Health, I am indebted to Joseph Hattey, bovine TB laboratory technician, and to students Kathleen Gray and Christy Sampson. Desiree Oliver contributed in the laboratory and the field and Kile Kucher assisted with data entry in the final stages of this work.

A large component of this research would not have been possible without the participation of cattle farmers and landowners in the bovine TB endemic region of Michigan. I am grateful for their willingness to take time out of their busy schedules to participate in the project. I would also like to acknowledge the staff at the Michigan Department of Agriculture (MDA) who assisted with the identification of bovine TB positive farms in the region, the members of the Gaylord, MI, United States Department of Agriculture (USDA), Animal Plant Health Inspection Service (APHIS), Wildlife Services (WS) team and the Michigan Department of Natural Resources (MDNR) field staff in northeast Michigan who assisted with environmental sampling.

The Michigan Agricultural Experiment Station, and the Departments of Fisheries and Wildlife (FW) and Large Animal Clinical Sciences at MSU supported this research and program. The MDA, USDA/APHIS/WS, and the USDA Bovine TB Special Grant provided additional research funding.

I would like to thank friends and colleagues from the FW and LCS departments at MSU and the multi-agency Michigan bovine TB team, which includes staff from the MDNR wildlife disease laboratory, Veterinary Services and WS of USDA/APHIS, the MDCH and faculty and researchers at MSU. I feel privileged to have had the opportunity to engage and interact with such a supportive, talented and dynamic group of individuals. Finally, I would like to thank my parents for instilling in me what will undoubtedly be a life-long love of learning.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

PROBLEM STATEMENT

Bovine tuberculosis (TB) caused by *Mycobacterium bovis* has been recognized as a threat to the livestock industry and public health in North America since the late 1800's. The bovine TB eradication program, which began in the United States in 1917, has resulted in a dramatic reduction in the prevalence of bovine TB in cattle (*Bos taurus*) herds across the country, but nationwide TB-free status has not been accomplished (Frye, 1995). A tremendous threat to the eradication effort in the United States today is the emergence of bovine TB in a population of free ranging white-tailed deer (*Odocoileus virginianus*) in northeast Lower Michigan and the subsequent detection of bovine TB in cattle herds in the same region (Schmitt et al., 2002). It is believed that this is the first time a reservoir for bovine TB has been established and maintained in a wildlife population in the mainland United States (Schmitt et al., 1997).

The emergence of a wildlife reservoir for bovine TB in North America, and evidence of disease transmission between infected free-ranging white-tailed deer populations and domestic cattle, has forced a reevaluation of our understanding of the epidemiology of bovine TB. The long-standing notion, that bovine TB is exclusively a directly transmitted disease requiring very close contact between an infected and susceptible host for disease transmission to occur, is under scrutiny. This reevaluation of the epidemiology of bovine TB will have necessary and important impacts on disease management and prevention strategies in both domestic and wildlife populations.

STUDY RATIONALE

The role and relative importance of the indirect transmission of *M. bovis* in the epidemiology of bovine TB in Michigan cattle and wildlife populations is unknown. A clearer understanding of this component of the epidemiology of bovine TB is essential for the improvement of protocols for cattle farm bio-security, the maintenance of appropriate restrictions on feeding and baiting free-ranging white-tailed deer and other wildlife, and for informing human health and safety regulations regarding the potential public health hazards of bovine TB.

An understanding of the presence and persistence of *M. bovis* in the environment is required to assess the role of indirect transmission in the epidemiology of *M. bovis*. Until this PhD project was implemented, little work had been done to test environmental substrates for the presence of *M. bovis* or to characterize the persistence of the Michigan strain of *M. bovis* in the environment under conditions typical of the bovine TB endemic region. Earlier attempts to detect and characterize *M. bovis* persistence in the environment were constrained by the limits of standard methodologies, time and financial resources.

Information regarding the persistence of *M. bovis* in the environment in Michigan is by stakeholders (State and Federal regulatory agencies, policy makers, cattle producers, recreational hunters, and the academic community) who are working toward the control and eventual eradication of bovine TB in Michigan.

OBJECTIVES

This PhD project was designed to further the understanding of the role of indirect transmission in the epidemiology of bovine TB in northeast Michigan. To accomplish this goal the specific objectives included: 1) Optimizing a technique for processing environmental samples for *M. bovis* detection by mycobacterial culture; 2) Investigating and testing environmental samples from sites of natural bovine TB transmission; and 3) Characterizing the persistence of *M. bovis* in the environment under conditions typical of the bovine TB endemic region of Michigan.

OVERVIEW

Chapter 1 is a review of the literature addressing the role of indirect transmission of bovine TB in domestic and wild populations of animals and a presentation of studies that have examined the persistence of *M. bovis* in the environment in Michigan and other regions of the world. **Chapter 2** describes the difficulties associated with the detection of *M. bovis* in the environment and the methods and techniques developed for processing environmental samples and detecting *M. bovis* during the course of this dissertation work. **Chapter 3** presents the results of a series of field investigations designed to detect *M. bovis* in environmental samples from known TB transmission sites including TB positive cattle farms and areas of the Michigan with the highest apparent prevalence of *M. bovis* in free-ranging white-tailed deer. **Chapter 4** describes an experimental study designed to determine the length of survival of *M. bovis* on a range of substrates (feed, soil, and water) exposed to environmental conditions in Michigan throughout a calendar year and the factors that influence the likelihood of survival.

The hypothesis being tested is that *M. bovis* can survive in environmental substrates for sufficient lengths of time to serve as a source of infection for cattle and/or free-ranging white-tailed deer and that specific conditions influence the persistence of *M. bovis* in the environment. The implications of the findings presented in Chapters 1 through 4 are discussed in the "overall conclusions" section at the end of this dissertation. The prospects for the control and eradication of bovine tuberculosis in Michigan, the effectiveness of the century-old bovine TB eradication program in the United States and our understanding of wildlife/livestock disease transmission dynamics are all influenced by this closer examination of the persistence of *M. bovis* in the environment and the role of indirect transmission in the epidemiology of bovine tuberculosis.

Chapter 1

LITERATURE REVIEW OF THE PERSISTENCE OF *MYCOBACTERIUM BOVIS* IN THE ENVIRONMENT AND THE POTENTIAL ROLE OF INDIRECT TRANSMISSION IN THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MICHIGAN

1.0 INTRODUCTION

The role of indirect transmission of *Mycobacterium bovis* in the epidemiology of bovine tuberculosis (TB) in Michigan has been a point of discussion since the current TB epidemic in Michigan was first described in 1997 (Schmitt et al., 1997). Spillover of bovine TB infection from domestic cattle to white-tailed deer in Michigan is thought to have occurred during the late 1950's when large numbers of cattle infected with *M. bovis* were present in the State (Frye, 1995). The establishment and persistence of *M. bovis* in free-ranging white-tailed deer in northeast Michigan today is thought to have been influenced by the long-term practice of winter feeding of deer in the region (Schmitt et al., 1997). The feed, set out to attract deer and improve their productivity and winter survival, is thought to contribute to the transmission of TB among white-tailed deer by 1) increasing local density and direct contact between animals, 2) increasing overall density of deer above biological carrying capacity making deer more susceptible to *M. bovis* infection, and 3) providing a site for the indirect transmission of TB through contamination of the feed by infected deer shedding *M. bovis* in their saliva or nasal

discharges and the subsequent infection of a naïve deer by consumption of contaminated feed (Hickling, 2002; Schmitt et al., 1997).

Michigan received its United States Department of Agriculture (USDA) "accredited-free of TB" status in 1979. Concern surrounding the role of indirect transmission in the epidemiology of bovine TB in Michigan increased when the first case of bovine TB in cattle was confirmed in northeast Michigan in 1998 (State of Michigan, 2005). Since 1998, thirty-seven cattle herds in Michigan have been confirmed as TB-positive and two of these herds have been found to be positive on two separate occasions (Judge, 2006). The spatial and temporal relationship between bovine TB in deer and cattle in Michigan, coupled with data from DNA and restriction fragment length polymorphism (RFLP) analyses of *M. bovis* isolates from both populations, indicate that deer and cattle are infected with the same strain of TB (Whipple et al., 1999a). Close "nose-to-nose" contact and interaction, necessary for aerosol transmission of *M. bovis*, are very rarely observed between wild white-tailed deer and cattle (DeLiberto et al., 2004). Indirect transmission of *M. bovis* has, therefore, been suggested as a possible mechanism for interspecies (deer to cattle or cattle to deer) TB transmission in Michigan.

Control of bovine TB in Michigan is of interest to individuals and agencies in the natural resources, veterinary and public health sectors. The presence of TB in free-ranging wildlife and domestic cattle is associated with economic costs at the local and national level (Corner, 2006; Holecek and Bristor, 2003; Thornton et al., 1998c). The economic costs in Michigan have included losses associated with the restriction of cattle trade, the cost of disease surveillance and loss of genetic stock. In addition economic losses have been noted in the tourism sector due to concerns regarding bovine TB in the

white-tailed deer population. The economic impact of bovine TB in Michigan has also extended to the public health and companion animal fields in which the risk of zoonotic disease transmission is being monitored (Kaneene et al., 2002a; Wilkins et al., 2003).

To address this multifaceted disease issue, scientists and disease control officials from Michigan State University (MSU) and MSU's Diagnostic Center for Population and Animal Health (DCPAH), the Michigan Departments of Natural Resources (MDNR), Agriculture (MDA) and Community Health (MDCH), and the USDA, Animal Plant Health Inspection Service (APHIS), Veterinary Services (VS) and Wildlife Services (WS), have initiated an extensive cooperative TB research and control program addressing the disease in both domestic and wildlife populations. Understanding the role of indirect transmission in the epidemiology of bovine TB in Michigan will assist policy makers in their efforts to control and eradicate bovine TB. Improved understanding of bovine TB transmission should increase public confidence in the policies developed and ensure their continued cooperation in efforts to eradicate bovine TB in Michigan.

The potential role of indirect transmission in the epidemiology of bovine TB in Michigan is explored below through a review of: 1) experimental and observational studies describing evidence of the persistence of *Mycobacterium bovis* in the environment and the role this may or may not have in inter- and/or intra-species transmission of bovine TB and; 2) experimental and observational studies examining the potential role of indirect transmission in the epidemiology of bovine TB in cattle and deer in Michigan.

1.1 EVIDENCE OF THE PERSISTENCE OF *MYCOBACTERIUM BOVIS* IN THE ENVIRONMENT

Attempting to detect and characterize the persistence of *M. bovis* in the environment has historically been the approach to elucidating the role of indirect transmission in the epidemiology of bovine tuberculosis in systems around the world. The role of indirect transmission in the epidemiology of *M. bovis* is of particular concern in systems in which a wildlife reservoir of *M. bovis* has been identified with the threat of the spillover (or spill back) of infection into domestic cattle populations (Corner, 2006). The role of indirect transmission in bovine TB epidemiology has been studied by attempting to detect *M. bovis* in the environmental substrates in a location where a *M. bovis* transmission event has occurred, and in experimental studies in which either the substrates or the species under investigation are infected with *M. bovis* and the persistence of *M. bovis* is assessed under varying conditions designed in a simulated setting.

1.1.1 Detection of *Mycobacterium bovis* under natural conditions

Few studies have been published that address the detection of *M. bovis* under natural conditions or discuss attempts to investigate environmental sources of *M. bovis*. In one study, Pillai and coworkers (2000) described an investigation of 14 dairy herds in El Paso County, Texas, with a prior history of bacteriologically confirmed *M. bovis* infection in cattle. Extensive soil, water and air sampling was carried out on each farm and the samples were processed for mycobacterial culture, but *M. bovis* was not isolated.

Young and coworkers (2005) detected *M. bovis* in the farm environment using molecular techniques. Specific polymerase chain reaction (PCR) primers targeting the *M. bovis* 16S rRNA gene were used to detect *M. bovis* in environmental soil samples collected from a cattle farm in Ireland with a history of bovine tuberculosis. The pathogen was detected in soil from the farm at 4 and 12 months after possible contamination. The source of contamination in this farm was presumably *M. bovis* shed by infected badgers (*Meles meles*) present on the property before the removal of all cattle and badgers according to disease control protocols in the region.

Other attempts to identify and isolate mycobacterial species from environmental substrates, i.e. soil and stream water, have failed to detect *M. bovis* (Cooney et al., 1997; Livanainen, 1995; Livanainen et al., 1999). Failure to detect, and therefore report, the presence of *M. bovis* in the environment is related to difficulties associated with isolating *M. bovis* from environmental substrates. An efficient and effective method for processing environmental samples for mycobacterial isolation is needed to support efforts to understand *M. bovis* transmission.

The challenges associated with the detection of *M. bovis* in the environment fall into three major categories. First, environmental samples (soil, feed, fecal material, pond/stream water, etc.) contain large numbers of saprophytic bacteria, molds and other infectious organisms. *Mycobacterium* are slow-growing in culture and other organisms interfere with the isolation of mycobacteria by overgrowing and out-competing the mycobacteria during the bacterial culture process. The essential decontamination process, designed to eliminate saprophytic organisms, also reduces the viability of

mycobacteria in the specimen and therefore interferes with the sensitivity of detection of *M. bovis* by bacterial culture methods (Kent and Kubica, 1985; Yajko et al., 1995).

Secondly, evidence suggests that the shedding of *M. bovis* from both infected deer (Palmer et al., 2001) and infected cattle (Goodchild and Clifton-Hadley, 2001; Neill et al., 1988) is intermittent. Intermittent or low level shedding of *M. bovis* coupled with the relatively small size—when compared with the scale of the potentially contaminated environment—of sample that can be collected and processed for mycobacterial culture results in a greatly reduced chance of collecting a contaminated substrate in the environment.

Thirdly, the properties of *M. bovis* make it particularly difficult to process and culture. Like other mycobacteria, *M. bovis* has a 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 (37° C, moist and free of other microbial contamination).

Improved protocols for processing samples collected from the environment for mycobacterial culture are necessary to further the investigation of *M. bovis* persistence in the environment and our understanding of the role of indirect transmission in the epidemiology of bovine TB in Michigan and other regions of the world.

1.1.2 Experimental *Mycobacterium bovis* persistence studies

A number of reports have been published that examine the persistence of *Mycobacterium bovis* in the environment by utilizing various experimental study designs. Early studies were concerned primarily with food hygiene and examined the persistence of *M. bovis* in meat and dairy products experimentally contaminated, or known to be contaminated, with *M. bovis* from infected cattle (Mitscherlich and Marth, 1984). Interestingly, one of the studies cited in the Mitscherlick and Marth textbook chapter on mycobacterial survival in the environment was an experiment performed by Soparkar in 1917 with "a purulent mass obtained from the lung of a deer which had died of bovine tuberculosis". Soparkar reported that *M. bovis* in the purulent mass survived for 10 but not 12 hours in direct sunlight and 30 days in diffuse sunlight.

Early studies of more relevance to concerns surrounding the persistence of *M. bovis* in the environment and the potential role of indirect transmission in the epidemiology of bovine TB in Michigan, are a series of studies published in the *Journal of Hygiene* in the early 1930's (Maddock, 1933, 1934, 1936; Williams and Hoy, 1930). William and Hoy were concerned with the persistence of *M. bovis* in feces shed by tuberculous cows in the south of England. Their attempts to isolate *M. bovis* from feces on pasture grazed by a naturally infected cow failed, despite the fact that the cow had culture-confirmed *M. bovis* in its feces at the start of the experiment. *M. bovis* was reported to survive for four months in naturally infected feces that was subsequently spread on pasture and monitored. The Williams and Hoy experiments with artificially infected cattle feces spread on pasture and monitored indicate that *M. bovis* can

predictably be recovered from feces on pasture for two to four months. Survival was reported to be longer during the winter (5 months) and shorter during the summer (< 2 months) but these conclusions must be accepted with caution given, 1) the fact that the source of the inoculums was emulsified tuberculous lungs; and 2) the fact that the dose of *M. bovis* inoculums used for the Williams and Hoy autumn, winter and spring experiments were 20 to 100 times larger than those used for summer experiments. Williams and Hoy also reported the recovery of *M. bovis* from naturally infected feces stored in cool dark conditions after 12 months and recovery of *M. bovis* from experimentally infected feces stored in a similar manner for 2 years (Williams and Hoy, 1930).

Maddock expanded on the work of Williams and Hoy by examining the survival of *M. bovis* in soil and on pasture grass as well as feces, and by using guinea pig inoculation to assess the viability of the *M. bovis* recovered from samples (Maddock, 1933). In the 1933 study, Maddock reported a 6-month survival time for *M. bovis* in soil, soil and feces mixtures and in feces exposed to the environment on pastures in south England. In 1934, Maddock reported the failure to infect guinea pigs or calves grazed on pasture known to be contaminated by the fecal shedding of *M. bovis* in the feces of cows with generalized TB (Maddock, 1934). The 1934 study was followed by one of similar design but in which pastures were artificially contaminated with an emulsion of tubercular bovine lungs. Both guinea pigs (*Cavia porcellus*) and calves that grazed on these *M. bovis* contaminated pastures and that were fed *M. bovis* contaminated grass indoors, contracted tuberculosis (Maddock, 1936).

The development of intensive livestock farming, and the disposal of animal waste from these operations, raised concerns about the survival and spread of pathogenic bacteria, including *M. bovis*, in the environment (Wray, 1975). The transmission of infectious mycobacteria in sewage effluent or sludge used in crop irrigation led to a study in the 1970's designed to determine the survival of *M. bovis* BCG in soil and on vegetables irrigated with effluent or sludge containing BGC. Reported findings indicated that BCG persisted for 11 days in effluent sprayed soil, 8 days in sludge treated soil, 4- 6 days on the surface of radishes and only intermittently on lettuce (Van Donsel and Larkin, 1977).

Although the studies outlined above do indicate that *M. bovis* survives for significant periods of time on soil or pasture, removal of tuberculous and exposed cattle from the premises, coupled with efforts to remove or expose any remaining organic matter to sunlight, should ensure that the farm is free of *M. bovis* in the environment within a 12-month period. This is, however, only true when cattle are the only source of *M. bovis* infection. In disease systems in which a wild animal population has been identified as a reservoir of infection for domestic cattle, traditional "cow-centered" disease management protocols alone will not protect a farm from TB re-infection. Recent studies designed to examine the persistence of *M. bovis* in the environment and the role of indirect transmission in the epidemiology of bovine TB have been focused in New Zealand, Australia, Kruger National Park in South Africa, Ireland and Great Britain, where a wildlife species has been identified as a reservoir of *M. bovis* infection (Corner, 2006).

Minimal environmental persistence of *M. bovis* was reported in an experimental study of *M. bovis* infection in badgers (*Mesles meles*) and cattle conducted over a four year period in south England (Little et al., 1982). TB transmission among badgers and between badgers and cattle was confirmed in these experiments but repeated attempts to isolate *M. bovis* from environmental samples were negative. Attempts were made to isolate *M. bovis* from the soil, hay, badger feces, scrapings from feed bowls and water in the experimental enclosures. *M. bovis* was isolated from badger feces and water on one occasion, and the authors concluded that *M. bovis* did not exist for long periods in the environment.

An experimental study of the survival of *M. bovis* in soil and bovine feces exposed to environmental conditions in north Queensland, Australia, indicated that *M. bovis* survived 4 weeks but less than 8 weeks (Duffield and Young, 1985). Rapid death of *M. bovis* was noted as a result of exposure to sunlight and the higher temperatures recorded in north Queensland were thought by the authors to be the reason behind the shorter *M. bovis* survival times in their study as compared to similar studies performed by Williams and Hoy (1930) and Maddock (1933, 1934, 1936) in south England.

A study performed in New Zealand, where the brushtail possum (*Trichosurus vulpecula*) is the wildlife reservoir of *M. bovis*, concluded that environmental contamination of pasture, the forest floor and possum dens was unimportant in the epidemiology of tuberculosis in cattle, deer (*Cervus elaphus*) and possums in New Zealand (Jackson et al., 1995). Cotton ribbons impregnated with *M. bovis* and set out at various sites in a typical farm environment were used to assess the environmental persistence of *M. bovis*. Weather data were collected to determine the effects on *M. bovis*

survival. *M. bovis* survived 2 days on pasture and 14 days on the forest floor and in possum dens. Survival time increased as daily temperatures decreased across all sites.

Researchers at Kruger National Park in South Africa, concerned about disease transmission among multiple species of wildlife, designed a study to determine the maximum survival time of *M. bovis* in tissues and feces (Tanner and Michel, 1999). Portions of tuberculous lung and lymph nodes from a naturally TB-infected African buffalo (*Syncerus caffer*), and cattle feces inoculated with *M. bovis*, were exposed to a range of environmental conditions throughout a 12-month period. Isolation of *M. bovis* was possible from infected tissues for 6 weeks, and from experimentally contaminated feces for 4 weeks, during winter months. The survival of *M. bovis* was limited to less than 2 weeks during the warmer seasons of the year and in the absence of moisture. Although indirect disease transmission seems feasible over the survival times reported, the authors conclude that the limiting factors present under natural conditions (withdrawal of water, predation, and the presence of competitive bacteria) would reduce the survival time below that recorded in their experimental studies to a point that *M. bovis* in the environment would not likely serve as a source of infection for cattle and wildlife in Kruger National Park.

1.2 THE ROLE OF INDIRECT TRANSMISSION IN THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN CATTLE AND WHITE-TAILED DEER IN NORTHERN LOWER MICHIGAN

Published reports on attempts to detect *Mycobacterium bovis* in the environment in Michigan are limited to the efforts described by Palmer et al. (2000), during a herd

depopulation process on a privately owned white-tailed deer farm in northeast Michigan (Palmer et al., 2000). The attempt involved collecting feed, soil, fecal and water samples for mycobacterial culture. Despite these efforts, *M. bovis* was not isolated from any of the samples. The remainder of reports in the published literature have addressed the issue of the environmental persistence and indirect transmission of *M. bovis* through experimental disease transmission studies and a series of observational epidemiologic studies. The relevant findings of these studies are discussed in the sub-sections below.

1.2.1 Experimental studies

Scientists and personnel from the Agricultural Research Service (ARS) of the USDA have performed a series of experiments in the bio-security level III facilities at the National Animal Disease Research Center (NADC) in Ames, Iowa, designed to characterize the transmission of *M. bovis* among and between white-tailed deer and cattle. Although the limitations of experimental transmission studies (stress and atypical behavior related to confinement of wild animals) apply, efforts were made to use white-tailed deer from northeast Michigan and a strain of *M. bovis* isolated from a wild white-tailed deer in northeast Michigan for these studies (Palmer et al., 1999).

An initial study was performed to develop an experimental model of natural *M. bovis* infection in white-tailed deer (Palmer et al., 1999). The deer were infected by instilling 1 X 10^3 colony forming units (CFU) of *M. bovis* (low dose) or 2 X 10^5 CFUs (high dose) in each tonsilar crypt. Of importance in determining the potential role of indirect transmission of *M. bovis* in the epidemiology of bovine TB, were the data that confirmed the presence of *M. bovis* in the saliva, nasal secretions and tonsilar swabs of

TB-infected deer. *M. bovis* shed in the saliva or nasal mucous of a TB-infected deer could serve as a source of contamination of shared feeding or watering sites. If *M. bovis* persists in the environment long enough, it could serve as a source of infection for previously unexposed deer visiting the feeding or watering sites. Also of note, are the data from this study that suggest that disseminated tuberculosis (gross lesions in multiple sites) is not a prerequisite for the presence of *M. bovis* in saliva or nasal secretions of infected deer.

The persistence of viable *M. bovis* on a variety of feeds often used for baiting white-tailed deer in Michigan (alfalfa hay, shelled corn, sugar beets, apples, carrots and potatoes) has been established in a laboratory setting (Whipple and Palmer, 2000). The feeds were inoculated with an unknown amount of *M. bovis* and held for 16 weeks at 75°F, 46°F and 0°F and sampled at daily and weekly intervals. The study demonstrated survival on all feed types stored at all temperatures at 1 week and for all feed types held at 0°F for at least 12 weeks. These data indicate "short-term" survival of *M. bovis* at temperatures consistent with the summer season in Michigan and prolonged persistence of *M. bovis* on feed types at temperatures that would be found in Michigan during the winter season.

Deer-to-deer transmission of *M. bovis* was studied by determining whether or not *M. bovis* can be transmitted from experimentally infected deer to uninfected in-contact deer (Palmer et al., 2001). Transmission of *M. bovis* was 100% in this sample of 8 in-contact deer indicating efficient disease transmission and significant susceptibility to *M. bovis* in this species. Of importance in elucidating the potential role of indirect transmission of *M. bovis* in this and other more natural situations are the following study

results: 1) Shedding of *M. bovis* was detected in the saliva and nasal secretions of experimentally infected deer and less commonly in in-contact deer; 2) *M. bovis* was detected in pelleted feed and hay, and the timing of the detection indicated that the feed was most likely contaminated by the "naturally" infected in-contact deer; and 3) Bacterial culture of feces from one pen and 1 of 8 fecal samples collected at necropsy and 1 of 4 urine samples collected at necropsy yielded *M. bovis*.

Although the TB transmission study described above produced data that indicate that indirect transmission of *M. bovis* between deer through feed or a contaminated environment might be possible, it was not designed to differentiate between routes of exposure. A follow-up study was designed to explicitly examine shared feed as a means of deer-to-deer transmission of *M. bovis* (Palmer et al., 2004b). Experimentally infected and uninfected deer were housed separately with no direct contact between the groups. Airflow was controlled to prevent room-to-room transfer of air and precautions were taken to eliminate any transfer of *M. bovis* between rooms by personnel. Only uneaten feed (complete pellet) was transferred from the pen holding the infected deer (4 individuals) to the pen holding the uninfected deer (4 individuals) for 136 days of feed sharing. All of the deer exposed to the feed previously offered to experimentally-infected deer exhibited gross and microscopic lesions consistent with TB at necropsy and isolation of *M. bovis* was confirmed from at least one cultured tissue in each animal. This study clearly demonstrates indirect transmission of *M. bovis* through contaminated feed from infected white-tailed deer to naïve "non-contact" deer.

The role of indirect transmission of *M. bovis* in interspecies disease dynamics in Michigan is of particular interest when attempting to explain deer to cattle and/or cattle to

deer transmission events. The presence of *M. bovis* infection in cattle from over ½ of the cattle herds confirmed as TB-positive in Michigan have had no relationships (fence-line contact, or cattle contact through movement) with other domestic cattle herds affected by bovine TB (O'Brien et al., 2006). It is presumed that the remaining herds have become infected by interspecies transmission of *M. bovis* from deer (O'Brien et al., 2002). Interspecies transmission of *M. bovis* from deer to cattle through indirect contact was examined by exposing cattle (9 six-month-old calves) to pens previously occupied by experimentally infected deer (Palmer and Whipple, 2000). The pen switching was carried out for 80 days. At day 77, all of the calves reacted positively to the comparative cervical skin test for bovine TB. At day 177, the calves were necropsied and all demonstrated gross and microscopic lesions consistent with TB and *M. bovis* was isolated from all calves. Neither the specific substrate (water, floor or feed) contaminated with *M. bovis* nor the specific discharge (nasal secretions, saliva, urine or feces) containing *M. bovis* can be determined by this study, but it confirms that *M. bovis* can be transmitted from deer to cattle through a contaminated environment and indirect contact.

An expansion of the study described above, was designed to determine whether or not cattle become infected with *M. bovis* after oral exposure and whether or not cattle become infected with *M. bovis* when exposed to feed previously offered to *M. bovis* infected white-tailed deer (Palmer et al., 2004a). In the oral exposure study, cattle were exposed to *M. bovis* by feeding shelled corn mixed with 8.6×10^5 CFU of *M. bovis* (high dose) or 9.9×10^2 CFU (low dose) over 5 consecutive days. At 133 days after inoculation, the calves were euthanatized and examined for signs of established *M. bovis* infection and lesions consistent with TB. Ingestion of *M. bovis* resulted in TB lesions or

M. bovis was isolated in 3 of 4 calves in the "low-dose" group and 1 of 4 calves in the "high dose" group, indicating that cattle are susceptible to infection via the oral route of exposure and at doses of *M. bovis* lower than those previously considered necessary for infection via ingestion (O'Reilly and Daborn, 1995; Palmer et al., 2004a). The final phase of the study, in which calves were exposed exclusively to feed previously offered to *M. bovis* infected deer, also confirmed the feasibility of contaminated feed acting as a vehicle in the indirect and interspecies transmission of *M. bovis* between white-tailed deer and cattle. Four of the nine calves exposed to the feed previously offered to *M. bovis* infected deer had lesions consistent with TB or had tissues from which *M. bovis* was isolated at the end of the study.

The studies presented in this section clearly demonstrate the feasibility of indirect transmission of *M. bovis* between white-tailed deer and cattle through *M. bovis* contaminated feed and/or environment. The application of the findings of these transmission studies, performed in a laboratory environment, to disease dynamics in the natural setting have been questioned given the fact that animals, both domestic and wild, housed in confinement are often under a level of stress that might compromise their immune systems. An additional drawback of transmission studies carried out in confined housing (unavoidable given the bio-security concerns when working with *M. bovis*) is that animals in confined housing will exhibit behaviors different from those observed in a natural setting and these behaviors in confinement (primarily close contact and feeding from a single source) will generally increase both direct and indirect transmission of *M. bovis*. Regardless of the limitations of these transmission studies, they do confirm the shedding of *M. bovis* in the secretions of infected deer and the subsequent contamination

of feed and the environment. The studies confirm the susceptibility of cattle to relatively low doses of *M. bovis* via the oral route and they demonstrate indirect transmission of *M. bovis* among white-tailed deer and between white-tailed deer and cattle.

1.2.2 Lesion distribution, character and route of disease transmission

1.2.2.1 Lesion Distribution in White-Tailed Deer

The distribution of tuberculosis lesions in an infected animal has often been used as an indication of the route by which the animal became infected with *M. bovis*. The majority of tuberculosis lesions in cattle occur in the respiratory lymph nodes and lungs and the most common and efficient route of disease transmission is thought to be the inhalation of aerosolized *M. bovis* on respiratory droplets produced by an infected animal (Drobniewski et al., 2003). The less common discovery of a tuberculosis lesion in the mesenteric lymph nodes of cattle has often been attributed to infection via the oral route (Neill et al., 1994). Attempts have, therefore, been made to examine the distribution of gross and microscopic tuberculosis lesions in Michigan white-tailed deer to assess, among other objectives, what can be determined about the route of pathogen transmission. Studies assessing the distribution of lesions in domestic cattle from TB-positive farms in Michigan have been more limited and instead research has been more focused on perfecting and assessing TB infection detection methods in cattle (Dunn et al., 2005; Norby et al., 2004; Norby et al., 2005).

The distribution of lesions and extent of tissues infected with *M. bovis* was examined in a population of captive white-tailed deer in Michigan that had been naturally infected with *M. bovis* (Palmer et al., 2000). Fourteen of the 116 captive deer examined

were determined to be TB-positive based on the isolation of *M. bovis* from one or more tissues. Nine of the 14 deer had lesions consistent with TB. Lesions were most commonly seen in the medial retropharyngeal lymph node followed by the lung. Lesions were also noted in the mediastinal lymph node, hepatic lymph node, mesenteric lymph node, small intestine and palatine tonsil. This distribution of lesions is similar to that described in the population of free-ranging white-tailed deer examined during the initial surveys for TB in northeast Michigan in 1995-1996 when the outbreak in the region was first described (Schmitt et al., 1997). The 1995-1996 survey relied on the submission of hunter-harvested deer so full carcasses were rarely (3 of 354) available for examination. However, the medial retropharyngeal lymph node was again identified as the tissue most likely to contain gross lesions consistent with TB in *M. bovis* infected deer. The medial retropharyngeal lymph node was also found to be the most commonly affected tissue among white-tailed deer that tested positive (58) in a total of 19,500 deer submitted to the Rose Lake Wildlife Disease Laboratory for *M. bovis* testing in 1999, as part of the on-going surveillance efforts of the Michigan Department of Natural Resources. The vast majority of these submissions also consisted only of the head, but when extracranial lesions were noted the thorax, specifically the costal pleura, was the most common lesion site (O'Brien et al., 2001).

The predominance of TB lesions in the medial retropharyngeal lymph nodes of naturally infected white-tailed-deer neither confirms nor refutes the possibility that these deer are infected with *M. bovis* indirectly via ingestion of *M. bovis* contaminated feed or other substrate found in the environment. As has been pointed out by other authors, involvement of medial retropharyngeal lymph node in deer is likely the result of drainage

from the palatine tonsil, and due to its anatomical location, the tonsil would process *M. bovis* in the oropharynx whether it was initially inhaled or ingested (Palmer et al., 2002d). A distinction, however, has been noted in TB lesions in white-tailed-deer infected with *M. bovis* experimentally via the aerosol or "oral" route. The TB lesion distribution recorded in deer known to be infected orally via intratonsilar instillation of *M. bovis* (low and high doses) or those presumed to be infected orally via exposure to contaminated feed, was similar to that seen in naturally infected deer in Michigan with lesions predominantly found in the medial retropharyngeal lymph nodes with the tonsil culturing positive for *M. bovis* throughout the course of infection (Palmer et al., 2002a, 2002b; Palmer et al., 1999). The TB lesions in deer experimentally infected with a low dose of aerosolized *M. bovis* were distributed primarily in the lungs, tracheobronchial and mediastinal lymph nodes, with fewer lesions in the medial retropharyngeal lymph nodes and fewer tonsils that cultured positive for *M. bovis* (Palmer et al., 2003).

1.2.2.2 Lesion Distribution in Cattle

The information available on the distribution of lesions in Michigan cattle involved in the current outbreak of bovine TB is based on a review of the necropsy records of the cattle culled from the first eight farms identified as TB-positive since 1998. Tuberculosis lesions were detected in 45 animals and the breakdown by location was 69% in the chest, 47% in the head and neck, and 24% in the abdomen. Animals with lesions limited to only one site included 33% with lesions only in the chest, 20% with lesions only in the head and neck and 2% with lesions only in the abdomen (Norby, personal communication). This distribution of lesions is similar but not identical to those reported in cattle from a TB-positive farm identified in the U.S. before the

Michigan outbreak (Whipple et al., 1996). The animals in the 1996 study were subjected to a similar full postmortem exam with bacterial culture of tissues for *M. bovis* as that performed for the first 8 herds identified as TB-positive in the Michigan epizootic. The breakdown of lesions by site in the 1996 non-Michigan herd was 60% in the chest, 26.7% in the head and 13% in the abdomen. Reports of lesion distribution in naturally infected TB-positive cattle from different regions of the world are based primarily on slaughter surveillance data. A study of 2,886 cattle with confirmed tuberculosis lesions identified in the slaughter houses of Northern Ireland over 6 year period indicated that 57% annually had lesions only in the chest, 23% annually had lesions only in the head and 3.2% annually had lesions only in the abdomen (mesenteric lymph nodes) (Neill et al., 1994).

As with the data on lesion distribution in naturally infected white-tailed deer in Michigan, the distribution of lesions in cattle identified as part of the current TB outbreak in Michigan provides little definitive information on the route of disease transmission. It is generally accepted that the presence of a primary tuberculosis lesion in the lungs and thoracic lymph nodes is the result of aerosol exposure of cattle to *M. bovis* and that lesions in the intestinal tract are the result of cattle ingesting food and/or water contaminated with *M. bovis* (Kaneene et al., 2002a). This strict delineation between lesions that are the result of ingestion of *M. bovis* in contaminated food vs. inhalation of via exposure to aerosolized *M. bovis* has been challenged by a number of the experimental transmission studies performed at the NADC in Ames, Iowa, and described in Section 1.3.1 (Palmer et al., 2004a; Palmer and Whipple, 2000). Both cattle that were inoculated orally with *M. bovis* in feed, and those presumed to be infected orally when

presented feed previously offered to *M. bovis* infected deer, developed lesions primarily in the lungs and associated lymph nodes (tracheobronchial and mediastinal) with few lesions reported in the medial retropharyngeal lymph nodes, no lesions reported in the mesenteric lymph nodes and only one lesion, in a hepatic lymph node, reported in the abdomen.

1.2.2.3 Lesion Character

Gross TB lesions in lymph nodes of experimentally infected white-tailed deer (Palmer et al., 2002a) and some naturally infected deer (O'Brien et al., 2001; Schmitt et al., 1997) in Michigan have been characterized as containing abundant purulent exudates surrounded by a layer of inflammatory cells and a light fibrous capsule. The frequent presence of liquefied lesions in the lymph nodes of the head of TB positive deer may be important in the dissemination of disease and the indirect transmission of *M. bovis* by increasing the likelihood that *M. bovis* is shed in the nasal exudates and oral secretions of infected deer. However, liquefied lesions are not consistently recorded, even in deer with disseminated disease.

1.2.2.4 Route of Disease Transmission

The distribution of lesions described in naturally infected deer and cattle in the Michigan TB epidemic support transmission of *M. bovis* through contaminated feed as a potentially important means of TB transmission among and between wild white-tailed deer and domestic cattle. The distribution of lesions seen in naturally infected white-tailed deer in Michigan is similar to the distribution seen in orally infected white-tailed deer, as compared to deer infected via inhalation of aerosolized *M. bovis*. This is an indication that inhalation (necessitating close contact between animals) is not the only

important route of disease transmission among white-tailed deer. Lesions recorded in cattle from TB farms in Michigan, and those experimentally infected through the oral route, were located primarily in the lungs and associated lymph nodes. It is possible that *M. bovis* can be aerosolized over feed piles or within the oral cavity during chewing and eructation. But the distribution of lesions in the lungs and associated lymph nodes does not necessarily indicate direct transmission of via nose-to-nose contact between animals. In addition, the time point in the course of infection at which an animal is examined has been shown to influence the pattern of TB lesion distribution in cattle (Goodchild and Clifton-Hadley, 2001; Menzies and Neill, 2000). Conclusions about routes of disease transmission based on the recorded distribution of TB lesions found within an animal should be addressed with caution. This is of particular concern in the population of cattle in northeast Michigan since they are frequently tested for exposure to *M. bovis* and will likely be identified as positive during the early stages of bovine TB disease. The absence of extra-pulmonary lesions in Michigan cattle should not rule out their exposure to *M. bovis* via the oral route.

1.2.3 Observational studies in Michigan

Epidemiological studies and disease modeling have been used to quantify the relative importance of various sources of *M. bovis* transmission in cattle and other species (Menzies and Neill, 2000). Observational case-control studies designed to identify risk factors for disease transmission can, when coupled with the findings of experimental studies, improve our understanding of disease dynamics. A number of studies have focused on Michigan's recent *M. bovis* outbreak and provide various clues to the

potential role of indirect transmission of bovine TB between and among cattle and wildlife populations in the state.

1.2.3.1 Observation Studies of White-tailed Deer

A retrospective study of *Mycobacterium bovis* in white-tailed deer in Michigan, based on data from 5 years of a field surveillance program, provides information on trends in apparent prevalence of disease and various factors associated with *M. bovis* detection in this population (O'Brien et al., 2002). The risk factors associated with *M. bovis* infection identified in this study were age, sex, age/sex interaction and geographic location; older male deer (≥ 2 years), female deer (≥ 3 years) and location within the TB "core"[1] area, significantly increased the odds of a deer being TB positive. Explanations for the disease risk factors identified do not necessarily point to indirect transmission of *M. bovis* as an important means of disease transmission among this population, however, the dispersal behavior of male deer would provide them the opportunity to contact a larger number of potentially *M. bovis* contaminated feed sites in addition to more potentially contaminated individuals during their lifetime. Data available on Michigan elk (*Cervus elaphus*), also included in the TB field surveillance program, provide evidence of the occurrence of indirect disease transmission (O'Brien et al., 2005). As pointed out by O'Brien et al., (2005), elk and deer will generally maintain spatial separation, so interspecies transmission of *M. bovis* more likely occurs through the contamination of feed and bait piles known to be visited by both deer and elk in the area. The likely occurrence of indirect disease transmission between white-tailed deer and elk supports other evidence (lesion distribution and historical practice of winter feeding) that

[1] The bovine TB "core" area is defined as the area where the four corners of Montmorency, Alpena, Oscoda and Alcona counties meet and includes Michigan Department of Natural Resources deer management unit number 452.

indirect transmission of *M. bovis* through contaminated feed, also contributes to bovine TB transmission among white-tailed deer and between deer and cattle.

The role of supplemental feeding of wild white-tailed deer, and the influence of the practice on the apparent prevalence of bovine TB in Michigan deer, was investigated by characterizing the association between the risk of *M. bovis* infection and supplemental feeding practices in the same geographic area (Miller et al., 2003). Although potentially biased by a high non-response rate among landowners in the region, the data collected suggest that supplemental feeding practices associated with an increasing risk for bovine TB in deer were generally indicators of large-scale feeding practices (number of deer fed, quantity of fruits and vegetables provided, quantity of grain provided and the spreading of grain). These feeding sites congregate deer and will facilitate both direct and indirect transmission of *M. bovis*. The provision of larger quantities of feed, however, would also result in the availability of feed at a single location over a longer period of time; circumstances that would enhance the likelihood of indirect transmission of *M. bovis* via the contamination and subsequent ingestion/inhalation of *M. bovis* in feed.

1.2.3.2 Observational Studies of Cattle

Factors associated with the occurrence of bovine TB in cattle on farms in northeast Michigan were assessed by performing a matched case-control study of 68 farms (17 "cases" farms and 51 "controls") (Kaneene et al., 2002b). The relationship between a farm's TB status and farm management practices and environmental factors were assessed with multivariable logistic regression analysis. Factors associated with a reduced risk of TB were the percentage of open land surrounding the farms and the deer exclusion index (a combination of factors relating to exclusion of deer from cattle

housing areas). Univariate analyses suggested an association between an increased risk of TB and the prevalence of TB in wild deer in the surrounding area, the number of other TB farms in the same TRS (square-mile township, range, section block), and the presence of ponds or creeks in cattle housing areas, although these factors did not maintain their significance when adjusted for confounding in the multivariable model.

The results of the case-control study described above contribute to our understanding of the potential role of indirect transmission in the epidemiology of bovine TB in northeast Michigan. The identification of "TB farms in the same TRS" as a risk factor for disease reminds us that fence-line contact and local "sharing" of cattle not necessarily identified as a "cattle purchase" may contribute to cattle-to-cattle *M. bovis* transmission in the region. The other factors identified as significant indicate that opportunities for indirect transmission of *M. bovis* between deer and cattle increase the likelihood of a TB positive farm. The protective "deer exclusion index" indicates that cattle not contained in housing, but presumably with access to open pastures and adjacent wood lots where deer are present, are more likely to become infected with TB. These data suggest that opportunities for overlap in infected deer and cattle feeding/grazing and watering areas enhances interspecies *M. bovis* transmission. Given the lack of observed close contact between cattle and deer, indirect transmission through a contaminated environment is the most likely route of disease transmission.

1.2.3.3 Observational Studies of Other Wildlife

The presence of a wildlife reservoir or maintenance host of *M. bovis* other than the white-tailed deer has been investigated by surveying for the presence of *M. bovis* in free-ranging wildlife (Bruning-Fann et al., 2001; Bruning-Fann et al., 1998; Payeur et al.,

2001; Schmitt et al., 2002) and performing experimental disease transmission studies with some of these species (Butler et al., 2001; Diegel et al., 2002; Fitzgerald et al., 2003; Palmer et al., 2002c). The same strain of *M. bovis* found in cattle and white-tailed deer in Michigan has also been detected in elk, black bear (*Ursus americanus*), bobcat (*Felis rufus*), coyote (*Canis lantrans*), opossum (*Didelphis virginiana*), raccoon (*Procyon lotor*), and red fox (*Vulpes vulpes*) in the bovine TB endemic area of the State (Payeur et al., 2001). The route of disease transmission in the furbearers is thought to be via ingestion of *M. bovis* from an infected deer carcass during natural scavenging events (Bruning-Fann et al., 2001; Bruning-Fann et al., 1998).

The scarcity of gross and histologic lesions in bovine TB positive furbearers, coupled with data from experimental inoculation studies, suggest that none of these species would likely serve as effective maintenance hosts or reservoir of *M. bovis*. Additionally, there is little evidence that they would act as a source of environmental contamination with *M. bovis*. The detection of *M. bovis* in Michigan elk does, however, raise concerns about the potential for the establishment of *M. bovis* in this population (O'Brien et al., 2006) and therefore its potential role in indirect transmission of *M. bovis*.

1.3 CONCLUSIONS

The prevalence of bovine TB in deer varies across the landscape of the endemic region of Michigan as do the environmental conditions and farm management practices that would facilitate or discourage the indirect transmission of *M. bovis* among or between livestock and wildlife species. The published literature on the epidemiology of bovine TB in other systems around the world contains reports that confirm the feasibility,

but do not necessarily emphasize the role, of indirect transmission of bovine TB among or between wildlife and livestock.

Experimental and observational epidemiologic studies, with a focus on the recent discovery of bovine TB in white-tailed deer and cattle in Michigan, have investigated various aspects of disease dynamics and the potential role of indirect transmission of *M. bovis* in the epidemiology of bovine TB in Michigan. Lacking in the investigative efforts in this area are more intensive environmental sampling in locations where bovine TB transmission is known to have occurred, and the determination of the length of time *M. bovis* remains in the environment as a potential source of infection for cattle and/or wildlife.

The epidemiology of bovine TB in every geographical location of its occurrence is complex (Morris et al., 1994). A comprehensive study of the epidemiology of bovine TB in Michigan requires investigating the role of indirect transmission in the dynamics of disease in both domestic livestock and wildlife populations. Understanding the persistence of *M. bovis* in the environment, and its potential role as a source of infection for susceptible hosts, is essential in the development of epidemiologically appropriate control and management policies. Informed decisions regarding the feeding and baiting of wildlife, repopulating cattle farms, designing surveillance programs, and assigning regional bovine TB accreditation zones can only be made with an improved understanding of the role of indirect transmission in the epidemiology of bovine TB in Michigan.

Chapter 2

OPTIMIZING THE ISOLATION OF *MYCOBACTERIUM BOVIS* FROM ENVIRONMENTAL SAMPLES

2.0 ABSTRACT

Objective: To optimize methods for detecting *Mycobacterium bovis* in samples collected from the environment by reducing culture contamination rates and increasing the minimum detection level of the process.

Design: A series of experimental inoculation studies designed to compare two techniques for processing environmental samples for *M. bovis* isolation by bacterial culture.

Sample Population: *M. bovis* inoculated substrates included hay, soil and water. CB-18 and NaOH-based methods were used to process the inoculated samples for isolation of *M. bovis* by mycobacterial culture.

Procedure: *M. bovis* recovery and culture contamination rates were compared in samples processed with CB-18 and NaOH sample processing methods. The minimum detection level of *M. bovis* in feed, soil and water was determined for the CB-18 based method.

Results: CB-18 sample processing methods markedly reduced culture contamination rates. The minimum detection level in the absence of contamination was similar for samples processed with the CB-18 and NaOH-based methods. The minimum detection level was 120 CFU per processed soil, water or hay sample.

Conclusions and Clinical Relevance: The development of a more efficient and effective method for examining samples from the environment for the presence of *M. bovis* is an important step in the endeavor to characterize the persistence of *M. bovis* in the

environment and the potential role of its persistence in the indirect transmission of bovine tuberculosis among and between susceptible species.

2.1 INTRODUCTION

Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, has become established in free-ranging wildlife in northeast lower Michigan (O'Brien et al., 2005). White-tailed deer have been identified as the current primary reservoir host for TB in the region and they are the presumed source of *M. bovis* infection in cattle in over 50% of the herds identified as TB-positive in Michigan (O'Brien et al., 2002). Understanding the role of indirect transmission of *M. bovis* in the epidemiology of bovine TB is of particular importance in Michigan where disease transmission between deer and cattle is thought to occur in the absence of observations of close contact between the species (DeLiberto et al., 2004). The ability to detect and quantify the presence of viable *Mycobacterium bovis* in the environment is an essential first step in understanding the role of indirect transmission in the epidemiology of bovine TB. Information regarding the persistence of *M. bovis* in the environment is essential for the improvement of protocols for cattle farm bio-security, the maintenance of appropriate restrictions on feeding and baiting free-ranging white-tailed deer and other wildlife, and for informing human health and safety regulations regarding the potential public health hazards of bovine TB.

The challenges associated with the detection of *M. bovis* in the environment fall into three major categories. First, environmental samples (soil, feed, fecal material, pond/stream water, etc.) contain large numbers of saprophytic bacteria, molds and other potentially infectious organisms. These other organisms interfere with the isolation of

mycobacteria by overgrowing and out-competing the mycobacteria during the bacterial culture process. A decontamination step is, therefore, 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, evidence suggests that the shedding of *M. bovis* from both infected deer (Palmer et al., 2001) and infected cattle (Goodchild and Clifton-Hadley, 2001; Neill et al., 1988) is intermittent. Intermittent or low level shedding of *M. bovis* coupled with the relatively small size—when compared with the scale of the potentially contaminated environment—of sample that can be collected and processed for mycobacterial culture results in a greatly reduced chance of collecting a contaminated substrate in the environment.

Thirdly, the properties of *M. bovis* make it particularly difficult to process and culture. Like other mycobacteria, *M. bovis* has a 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 (37° C, moist and free of other microbial contamination).

A few attempts have been made to isolate *M. bovis* from environmental substrates in areas identified as sites of natural transmission of bovine TB in Michigan including a captive white-tailed deer facility (Palmer et al., 2000) and one of the first cattle farms identified as TB-positive in the current outbreak of *M. bovis* in Michigan (Kaneene,

personal communication). Environmental samples (feed, soil, water and fecal material) collected from these sites were all negative for *M. bovis*. It has been difficult to determine whether the lack of positive results is due the limited persistence of *M. bovis* in the environment or the challenges associated with detection of *M. bovis* in environmental substrates. Studies performed in indoor bio-safety level III pen facilities have been designed to examine *M. bovis* transmission among deer (Palmer et al., 2004b; Palmer et al., 2001) and between deer and cattle (Palmer et al., 2004a). These studies have yielded positive *M. bovis* cultures from environmental samples collected from the pen floors or feeding areas. The *M. bovis* found in the environment is thought to have played a role in the indirect transmission of TB under these experimental conditions.

Epidemiologic studies of bovine TB in other regions have also failed in efforts to isolate viable *M. bovis* from environmental samples collected under natural disease transmission conditions (Pillai et al., 2000). Researchers interested in the persistence of *M. bovis* in the environment have turned to experimental inoculation studies and an assessment of the conditions that support or inhibit *M. bovis* survival (Duffield and Young, 1985; Jackson et al., 1995; Little et al., 1982; Maddock, 1933, , 1934, , 1936; Tanner and Michel, 1999; Williams and Hoy, 1930; Wray, 1975). A recent study has described the successful identification of *M. bovis* DNA in the environment under natural disease transmission conditions (Young et al., 2005) but this does not address the epidemiologically important question of the persistence of viable and infectious *M. bovis* in the environment.

The standard procedure for processing samples for mycobacterial isolation involves the mixing and grinding of the substrates, settling and the removal of the

supernatant for further processing, a NaOH-based decontamination step, and centrifugation to concentrate any mycobacteria in the sample before inoculating culture media for mycobacterial isolation (Payeur et al., 2001). This standard procedure was followed for processing the environmental samples collected from a large enclosure holding white-tailed deer infected with *M. bovis* (Palmer et al., 2000).

A new method for processing respiratory specimens for mycobacterial detection with C_{18}-Carboxypropylbetaine was published in 1998 (Thornton et al., 1998a). The C_{18}-Carboxypropylbetaine is a zwitterionic detergent. It replaces NaOH as the primary decontamination agent and its addition to processed samples is thought to decrease surface tension and counteract the natural buoyancy of mycobacteria and facilitate the more efficient collection of the bacilli (Thornton et al., 1998a).

The CB-18 method was initially reported to significantly increase the mycobacterial detection sensitivity of both culture and acid-fast smear methods, but it also resulted in an increase in the rate of non-mycobacterial contamination of liquid cultures (Thornton et al., 1998a). A sediment resuspension buffer with lecithin, to reduce toxicity, and a mixture of lytic enzymes (lysosyme, zymolyase, *Cytophaga* and *Trichoderma* extracts) to reduce contamination was, therefore, added to the CB-18 sample processing protocol (Thornton et al., 1998c).

A follow-up study by Thornton, et al, 1998b, produced results that suggest that *M. bovis* BCG is particularly sensitive to NaOH, and that processing with this method may generate a large number of false-negative culture results (Thornton et al., 1998b). The effect of CB-18 replacement of NaOH as the primary decontamination agent on the recovery rates of 5 *M. tuberculosis* complex isolates and 4 non-*M. tuberculosis*

mycobacterial isolates was studied. The recovery rate of mycobacteria were significantly higher in those specimens processed with CB-18 as compared to NaOH across isolates, but the difference in recovery rates (1.6% to 2.3 % for NaOH, and 70.9% to 84.4% for CB-18) was markedly higher for the *M. bovis* BCG isolate when compared to other *M. tuberculosis* complex bacteria, and this may be true for all *M. bovis* isolates.

An improved protocol for processing samples collected from the environment for mycobacterial culture is necessary for the further investigation of the role of *M. bovis* persistence in the environment and indirect transmission in the epidemiology of bovine TB in Michigan. The CB-18 sample processing method has resulted in improved mycobacterial detection when applied to various specimens in a number of different settings (Cornejo et al., 1998; Laserson et al., 2005; Manterola et al., 2003; Ozbek et al., 2003; Padilla et al., 2005; Thornton et al., 1999; Thornton et al., 1998a). Experimental *M. bovis* inoculation studies were, therefore, designed to create and evaluate a method for processing environmental samples collected from areas of suspected bovine TB transmission in Michigan and elsewhere. The specific hypothesis tested is that the CB-18 method significantly reduces the rate of contamination of *M. bovis* inoculated environmental substrates and reduces the probability of false-negative results associated with the standard NaOH-based method. To test the stated hypothesis, *M. bovis* recovery and culture contamination rates were compared in samples processed with both CB-18 and NaOH. The minimum detection level of *M. bovis* in feed, soil and water was determined for the CB-18 based method.

2.2 MATERIALS AND METHODS

2.2.1 Facility, culture, media, and growth conditions

All *Mycobacterium bovis* experimental inoculation studies were performed in the mobile bio-safety level III (BL3) laboratory on the campus of Michigan State University (MSU). *M. bovis* was obtained from a frozen culture of *M. bovis* originally isolated in 2001 from a 5-year-old Charolais cow from northeast Michigan with gross and microscopic lesions consistent with bovine TB. One ml of the previously frozen culture was added to 10 ml of Middlebrook 7H9 Broth with Middlebrook ADC Enrichment for cultivation of mycobacteria (Becton-Dickenson, Cockeysville, Maryland 21030, USA). Multiple 10 ml vials of *M. bovis* inoculated 7H9 Broth were incubated at 37° C for 21 to 30 days or until the cell density reached 1 X 10^8 to 5 X 10^8 colony forming units (CFU)/ml. The culture was then distributed into 1.5 ml portions, used immediately or frozen at -80° C.

2.2.2 Environmental substrates

Grass hay, water and soil were selected as environmental substrates for *M. bovis* inoculation, sample processing and isolation experiments. Grass hay was collected from the feed storage area of the MSU Large Animal Veterinary Teaching Hospital in East Lansing, Michigan. Soil was collected from the Baker Woodlot (Rachana Rajendra Neotropical Bird Sanctuary) located in the south central section of the MSU campus. Water was collected from the large pond at the center of the Baker Woodlot and from the Red Cedar River at the Farm Lane Bridge on the MSU campus.

Samples of soil and hay were stored in 5-gallon capacity black plastic bags and water was stored in clear 1 liter plastic bottles. Once collected all environmental substrates were stored at 4° C with no exposure to light.

Sets of environmental substrates for the *M. bovis* inoculation experiments were prepared as follows. Soil samples were divided into 2 gm portions and placed in 3 X 7 inch Whirl-Pak® bags. Hay samples were chopped with scissors and 2 gm portions were placed in sterilized Ball® pint regular mason jars. Water was divided into 7.5 ml portions and place in 50 ml conical centrifuge tubes.

Approximately half of the bulk samples of soil, hay and water were autoclaved for 2 hours at 121° C and 20 psi before they were apportioned into the experimental samples sets described above. The autoclaved environmental substrates were used to set up the "pre-sterilized" environmental substrates used in the *M. bovis* inoculation studies.

2.2.3 Inoculation Experiment 1 (CB18 vs. NaOH Processing)

This experiment included 8 soil samples (4 sterilized; 4 non-sterilized), 8 hay samples (4 sterilized; 4 non-sterilized), and 8 water samples (4 sterilized; 4 non-sterilized). Two ml of thawed *M. bovis* liquid culture were added to each sample. Four replicates of each sample type, 2 sterilized before *M. bovis* inoculation and 2 not sterilized before inoculation, were processed with CB-18. The remaining 12 samples were processed with NaOH.

2.2.4 Inoculation Experiment 2 (CB18 vs. NaOH Processing at Different *M. bovis* Concentrations)

This experiment included 16 soil samples (8 sterilized; 8 non-sterilized), 16 hay samples (8 sterilized; 8 non-sterilized), and 16 water samples (8 sterilized; 8 non-sterilized). The 48 samples were divided into 8 sets of 2 samples (1 sterilized; 1 non-sterilized) of each substrate. The sample sets were inoculated with either 2 ml of *M. bovis* liquid culture, a 1:10 dilution, a 1:100 or a 1:1000 dilution. The *M. bovis* liquid culture was diluted with Middlebrook 7H9 Broth. The inoculated samples were divided into two equivalent sets and processed with either the CB-18 or NaOH. Samples of the *M. bovis* liquid culture and 3 dilutions were inoculated onto plates with modified Middlebrook 7H11 Agar (Diagnostic Center for Population and Animal Health (DCPAH), Lansing, MI, USA) and Selective 7H11 agar (Becton-Dickinson) to monitor for *M. bovis* growth.

2.2.5 Inoculation Experiment 3 (CB18 vs. NaOH Processing 4 Different Known *M. bovis* Concentrations)

This experiment included 16 soil samples (8 sterilized; 8 non-sterilized), 16 hay samples (8 sterilized; 8 non-sterilized), and 16 water samples (8 sterilized; 8 non-sterilized). The 48 samples were divided into 8 sets of 2 samples (1 sterilized; 1 non-sterilized) of each substrate. The sample sets were inoculated with either Dilution 1 (1 ml of *M. bovis* liquid culture + 50 ml of 7H9), Dilution 2 (1 ml of Dilution 1 + 50 ml of 7H9), Dilution 3 (1 ml of Dilution 2 + 50 ml of 7H9) or Dilution 4 (1 ml of Dilution 3 +

50 ml of 7H9). Four (Dilution 1-4) inoculated sample sets were processed with CB-18 and the remaining 4 sample sets were processed with NaOH. Exactly 100 µl of the 4 dilutions of *M. bovis* were inoculated onto plates with modified Middlebrook 7H11 Agar (DCPAH) and Selective 7H11 agar (Becton-Dickinson) to monitor for *M. bovis* growth and to determine the *M. bovis* concentration (CFU/ml) of each dilution.

2.2.6 Inoculation Experiment 4 (CB18 Processing, with and without Liquifaction, of Samples with 2 Different Known *M. bovis* Concentrations Stored for Varying Times Under Different Conditions)

This experiment included 32 soil samples (16 sterilized; 16 non-sterilized), 32 hay samples (16 sterilized; 16 non-sterilized), and 32 water samples (16 sterilized; 16 non-sterilized). The 96 samples were divided into 4 sample sets. Each set contained 4 samples (2 sterilized; 2 non-sterilized) of each substrate that were inoculated with 1 ml of Dilution 1 (5 ml of *M. bovis* liquid culture + 75 ml of 7H9) and 4 samples (2 sterilized; 2 non-sterilized) of each substrate inoculated with 1 ml of Dilution 2 (1 ml of *M. bovis* liquid culture + 75 ml of 7H9) of *M. bovis* liquid culture. One sample set was processed for mycobacterial isolation at time 0 (on the same day as the inoculation), one sample set was held for 72 hours at room temperature before processing, one sample set was held for 72 hours at 4° C before processing and one sample set was stored for 18 days at –20 ° C before processing. All samples were processed with the CB-18 method. The samples were further divided into 1 set treated with liquifaction solution and another set in which water was substituted for liquifaction in the sample processing procedure.

Samples of the 2 *M. bovis* dilutions were stored alongside the inoculated environmental substrates. Exactly 100 µl of the 2 dilutions of *M. bovis* were inoculated onto plates with modified Middlebrook 7H11 Agar (DCPAH) and Selective 7H11 agar (Becton-Dickinson) to monitor for *M. bovis* growth and to determine the *M. bovis* concentration (CFU/ml) of each dilution at time 0, after 72 hours at room temperature, after 72 hours at 4° C and after 19 days at –20 ° C

2.2.7 Inoculation Experiment 5 (CB18 Processing of Samples with 2 Different Known *M. bovis* Concentrations Stored for Varying Times Under Different Conditions)

Inoculation experiment 5 was a repeat of inoculation experiment 4 with ½ the number of samples per set (12). Each set contained 2 samples (1 sterilized; 1 non-sterilized) of each substrate that were inoculated with 1 ml of Dilution 1 (2.5 ml of *M. bovis* liquid culture + 37.5 ml of 7H9) and 2 samples (1 sterilized; 1 non-sterilized) of each substrate that were inoculated with 1 ml of Dilution 2 (0.5 ml of *M. bovis* liquid culture + 39.5 ml of 7H9) of *M. bovis* liquid culture. One sample set was processed for mycobacterial isolation at time 0 (on the same day as the inoculation), one sample set was stored for 72 hours at room before processing, one sample set was stored for 72 hours at 4° C before processing and one sample set was stored for 16 days at –20 ° C before processing. All samples were processed with the CB-18 method. Exactly 100 µl of the 2 dilutions of *M. bovis* were inoculated onto plates with modified Middlebrook 7H11 Agar (DCPAH) and Selective 7H11 agar (Becton-Dickinson) to monitor for *M. bovis* growth and to determine the *M. bovis* concentration (CFU/ml) of each dilution. In addition,

exact amounts (100 µl) of the processed samples were inoculated onto mycobacterial culture plates to facilitate more precise determination of *M. bovis* recovery rates and minimum detection levels.

2.2.8 CB-18 Sample Processing

The CB-18™ TB Culture Kit with Lytic Decon™ II (Integrated Research Technology, LLC, Quest Diagnostics Inc., Baltimore, MD) was used to process *M. bovis* inoculated environmental substrates in the "CB-18" sample sets of the experimental studies. The sample processing procedure used was an adaptation of the protocol for processing tissue specimens outlined in the official CB-18™ TB Culture Kit with Lytic Decon™ II instruction booklet (Technology). The solutions and reagents necessary for the CB-18™ processing were made up according to the procedures outlined in the instruction booklet. These included the following: 1) Decontamination Solution (20X Tris-citrate Buffer, CB-18™ Stock, N-acetyl-L-cysteine or NALC and water); 2) 2X-Resuspension Solution (10X-Enzyme Stock-*Trichoderma harzianum* extract, lysozyme and *Lysobacter* extract and NALC); and 3) NALC-Citrate Liquefaction Solution trisodium citrate dehydrate and NALC).

Environmental substrate specimens were processed in the following 10 steps:

1. Pulverize/Homogenize Sample
    a. Add 5 ml liquefaction solution (all samples)
    b. Add 7.5 ml sterile water to soil and hay samples
    c. Mix
        i. Water Samples: Vortex on high for 30 seconds

ii. Soil Samples: Place in Stomacher® 80 (laboratory blender) on medium for 30 seconds

iii. Hay Samples: Secure blade unit head and gasket on jar, invert and blend with household blender on high for 30 seconds

2. Place specimens upright and allow to settle for 30 minutes.
3. Remove top 5 ml of fluid for each specimen and transfer to 50 ml conical tube containing 10 ml of Decontamination Solution.
4. Vortex well and incubate at 37° C for 75 minutes.
5. Add sterile water to 50 ml mark on each tube and mix.
6. Centrifuge specimens at 3,000 g for 20 minutes.
7. Decant pellet-containing tubes completely; use a pipette to remove all but 1-3 ml of liquid from samples without a visible pellet. Resuspend the pellet by vortexing in supernatant backwash.
8. Add 1 ml of sterile water and mix. Remove 0.5 ml of sample and transfer to 1.5 ml labeled cryo tube and freeze at –80° C.
9. Add 1 ml of 2X Resuspension solution to each sample, mix and incubate for 45 minutes at 37° C.
10. Remove samples from incubator and prepare to inoculate on mycobacteria isolation media.

2.2.9 NaOH Sample Processing

An adaptation of the standard NaOH-based procedure for processing tissue samples for mycobacteria isolation (Payeur et al., 2001) was used to process the *M. bovis* inoculated environmental substrates in the "NaOH" sample sets of the experimental studies. Briefly: An equal volume of phenol red nutrient broth (Becton-Dickinson) was added to each sample. Sample containers were capped and/or sealed and the contents were thoroughly mixed. Ten ml of the mixed sample suspension was transferred with a large orifice pipette to a 50 ml screw-cap conical centrifuge tube containing 5 ml of 0.5 N NaOH. The samples remained in the NaOH for 20-30 minutes. Drops of 6.0 N HCl were added to the sample suspension until the mixture turned yellow and then the suspension color was brought back to pale pink with 1.0 N NaOH. The neutralized sample suspension was then centrifuged at 6,000g (4540 rpm) for 20 minutes. The pellet and approximately 85% of the overlaying fluid was decanted. The sample sediment was resuspended in the residual fluid and used to inoculate the *M. bovis* isolation media.

2.2.10 *Mycobacterium bovis* Isolation and Identification

A range of media, solid and liquid culture systems were used to isolate *Mycobacterium bovis* in these experiments. For initial experiments (*M. bovis* inoculation experiments 1-3) both samples processed with NaOH and those processed with CB-18 were inoculated onto solid media slants containing modified Middlebrook 7H11 agar (Becton-Dickinson) with sodium pyruvate (DCPAH), modified Middlebrook 7H10 (Becton-Dickinson) with sodium pyruvate (DCPAH), Lowenstein Jensen and Mitchison 7H11 Selective agar (Remel, Lenexa, Kansas 66215, USA). Sample sediments were also

inoculated into Bactec 12B liquid media (Becton-Dickinson) supplemented with the antimicrobial PANTA™ (Becton-Dickenson) and erythromycin. In later experiments (*M. bovis* inoculatoin experiments 4-5) plates containing modified Middlebrook 7H11 agar (Becton-Dickinson) with sodium pyruvate (DCPAH) and 7H11 Selective plates (Becton-Dickinson) were used exclusively.

Solid media slants and plates were incubated at 37°C for 8-12 wk and examined weekly for colony formation. Inoculated Bactec 12B vials were placed in a BACTEC 460TB system and monitored daily for indications of mycobacterial growth.

Positive mycobacterial cultures and colonies on solid media were subjected to an acid-fast smear analysis to confirm the presence of acid-fast bacteria using standard protocols for slide preparation, staining and examination (Kent and Kubica, 1985). Random samples were identified to species group, *Mycbocaterium tuberculosis* complex, using a genetic probe (AccuProbes, Gen-Probe, San Diego, California 92121, USA).

2.2.11 Recording of Contamination and Calculation of *M. bovis* Recovery Rates and Minimum Detection Levels

The presence of contamination with non-mycobacteria in liquid and solid media, on slants and plates, was recorded for every processed sample. The presence of contamination on solid agar plates was further characterized according to the percent of total area of the plate affected. "None" (corresponding to 0% of the plate), "some" (corresponding to $\leq$ 25% of the plate), "yes" (corresponding to >25% and $\leq$ 50% of the plate) or "very" (corresponding to >50% of the plate).

An *M. bovis* growth curve experiment (concentration of CFU recorded over time) was used to predict the concentrations of *M. bovis* dilutions used in the inoculation experiments. This was, however, always confirmed by quantitative culture at the time of sample inoculation. The confirmed concentration of *M. bovis* dilutions were compared to the mycobacterial culture results of the processed specimens to determine the minimum number of *M. bovis* CFUs necessary per sample to detect their presence with mycobacterial culture. The mycobacterial culture results of the inoculated pre-sterilization substrates were used to compare sample processing recovery rates in the absence of contamination.

2.2.12 Data Analysis

Descriptive statistics were generated in Excel (Microsoft Office XP Professional). Comparisons of contamination rates in the CB-18 and NaOH-processed samples were conducted with SAS software (SAS version 9.0, Cary N.C.: SAS Institute, Inc., 2003) using the Cochran-Mantel-Haenszel Chi-square or 2-tailed Fisher's exact test where appropriate. Odds ratios with 95% confidence intervals that did not contain 1 were considered significant.

2.3 RESULTS

2.3.1 Comparison of CB-18 and NaOH-based Processing of Environmental Substrates for *M. bovis* Isolation

The results of *M. bovis* inoculation experiments 1 – 3 are presented in tables 2.1, 2.2 and 2.3. Environmental substrates (hay, soil and water) are significantly less likely to be contaminated if processed for mycobacterial culture with the CB-18 method as compared to the NaOH-based method. The odds of contamination in NaOH processed *M. bovis* inoculated environmental substrates that were not pre-sterilized was 11 times that of the same substrates processed with the CB-18 method [OR=11.0; 95% C.I. (3.2, 37.9)]. The actual concentration of *M. bovis* used in dilutions 1-4 in inoculation experiment 3 was 100 fold lower than intended. Relevant data on rates of contamination are presented, however, *M. bovis* detection in dilutions 2-4 would not be expected irrespective of sample processing method.

The presence of contamination resulted in the inability to detect *M. bovis* and the reporting of false negative results. There was a negative correlation between the number of contaminated samples and the number of positive results. CB-18 processing outperformed NaOH processing methods for *M. bovis* detection in the inoculated environmental substrates that were not pre-sterilized because a greater number of false negative results were produced by the samples processed with the NaOH based method.

The substrate that was most likely to produce contamination, regardless of sample processing method, was soil.

2.3.2 Effect of Sample Storage Time and Conditions on *M. bovis* Recovery and Contamination Rates with CB-18 Processing

The results of *M. bovis* inoculation experiments 4-5 are presented in Tables 2.4.1, 2.4.2 and 2.5. The least contamination and least number of false negative results were found in the pre-sterilized environmental substrates processed with CB-18 immediately after inoculation (time 0). The number of contaminated samples and the number of false negative results was highest for inoculated substrates held at room temperature for 72 or 24 hours before processing. The storage of samples at 4° C and -20° C before CB-18 processing resulted in an intermediate number of contaminated samples and false negative results. Samples held at -20° C in inoculation experiment 5 were all contaminated during sample processing. *M. bovis* isolation and contamination data were not available for these samples.

False negative *M. bovis* culture results in experiments 4 and 5 are associated with the presence of contamination. Colony count data (not presented in the tables) suggests that storage at room temperature and 4° C does not have an affect on the numbers of viable *M. bovis* bacilli, however, testing of *M. bovis* stored at –20° C indicated a 5 fold reduction in recoverable *M. bovis* after a single freeze-thaw cycle.

The substitution of water for liquefaction in the CB-18 sample processing procedure (inoculation experiment 4) did not affect sample contamination or *M. bovis* detection.

2.3.3 Minimum Detection Levels

In the absence of contamination, samples processed with CB-18 and those processed with NaOH performed equally in the detection of *M. bovis* via mycobacterial culture. Both the NaOH and CB-18 sample processing methods, regardless of the substrate type, decreased the number of viable *M. bovis* detectable by mycobacterial culture. In inoculation experiment 2 approximately 120 CFUs of *M. bovis* were delivered to each substrate. Both CB-18 and NaOH-processed samples consistently detect *M. bovis* at this original concentration. Colony counts on plates inoculated with exactly 100 µl of processed specimens originally inoculated with Dilution 1 (120 CFU) yield 1-2 CFU. The final volume of processed specimen was approximate 1 ml, indicating a 80%-90% loss of *M. bovis* bacilli during processing. The minimum detection level of *M. bovis* in the volume of environmental substrates processed in this series of experiments was 120 CFUs.

2.3.4 Mycobacterial Culture Media

The use of Bactec 12B liquid media (Becton-Dickinson) resulted in high rates of contamination in samples processed with NaOH and CB-18 and the culture media was not used in inoculation experiments 4-5. The least contamination was recorded on plates containing modified Middlebrook 7H11 agar (Becton-Dickinson) with sodium pyruvate (DCPAH) and 7H11 Selective plates (Becton-Dickinson). The quantification of contamination and the differentiation and identification of individual *M. bovis* colonies was difficult on slants.

Table 2.1 Inoculation Experiment 1: Record of contamination and culture results of sterilized and non-sterilized hay, water and soil samples inoculated with 1 X 10^6 to 1 X 10^8 CFU of *M. bovis* and processed with the CB-18 or NaOH-based method.

Substrate	Sterilized/Non	CB-18 Contamination	CB-18 Culture Result	NaOH Contamination	NaOH Culture Result
Hay	Sterilized	None	Positive	None	Positive
Hay	Sterilized	None	Positive	None	Positive
Water	Sterilized	None	Positive	None	Positive
Water	Sterilized	None	Positive	None	Positive
Soil	Sterilized	None	Positive	None	Positive
Soil	Sterilized	None	Positive	None	Positive
		% Cont. = 0%	% Pos. = 100%	% Cont. =0%	% Pos. =100%
Hay	Non-Sterilized	None	Positive	Yes	Negative
Hay	Non-Sterilized	None	Positive	None	Positive
Water	Non-Sterilized	None	Positive	Yes	Positive
Water	Non-Sterilized	None	Positive	None	Positive
Soil	Non-Sterilized	None	Positive	Yes	Negative
Soil	Non-Sterilized	None	Positive	Yes	Negative
		% Cont. = 0%	% Pos. = 100%	% Cont. = 66.6%	% Pos. = 50%

Table 2.2 Inoculation Experiment 2: Record of contamination and culture results of sterilized and non-sterilized hay, water and soil samples inoculated with either Dilution 1 (>10,000 CFU); Dilution 2 (> 1,000 CFU); Dilution 3 (>100 CFU) or Dilution 4 (<10 CFU) of *M. bovis* and processed with the CB-18 or NaOH-based method.

Dilution	Substrate	Sterilized/Non	CB-18 Contamination	CB-18 Culture Result	NaOH Contamination	NaOH Culture Result
1	Hay	Sterilized	None	Positive	None	Positive
2	Hay	Sterilized	None	Positive	None	Positive
3	Hay	Sterilized	None	Positive	None	Positive
4	Hay	Sterilized	None	Negative	None	Negative
1	Water	Sterilized	None	Positive	None	Positive
2	Water	Sterilized	None	Positive	None	Positive
3	Water	Sterilized	None	Positive	None	Positive
4	Water	Sterilized	None	Negative	None	Negative
1	Soil	Sterilized	None	Positive	None	Positive
2	Soil	Sterilized	None	Positive	None	Positive
3	Soil	Sterilized	None	Positive	None	Positive
4	Soil	Sterilized	None	Negative	None	Negative
1	Hay	Non-Sterilized	None	Positive	Yes	Positive
2	Hay	Non-Sterilized	None	Positive	Yes	Positive
3	Hay	Non-Sterilized	None	Positive	Yes	Positive
4	Hay	Non-Sterilized	None	Negative	Yes	Negative
1	Water	Non-Sterilized	None	Positive	None	Positive
2	Water	Non-Sterilized	None	Positive	Yes	Positive
3	Water	Non-Sterilized	None	Positive	None	Positive
4	Water	Non-Sterilized	None	Negative	Yes	Negative
1	Soil	Non-Sterilized	None	Positive	Yes	Positive
2	Soil	Non-Sterilized	None	Positive	Yes	Positive
3	Soil	Non-Sterilized	None	Positive	Yes	Positive
4	Soil	Non-Sterilized	None	Negative	Yes	Negative
			% Cont. = 0%		% Cont. = 83.3 % (Non-Sterilized)	

Table 2.3 Inoculation Experiment 3: Record of contamination and culture results of sterilized and non-sterilized hay, water and soil samples inoculated with either Dilution 1* (120 CFU); Dilution 2* (0 CFU); Dilution 3* (0 CFU) and Dilution 4* (0 CFU) of *M. bovis* and processed with the CB-18 or NaOH-based method. *100 fold less than the intended concentration of *M. bovis*

Dilution	Substrate	Sterilized/Non	CB-18 Contamination	CB-18 Culture Result	NaOH Contamination	NaOH Culture Result
1	Hay	Sterilized	None	Negative	None	Positive
2	Hay	Sterilized	None	Negative	None	Negative
3	Hay	Sterilized	None	Negative	None	Negative
4	Hay	Sterilized	None	Negative	None	Negative
1	Water	Sterilized	None	Positive	None	Positive
2	Water	Sterilized	None	Negative	None	Negative
3	Water	Sterilized	None	Negative	None	Negative
4	Water	Sterilized	None	Negative	None	Negative
1	Soil	Sterilized	None	Positive	None	Positive
2	Soil	Sterilized	None	Negative	None	Negative
3	Soil	Sterilized	None	Negative	None	Negative
4	Soil	Sterilized	None	Negative	None	Negative
1	Hay	Non-Sterilized	None	Positive	Yes	Positive
2	Hay	Non-Sterilized	Yes	Negative	Yes	Negative
3	Hay	Non-Sterilized	None	Negative	Yes	Negative
4	Hay	Non-Sterilized	None	Negative	Yes	Negative
1	Water	Non-Sterilized	None	Positive	None	Positive
2	Water	Non-Sterilized	None	Negative	None	Negative
3	Water	Non-Sterilized	Yes	Negative	None	Negative
4	Water	Non-Sterilized	None	Negative	None	Negative
1	Soil	Non-Sterilized	Yes	Negative	Very	Negative
2	Soil	Non-Sterilized	Yes	Negative	Very	Negative
3	Soil	Non-Sterilized	Yes	Negative	Very	Negative
4	Soil	Non-Sterilized	Yes	Negative	Very	Negative
			Non-Sterilized % Cont. = 50%		Non-Sterilized % Cont. = 66.6%	

Table 2.4.1 Inoculation Experiment 4: Record of contamination and culture results of sterilized hay, water and soil samples inoculated with either Dilution 1 (6 X 10^3 CFU) or Dilution 2 (3 X 10^3 CFU) of *M. bovis* and processed with CB-18 immediately (Time 0); after storage for 72 hr. at Room Temperature; after storage for 72 hr. at 4° C and after storage for 19 days at –20 ° C. "L" denotes samples processed with liquefaction and "W" indicates the substitution of water for liquefaction in the mixing process.

			Time 0		R Temp.; 72 hr.		4° C; 72 hr.		-20° C; 19 days	
Dilution	Substrate	Sterilized/Non	Cont.	Result	Cont.	Result	Cont.	Result	Cont.	Result
1L	Hay	Sterilized	None	Pos	None	Pos	Some	Pos	Yes	Pos
1W	Hay	Sterilized	Some	Pos	Yes	Neg	None	Pos	None	Pos
2L	Hay	Sterilized	Some	Pos	Some	Pos	None	Pos	None	Pos
2W	Hay	Sterilized	None	Pos	Some	Neg	None	Neg	Some	Pos
1L	Water	Sterilized	None	Pos	None	Pos	None	Pos	None	Pos
1W	Water	Sterilized	None	Pos	None	Pos	None	Pos	None	Pos
2L	Water	Sterilized	None	Pos	None	Pos	None	Pos	Yes	Neg
2W	Water	Sterilized	None	Pos	None	Pos	None	Pos	None	Neg
1L	Soil	Sterilized	None	Pos	Yes	Neg	None	Pos	Some	Pos
1W	Soil	Sterilized	None	Pos	Yes	Neg	None	Pos	None	Pos
2L	Soil	Sterilized	Some	Pos	None	Pos	None	Pos	Some	Pos
2W	Soil	Sterilized	None	Pos	Yes	Neg	None	Pos	None	Pos
% Contamination/% Positive:			25%	100%	50%	58.3%	8.3%	91.7%	41.7%	83.3%

Table 2.4.2 Inoculation Experiment 4 continued: Record of contamination and culture results of non-sterilized hay, water and soil samples inoculated with either Dilution 1 (6 X 10^3 CFU) or Dilution 2 (3 X 10^3 CFU) of *M. bovis* and processed with CB-18 immediately (Time 0); after storage for 72 hr. at Room Temperature; after storage for 72 hr. at 4° C and after storage for 19 days at –20 ° C. "L" denotes samples processed with liquefaction and "W" indicates the substitution of water for liquefaction in the mixing process.

			Time 0		R Temp.; 72 hr.		4° C; 72 hr.		-20° C; 19 days	
Dilution	Substrate	Sterilized/Non	Cont.	Result	Cont.	Result	Cont.	Result	Cont.	Result
1L	Hay	Non-Sterilized	Some	Pos	Yes	Neg	None	Pos	Yes	Neg
1W	Hay	Non-Sterilized	Some	Pos	Very	Neg	Some	Neg	Some	Pos
2L	Hay	Non-Sterilized	Some	Pos	Very	Neg	Some	Neg	None	Pos
2W	Hay	Non-Sterilized	Some	Pos	Very	Neg	Very	Neg	Some	Neg
1L	Water	Non-Sterilized	None	Pos	None	Pos	None	Pos	None	Pos
1W	Water	Non-Sterilized	None	Pos	None	Pos	None	Pos	None	Pos
2L	Water	Non-Sterilized	None	Pos	None	Pos	None	Pos	None	Pos
2W	Water	Non-Sterilized	None	Pos	None	Pos	None	Pos	None	Pos
1L	Soil	Non-Sterilized	Yes	Neg	Very	Neg	Very	Neg	Yes	Neg
1W	Soil	Non-Sterilized	Yes	Neg	Very	Neg	Very	Neg	Very	Neg
2L	Soil	Non-Sterilized	Yes	Neg	Very	Neg	Very	Neg	Yes	Neg
2W	Soil	Non-Sterilized	Yes	Neg	Very	Neg	Very	Neg	Yes	Neg
% Contamination/% Positive:			66.7%	66.7%	66.7%	33.3%	58.3%	41.7%	58.3%	50%

Table 2.5 Inoculation Experiment 5: Record of contamination and culture results of non-sterilized hay, water and soil samples inoculated with either Dilution 1 (5 X 10^3 CFU) or Dilution 2 (8 X 10^2 CFU) of *M. bovis* and processed with CB-18 immediately (Time 0); after storage for 24 hr. at Room Temperature; after storage for 24 hr. at 4° C and after storage for 16 days at –20 ° C*.

			Time 0		R Temp.; 24 hr.		4° C; 24 hr.		-20° C; 16 days	
Dilution	Substrate	Sterilized/Non	Cont.	Result	Cont.	Result	Cont.	Result	Cont.	Result
1	Hay	Sterilized	None	Pos	Yes	Pos	None	Pos	--	--
2	Hay	Sterilized	None	Pos	None	Pos	None	Pos	--	--
1	Water	Sterilized	None	Pos	None	Pos	None	Pos	--	--
2	Water	Sterilized	None	Pos	None	Pos	None	Pos	--	--
1	Soil	Sterilized	None	Pos	None	Pos	None	Pos	--	--
2	Soil	Sterilized	None	Pos	None	Pos	None	Pos	--	--
% Contamination/% Positive:			0%	100%	16.7%	100%	0%	100%		

			Time 0		R Temp.; 24 hr.		4° C; 24 hr.		-20° C; 16 days	
Dilution	Substrate	Sterilized/Non	Cont.	Result	Cont.	Result	Cont.	Result	Cont.	Result
1	Hay	Non-Sterilized	None	Pos	Some	Pos	Some	Pos	--	--
2	Hay	Non-Sterilized	Some	Pos	Some	Pos	None	Pos	--	--
1	Water	Non-Sterilized	None	Pos	None	Pos	None	Pos	--	--
2	Water	Non-Sterilized	None	Pos	None	Pos	None	Pos	--	--
1	Soil	Non-Sterilized	Yes	Neg	Very	Neg	Yes	Neg	--	--
2	Soil	Non-Sterilized	Yes	Neg	Yes	Neg	Very	Neg	--	--
% Contamination/% Positive:			50%	33.3%	66.7%	33.3%	50%	33.3%		

* All samples contaminated during processing. No data available.

2.4 DISCUSSION

The detection of *Mycobacterium bovis* in substrates collected from the environment under natural conditions of bovine TB transmission will always be a challenge. The low level and intermittent shedding of *M. bovis* by infected animals (Neill et al., 1988; Palmer et al., 1999), and the limits on the volume of substrate that can be collected and processed for mycobacterial detection, reduce the probability that *M. bovis* will be present in a sample collected for testing from a large potentially contaminated landscape. The detection of *M. bovis* in a contaminated environmental substrate can, however, be improved by the development of sample processing and bacterial culturing methods that limit the growth of non-mycobacterial species and improve the recovery of *M. bovis*.

The standard method for processing veterinary samples, primarily tissues, for mycobacterial detection by culture utilizes NaOH in its decontamination step (Payeur et al., 2001). This method has been applied to the environmental samples collected in the field under natural TB transmission conditions in Michigan (Palmer et al., 2000) and it has been applied to environmental samples collected as part of bovine TB experimental transmission studies with the Michigan TB strain of *M. bovis* performed with penned deer in an indoor bio-safety level II facility (Palmer et al., 2002a, , 2004a, 2004b; Palmer et al., 1999; Palmer et al., 2001). Although *M. bovis* was detected in a subset of the environmental samples collected during the experimental bovine TB transmission studies performed indoors in penned animals, the authors have noted that an improved and more

sensitive method for *M. bovis* detection may be necessary to accurately assess environmental contamination (Palmer et al., 2004a).

The CB-18™ TB Culture Kit with Lytic Decon II (Integrated Research Technology, LLC) is a commercially available set of reagents and instructions for processing specimens for the detection of mycobacteria by culture. The kit contains C_{18}-Carboxypropylbetaine (CB-18), a zwitterionic detergent that replaces NaOH in the decontamination step and is also thought to decrease surface tension and counteract the natural buoyancy of mycobacteria and facilitate the more efficient collection of the bacilli (Thornton et al., 1998a). The kit also contains the components of a resuspension buffer with lecithin and a mixture of lytic enzymes (lysosyme, zymolyase, *Cytophaga* and *Trichoderma* extracts). The resuspension buffer is added to the sample sediment before the inoculation of mycobacteria isolation media to reduce contamination (Thornton et al., 1998c).

The performance of the standard NaOH-based sample processing method and the CB-18 protocol for processing environmental substrates (hay, water and soil) were compared. The performance of the two methods was assessed by processing replicate samples of environmental substrates experimentally "contaminated" or inoculated with *M. bovis*. In each study, one of the sample replicates processed by both methods was sterilized before *M. bovis* inoculation to remove the effect of competition from saprophytic organisms in the environmental substrates.

Data from the first set of experiments (Table 2.1 – 2.3) clearly indicate that the CB-18 method reduced the number of samples contaminated with non-mycobacteria as well as the level of contamination in individual samples. The number of contaminated

samples was negatively correlated with the number of positive *M. bovis* culture results. When contamination was examined by analyzing the results of the processed environmental substrates that were not sterilized before *M. bovis* inoculation, the NaOH processed samples were found to have 11 times the odds of being contaminated. This level of contamination led to the reporting of false negative results in 13% of the samples processed with the NaOH method vs. 3% of the samples processed with the CB-18 method.

A comparison of the NaOH and CB-18 methods for processing environmental samples did not reveal a difference in the minimum detection level of these two methods for isolation of *M. bovis* by culture. Both sample processing methods reduced the number of *M. bovis* bacilli, or CFU, by 80% to 90%. The minimum detection level for both methods of sample processing was approximately 100 CFU/sample. The data from these comparative studies did not reveal the increased sensitivity of *M. bovis* to NaOH reported by Thornton et al (Thornton et al., 1998b), however, the quantitative culturing methods used in these studies may not have been sensitive enough to detect a difference.

The final two *M. bovis* experimental inoculation studies evaluated the effect of sample storage conditions on the recovery of *M. bovis* from samples processed with CB-18 and Lytic Decon II (Table 2.4-2.5). Recovery rates, based on the number of positive samples, were highest for those samples processed immediately. Storage of the samples at room temperature led to an increase in contamination and the reporting of false negative results. When compared to storage at room temperature, refrigerating the samples at 4° C or freezing the samples at –20° C reduced contamination of samples and therefore the reporting of false negative results. A separate analysis of the effect of the

freeze (-20° C) and thaw cycle on the viability of *M. bovis* stored in liquid culture media indicated a 5-fold reduction in the number of viable *M. bovis* bacilli. This indicates that the minimum detection level of *M. bovis* in samples with lower amounts of *M. bovis* than those used in these experiments would be increased if the specimens were subjected to a freeze-thaw cycle.

The final two studies also reveal the variability of results when using sample substrates collected from the field for experimental inoculation studies and the particular difficulty of producing a non-contaminated result from soil samples. Some degree of contamination was encountered when processing the samples with the CB-18 method at all time points and under all storage conditions. The unpredictability of the range of saprophytic organisms encountered in environmental samples necessitates the use of a range of selective media when attempting to isolate mycobacterial species. A commercially available Selective 7H11 agar plate (Becton-Dickenson) specifically designed to isolate pathogenic bacteria and inhibit the growth of other bacteria and molds, produced the "cleanest" *M. bovis* cultures from environmental samples. If additional efforts are made to keep the agar plates and *M. bovis* cultures hydrated, the plates allow for the visual identification and selection of *M. bovis* colonies for further testing in the event that a sample is contaminated with a mix of bacteria and mold.

Despite the difficulties with contamination encountered when using culture as the means of detecting mycobacteria in the environment, it is the only routine way to assess the viability and potential infectivity of *M. bovis* present in environmental substrates. The application of the CB-18 method to the processing of environmental samples does increase the sensitivity of mycobacterial culture by decreasing the contamination that

often results in the reporting of false negative results. The CB-18 sample processing method coupled with the use of selective culture media and special attention applied to the identification of *M. bovis* colonies in mixed cultures should improve the efficiency and success of efforts to identify and characterize the persistence of *M. bovis* in the environment.

2.4.1 Conclusions

Optimized methods for processing environmental samples, and isolating viable *M. bovis* with bacterial culture, include the following: 1) Rigorous mixing of the substrate; 2) Use of CB-18™ TB Culture Kit with Lytic Decon™ II (Intergrated Research Technology, LLC); and 3) Isolation with Selective 7H11 agar plates (Becton-Dickenson). This method reduced contamination and improved the sensitivity of *M. bovis* detection in environmental substrates with bacterial culture.

Chapter 3

AN INVESTIGATION OF BOVINE TUBERCULOSIS TRANSMISSION SITES (CATTLE FARMS AND WILDLIFE AREAS) IN MICHIGAN, USA

3.0 ABSTRACT

Objective: To culture *Mycobacterium bovis* from environmental substrates collected from bovine tuberculosis transmission sites (Michigan cattle farms with confirmed *M. bovis* infection or Michigan townships with the highest recorded apparent prevalence of *M. bovis* in white-tailed deer) in an attempt to document the persistence of *M. bovis* in the environment.

Design: Cross-Sectional Study

Sample Population: 13 cattle farms and 5 wildlife sites within townships with a high apparent prevalence of bovine TB in white-tailed deer.

Procedure: Bovine tuberculosis (TB) positive cattle farms were those farms with a culture confirmed case of *M. bovis* infection identified through the State and Federal bovine TB surveillance program. Wildlife sites were all within the 5 Michigan townships with the highest apparent prevalence of bovine TB in white-tailed deer. The sites sampled were either, the capture locations of *M. bovis*-infected small mammals, or areas of known white-tailed deer congregation. Environmental samples were collected from the sites and processed for mycobacterial culture.

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

Conclusions and Clinical Relevance: White-tailed deer incursion on cattle farms, wildlife feeding, and livestock management practices in Michigan create potential opportunities

for *M. bovis* contamination of environmental substrates by infected hosts. Agent, host and landscape level factors decrease the probability of detecting *M. bovis* in the environment through environmental sampling and conventional mycobacterial culture.

3.1 INTRODUCTION

The State of Michigan lost its United States Department of Agriculture designation as "Free from Tuberculosis (TB)" in 2000. This reversal of the State's TB Free status, originally achieved in 1979, was the result of the detection of bovine TB in Michigan cattle in 1998, and confirmation of the establishment of bovine TB in free-ranging white-tailed deer (*Odocoileus virginianus*) in northeast lower Michigan in 1995 (Schmitt et al., 1997). Thirty-seven farms with *Mycobacterium bovis* infected cattle have been detected in Michigan since intensive surveillance for TB in livestock was reinitiated in 1998 (Judge, 2006).

The occurrence of bovine TB in cattle and white-tailed deer in Michigan has a similar temporal and spatial distribution (Schmitt et al., 2002). In addition, DNA fingerprinting techniques have revealed that cattle and deer are infected with an identical strain of *M. bovis* (Whipple et al., 1999c). Evidence supports interspecies transmission of *M. bovis* from the wildlife reservoir (white-tailed deer) to cattle but the exact mechanism and route of disease transmission is unknown. White-tailed deer are the presumed source of *M. bovis* infection in cattle in over 50% of the herds identified as TB positive in Michigan (O'Brien et al., 2002). Disease transmission in these instances is thought to occur in the absence of close contact between cattle and deer, since nose-to-nose contact between the two species is rarely observed (DeLiberto et al., 2004).

Bovine TB in Michigan's white-tailed deer population has been characterized as having an endemic focus within the five-country area of Presque Isle, Montmorency, Alpena, Oscoda and Alcona counties, where 97% of the TB positive deer have been found (Hickling, 2002; O'Brien et al., 2002; O'Brien et al., 2006). The detection of bovine tuberculosis in Michigan cattle has been concentrated in the same area of Michigan. This region of the State, encompassing the five counties that make up the endemic focus of bovine TB in deer in addition to Antrim, Charlevoix, Cheboygan, Crawford, Emmet, Otsego, and portions of Iosco and Ogemaw counties have been designated as "infected" with bovine tuberculosis and classified as "modified accredited" under the guidelines of the Federal Bovine Tuberculosis Eradication Uniform Methods and Rules (USDA, 2004).

This study was designed to investigate potential sites of bovine TB transmission in an effort to detect *M. bovis* in environmental substrates. Potential sites of bovine TB transmission were defined as Michigan cattle farms with confirmed *M. bovis* infection or Michigan townships with the highest recorded apparent prevalence of *M. bovis* in white-tailed deer. Bovine TB positive cattle farms were those farms with a culture confirmed case of *M. bovis* infection identified through the State and Federal bovine TB surveillance program. Wildlife sites were all within the 5 Michigan townships with the highest apparent prevalence of bovine TB in white-tailed deer. The sites sampled were either, the capture locations of *M. bovis*-infected small mammals, or areas of known white-tailed deer congregation. Specific substrates targeted for collection within the sites were selected based on environmental and farm management factors identified as risk factors for bovine TB infection on Michigan cattle farms (Kaneene et

al., 2002b) and previous evaluations of the practice of supplemental feeding of white-tailed deer (Hickling, 2002; Miller et al., 2003; Schmitt et al., 1997).

The overall goal of this study was to apply targeted sampling and an optimized technique for processing environmental samples for *M. bovis* detection to characterize the persistence of *M. bovis* in the environment and the role of indirect transmission of *M. bovis* in the epidemiology of bovine TB in Michigan. The hypothesis being tested is that *M. bovis* can be detected in the environment where it can survive for sufficient lengths of time to serve as a source of infection for cattle and/or wild deer.

3.2 MATERIALS AND METHODS

3.2.1 Study design

All cattle farms in Michigan identified as bovine TB positive between June 2002 and September 2004 were included in the study. Two additional cattle farms, one identified as bovine TB positive in 2000, and the other identified in 2001, were added to the sample. Five wildlife areas were also investigated.

3.2.2 Identification of bovine tuberculosis transmission sites

Data generated by on-going bovine tuberculosis surveillance efforts in domestic livestock and wildlife in Michigan were used to identify specific sites of potential bovine TB transmission.

3.2.2.1 Cattle Farms

Cattle farms identified as bovine TB positive (confirmation of the presence of cattle infected with *M. bovis* on the premises) were presumed to be sites of bovine TB

transmission. The farms were identified with the assistance of the Michigan Department of Agriculture (MDA). The farm owners were contacted and permission to conduct an in-person interview and perform a field investigation on their property was sought. The approved protocol for farm identification is presented in Appendix 1. The survey used was reviewed and approved by the Michigan State University Committee on Research Involving Human Subjects.

All of the farms investigated were located in the region of the State of Michigan designated as "infected" with bovine tuberculosis and classified as "modified accredited" under the guidelines of the Federal Bovine Tuberculosis Eradication Uniform Methods and Rules (USDA, 2004). The "modified accredited" zone encompasses Alcona, Alpena, Antrim, Charlevoix, Cheboygan, Crawford, Emmet, Montmorency, Oscoda, Otsego, and Presque Isle counties, and those portions of Iosco and Ogemaw counties that are north of the southernmost boundaries of the Huron National Forest and the Au Sable State Forest (Figure 3.1).

3.2.2.2 Wildlife Areas

Presumed sites of bovine TB transmission among wildlife species were selected in two ways. The 1996-2002 Michigan Department of Natural Resources (MDNR) database of bovine TB surveillance in white-tailed deer in the 4-county TB "core area" (Montmorency, Alpena, Oscoda and Alcona counties) was used to identify individual townships with the highest apparent prevalence of bovine TB positive white-tailed deer. Opportunities to accompany MDNR biologists during their yearly surveys of the white-tailed deer population in these high prevalence townships were used to select sites for environmental sampling as described below in section 3.2.3.

The trap locations of small mammals identified as TB positive in a study performed by the United States Department of Agriculture (USDA), Animal Plant Health Inspection Service (APHIS), Wildlife Services (WS), were used as the second method to identify sites of presumed TB transmission among wildlife. The sites were identified with the assistance of USDA/APHIS/WS personnel. Permission to access the sites was requested from landowners if the trap site was on private land.

Two of the areas were selected based on their location within the range of townships with the highest apparent prevalence of bovine TB in white-tailed deer from 1996-2001, and three of the areas were selected based on the reported trap location of a small mammal with culture-confirmed *M. bovis* infection.

Five wildlife areas were selected for further investigation and environmental sampling. They were all located in the region designated as the "core" of the endemic area of bovine TB currently affecting white-tailed deer (O'Brien et al., 2002). The "core" is defined by the administrative boundaries of the Michigan Department of Natural Resources Deer Management Unit number 452 (Figure 3.2).

The wildlife areas selected for sampling were primarily forested habitat on private land. Samples were collected from five of the townships with the highest apparent prevalence of bovine TB in white-tailed deer surveyed from 1995-2002. Three of the areas selected within the high apparent prevalence townships were also selected based on a history of trapping a bovine TB positive small mammal in the immediate vicinity. Specific locations for substrate collection within the wildlife areas selected for sampling included locations with evidence of deer feeding activity, areas of open water with

evidence of wildlife congregation along the shores, deer and wildlife fecal material and soil and vegetation in the immediate surroundings.

3.2.3 Sampling site and substrate selection

Following the identification of a potential TB transmission site, the area was investigated to identify specific locations for substrate collection within the identified site that could be contaminated with *M. bovis* and facilitate the indirect transmission of bovine TB among or between cattle and wildlife.

3.2.3.1 Cattle Farms

Data generated through the administration of a structured questionnaire and observations recorded during a walk through of the farm were used to identify specific sites for sample collection on TB positive cattle farms. An in-person interview was conducted with the individual most familiar with the management of cattle and the general practices on the farm. Part I of the structured questionnaire (Appendix 2) was used to record farm characteristics including the number, species and type of livestock and other domestic animals present on the farm, the size and location of the farm relative to other livestock operations, and the general production cycle on the farm. Specific questions about water sources, feeding practices, livestock containment (housing and fencing), feed storage and cattle movement were also included.

Part II of the structured questionnaire (Appendix 2) was used to gather specific information on reported wildlife incursions on the farm. The questions were designed to identify the sites and patterns of white-tailed deer incursions on the farm, however,

information on the presence and observed behavior of other wildlife on the farm was also recorded.

Following the interview, a map of the farm was drawn with the assistance of the landowner. The structures on the premises, locations of pastures, crop fields, woodlots, open water, feed storage and any cattle or wildlife feeding sites were recorded. The location of cattle on the farm and any seasonal movement patterns were noted, and all locations of observed wildlife incursions were recorded.

A farm walk through was performed to identify and confirm the locations indicated on the map. Any deviations from the information recorded on the map were noted. Observations of wildlife incursion, including the presence of wildlife feces, footprints, trails and bedding areas, were recorded.

A final list of approximately 20 sampling locations was produced based on the results of the questionnaire and farm walk through. Targeted sites included those with evidence of animal concentration (mixed and single species), feed and water sites with open access to livestock and wildlife, the location of the infected cattle if known, and sites of wildlife incursion including pastures and woodlots. Substrates from the specified locations selected for sampling included feed (hay, grain and silage), pasture grass, soil, fecal material, bedding and water. Figure 3.3 depicts three sites selected for sampling on bovine TB positive farms.

3.2.3.2 Wildlife Areas

The locations for environmental sampling within the wildlife areas selected as potential bovine TB transmission sites were identified with the assistance of MDNR and USDA/APHIS/WS biologists. Deeryards (naturally sheltered areas used by deer during

severe winters with significant snow fall), deer feeding sites and adjacent areas of open water were selected within the townships identified as having a high prevalence of bovine TB. The landscape surrounding the trap location of small mammals identified as TB positive was surveyed to identify specific substrates for sampling. Substrates selected for sampling from both white-tailed deer and small mammal sites included fecal material, soil, vegetation and water. Figure 3.4 depicts three sites selected for sampling in the wildlife bovine TB transmission areas.

3.2.4 Sample collection

Approximately 500 grams of substrate was collected from each of the sampling locations identified. Latex gloves or a cleaned and betadine-disinfected shovel were used to collect each sample to prevent cross contamination. Water samples were collected in 0.5 liter sterile plastic bottles and capped. All other substrates were placed in large capacity Whirl-Pak™ bags and sealed. The sample containers were labeled with a unique identification number. Additional data corresponding to the sample identification number and a description of the sampling site were collected and recorded on the field data sheets (Appendix 3). Recorded data included a description of the sample collected, a description and the GPS coordinates of the sampling collection location, and a digital photograph of the sampling site.

The samples were stored in an insulated cooler surrounded with cold packs. They were transported by vehicle to the Bio-safety Level III laboratory at Michigan State University within 8 hours of collection. The samples were stored at 4° C for 12 hours before processing.

3.2.5 Sample Processing

All samples were processed for mycobacterial culture with the CB-18™ TB Culture Kit with Lytic Decon™ II (Integrated Research Technology, LLC, Quest Diagnostics Inc., Baltimore, MD). Collected substrates were thoroughly mixed and approximately 5 gm of the solid substrates and 7.5 ml of water were transferred for further processing. Soil and fecal samples were placed in 3 X 7 inch Whirl- Pak™ bags. Feed and vegetation samples were chopped with scissors when necessary and placed in sterilized Ball® pint regular mason jars. Water samples were transferred to 50 ml conical centrifuge tubes.

Sterile water (7.5 ml) and 5 ml of Liquefaction Solution (trisodium citrate dehydrate and N-acetyl-L-cysteine or NALC) were added to the solid substrates. Samples were then pulverized and homogenized by placing the Whirl-Pak® bags in a Stomacher® 80 laboratory blender for 30 seconds, and securing a blade unit and gasket on the jars, inverting and blending them for 30 seconds on high with a household blender. Five ml of Liquifaction Solution were added to the water samples and they were mixed on high for 30 seconds with a vortex machine.

The 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 (20X Tris-citrate Buffer, CB-18™ Stock, or NALC and water). Samples were mixed with a vortex machine and incubated at 37° C 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 decanted completely.

A pipette was used to remove all but 1-3 ml of liquid from samples without a visible pellet. The pellet was resuspended 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° C. One ml of 2X Resuspension Solution (10X-Enzyme Stock-*Trichoderma harzianum* extract, lysozyme and *Lysobacter* extract and NALC) was added to each sample and they were incubated for 45 minutes at 37° C.

3.2.6 Mycobacterial culture and isolation

CB-18 processed samples were inoculated onto solid media slants and plates containing modified Middlebrook 7H11 agar (Becton-Dickinson) with sodium pyruvate (Diagnostic Center for Population and Animal Health, Lansing, MI, USA) and 7H11 Selective plates (Becton-Dickinson). Solid media slants and plates were incubated at 37°C for 8-12 weeks and examined weekly for colony formation. Positive mycobacterial cultures and colonies on solid media were subjected to an acid-fast smear analysis to confirm the presence of acid-fast bacteria using standard protocols for slide preparation, staining and examination (Kent and Kubica, 1985). Acid-fast positive isolates were identified to the *Mycbocaterium tuberculosis* complex species group using a genetic probe (AccuProbes, Gen-Probe, San Diego, California 92121, USA). Biochemical tests and high performance liquid chromatography was performed by the Michigan Department of Community Health (MDCH) Tuberculosis/Mycology laboratory to speciate non-*M. tuberculsosis* complex mycobacteria or to differentiate between *Mycobacterium bovis* and other members of the *M. tuberculosis* complex.

3.2.7 Statistical analysis

Excel spreadsheets and statistical functions (AVERAGE, AVEDEV, MEDIAN, MAX, MIN) were used to generate simple descriptive statistics characterizing the bovine tuberculosis transmission sites and the specific locations identified as sites of potential environmental contamination with *Mycobacterium bovis* (Excel, Microsoft Office XP Professional). Excel spreadsheets were also used to record mycobacterial culture results and summarize the data on the isolation of acid-fast bacteria and the presence or absence of contamination. Comparisons between mycobacterial culture result, substrate type and presence or absence of contamination were made using the Cochran-Mantel-Haenszel test for *n*–way cross tabulation tables and the SAS software program (Proc Freq; Tables/CMH, SAS version 9.0, Cary N.C.: SAS Institute, Inc.).

3.3 RESULTS

3.3.1 Identification of potential sites of bovine tuberculosis transmission

Between June 2002 and September 2004, 12 cattle farms in Michigan were declared bovine tuberculosis positive by State (Michigan Department of Agriculture) and Federal (USDA/APHIS/Veterinary Services) animal health officials. DNA fingerprinting of the *M. bovis* isolates associated with bovine tuberculosis (TB) on all of these farms was confirmed as the Michigan *M. bovis* strain, first characterized in the state in 1999 (Judge, 2006; Whipple et al., 1999c). Access to collect environmental samples potentially contaminated with *M. bovis* was granted for 11 of the 12 farms. Two

additional cattle farms, one identified as bovine TB positive in 2000, and the other identified in 2001, were also investigated at the request of the farm owners.

Farm investigations were scheduled within an average of two months of the officially recorded bovine TB positive date for each of the farms identified between June 2002 and September 2004 (Table 3.1). One farm was investigated 10 days before the official TB positive date and the remaining 10 farms were investigated after their officially recorded TB positive date. The average time between environmental sampling and the official TB positive date was 56.18 days (average deviation=27.47; median=55, max=107; min=10). The two farms identified in 2000 and 2001 were investigated 929 days, and 612 days respectively, after the officially recorded TB positive date for each farm. The cattle from 9 of the 10 farms identified as TB positive between 2002 and 2004 were depopulated. The investigation and sampling of the TB positive farms was accomplished before the cattle were depopulated on five farms and after the cattle were depopulated for the remaining 4 farms (Table 3.1). The average time between farm sampling and cattle depopulation was 32.67 days (average deviation=11.56; median=31; max=55; min=7).

3.3.2 Cattle Farms: General characteristics, farm management practices and description of potential sites of *M. bovis* contamination

Of the thirteen bovine tuberculosis cattle farms investigated, four were dairy operations, seven were beef cow/calf operations, one was a small beef feeder operation, and one was a combination cow/calf and feeder operation. The number of adult cattle on the farms ranged from 14 to 239 and averaged 74. The average size of the farm

properties was 251 acres. Only 1 farm reported fence line contact with another cattle farm. Approximately ½ of farms that were identified as bovine TB positive between June 2000 and September 2004 purchased 100% of their cattle from outside sources. The majority of the farms did not raise any other kind of livestock, with the exception of chickens, and all but one reported the presence of pet dogs and cats on the farm (Table 3.2).

Full farm investigations (interviews with farm owners or primarily cattle managers and farm walk-through) were performed on the first 12 farms sampled. USDA/APHIS/VS personnel collected environmental samples from the 13th farm, but only very general farm characteristics were recorded. Although 10 of the 12 farms had cattle housing (barn, feedlot, or barn yard) facilities, cattle spent more than 50% of their time outside on 9 of the 12 farms (Table 3.3). The three farms on which cattle spent more than 50% of their time inside were dairy herds. Only 1 farm, a dairy, never fed cows outside. Cattle on all of the farms had access to water sources outside and on 10 of the farms cattle had access to surface water (ponds, streams or water ways). Hay was provided to cattle in a feeder on 4 of the farms but it was also provided on the ground on 2 of these. Ten of the 12 farms provided hay to their cattle outside on the ground. A summary of the cattle management practices on each of the farms is summarized in Table 3.3.

An examination of feed storage and general fencing practices on the farms revealed that hay (round bales) were stored outside and unprotected on 8 of the 12 farms. Round bales of hay were stored in the fields, along fencerows or in the woods on 4 of the farms. Five of the farms used some barbed wire and 9 of the farms used electric fencing.

Two of the farms had "deer proof" fencing around their hay that had been installed through a USAID/APHIS/WS program after their cattle were identified as bovine TB positive. A summary of the fencing and feed storage practices is provided in Table 3.4. All of the farmers interviewed reported observing deer on their property. All but one of the 12 farms reported observing deer in their cattle pastures and all 12 farms reported observing deer feeding on either their pastures or crop fields. Deer were not observed drinking water from outdoor tanks but 5 of the farms reported observing evidence of deer using the same open water sources accessible to cattle on the farm. Six of the 12 farms reported observing deer feeding on harvested hay intended for cattle and all but 1 farm reported the presence of deer attractants (orchards or cedar swamps) on or adjacent to their property (Table 3.5). Producers on all farms surveyed reported observations of wildlife other than deer. The species observed included raccoons (*Procyon lotor*), opossum (*Didelphis virginiana*), fox (*Vulpes vulpes*), coyote (*Canis lantrans*), turkeys (*Meleagris gallopavo*), skunks (*mephitis mephitis*), bobcats (*Felis rufus*), bears (*Ursus americanus*) and porcupine (*Erethizon dorsatum*).

3.3.3 Cattle farms (samples collected)

A total of 409 samples were collected from 13 farms. Approximately 20 sites were selected for sampling on each farm. One sample was collected per site for the first 10 farms. An effort was made to intensify sampling on the final two farms and more samples were collected per site. A total of 140 samples were collected from farm 112 and a total of 52 samples were collected from farm 113 (Table 3.6).

3.3.4 Wildlife areas (samples collected)

A total of 97 samples were collected from 5 wildlife areas. Approximately 20 locations were selected for sampling in each wildlife area and approximately 1 sample was collected per site. An attempt was made to distribute the sampling across substrate type (Table 3.7).

3.3.5 *Mycobacterium bovis* culture results

None of the samples collected from the bovine TB positive farms were positive for *Mycobacterium bovis* based on mycobacterial culture. A number of acid-fast organisms were isolated but further testing revealed that none of these were members of the *M. tuberculosis* complex (Table 3.6). Acid-fast organisms were most commonly isolated from soil, vegetation, manure and cattle feed samples but an association between the isolation of acid-fast bacteria and sample type was not found (Mantel-Haenszel X^2 = 0.04; degree freedom = 1; p-value = 0.84). Samples processed from wildlife areas produced similar results. Acid-fast bacteria were isolated from samples of soil and vegetation but no significant associations between substrate type and acid-fast bacterial isolation were found. The isolates of the non-*M. tuberculosis* complex acid-fast bacteria that could be identified to species included *M. fortuitum*, *M. avium*, *M. fortuitum-chelonae*, and *Mycobacterium sp.* Group IV.

The prevalence of contamination (overgrowth of the cultures with mold and non-mycobacteria) was high. Twenty-seven percent of the samples collected from bovine TB positive farms (Table 3.6) and 11% of the samples collected from wildlife areas (Table 3.7) were contaminated. Soil samples and substrates mixed with manure were most

likely to be contaminated but no significant association between sample substrate and the presence of contamination was found (Mantel-Haenszel $X^2 = 0.01$; degree freedom = 1; p-value = 0.92)

Figure 3.1 Map of Michigan indicating the Bovine Tuberculosis State Status Designations: 1) Modified Accredited (Infected Zone), and 2) Modified Accredited Advanced (Disease Free Zone). Source: Michigan Department of Agriculture **www.michigan.gov/bovinetb**

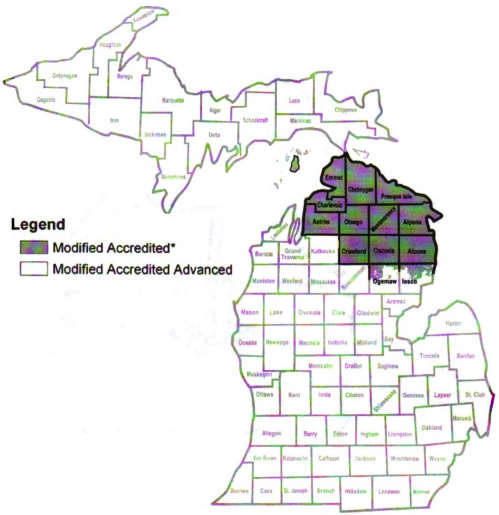


Figure 3.2 Map of the bovine TB "core" area within the counties of Montmorency, Alpena, Oscoda and Alcona, Michigan. Images in this dissertation are presented in color.

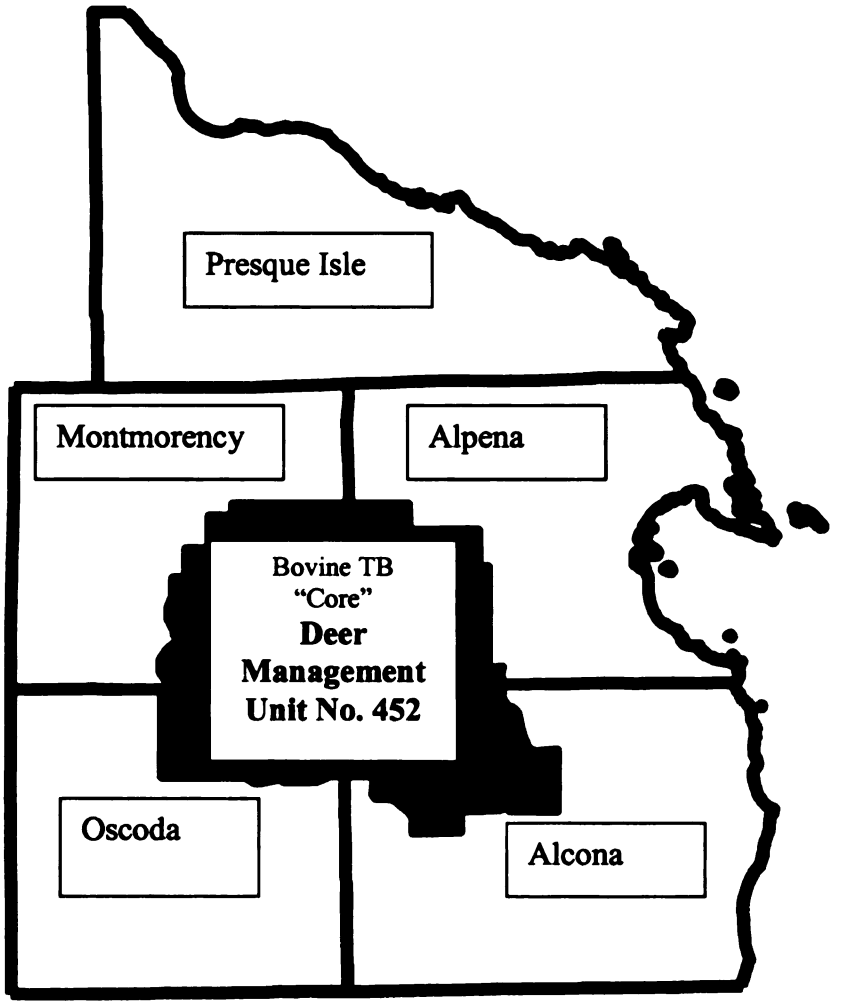


Figure 3.3 Three photographs of sampling locations selected during investigations of bovine tuberculosis positive cattle farms: 1) an unprotected hay bale with evidence of deer feeding activity; 2) a pond in a pasture that both deer and cattle have access to; and 3) an example of feeding cattle hay on the ground in the woods. Images in this dissertation are presented in color.

Figure 3.4 Three photographs of sites selected for sampling in the wildlife bovine TB transmission areas: 1) an oak forest where deer fecal pellets were collected; 2) a deer feeder and a plot of forage planted for deer; and 3) a pond near the trap location of a bovine TB positive small mammal.

Table 3.1 Time between farm investigation (environmental sampling), official TB positive date, and date of cattle depopulation.

Farm Number	TB+ Date	Depopulation Date	Sampling Date	TB+ to Sampling Days	Depopulation to Sampling Days
101	09/20/02	10/02/02	09/10/02	-10	-22
102	07/17/02	09/17/02	09/10/02	55	-7
103	07/16/02	08/21/02	09/13/02	59	23
104	06/12/02	10/29/02	09/27/02	107	-32
105	04/06/01	05/01/01	12/09/02	----	----
106	06/02/00	06/12/00	12/18/02	----	----
107	01/09/03	03/31/03	02/28/03	50	-31
108	11/27/02		03/04/03	97	
109	01/27/03	03/11/03	05/05/03	98	55
110	05/27/03	06/03/03	07/02/03	36	29
111	11/10/03	01/21/04	12/02/03	22	-50
112	12/23/03		03/03/04	71	
113	08/20/04	07/19/04	09/02/04	13	45
			Average:	**56.18 days**	**32.67 days**

Table 3.2 General characteristics of bovine TB positive farms selected for environmental sampling.

Farm Number	Farm Type	Herd Size No. Adults	Farm Size Acres	% Purchased	Fence Line Contact	Other Livestock	Pets Dogs/Cats
101	Beef Feeder	31	110	100	No	No	Yes
102	Dairy	79	428	0	No	No	Yes
103	Cow/Calf	37	102	100	No	No	Yes
104	Cow/Calf	40	128	100	No	No	No
105	Cow/Calf	108	330	-----*	Yes	No	Yes
106	Cow/Calf	103	540	-----*	No	Horses	Yes
107	Cow/Calf	23	188	5	No	Chickens	Yes
108	Dairy	239	500	100	No	No	Yes
109	Cow/Calf	19	80	>50	No	Chickens	Yes
110	Cow/Calf	48	300	25	No	Horses/Sheep	Yes
111	Cow/Calf-Feeder	14	244	<5	No	Chickens	Yes
112	Dairy	68	65	<5	No	No	Yes
113	Dairy	148					
	Average:	**73.62**	**251.25**	***Farm 105 and 106 were TB-positive before the study period**			

Table 3.3 A summary of cattle management practices on bovine TB positive farms selected for environmental sampling.

Farm No.	Housing Barn/Lot	>50% Outside	Feed Outdoors	Feed Outdoors Only	Hay Feeder	Hay Ground	Water Tank Outdoors	Water Open Outdoors
101	Yes	Yes	Yes	Yes (Adults)	No	Yes	Yes	No
102	Yes	No	Yes	No	Yes	Yes	No	Yes
103	Yes	Yes	Yes	No	No	Yes	No	Yes
104	No	Yes	Yes	Yes	Yes	Yes	No	Yes
105	Yes	Yes	Yes	Yes	No	Yes	No	Yes
106	Yes	Yes	Yes	Yes (Adults)	No	Yes	No	Yes
107	Yes	Yes	Yes	Yes	No	Yes	No	Yes
108	Yes	No	No	No	Yes	No	Yes	Yes
109	Yes	Yes	Yes	No	No	Yes	Yes	Yes
110	No	Yes	Yes	Yes	No	Yes	Yes	Yes
111	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
112	Yes	No	Yes	No	Yes	No	Yes	No
% Yes:	**83%**	**75%**	**92%**	**58%**	**33%**	**83%**	**50%**	**83%**

Table 3.4 A summary of feed storage and fencing practices on bovine TB positive farms selected for environmental sampling.

Farm No.	Hay Unprotected Outdoors	Round Bale Field/Fence/Woods	Feed Fenced "Deer Proof"	Cattle Fencing Barbed	Cattle Fencing Electric
101	Yes	Yes	No	Yes	Yes
102	No	Yes	No	No	Yes
103	Yes	No	No	No	Yes
104	Yes	No	No	No	Yes
105	Yes	Yes	Yes	No	Yes
106	No	No	Yes	Yes	Yes
107	Yes	Yes	Inside	No	No
108	Yes	No	No	No	Yes
109	Yes	No	No	Yes	No
110	Yes	No	No	No	No
111	No	No	Inside	Yes	Yes
112	No	No	No	Yes	Yes
% Yes:	**67%**	**33%**	**25%**	**42%**	**75%**

Table 3.5 A summary of reported white-tailed deer incursion on bovine TB positive farms selected for environmental sampling.

Farm No.	In Pasture	Near Cattle Housing	Near Home	Drinking Tank Water	Drinking Open Water	Feeding on Pasture/Crop	Feeding on Hay	Deer Attractants
101	Yes	No	No	No	No	Yes	No	No
102	Yes	No	No	No	No	Yes	No	Yes
103	Yes	No	No	No	Yes	Yes	Yes	Yes
104	Yes	No	Yes	No	No	Yes	Yes	Yes
105	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
106	Yes	Yes	Yes	No	Yes	Yes	No	Yes
107	Yes	No	No	No	Yes	Yes	Yes	Yes
108	No	No	No	No	No	Yes	No	Yes
109	Yes	No	No	No	No	Yes	Yes	Yes
110	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
111	Yes	No	No	No	No	Yes	No	Yes
112	Yes	No	Yes	No	No	Yes	No	No
% Yes:	**92%**	**25%**	**42%**	**0%**	**42%**	**100%**	**50%**	**83%**

Table 3.6 Mycobacterial culture results of environmental substrates collected from bovine tuberculosis positive farms.

	TOTAL	AFB + (%)	Contamination + (%)
Pasture & Vegetation	79	13 (16%)	15 (19%)
Soil	75	13 (17%)	32 (43%)
Open Water	78	2 (3%)	12 (30%)
Hay	50	5 (10%)	10 (20%)
Cattle Feed	10	3 (30%)	2 (20%)
Barn Water	4	0 (0%)	0 (0%)
Bedding	22	0 (0%)	9 (41%)
Manure	40	1 (3%)	12 (30%)
Manure Mix	23	6 (26%)	12 (52%)
Deer Feces	10	0 (0%)	4 (40%)
Wildlife Feces	17	1 (6%)	3 (18%)
Bear Hair	1	0 (0%)	0 (0%)
	409	**44 (11%)**	**111 (27%)**

Table 3.7 Mycobacterial culture results of environmental substrates collected from potential wildlife bovine tuberculosis transmission sites.

	TOTAL	AFB + (%)	Contamination + (%)
Pasture & Vegetation	17	1 (6%)	4 (24%)
Soil	24	2 (8%)	5 (21%)
Open Water	22	0 (0%)	0 (0%)
Wildlife Feces	5	0 (0%)	1 (20%)
Deer Feces	28	0 (0%)	0 (0%)
Grain	1	0 (0%)	1 (100%)
	97	**3 (3%)**	**11 (11%)**

3.4 DISCUSSION

Mycobacterium bovis, the causative agent of bovine TB, continues to circulate among cattle and white-tailed deer in Michigan. Over the two-year span of this study, from June 2002 until September 2004, 12 cattle farms in northern Lower Michigan were identified as bovine TB positive (Michigan Department of Agriculture). The cattle herds identified as bovine TB positive during this period were all in the USDA designated "Modified Accredited Zone" (Figure 3.1) where annual whole-herd bovine TB testing is required (USDA, 2004). The farms identified, therefore, likely represent new bovine TB infection and relatively recent transmission events. Similarly, on-going surveillance for bovine TB in the white-tailed deer population during the same period revealed an apparent prevalence fluctuating around 2.0% in animals originating in MDNR, Deer Management Unit # 452, thought to be the endemic focus of bovine TB in white-tailed deer in the State (Figure 3.2) (O'Brien et al., 2006). A number of the bovine TB positive white-tailed deer identified during this routine surveillance were yearlings, indicating new infection and recent bovine TB transmission events.

Despite evidence of on-going bovine TB transmission in northeast Michigan, efforts to isolate *Mycobacterium bovis* from environmental substrates collected from bovine TB positive farms and areas of wildlife TB transmission failed. The field investigations did reveal circumstances (location and practice) that would facilitate the indirect transmission of bovine TB through the contamination of environmental substrates among and between cattle and white-tailed deer populations.

3.4.1 Potential sites of *M. bovis* contamination of the environment

3.4.1.1 Bovine TB positive farms

The general characteristics of the 13 bovine TB positive farms selected for investigation and environmental sampling in this study were similar to those of the initial group of bovine TB positive farms identified between 1998 and 2002 (Schmitt et al., 2002). The majority of the farms were small beef cattle operations and they were all located in northeast lower Michigan. Particular cattle management practices that have been identified as risk factors associated with tuberculosis on cattle farms in northeast Michigan in the past (Kaneene et al., 2002b), and those that would facilitate the indirect transmission of bovine TB from deer to cattle via *M. bovis* contaminated substrates included 1) maintenance of cattle outside for more than 50% of the time outside (75% of farms); 2) feeding cattle outside (92%) and feeding cattle outside exclusively (58%); 3) watering cattle outside with access to open water (streams, ponds, etc.) (83%); and 4) feeding cattle hay on the ground (83%).

The practices outlined above are only a bovine TB risk to cattle if infected white-tailed deer in the area also have access to the hay, pasture and water sources identified as cattle feeding and watering sites. Answers to "deer incursion" survey questions indicated that deer were seen on the premises of 100% of the farms identified. Ninety-two percent of respondents observed deer in pastures, 50% observed evidence of deer feeding on hay intended for cattle, and 42% observed evidence of deer drinking from open water sources on the farms. Electric (75%) and barbed wire (42%) fencing was used on these cattle farms but feed was only stored in "deer proof" facilities on 25% of the farms surveyed.

Dairy cattle operations generally maintained their cattle inside housing more than 50% of the time, fed their cattle inside and used hay feeders, however, cattle had access to open water and hay was stored outside and unprotected on many of the premises investigated.

3.4.1.2 Wildlife bovine TB transmission sites

The investigations of wildlife areas were performed in the summer and spring. In the spring and summer months particular wildlife management practices that would facilitate the indirect transmission of bovine TB were not observed with the exception of the identification of small fields in wooded areas planted with alfalfa grass to attract deer and a limited number of empty deer feeding stations which would likely have been active in winter.

3.4.2 Detection of *M. bovis* in the environment: Study related factors

Every attempt was made to investigate cattle farms as soon as they were identified as bovine TB positive with the goal of collecting environmental samples from the premises as close to the bovine TB transmission event as possible. The combination of State and Federal protocols for identifying a herd as bovine TB positive, and the arrangements necessary for gaining access to the farm, resulted in an average delay of 56 days between the date the farm was recorded as bovine TB positive and the date that sampling occurred. For farms that were depopulated, the average time between sampling and depopulation was 33 days. The circumstances that facilitated the initial bovine TB transmission event on the farm were likely still in place at the time of environmental sampling, but the unknown timing of the *M. bovis* transmission event, and delayed access

to bovine TB positive cattle farms, is and will continue to be a weakness of field investigations of natural bovine TB transmission events.

3.4.3 Detection of *M. bovis* in the environment: Agent related factors

Properties of *M. bovis* contribute to difficulties associated with isolating the organism from environmental substrates. *M. bovis* is particularly difficult to culture. The necessity of a bacteriacidal decontamination step, coupled with the cording behavior and natural buoyancy of *M. bovis*, reduces the success of mycobacterial culture methods (Kent and Kubica, 1985; Yajko et al., 1995). The additional presence of large numbers of saprophytic bacteria, molds and other infectious organisms in environmental samples further interferes with the sensitivity of detection of *M. bovis* by bacterial culture. Attempts were made to improve the success of isolating *M. bovis* with bacterial culture methods by processing specimens with CB-18™ TB Culture Kit with Lytic Decon™ II (Integrated Research Technology, LLC, Quest Diagnostics Inc., Baltimore, MD). Although *M. bovis* was not isolated, other mycobacterial species were successfully identified from environmental substrates collected suggesting the techniques used were capable of detecting mycobacterial species from environmental samples in the presence of other competing microbes.

Culture contamination, an overgrowth of mold and non-mycobacterial species, affected our attempts to isolate *M. bovis* from approximately 25% of the samples collected. Although no significant associations were found between contamination rates and sample substrate type, the distribution of contamination in samples suggested that soil, vegetation samples and those mixed with cattle manure were more likely to produce

contaminated mycobacterial culture results. These data indicate that the sensitivity of isolation of *M. bovis* from these particular substrates types may be further reduced.

The challenges associated with detection of *M. bovis* in environmental substrates has been cited as a potential cause of failure to isolate *M. bovis* from environmental substrates from other areas identified as sites of natural transmission of bovine TB in Michigan. These include a captive white-tailed deer facility (Palmer et al., 2000) and one of the first cattle farms identified as TB-positive in the current outbreak of *M. bovis* in Michigan (Kaneene, personal communication). *M. bovis* has been isolated from environmental substrates contaminated in the course of experimental transmission studies among deer (Palmer et al., 2004b; Palmer et al., 2001) and between deer and cattle (Palmer et al., 2004a), however, even under these "ideal" circumstances, success has been intermittent.

3.4.4 Detection of *M. bovis* in the environment: Host related factors

As previously discussed, one of the limitations of opportunistic environmental sampling of bovine TB positive cattle herds and identified wildlife bovine TB transmission areas, is that a time lag likely exists between the time at which the *M. bovis* shedding animal (cattle or deer) was present on the premises and the time of sample collection. This time lag is likely exacerbated by intermittent shedding of *M. bovis* from both infected deer (Palmer et al., 2001) and infected cattle (Goodchild and Clifton-Hadley, 2001; Neill et al., 1988). The probability of collecting an environmental sample from an identified bovine TB transmission site in the time period the infected animal is present and at a time when it is shedding *M. bovis* is probably very low.

The denning behavior and patterns of movement of other wildlife reservoirs of bovine TB, primarily brushtail possums (*Trichosurus vulpecula*) in New Zealand (Jackson et al., 1995) and European badgers (*Meles meles*) in Ireland and Great Britain (Anderson and Trewhella, 1985; Hutchins and Harris, 1997), allow for a closer approximation of the opportunities for their potential direct and indirect contact with cattle on bovine TB affected farms. Although somewhat predictable, free-ranging white-tailed deer have much larger home ranges and their presence on cattle farms is more transient (Garner, 2001). The behavior of potentially bovine TB infected white-tailed deer does not allow for a fine level of targeted sampling of environmental substrates for the detection of *M. bovis*.

3.4.5 Detection of *M. bovis* in the environment: Landscape related factors

Efforts to isolate *M. bovis* in other regions have yielded similar results when samples were collected under natural disease transmission conditions (Pillai et al., 2000). Researchers interested in the persistence of *M. bovis* in the environment have turned to experimental inoculation studies and an assessment of the conditions that support or inhibit *M. bovis* survival (Duffield and Young, 1985; Jackson et al., 1995; Little et al., 1982; Maddock, 1933, , 1934, , 1936; Tanner and Michel, 1999; Williams and Hoy, 1930; Wray, 1975). This is due primarily to the difficulty of identifying the exact location of *M. bovis* contamination over what is often a very large potentially contaminated site. This study faced the same challenge. Financial and time constraints limited the number and volume of environmental substrates that could be collected and processed from sites potentially contaminated with *M. bovis*. These constraints limited

the total surface area of both bovine TB positive farms and wildlife areas that could be sampled effectively.

3.4.6 Persistence of *M. bovis* in the environment: Overall conclusions

It is generally accepted that *M. bovis* is transmitted within deer populations, and within cattle herds, primarily through close contact and direct routes of disease transmission (Kaneene et al., 2002a; Palmer et al., 1999). Interspecies transmission of bovine TB between white-tailed deer and cattle, however, likely occurs through indirect routes of transmission since little evidence of direct contact between the species exists (DeLiberto et al., 2004). This study, therefore, focused on characterizing the potential for the indirect transmission of *M. bovis* through the contamination of environmental substrates.

This field investigation of bovine TB transmission sites confirmed the findings of earlier studies that have identified environmental and cattle farm management practices (Kaneene et al., 2002b) in northeast Michigan that may facilitate indirect interspecies transmission of bovine TB between deer and cattle. Investigations of wildlife TB transmission areas also produced evidence of deer feeding and baiting practices that have been identified by other authors as likely contributing to the indirect transmission of bovine TB among white-tailed deer (Hickling, 2002; Miller et al., 2003; O'Brien et al., 2006; Schmitt et al., 1997). The failure to isolate *M. bovis* from environmental substrates collected from bovine TB positive cattle farms and wildlife areas was likely due to agent, host and landscape factors that contribute to the difficulty of identifying specific sites of *M. bovis* contamination and recovering *M. bovis* from environmental substrates.

Chapter 4

A STUDY OF THE PERSISTENCE OF *MYCOBACTERIUM BOVIS* IN THE ENVIRONMENT

4.0 ABSTRACT

Objective: To characterize the persistence of the Michigan strain of *Mycobacterium bovis* in the environment under natural weather conditions.

Design: Experimental Study

Sample Population: *Mycobacterium bovis* inoculated environmental substrates (corn, hay, soil and water) examined for persistence during three seasons and over a 12-month period.

Procedure: Re-isolation of *M. bovis* from experimentally inoculated substrates was used to follow the persistence of viable *M. bovis* bacteria in soil, hay, water and corn exposed to natural weather conditions. Environmental factors (humidity, temperature, rainfall, and levels of solar radiation) were recorded continuously during the experimental persistence studies. Mean and maximum survival time for *M. bovis* in each of the environmental substrates was determined for each of 3 seasons (Fall/Winter, Winter/Spring, Spring/Summer) of experiments. Factors affecting *M. bovis* persistence (substrate type, weather conditions, presence of shade, etc.) were studied using survival analysis and Cox's proportional hazards regression.

Results: The Michigan strain of *M. bovis* persisted in the environment for up to 88 days in soil, 58 days in water and hay and 43 days on corn. The time period, or season, in

which the *M. bovis* was exposed significantly affected its survival. The shortest survival across all substrate types was seen in the spring/summer season in which average daily temperatures and levels of solar radiation were highest.

Conclusions and Clinical Relevance: Viable *M. bovis* bacteria persists in environmental substrates (corn, hay soil and water) under natural Michigan weather conditions for up to 6-10 weeks in the cooler fall/winter and winter/spring seasons. Cattle and/or wildlife with access to environmental substrates contaminated with *M. bovis* within days to weeks of the contamination event are at risk of exposure to bovine TB. These findings strengthen evidence that suggests that cattle farm bio-security and efforts to eliminate supplemental feeding of white-tailed deer will decrease the risk of bovine TB transmission among and between cattle and deer populations.

4.1 INTRODUCTION

An endemic focus of bovine tuberculosis (TB), caused by a single strain of *Mycobacterium bovis*, has been identified in white-tailed deer (*Odocoileus virginianus*) in northeast lower Michigan (O'Brien et al., 2006; Schmitt et al., 1997; Whipple et al., 1999b). Spillover of *M. bovis* infection from white-tailed deer to cattle is suspected in the majority of the 33 cattle farms in the same region of the State identified as bovine TB positive since intensive surveillance for TB in Michigan livestock was reinitiated in 1998 (Michigan Department of Agriculture; USDA/APHIS/VS). The emergence of a wildlife reservoir for bovine TB in Michigan, and evidence of disease transmission between infected free-ranging white-tailed deer populations and domestic cattle, has forced a reevaluation of the understanding of the epidemiology of bovine TB in North America.

Disease transmission between deer and cattle in Michigan is thought to occur in the absence of close contact between the species (DeLiberto et al., 2004). This has raised questions about the role of indirect transmission of *M. bovis* in the epidemiology of bovine TB and identified a need to investigate the persistence of *M. bovis* in the environment and the potential role of contaminated substrates in the transmission of *M. bovis* among and between wildlife and cattle populations.

The persistence of *M. bovis* in the environment and role of indirect transmission in the epidemiology of bovine TB in Michigan has been debated since the current TB epidemic in Michigan was first described in 1997 (Schmitt et al., 1997). Bovine TB infection in white-tailed deer today is likely linked to the large number of cattle infected with *M. bovis* in Michigan during the late 1950's (Frye, 1995), however, the establishment and persistence of *M. bovis* in free-ranging white-tailed deer in northeast Michigan is thought to have been influenced by the long-term practice of winter feeding of deer in the region (Schmitt et al., 1997). These piles of feed, set out to attract deer and improve their productivity and winter survival, are thought to contribute to the transmission of TB among white-tailed deer by 1) increasing local density and contact between animals and 2) providing a site for the indirect transmission of TB through contamination of the feed by infected deer shedding *M. bovis* in their saliva or nasal discharges and the subsequent infection of a naïve deer by consumption of contaminated feed (Hickling, 2002; Schmitt et al., 1997). This is supported by evidence that suggests that specific supplemental feeding practices, generally indicative of large-scale feeding operations, are associated with an increasing risk for bovine TB in deer in Michigan (Miller et al., 2003).

The role *M. bovis* contaminated environmental substrates in the interspecies transmission of bovine TB between cattle and deer has also been investigated (Fine et al. 2006—chapter 3). Although *M. bovis* was not identified from any of the environmental substrates tested, particular cattle management practices and environmental factors were identified that have been shown to be associated with tuberculosis on cattle farms in northeast Michigan in the past (Kaneene et al., 2002b). These practices are likely facilitate the indirect transmission of bovine TB from deer to cattle via *M. bovis* contaminated substrates. The factors and practices identified included the presence of ponds or open water in cattle areas, maintaining cattle outside more than 50% of the time, feeding and watering cattle outside and not protecting feed intended for cattle from deer.

Evidence suggests that opportunities for the indirect transmission of *M. bovis* between white-tailed deer and cattle exist in northeast Michigan under current cattle and deer management practices. Data on the persistence of *M. bovis* on various environmental substrates and the factors that influence its survival are essential to the further understanding of the complexity bovine TB transmission and epidemiology in Michigan. In addition to contributing to our understanding of bovine TB dynamics in this system, information regarding the persistence of *M. bovis* in the environment will support efforts to improve protocols for cattle farm bio-security and the maintenance of appropriate restrictions on feeding and baiting free-ranging white-tailed deer and other wildlife.

Experimental studies conducted recently in New Zealand, Australia, South Africa, Great Britain and Ireland, have shown that *M. bovis* persists in typical environmental substrates for varying amounts of time (Duffield and Young, 1985; Jackson et al., 1995; Little et al., 1982; Tanner and Michel, 1999; Young et al., 2005).

This study was designed to describe the persistence of the Michigan strain of *M. bovis* in typical environmental substrates (corn, hay, soil and water) exposed to natural weather conditions in Michigan. Factors affecting the length of persistence, or survival time, of *M. bovis* in the environment were also investigated. The hypothesis being tested is that *M. bovis* can survive in environmental substrates for sufficient lengths of time to serve as a source of infection for cattle and/or wild deer.

4.2 MATERIALS AND METHODS

4.2.1 Culture, media, and growth conditions

A Michigan strain of *Mycobacterium bovis* was obtained from a frozen culture of *M. bovis* originally isolated in 2002 from the retropharyngeal lymph node of a naturally infected 2-year-old Holstein cow from Michigan. The animal was classified as a reactor on a comparative cervical test and had gross and microscopic lesions consistent with bovine tuberculosis (TB) at necropsy. The frozen *M. bovis* culture was added to 10 ml of Middlebrook 7H9 Broth with Middlebrook ADC Enrichment for cultivation of mycobacteria (Becton-Dickinson, Cockeysville, MD, USA). Multiple 10 ml vials of *M. bovis* inoculated 7H9 Broth were incubated at 37° C for 21 to 30 days. A series of growth curve experiments were used to estimate the concentration (colony forming units (CFU) of *M. bovis* per ml) of the liquid cultures. The exact final concentration (CFU/ml) of the *M. bovis* liquid culture stock was determined by monitoring growth and performing colony counts on Selective 7H11 agar (Becton-Dickinson) plates inoculated with exactly 100 µl of *M. bovis* liquid stock, a 1:10, 1:100, 1:1000 and a 1:10000 dilution. Blood and

CNA agar plates were inoculated with *M. bovis* liquid culture stock to monitor for contamination. Only pure *M. bovis* cultures were used for inoculation experiments.

4.2.2 Environmental substrates

Substrates selected for testing included grass hay, soil, water and shelled corn. Grass hay was collected from the feed storage area of the Michigan State University (MSU) Large Animal Veterinary Teaching Hospital in East Lansing, Michigan. Soil was collected from the Baker Woodlot (Rachana Rajendra Neotropical Bird Sanctuary) located in the south central section of the MSU campus. Water was collected from the large pond at the center of the Baker Woodlot and from the Red Cedar River at the Farm Lane Bridge on the MSU campus. Shelled corn was purchased in 20-pound bags from a local feed store. Environmental substrates were stored at 4° C with no exposure to light.

A set of environmental substrates consisted of 4 samples each of grass hay, soil, water and shelled corn for a total of 16. Ball® Half-Pint Regular Can-or-Freeze Jars were filled with 5 gm of hay, 10 gm of soil, 10 gm of corn or 10 ml of water. Half of the sample-filled jars were autoclaved for 2 hours at 121° C and 20 psi to sterilize the contents. Each sample set of 16 was identified with uniquely colored tape and a label denoting the sample type, sample set number, the autoclave status, and shade or non-shade treatment. The make up of a full sample set is presented in Table 4.1.

4.2.3 Facility (laboratory and outdoor enclosure)

Mycobacterium bovis sample inoculation, sample processing and *M. bovis* isolation procedures were all performed in the bio-safety level III (BL3) laboratory in the

Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University (MSU).

The *M. bovis* environmental persistence studies were carried out in a structure erected within the fenced livestock containment facility south of the DCPAH at MSU. The structure was erected on a concrete slab along the north fence of the livestock containment facility (Figure 4.1). The structure consisted of an enclosed "cage" 16-ft. X 25-ft. X 8-ft. with a galvanized steel frame covered with $3/8^{th}$-inch fencing (chain link-type fence with $3/8^{th}$ inch holes) on all sides including the top. The bottom rail of the cage was flush with the concrete slab or buried below the ground surface. Any gaps below the bottom rail were closed with 2-ft. X 4-ft. wooden beams. A locked door was built into one side of the enclosure with a minimum clearance with the concrete slab and doorframes.

The fencing excluded all birds and small mammals. The structure was built to exclude livestock and/or deer in the unlikely event that they gain entrance to the fenced containment facility and access to the experimental enclosure was limited to authorized individuals.

Specified sets of *M. bovis* inoculated environmental substrates were placed within secondary plastic containers on 2 lines of steel tables set up within the enclosure (Figure 4.2). Sample containers on 1 line of steel tables were covered with black shade cloth. All secondary sample containers were lined with gravel and sand and secured with wire mesh covers.

4.2.4 Environmental monitoring

A WeatherHawk™ weather station, Division of Campbell Scientific, Inc., was positioned at the center of the enclosure. The station was powered by a solar panel charged battery pack. Environmental data collected included rainfall (mm), wind speed (m/sec), temperature (°C), humidity level (%), and solar radiation (W/m^2). Evapotranspiration, a combination of solar radiation, temperature, wind speed, and humidity, was also calculated with the WeatherHawk™ Virtual Weather software. The weather station was programmed to record data at 20-minute intervals, 24 hours a day. Environmental data was downloaded from the weather station to an Excel (Microsoft Corporation, Redmond, WA, USA) computer database on a desktop computer using a wireless system.

4.2.5 Inoculation with *M. bovis*

Each sample was inoculated with 50,000 CFUs of a clinical strain of *Mycobacterium bovis* isolated from a cow in Michigan. Samples were inoculated in the BL3 laboratory. Sample jars were sealed with a plastic, leak-proof lids and transported to the outdoor experimental enclosure, located 500 meters from the BL3 laboratory, in sealed and labeled coolers.

4.2.6 Study design and sampling

The persistence of *M. bovis* in environmental substrates was evaluated over 4 sampling periods. The first sample period spanned 12 months from November 2004 to

December 2005. Twelve sets of 16 samples were inoculated with *M. bovis* as described above and placed in their assigned positions in the experimental enclosure. One sample set was collected at the end of the 1st through 12th month of the experiment and processed for *M. bovis* isolation. The remaining three sampling periods covered 12 weeks and began on November 8, 2004, February 4, 2005 and May 20, 2005 (Figure 4.3). At the start of each sampling period twelve sets of 16 samples were prepared and inoculated with *M. bovis* and placed in the environmental sample enclosure. The persistence of *M. bovis* over time was determined by processing sample sets for mycobacterial culture at the time of inoculation, every other day and then at weekly intervals for 12 weeks.

4.2.7 Environmental sample processing

All samples were processed for mycobacterial culture with the CB-18™ TB Culture Kit with Lytic Decon™ II (Integrated Research Technology, LLC, Quest Diagnostics Inc., Baltimore, MD). Collected samples were processed within their original container (Ball® 1/2 pint regular jar). If necessary sterile water (5-10 ml) was added to the solid substrates. Samples were pulverized and homogenized by securing a blade unit and gasket on the jars, inverting and blending them for 30 seconds on high with a household blender.

The 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 (20X Tris-citrate Buffer, CB-18™ Stock, NALC and water). Samples were mixed with a vortex machine and incubated at 37° C 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 decanted completely. A pipette was used to remove all but 1-3 ml of liquid from samples without a visible pellet. The pellet was resuspended 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° C. One ml of 2X Resuspension Solution (10X-Enzyme Stock-*Trichoderma harzianum* extract, lysozyme and *Lysobacter* extract and NALC) was added to each sample and they were incubated for 45 minutes at 37° C.

4.2.8 Mycobacterial culture and isolation

CB-18 processed samples were inoculated onto solid 7H11 Selective plates (Becton-Dickinson) and incubated at 37°C for 8-12 weeks and examined weekly for colony formation. Typical *M. bovis* colonies were counted and recorded on laboratory data sheets. Acid-fast smear analysis was performed to confirm the presence of acid-fast bacteria using standard protocols for slide preparation, staining and examination (Kent and Kubica, 1985). A subset of the acid-fast positive isolates were confirmed to be *Mycobacterium tuberculosis* complex species group using a genetic probe (AccuProbes, Gen-Probe, San Diego, CA, USA).

4.2.9 Data analysis

Mycobacterial culture results, recorded on laboratory data sheets for each sampling period, were entered in an Excel spreadsheet database (Excel, Microsoft Office XP Professional, Redmond, WA, USA). Excel data from the weather records and mycobacterial culture results were imported into the SAS software program (SAS version

9.0, SAS Institute, Inc., 2003, Cary, NC, USA) and combined. Imported Excel spreadsheet data were analyzed using the SAS software program. Summaries of the weather records for each sampling period were created and descriptive statistics were generated for the persistence of *M. bovis* on each sample type for all sampling periods (SAS Institute, Inc., 2003).

The t-test (Proc TTEST) was used to test for significant differences between mean *M. bovis* survival time (persistence) in shade/non-shade treated samples and sterilized/non-sterilized treated substrates. An ANOVA (Proc ANOVA), using the Bonferroni approach for multiple comparisons, was performed to test for significant differences in the mean survival time (persistence) in sample types and sampling seasons (fall/winter, winter/spring and spring/summer). Assessment of associations between the bovine TB status of experimental samples (positive vs. negative) and covariates (sample type, shade/non-shade, sterilized/non-sterilized substrate) were conducted using the Cochran-Mantel-Haenszel Chi-square (Proc FREQ/CMH) or 2-tailed Fisher's exact test where appropriate.

Survival analyses to compare the persistence of *M. bovis* in the environment between seasons, and to study the effects of environmental factors on *M. bovis* survival across the seasons, were conducted in SAS. The time from sample inoculation to the first negative bovine TB culture after the last positive bovine TB culture was used as the survival, or *M. bovis* persistence period, for each sample. Parametric (Proc LIFEREG) procedures, assuming an exponential distribution were used. The Log rank and Wilcoxon tests were used to compare the survival distributions for *M. bovis* persistence in the environment across the 3 seasons tested. The survival function or Kaplan-Meier curves

for *M. bovis* persistence in each of the three seasons and in each of the 4 substrates across the three seasons were plotted. Cox's proportional hazards regression procedure (Proc PHREG) was used to study the effects of the non-weather related covariates (sample type, shade/non-shade, sterilized/non-sterilized substrate) and season on *M. bovis* survival in the environment.

Model selection in Cox regression (Proc PHREG) was used to identify specific weather or seasonal factors that influenced the survival of *M. bovis* in the environment. The weather data were summarized as daily means, maxima and minima for rainfall, wind speed, temperature, humidity, barometer readings, solar radiation and evapotranspiration. Univariable Cox proportional hazards regression was conducted for each weather-related risk factor and the likelihood ratio test was used to determine the influence of each covariate on the outcome variable. Spearman correlation coefficients (r) were computed to identify potential areas of multicollinearity between the weather related risk factors.

A multivariable Cox proportional hazards regression model was then developed (SAS Institute Inc., 2003). The model contained all weathered-related risk factors that had a univariable model Likelihood ratio statistic (LRS) p-value <0.15. Highly correlated weather-related risk factors were removed due to redundancy of information and multicollinierity (e.g. solar radiation and evapotranspiration were both highly correlated with temperature). Purposeful selection of covariates and a modified stepwise method of variable evaluation with the entry criteria set at LRS p-value of 0.15 and the "stay" or removal criteria set at LRS p-value of 0.20 was used to identify the final multivariable model for *M. bovis* survival in the environment.

Table 4.1 The make up of one sample set used in the bovine TB environmental persistence study

TREATMENT	Shade Sterilized	Shade Non-Sterilized	Non-Shade Sterilized	Non-Shade Non-Sterilized
Soil	1	1	1	1
Water	1	1	1	1
Hay	1	1	1	1
Corn	1	1	1	1

Total = 16 samples

Figure 4.1 A diagram of the location of the enclosure used for the bovine TB environmental persistence study on the campus of Michigan State University.

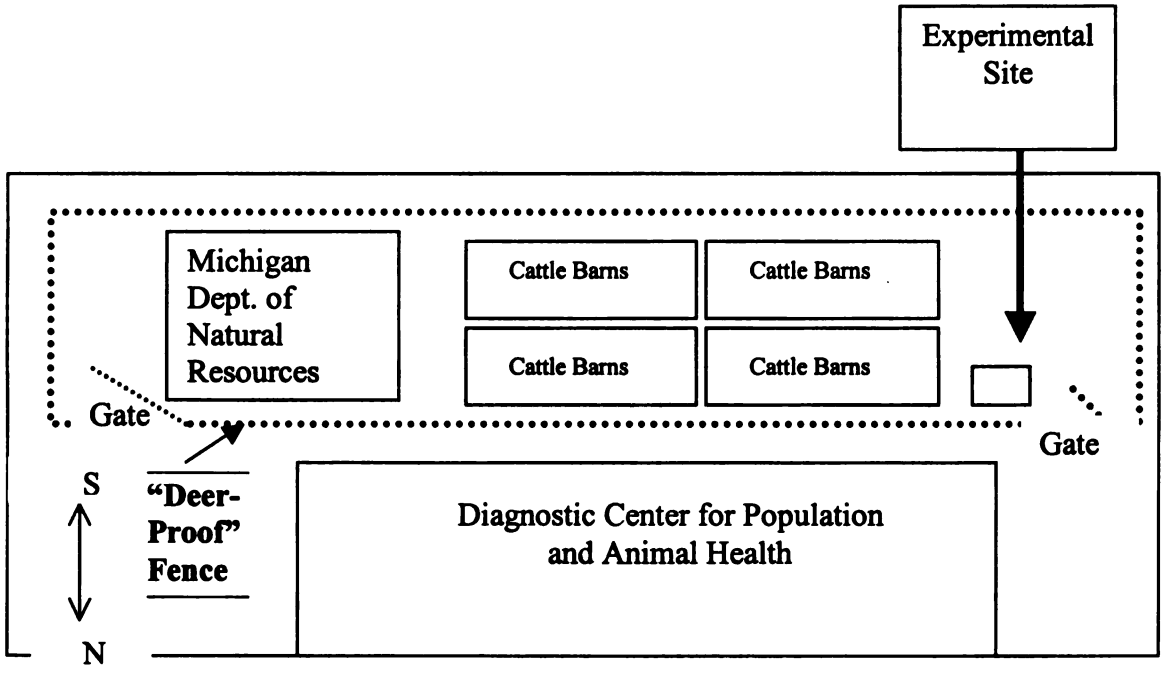
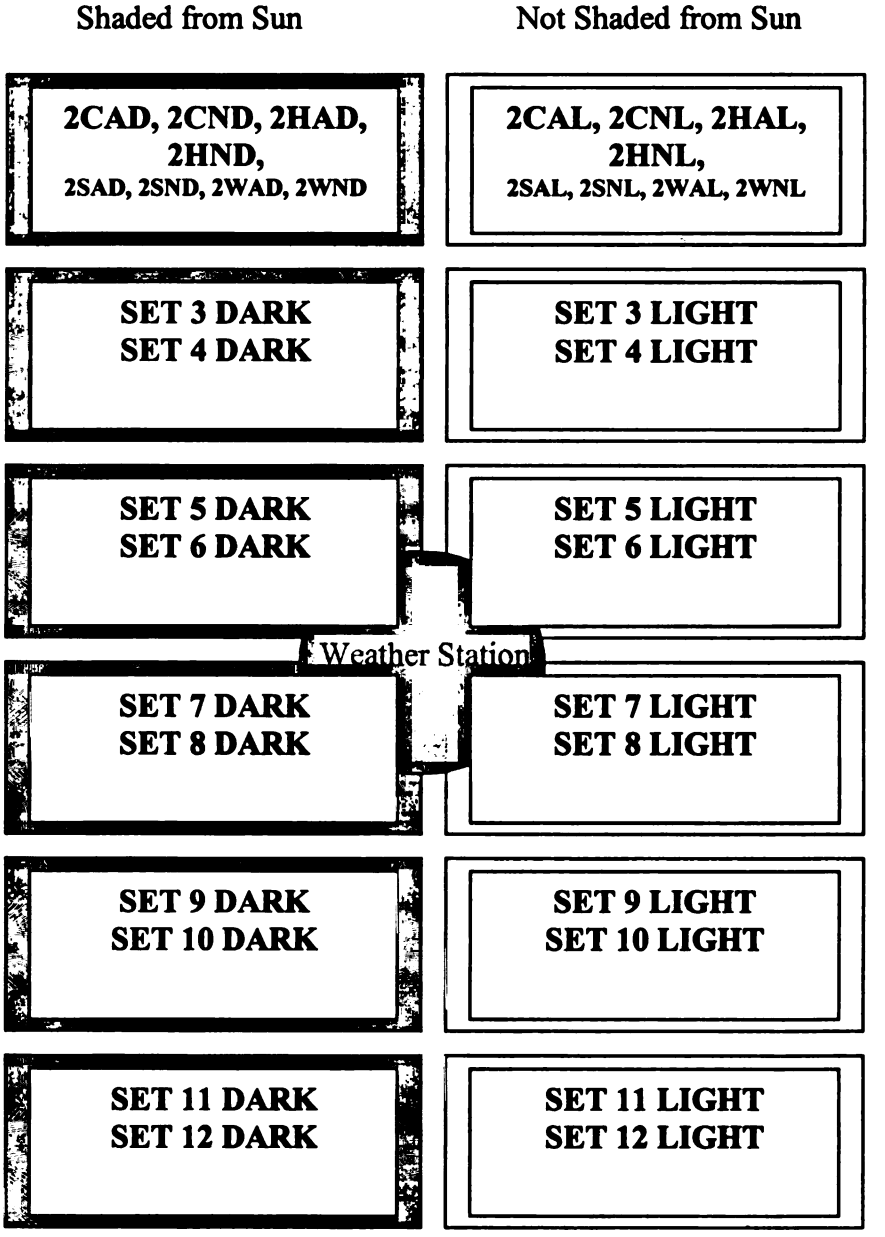
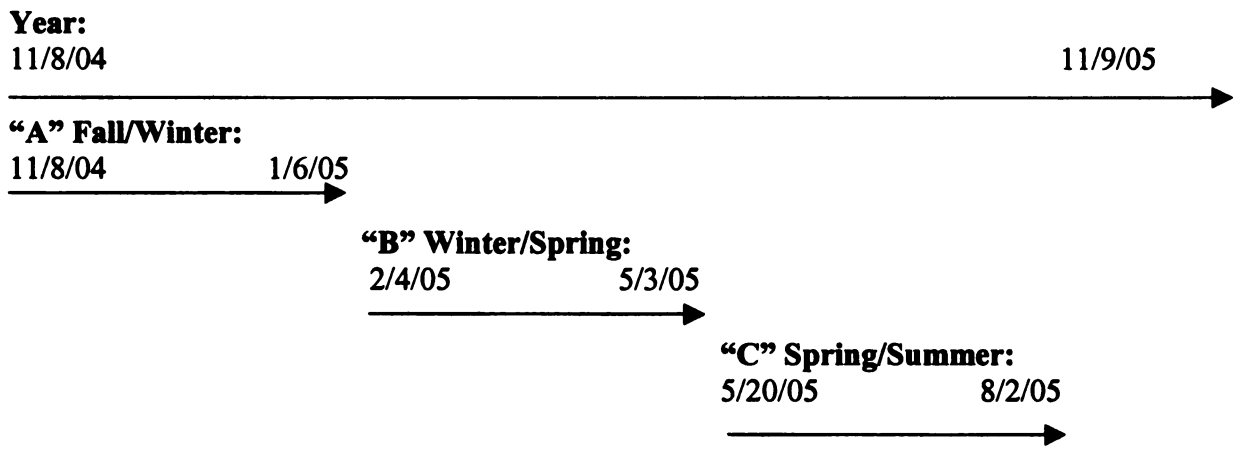


Figure 4.2 Placement of samples and weather station within the bovine TB environmental persistence study enclosure.



Key: C=corn; H=hay; S=soil; W=water; A=autoclaved; N=non-autoclaved; D=dark; L=light

Figure 4.3 Timing of sampling periods: Monthly over 1 year and weekly over 3 seasons.



4.3 RESULTS

Samples of *M. bovis* inoculated substrates set up for the twelve-month study were negative for months three through twelve. Data from the initial two months of the twelve-month study are represented by the fall/winter season "A".

4.3.1 Mycobacterial culture

One hundred and ninety-two sample replicates from each of the three sampling periods (total 576) were processed for *M. bovis* isolation. Contamination of cultures with mold and other non-mycobacterial species was detected in 13% of the samples processed in sampling period fall/winter "A" and winter/spring "B" and 50% of the samples processed in sampling period spring/summer "C". Sample substrates that were sterilized before *M. bovis* inoculation had significantly lower odds of contamination across all sampling periods ("A": $X^2 = 20.28$, $p < 0.01$; "B": $X^2 = 10.29$, $p < 0.01$; "C": $X^2 = 4.7$, $p < 0.05$).

4.3.2 Environmental conditions

The coldest sampling period was the fall/winter "A", winter/spring "B" was intermediate and spring/summer "C" was the warmest with the greatest amount of precipitation, highest solar radiation and greatest degree of evapotranspiration (Table 4.2). Weekly average temperatures and weekly average rainfall recorded at a Michigan Automated Weather Network weather station in the endemic bovine TB area were identical to the trends recorded at the study site. Average weekly temperature at the

northern weather station in the bovine TB endemic region was slightly higher across all sampling periods.

4.3.3 Persistence of *M. bovis* in the environment

M. bovis persisted in substrates exposed to environmental conditions for an average of one month in cool fall/winter and winter/spring conditions and for an average of 7 days in warmer spring/summer conditions (Table 4.3). Both the time from inoculation to the last positive *M. bovis* sample and the time from inoculation to first negative after last positive *M. bovis* sample are presented. The average and maximum survival times were lowest in the spring/summer sampling period "C". The maximum survival time across all substrate types and sampling seasons was recorded in a soil sample in the winter/spring "B" period. The soil sample was *M. bovis* positive at the final sampling point of 88 days. The shortest survival period was recorded in a hay sample in the spring/summer "C" period. The sample was positive at time 0 but negative at the 1st sampling point at 3 days. The mean and maximum survival time in each substrate type in each season is presented in Figure 4.4. The overall mean and maximum survival time across all substrate types in each season is presented in Figure 4.5.

The number of *M. bovis* positive replicate samples, and the number of *M. bovis* colonies recovered per sample, drops off quickly over the first 7 to 14 days of exposure to environmental conditions. The isolation of *M. bovis* from substrates exposed to environmental conditions was more intermittent after 14 days and positive samples were often identified based on the isolation of less than 5 *M. bovis* colonies per 100 µl of

sample. The percent of *M. bovis* positive replicates recorded at each sampling point and the number of colony forming units isolated are displayed in Figures 4.6 and 4.7.

4.3.4 Effects of non-seasonal factors on the persistence of *M. bovis* in the environment

The effect of substrate type on the persistence of *M. bovis* in the environment is variable. Symbols indicating significantly different mean survival times are displayed in Table 4.3 next to the mean survival period for *M. bovis* in each of the tested substrates. In the spring/summer period "C" survival is significantly longer in water. In the fall/winter "A" period survival appears to be significantly longer in hay and in the winter/spring "B" period survival appears to be significantly longer in soil. Survival probability curves for *M. bovis* in soil, corn, hay and water across all seasons are illustrated in Figure 4.8. The curves appear similar and log rank statistics confirm that the survival curves for the different substrates types are not significantly different from one another ($X^2 = 5.03$, p=0.17). Among all seasons there is no significant difference in the survival of *M. bovis* in one sample type versus another.

No significant associations were found between the bovine TB status of a sample and whether or not it was sterilized before *M. bovis* inoculation. The placement of the inoculated samples in shade or direct sunlight did not have a statistically significant effect on *M. bovis* survival but the mean survival time was longer across all samples and seasons in those placed under shade. The difference in mean survival time in the shaded and non-shaded samples approached significance in the fall/winter "A" and spring/summer "C" seasons (Table 4.4).

4.3.5 Effects of season on *M. bovis* persistence in the environment

Figure 4.9 illustrates the survival probability curves of *M. bovis* organisms exposed to environmental conditions in fall/winter "A", winter/spring "B", and spring/summer "C". The log rank statistics were associated with highly significant differences (chi-square = 19.88, $p<0.0001$) for between season probabilities. An analysis of maximum likelihood estimates when other covariates (shade/non-shade, substrate type, sterilize/non-sterilized substrates and interaction between shade/non-shade and season) are added to the model makes it clear that it is the season that drives the difference in the survival probability (Table 4.5).

4.3.6 Effects of weather on *M. bovis* persistence in the environment across seasons

The Cox's proportional hazard regression model, used to determine the relative influence of various weather related factors that together contribute to seasonal differences in the environmental persistence of *M. bovis*, revealed that temperature is the most influential factor in *M. bovis* survival. A number of the weather-related factors recorded throughout the sampling periods were significantly associated with the survival of *M. bovis* in the environment, however, many of these factors correlated with one another. The univariable hazard ratios and 95% confidence intervals for the weather related factors tested are presented in Table 4.6. Although all of these variables were significant at the $p < 0.15$ level, evapotranspiration and solar radiation were removed due to redundancy. The final multivariable Cox proportional hazard regression model is presented in Table 4.7.

Table 4.2 Weather conditions recorded over the three sampling periods of the *Mycobacterium bovis* environmental persistence study

	Fall/Winter "A" 11/8/04 – 1/6/05	**Winter/Spring "B" 2/4/05 – 5/3/05**	**Spring/Summer "C" 5/20/05 – 8/2/05**
	Average (Min. – Max)	Average (Min. – Max)	Average (Min. – Max)
Temperature (°C)	0.35 (-20.83 – 21.06)	2.82 (-14.44 – 29.72)	21.72 (4.83 – 36.94)
Precipitation (mm)	1.71 (0.00 – 20.83)	0.91 (0.00 – 18.54)	3.13 (0.00 – 30.48)
Humidity (%)	85.92 (27.00 – 100.00)	66.07 (4.00 – 100.00)	62.44 (10.00 – 100.00)
Solar Radiation (W/m^2)	24.29 (0.00 – 347.00)	79.99 (0.00 – 1040.00)	149.95 (0.00 – 1170.00)
Evapotranspiration (mm)	0.03 (0.00 – 1.02)	0.18 (0.00 – 2.29)	0.38 (0.00 – 3.30)

Table 4.3 Duration of *M. bovis* persistence in the environment on corn, hay, water and soil samples in season "A", "B" and "C".

Fall/Winter "A"	Days to last positive Mean (Standard Deviation)	Maximum (days)	Days to 1st negative after last positive Mean (Standard Deviation)	Maximum (days)
Corn	24.00 (11.75)	37.00	29.75 (13.94)	43.00
Hay	41.50 (3.00)*	43.00	54.25 (7.5)*	58.00
Soil	21.75 (5.32)	28.00	28.75 (6.18)*	37.00
Water	32.25 (18.23)	58.00	38.50 (14.80)	58.00
All Samples	29.88 (12.88)	58.00	37.81 (14.59)	58.00

Winter/Spring "B"	Days to last positive Mean (Standard Deviation)	Maximum (days)	Days to 1st negative after last positive Mean (Standard Deviation)	Maximum (days)
Corn	15.25 (7.89)*	26.00	24.50 (12.12)*	42.00
Hay	26.00 (0.00)	26.00	42.00 (0.00)	42.00
Soil	62.75 (30.63)*	88.00	68.25 (23.41)*	88.00
Water	12.25 (10.50)*	21.00	18.25 (9.39)*	26.00
All Samples	29.06 (25.56)	88.00	38.25 (23.61)	88.00

Spring/Summer "C"	Days to last positive Mean (Standard Deviation)	Maximum (days)	Days to 1st negative after last positive Mean (Standard Deviation)	Maximum (days)
Corn	1.50 (1.73)	3.00	7.00 (4.62)	11.00
Hay	0.00 (0.00)	0.00	3.00 (0.00)	3.00
Soil	8.25 (5.50)	11.00	15.75 (8.50)	20.00
Water	17.75 (22.01)*	48.00	23.50 (22.05)*	53.00
All Samples	6.88 (12.48)	48.00	12.31 (13.53)	53.00

* Significant difference in means (ANOVA) among substrate type Bonferoni $p \leq 0.05$.

Figure 4.4 The mean and maximum survival of *M. bovis* in corn, hay, soil and water exposed to environmental conditions in Fall/Winter "A", Winter/Spring "B", and Spring/Summer "C". Error bars represent standard deviation.

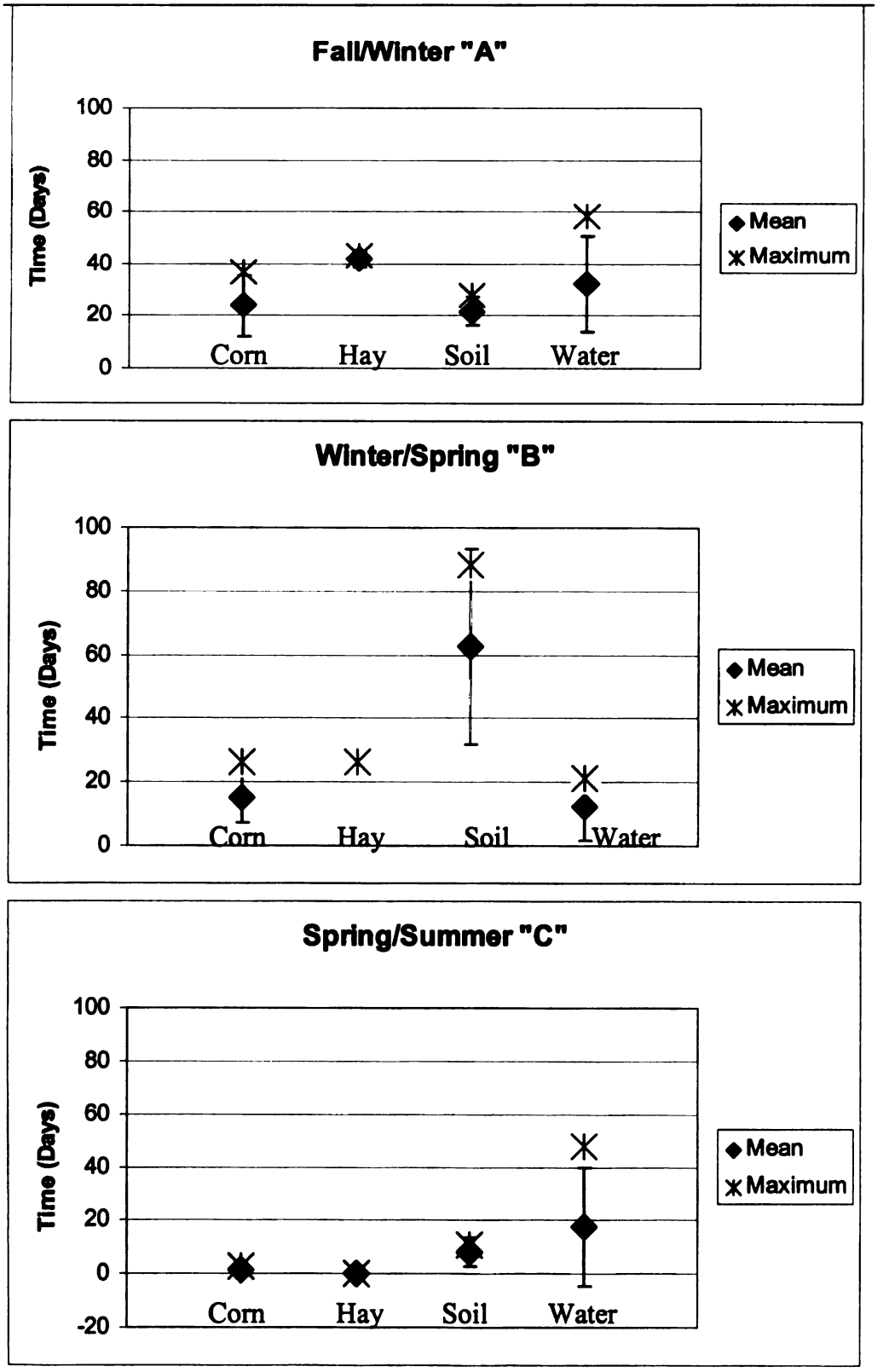


Figure 4.5 The overall mean and maximum survival time (persistence) of *M. bovis* in all substrates exposed to natural environmental conditions in Fall/Winter "A", Winter/Spring "B", and Spring/Summer "C". Error bars represent standard deviation.

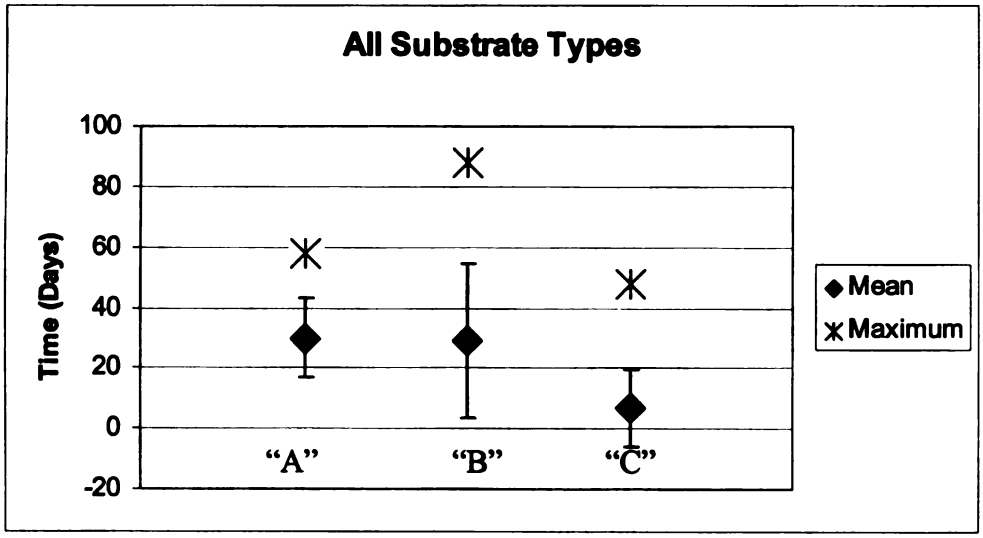
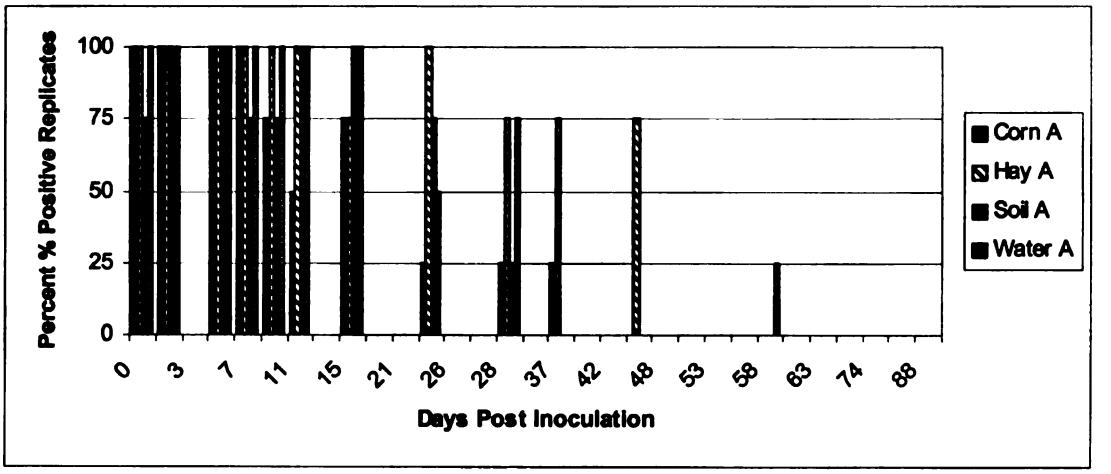
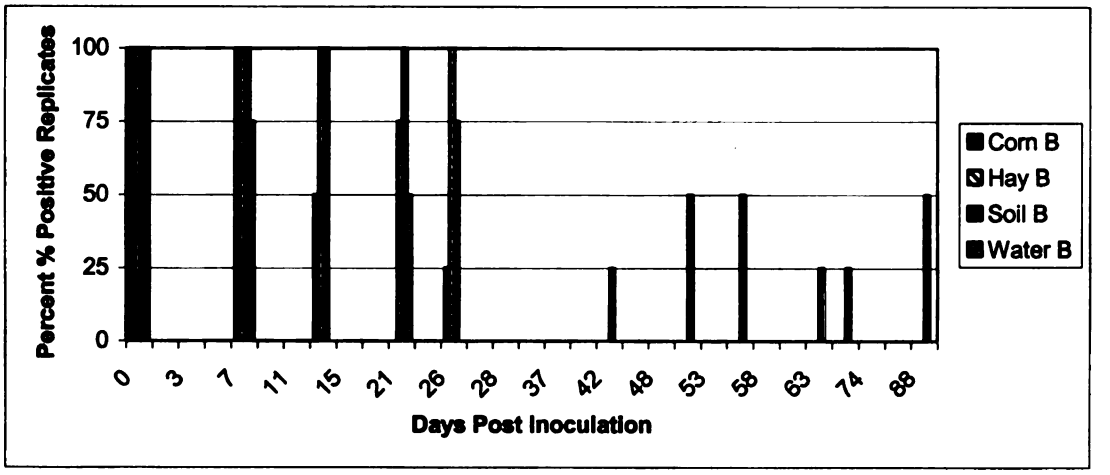


Figure 4.6 The percent (n=4 of each substrate) of *M. bovis* positive replicates of corn, hay, soil and water at each sampling point in Fall/Winter "A", Winter/Spring "B", and Spring/Summer "C".





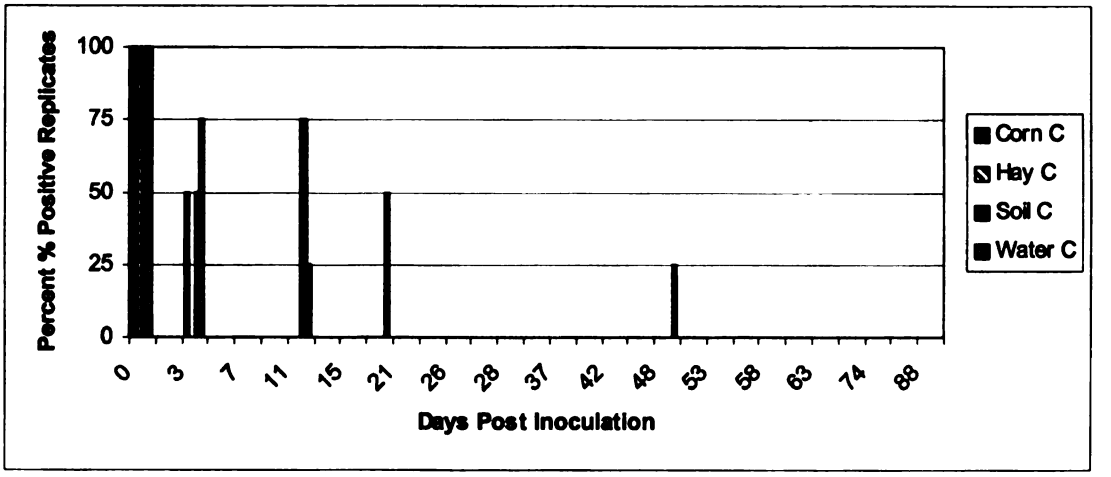
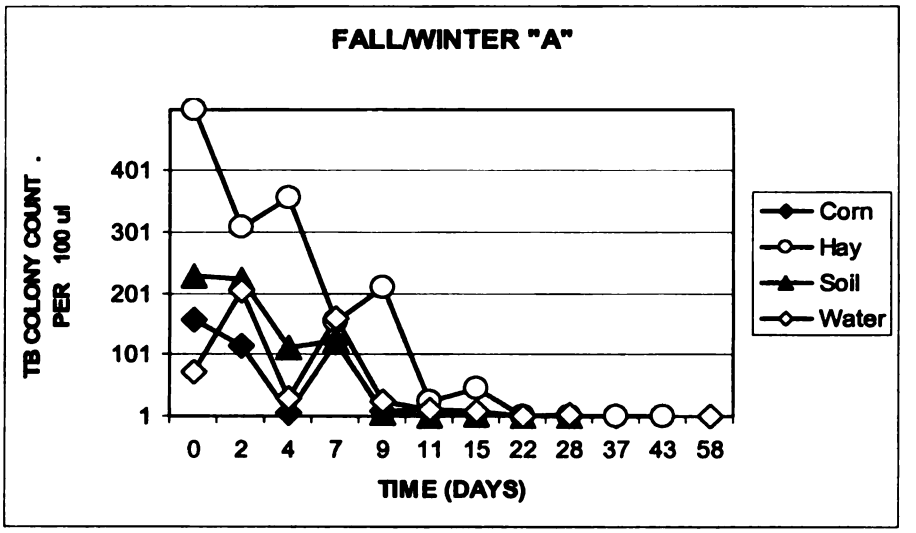
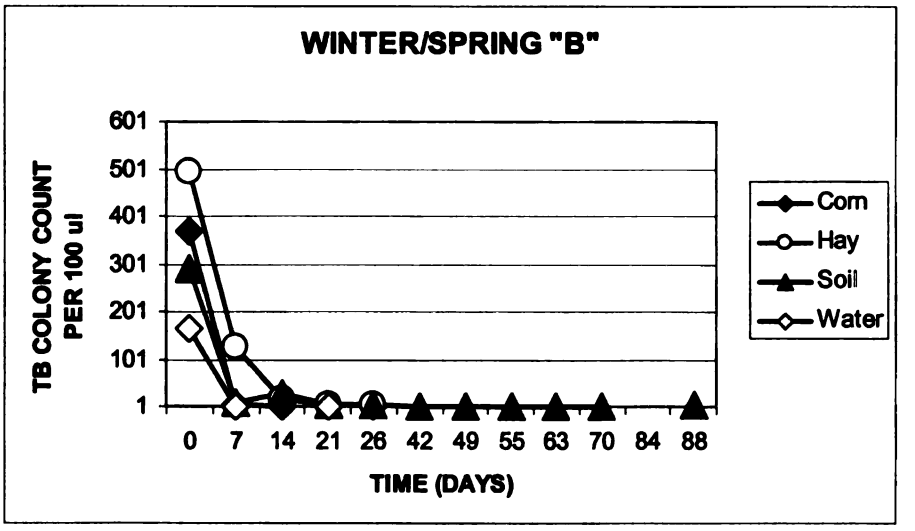


Figure 4.7 The average number of *M. bovis* colony forming units isolated from processed replicates of corn, hay, soil and water samples over time in Fall/Winter "A", Winter/Spring "B", and Spring/Summer "C".





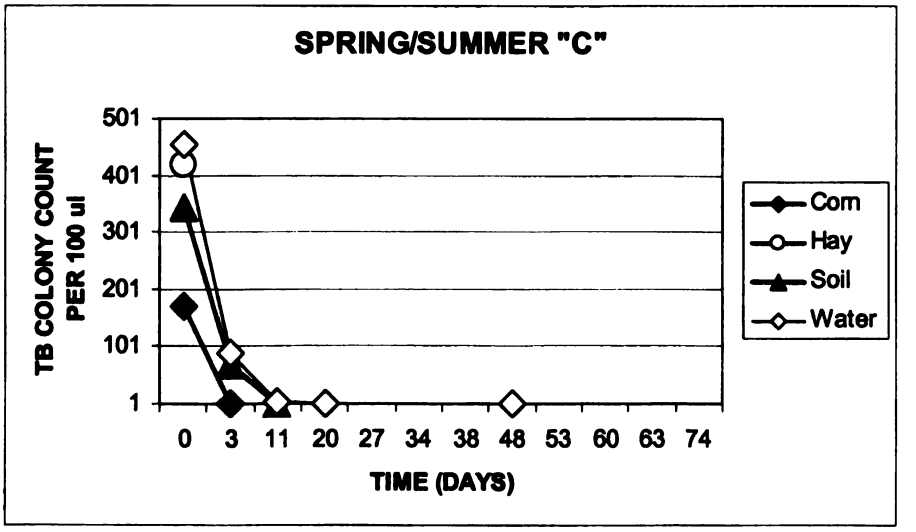


Figure 4.8 Survival probabilities of *M. bovis* in water, corn, hay and soil across all seasons tested. Images in this dissertation are presented in color.

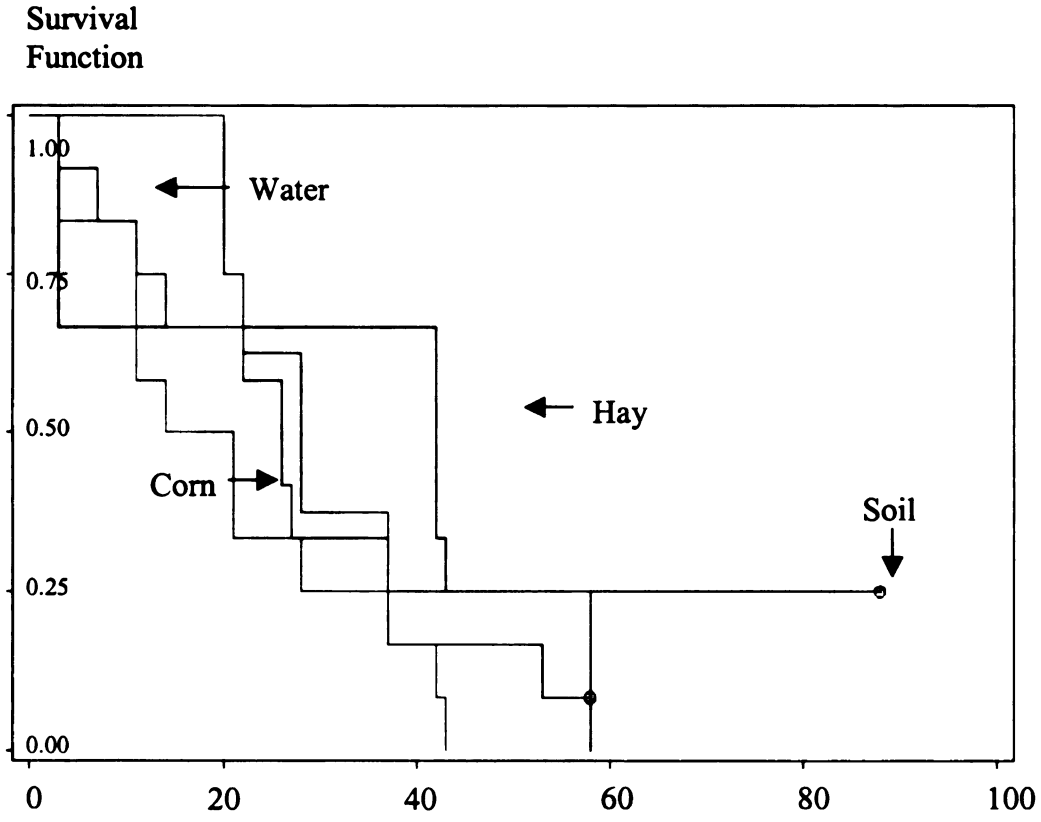


Table 4.4 Mean duration in days of *M. bovis* persistence in the environment on corn, hay, water and soil samples in season "A", "B" and "C" in shaded and non-shaded conditions

	Shade Mean (Std. Dev.)	No Shade Mean (Std. Dev.)	t statistic	p-value
Fall/Winter "A"	12.79 (12.27)	9.58 (9.38)	-1.68	0.10
Winter/Spring "B"	18.40 (20.65)	17.41 (20.48)	-0.20	0.84
Spring/Summer "C"	7.18 (12.52)	2.38 (4.03)	-1.48	0.15

Figure 4.9 Survival probabilities of *M. bovis* exposed to natural environmental conditions in "A", "B", and "C" sampling periods. N=16 samples per season. Images in this dissertation are presented in color.

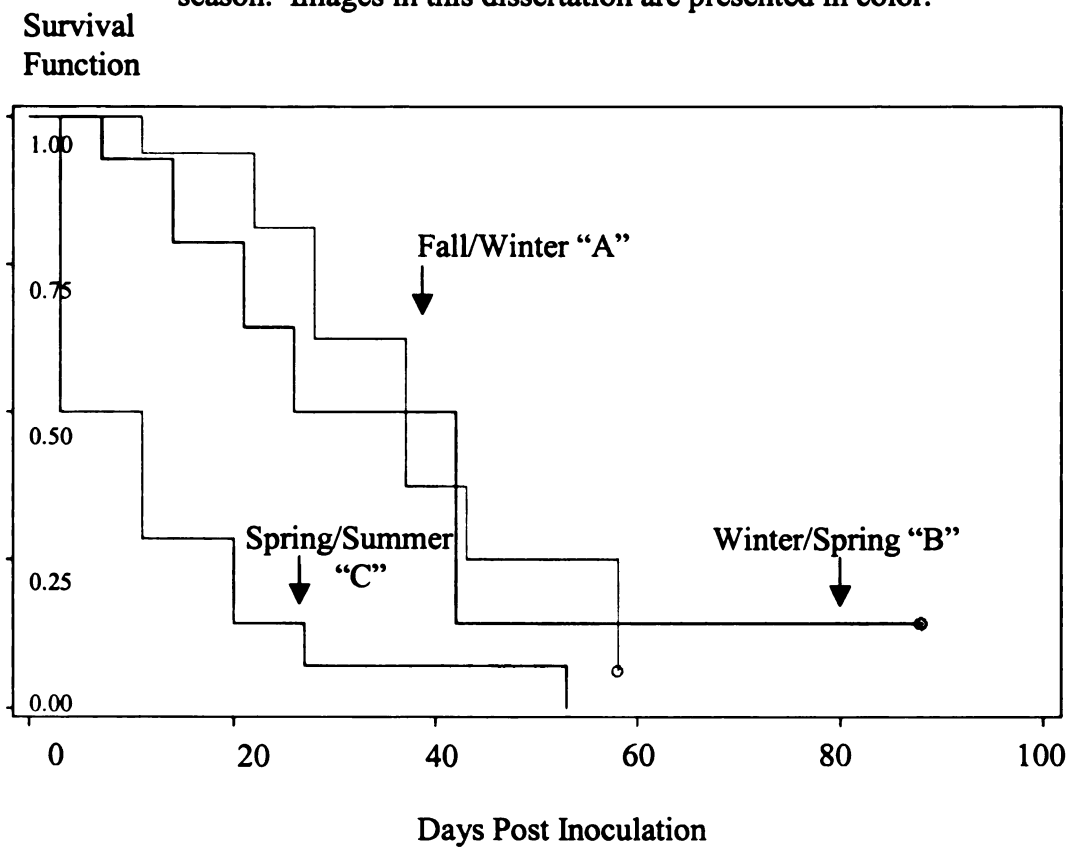


Table 4.5 Cox's proportional hazard regression model for persistence of *M. bovis* over three different sampling periods (fall/winter "A", winter/spring "B" and spring/summer "C") or seasons

Risk Factor	Parameter Estimate	Standard Error	Hazard Ratio (95% Confidence Interval)
Season A	-2.13	0.49	0.12 (0.05 – 0.32)
Season B	-1.94	0.49	0.14 (0.5 – 0.38)
Season C	0	--	--
Sample Type	-0.28	0.16	0.76 (0.55 – 1.05)
Sterilized	-0.50	0.35	0.61 (0.31 – 1.20)
Shade	-0.68	0.33	0.51 (0.26 – 1.05)

Table 4.6 Univariable analysis of the influence of each weather related variable on the hazard (survival) of *M. bovis* in the environmental using the score test in Cox regression.

Weather Factor (Average)	Score Chi-Square	p-value
Solar Radiation	7.50	0.0062
Humidity	2.46	0.1165
Temperature	9.85	0.0017
Precipitation	5.44	0.0197
Evapotranspiration	17.19	<.0001

Table 4.7 Multivariable Cox proportional hazards model of weather related factors associated with the survival of *M. bovis* in the environment.

Risk Factor	Parameter Estimate	Standard Error	Hazard Ratio (95% Confidence Interval)
Temperature*	0.06	0.02	1.06 (1.02 – 1.10)

* Results of purposeful selection of covariate and modified stepwise modeling in Cox regression. Only temperature remains in the model.

4.4 DISCUSSION

Mycobacterium bovis is an obligate intracellular pathogen but it has been shown to survive in the environment, outside a host, for substantial periods of time under favorable conditions (Duffield and Young, 1985; Jackson et al., 1995; Little et al., 1982; Maddock, 1933, , 1934, , 1936; Mitscherlich and Marth, 1984; Tanner and Michel, 1999; Van Donsel and Larkin, 1977; Williams and Hoy, 1930; Wray, 1975; Young et al., 2005). Although all of these studies demonstrated the persistence of *M. bovis* in the environment, their experimental nature, the use of high bacterial loads, and the variability of results have generally led to the conclusion that the environmental persistence of *M. bovis* does not play a significant role in the epidemiology of bovine tuberculosis through the indirect transmission of the pathogen among or between susceptible species.

The outbreak of bovine tuberculosis (TB) in northeast Michigan, and the establishment of a wildlife reservoir (white-tailed deer) for *M. bovis* in the region, has renewed interest in the characterization of *M. bovis* persistence in the environment and its role in the epidemiology of bovine TB in North America. This study clearly demonstrates that the Michigan strain of *M. bovis* persists in the environment under typical Michigan weather conditions. The study replicated the natural conditions under which *M. bovis* would be deposited on substrates in the environment and the weather to which the organisms would be exposed. Since the study site was approximately 200 miles to the south of the endemic bovine TB area in Michigan, basic indicators of environmental conditions (average weekly temperature and precipitation) were compared between the two areas by using data from the Michigan Automated Weather Network. The trends in temperature and precipitation were found to be identical with the

temperature in the northern TB endemic area an average of 1-2 °C cooler throughout the sampling periods. These slightly cooler temperatures in the north could be expected to support slightly longer persistence of *M. bovis* in the environment.

4.4.1 The Persistence of *M. bovis* in the Environment

The general pattern of *M. bovis* persistence in the environment, across all seasons and in all substrate types, was an initial decline in the numbers of recoverable bacilli over a 7 to 14 day period and then the persistence of small numbers of *M. bovis* bacilli for up to 4 to 10 weeks. The persistence of the Michigan strain of *M. bovis* on corn, hay, soil and water recorded in this natural environmental exposure study confirms the findings of laboratory-based studies conducted earlier in which the Michigan strain of *M. bovis* was found to persist for up to 12 weeks on feed (hay, corn, sugar beets, apples, carrots and potatoes) stored at 46° F and 0° F and for shorter periods when stored at 75° F (Whipple and Palmer, 2000). The similarity between the findings of the laboratory-based studies on *M. bovis* persistence and this one, in which the experimentally infected substrates were exposed to natural environment conditions, indicates that failure to detect *M. bovis* in the environment in known areas of *M. bovis* transmission is likely associated with the highly clustered spatial distribution of contaminated substrates under natural conditions and the inability to pin point the exact location of a *M. bovis* contaminated substrate for sampling, and not the inability of *M. bovis* to survive in the environment in a viable state.

The results of early studies performed in Europe and designed to characterize the persistence of *M. bovis* in the environment, have been scrutinized due to the large numbers of *M. bovis* bacilli used to experimentally inoculate of substrates (Maddock,

1933, 1934, 1936; Williams and Hoy, 1930). In this study the initial bacterial load used to inoculate the substrates was 50,000 CFU of *M. bovis*. This amount of *M. bovis* is more than the minimum infective oral dose of *M. bovis* established through experimental studies with the Michigan strain of *M. bovis* in both cattle (5,000 CFU) and white-tailed deer (300 CFU) (Palmer et al., 2002a, 2004a). The 50,000 CFU inoculums used is thought to be indicative of the amount of *M. bovis* that could be deposited by a bovine TB infected and shedding animal. The 50,000 CFU inoculum is smaller than the number of *M. bovis* bacilli recorded in the exudates from a lesion in an infected brush-tailed possum in New Zealand (5 X 10^8 CFU/ml) (Smith, 1972) but larger than number of bacilli (approximately 70 CFU) detected in the nasal mucous of an experimentally infected cow (McCorry et al., 2005). The amount of *M. bovis* used to inoculate substrates is believed to be relevant both in terms of the probability of contamination of environmental substrates through shedding of *M. bovis* from an infected animal and the likelihood of ingestion of *M. bovis* bacilli present in the environment by a susceptible host. This is particularly true in the 1st 7 days of exposure of *M. bovis* to environmental conditions when the number of CFUs recovered from the experimentally inoculated samples remained high.

4.4.2 Factors Influencing the Persistence of *M. bovis* in the Environment

This study was designed to characterize the persistence of *M. bovis* in a number of substrates exposed to natural environmental conditions over a 12-month period. A review of the literature indicated the necessity to supplement the 12-month study with a number of seasonal experiments designed to capture the effects on the persistence of *M.*

bovis under different weather conditions. The persistence of *M. bovis* during the Michigan spring/summer season (May 20th to August 2nd) was significantly shorter than the persistence recorded in the fall/winter season (November 8th to January 6th) and winter/spring season (February 4th to May 3rd). The spring/summer season was associated with higher average daily temperatures, greater intensity of solar radiation and higher loss of moisture through evapotranspiration. This findings are in agreement with those reported elsewhere in which an increase in temperature and a loss of moisture were found to be associated with a decease in the persistence of *M. bovis* in the environment (Duffield and Young, 1985; Jackson et al., 1995; Tanner and Michel, 1999; Wray, 1975).

Other factors, including substrate type, did not significantly affect the pattern of *M. bovis* persistence. The detection of *M. bovis* in a soil sample at 88 days (the final sampling point) in the winter/spring sampling period is in agreement with other studies that recorded the longest survival of *M. bovis* in cool, moist soil that presumably protects the bacilli from desiccation and provides an organic environment that supports its persistence (Duffield and Young, 1985; Maddock, 1933, 1934). Detection of *M. bovis* in a water sample at 48 days in the spring/summer sampling period, in which all other substrate types were negative at 20 days, indicates that even in the presence of high temperatures and intense solar radiation, viable *M. bovis* can persist under moist conditions.

The sterilization of substrates before *M. bovis* inoculation did not affect the persistence of *M. bovis* but it did significantly decease the occurrence of contaminated bacterial cultures. Pre-sterilized substrates were not used exclusively in this study

because decreased survival of *M. bovis* in sterile substrates has been reported (Duffield and Young, 1985; Young et al., 2005).

The location of the *M. bovis* inoculated substrates under shade had an effect on persistence. The mean survival time was longer for samples under shade than those exposed to direct sunlight. This was true in the fall/winter season and the spring/summer season but not in the winter/spring season. This apparent lack of a protective effect of shade during the winter/spring season is likely due to the fact that the cloth used to cover the "shaded" samples during this sampling period was severely damaged by wind and removed. It was replaced before the final spring/summer sampling period.

Survival analysis was used to evaluate the impact of weather over the three seasons on the persistence of *M. bovis* in the environment. Univariate analysis using Cox's proportional hazard regression indicated that the survival probability or persistence of *M. bovis* was decreased as temperatures increased, solar radiation intensified and evapotranspiration (a measure of moisture loss from a system) increased. The effects of average precipitation and percent humidity lost their significance in the presence of temperature. Since solar radiation and evapotranspiration are both directly related to temperature, the final conclusion was that temperature drives the seasonal effect seen in *M. bovis* persistence in the environment.

4.4.3 Implications of the Persistence of *M. bovis* in the Environment

The evidence that viable *M. bovis* persists in the Michigan environment under near natural conditions has significant implications on the efforts to control and eventually eliminate the occurrence of bovine TB in the region. Authors have argued

when examining other systems of bovine TB in other parts of the world that the conditions that contribute to *M. bovis* persistence in the environment also make the organisms inaccessible to susceptible hosts (Jackson et al., 1995; Morris et al., 1994). This is not the case in Michigan. The types of substrates tested (soil, water, hay and corn) are present in and around cattle farms in northeast Michigan. Additionally, white-tailed deer have access to these substrates on many cattle operations. Although many feed piles are exposed to sunlight and summer temperatures, there are periods of the year throughout the region in which low temperatures, cloud cover and the location of feed and water sources under the cover of a forest canopy or otherwise protected from the elements, would facilitate the long-term (4-12 weeks) survival of *M. bovis* bacilli deposited by a bovine TB infected animal.

The elimination of feeding and baiting sites for white-tailed deer and other wildlife should remain a component of the efforts to reduce bovine TB prevalence in this population and part of the management recommendations designed to reduce deer-to-deer bovine TB transmission events. Bovine TB eradication programs designed to eliminate the occurrence of bovine TB in cattle must consider *M. bovis* contaminated feed or water as a possible route for the indirect transmission of bovine TB between infected white-tailed deer and cattle. Farm bio-security measures focused on the elimination of the possibilities of cross contamination of feed and water sources should be added to the current protocols designed to eliminate cattle-to-cattle and the direct transmission of bovine TB. Specifically, cattle should be fenced out of open water sources and they should be provided an alternative source of water. Cattle should not be fed hay on the ground in the woods or in pasture adjacent to wooded areas and crops fields known to

attract white-tailed deer. The programs designed to encourage the fencing of feed storage areas should continue but emphasis should also be placed on the fencing and protection of cattle feeding areas.

4.4.4 Conclusions

These studies on the environmental persistence of *M. bovis* were designed to mimic natural conditions in the endemic region of bovine TB transmission in Michigan. The data clearly indicate that there is a real potential for the indirect transmission of *M. bovis* among and between cattle and white-tailed deer populations in Michigan. Persistence of *M. bovis* can be expected to be longer in cooler seasons. Practices that facilitate the cross contamination of substrates by infected and susceptible animals should be restricted at all times but especially during the cooler seasons of the year.

Failure to isolate *M. bovis* from environmental substrates collected from areas with known bovine TB transmission, is likely due to the inability to pin point the exact location of environmental contamination for sampling and less to do with the persistence of the *M. bovis* bacilli in the environment. Difficulties in isolating viable *M. bovis* from environmental substrates due to the limitations of sample processing and mycobacterial culture will also continue to hinder the accurate assessment of the quantity of *M. bovis* organisms in the environment. However, this study indicates that the organisms do persist over a time period that would allow a susceptible animal to become exposed and infected with *M. bovis* from an environmental source. Indirect transmission of *M. bovis* plays a role in the interspecies transmission of bovine TB and will continue to hinder programs designed to eliminate the disease if not addressed.

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

SUMMARY

This dissertation has examined the persistence of *Mycobacterium bovis* in the environment and the potential role of indirect transmission in the epidemiology of bovine tuberculosis (TB). Special attention has been paid to systems in which a wildlife species is believed to act as the reservoir of *M. bovis* infection. Experimental and observational studies with a focus on the Michigan outbreak of bovine TB in cattle and white-tailed deer have been discussed with the aim of enhancing our understanding of the role of indirect transmission in the epidemiology of bovine TB in the region.

One of the primary limitations to assessing the persistence of *M. bovis* in the environment was addressed by optimizing a technique for processing environmental substrates for the detection of *M. bovis* with mycobacterial culture, the only real testing option available for determining the viability of *M. bovis* bacilli in the environment.

Attempts were made to isolate *M. bovis*, with the optimized techniques, from environmental substrates collected for cattle farms with a known history of *M. bovis* infection. Opportunistic sampling of areas with a high prevalence of *M. bovis* infection in white-tailed deer was also performed. Though mycobacterial isolation was successful, none of the isolates were identified as *M. bovis*.

To address the question of *M. bovis* persistence in the environment, given the limitations of detecting the bacilli under natural conditions, a 12-month experimental study was designed. Environmental substrates were inoculated with *M. bovis* and

exposed to natural weather conditions. The effect of environmental conditions on the survival of *M. bovis* over time was assessed.

CONCLUSIONS

The recorded persistence of *M. bovis* in the Michigan environment under natural weather conditions, strongly suggests the potential contribution of indirect means to the transmission of bovine TB in the region. These data supplement those produced through experimental *M. bovis* disease transmission studies that have proven the feasibility of indirect transmission of *M. bovis* among and between cattle and white-tailed deer. They also support the analyses of observational data on *M. bovis* infection in cattle and white-tailed deer in Michigan that indicate the importance of indirect transmission in the interspecies transmission of *M. bovis* in the region.

Conclusions specific to this dissertation research include the following:

1. It is possible to improve methods for processing environmental samples for *M. bovis* isolation by bacterial culture, however, the necessity of decontaminating samples limits the sensitivity of *M. bovis* detection.
2. *Mycobacterium bovis* is not distributed across the landscape of known sites of bovine TB transmission and challenges associated with identifying the specific point of contamination make detecting *M. bovis* in the environment under natural disease transmission extremely unlikely.
3. Under experimental conditions *Mycobacterium bovis* does persist in the environment for a long enough period to serve as a source of infection to a susceptible host.

4. Survival of *M. bovis* is longest in the cooler months of the year, in which persistence of *M. bovis* in a soil sample was recorded at 88 days.
5. Indirect transmission of *M. bovis* is likely to occur if the host and environmental factors provide an opportunity for substrate contamination by an infected animal and subsequent contact by a susceptible individual.

RECOMMENDATIONS

Local, State and Federal bovine TB control and eradication policy needs to consider indirect transmission of *M. bovis* through contaminated environmental substrates in the creation and implementation of epidemiologically appropriate disease management plans. In the bovine TB endemic region of Michigan, interspecies transmission of bovine TB should be considered by both wildlife and livestock health agencies. If this component of the epidemiology of *M. bovis* transmission in the region is ignored, efforts to control and eventually eradicate the disease will fail.

Regulations restricting the supplemental feeding and baiting of wildlife should be upheld and approaches to cattle farm bio-security should be reviewed with the intent of preventing indirect transmission of *M. bovis* between white-tailed deer and cattle. Cattle farm repopulation protocols that rely only on time, in the absence of management changes, to reduce the probably of TB re-infection are inadequate. The current focus on preventing access of deer to feed intended for cattle is a step in the right direction, however, that emphasis needs to be carried beyond issues surrounding feed storage to those surrounding feeding sites and the opportunities for cross contamination of environmental substrates in and around cattle operations. Specifically, cattle should be

fenced out of open water sources and they should be provided an alternative source of water. Cattle should not be fed hay on the ground in the woods or in pasture adjacent to wooded areas and crops fields known to attract white-tailed deer. The programs designed to encourage the fencing of feed storage areas should continue but emphasis should also be placed on the fencing and protection of cattle feeding areas.

Appendix A

Bovine Tuberculosis: Identification of Environmental Reservoirs of *Mycobacterium bovis* in Michigan

On-Farm Environmental Sampling Protocol

1. The Michigan Department of Agriculture will inform the owners of bovine tuberculosis (TB) affected cattle herds of the opportunity to have environmental testing done on their farm by the research group at Michigan State University.

2. The names and contact information of interested bovine TB-affected herd owners will be forwarded to Dr. Amanda Fine, Population Medicine Center, Department of Large Animal Clinical Sciences, Michigan State University.

3. Dr. Amanda Fine will contact the TB-affected cattle herd owners by phone and discuss the project. An appointment for a farm visit will be made at a time that is convenient for the cattle farm owners.

4. During the initial farm visit the project will be explained, the herd owners will have the opportunity to ask any questions and they will be presented with a waiver form to sign allowing the collection of environmental samples from their farm.

5. A walk through of the premises will take place with the herd owner's guidance. A sampling plan will be formed after observations and discussions with the farm owners. Sites for sampling will be marked on a rough map of the premises.

6. Approximately 20 samples will be collected from different sites around the farm. It is expected that these samples will be of feed, water, soil, manure, bedding and pasture grass.

7. Dr. Amanda Fine, a veterinarian trained in biological hazards and safety, will collect all samples.

8. The samples will be placed in sterile, leak-proof containers or Whirl-Pak bags ranging in size from 50 ml to 1,000 ml.

9. The containers will be sealed with their screw top caps and plastic Whirl-Pak bags will be sealed with additional tape.

10. Smaller containers will be placed in larger plastic bags or containers and sealed.

11. All samples will be placed in a cooler on ice.

12. The samples will be transported by car to the Michigan State and will be delivered within 12 hours of collection.

Appendix B

Bovine Tuberculosis: Identification of Environmental Reservoirs of *Mycobacterium bovis* in Michigan: Farm Management Survey (Part I)

Farm # _____ Years cattle have been on this farm: _____ years

1. Operation type (check all that apply): ~ Dairy ~ Beef cow/calf ~ Beef feedlot

Type of cattle	# in herd
Dairy milking herd	
Beef cow-calf pairs	
Beef feeders	
Bulls	

2. Herd Size:

3. How large is your farm? _____ acres total
   a) Acres of pasture: _____
   b) Acres of crop fields (no cattle access): _____
   c) Acres of cattle housing (besides pasture): _____
   d) Acres of homestead (yard, garden, etc): _____

4. Do you raise any other livestock on your farm?

   ~ Yes, list: _____
   ~ No

5. Do you have any pets or barn cats on your farm?

   ~ Yes, list: _____
   ~ No

6. Do your cattle have fence line (nose to nose) contact with any livestock you do not own or manage?

   ~ Yes
   ~ No

Notes:

7. Please describe the movement of cattle on your farm as they age from birth to adult.

AGE (months)	LOCATION

8. Please describe the seasonal cycle of the movement of adult cattle on your farm. We are interested in knowing when and for what period of time cattle are housed in or have access to the following:

Beef Cow/Calf Pairs:

Season	Indoors (%)	Feedlot/ DryLot (%)	Pasture (%)	Fenced Cropland (%)	Fenced Woodlot (%)	Other (describe) (%)
Summer						
Fall						
Winter						
Spring						

Beef Bulls:

Season	Indoors (%)	Feedlot/ Dry Lot (%)	Pasture (%)	Fenced Cropland (%)	Fenced Woodlot (%)	Other (describe) (%)
Summer						
Fall						
Winter						
Spring						

Beef Feeders:

Season	Indoors (%)	Feedlot/ Dry Lot (%)	Pasture (%)	Fenced Cropland (%)	Fenced Woodlot (%)	Other (describe) (%)
Summer						
Fall						
Winter						
Spring						

Notes:

8. Seasonal cycle of the movement (cont.)

Dairy Cattle: period of time cattle are housed or have access to the following, in:

Summer

Cattle Group	Indoors (%)	Feedlot/ Dry Lot (%)	Pasture (%)	Fenced Cropland (%)	Fenced Woodlot (%)	Other (describe) (%)
Pre-weaned calves						
Weaned calves & Heifers						
Lactating dairy cows						
Dry cows						
Bulls						
Other (describe):						

Winter

Cattle Group	Indoors (%)	Feedlot/ Dry Lot (%)	Pasture (%)	Fenced Cropland (%)	Fenced Woodlot (%)	Other (describe) (%)
Pre-weaned calves						
Weaned calves & Heifers						
Lactating dairy cows						
Dry cows						
Bulls						
Other (describe):						

8. Seasonal cycle of the movement (cont.)

Dairy Cattle: What housing facilities did this operation use during the last 12 months for the following (check all that apply):

	Hutch (single animal)	Freestall	Tie Stall or Stanchion	Single Animal housing	Multiple Animal housing
A. Preweaned calves					
B. Weaned calves & heifers					
C. Lactating dairy cows					
D. Dry dairy cows					
E. Bulls					
H. Other (describe)					

9. Animals from outside the operation:

a) What percent of your animals were purchased from outside your operation?

Less than 5% ~ 5 to 10% ~ 11 to 25% ~ 26 to 50% ~ More than 50% ~

Notes:

b) What percent of your animals spend any time at a location other than your farm?

Less than 5% ~ 5 to 10% ~ 11 to 25% ~ 26 to 50% ~ More than 50% ~

Notes:

9. c) Were any of the following groups of animals brought onto this operation from outside sources during the last 12 months?

	Brought onto operation?		Percent were brought onto operation?
A. Preweaned (milk-fed) calves?	~ Yes	~ No	%
B. Weaned calves or heifers?	~ Yes	~ No	%
C. Dairy cows?	~ Yes	~ No	%
D. Bulls?	~ Yes	~ No	%
E. Beef cow-calf pairs?	~ Yes	~ No	%
F. Feeder cattle?	~ Yes	~ No	%
G. Other (describe)?	~ Yes	~ No	%

Water Sources

10. Where do your livestock drink? (mark all that apply)

- ~ Inside water tank/trough/individual waters
- ~ Outside water tank/trough
- ~ Surface Water (Pond, Lake or Stream)
- ~ Other, please describe:

Feeding Practices

11. What percent of the following feeds were purchased (rather than grown at your farm):

Forages: _____ %

Grains: _____ %

12. How are your livestock fed? (mark all that apply)

	Indoors		Outdoors	
	on Ground	In Feeder	on Ground	In Feeder
Harvested forage, in whole bales:	~	~	~	~
Harvested forage unrolled or ground up:	~	~	~	~
Silage or Haylage:	~	~	~	~
Concentrates (grain or pellets)	~	~	~	~
Supplements (salt and minerals)	~	~	~	~

Feed Storage

13. What type of hay bales do you use? (mark all that apply)
    - ~ Round bales
    - ~ Square bales
    - ~ Other, please describe: _____

14. How do you store your hay? (mark all that apply)

    In a building: ~ Outside: ~ Uncovered
    ~ Covered

    Round bales: ~ Left in field Fencing: ~ Fenced
    ~ Along fence rows ~ Unfenced

    Notes:

15. How do you store grain? (mark all that apply)

    - ~ In a grain bin
    - ~ Bagged, indoors
    - ~ Bagged outside
    - ~ On the ground outside, uncovered/unfenced
    - ~ On the ground outside, covered/unfenced
    - ~ On the ground outside, covered/fenced
    - ~ Other, please specify: _____

16. How do you store your silage/haylage? (mark all that apply)

~ Upright silo
~ Bunk with sides
~ Silage bags
~ Covered pile on ground
~ Uncovered pile on ground
~ Other, please describe: _____

Fencing:

17. What kind of fencing do you use for your livestock enclosures? (please specify units!)

Type of Fence	Used?	Average fence height	Length of type of fence used
Electric	~		
High tensile wire	~		
Woven wire	~		
Barbed wire	~		
Board/pipe	~		
Building wall (barn, shed, etc.)	~		
Other, please describe:	~		

18. What kind of fencing do you use for your feed storage areas?

Type of Fence	Used?	Average fence height	Length of type of fence used
Electric	~		
High tensile wire	~		
Woven wire	~		
Barbed wire	~		
Board/pipe	~		
Building wall (barn, shed, etc.)	~		
Other, please describe:	~		

Bovine Tuberculosis: Identification of Environmental Reservoirs of *Mycobacterium bovis* in Michigan: Wildlife Incursion (Part 2)

19. **Deer Incursions**

Homestead

Ave. #/day	Summer	Fall	Winter	Spring
None				
1-5				
5-20				
>20				

Cattle Housing

Ave. #/day	Summer	Fall	Winter	Spring
None				
1-5				
5-20				
>20				

Pasture/Wood Lot (Cattle Access)

Ave. #/day	Summer	Fall	Winter	Spring
None				
1-5				
5-20				
>20				

Cropland (No Cattle Access)

Ave. #/day	Summer	Fall	Winter	Spring
None				
1-5				
5-20				
>20				

Feed Storage Areas

Ave. #/day	Summer	Fall	Winter	Spring
None				
1-5				
5-20				
>20				

Other Land (No Cattle Access)

Ave. #/day	Summer	Fall	Winter	Spring
None				
1-5				
5-20				
>20				

20. Have you seen deer or evidence of deer using your livestock water sources?
    - ~ No
    - ~ Yes

21. If yes, what was the water source? (mark all that apply)
    - ~ Water tank/trough/individual waterers
    - ~ Surface Water (Pond, Stream, Lake)
    - ~ Other

22. Where was the water source located?
    - ~ In barn/shed
    - ~ Barnyard
    - ~ Pasture
    - ~ Wooded lot
    - ~ Other, please specify:

23. Where have you seen deer feeding activity on your farm? (mark all that apply)
    - ~ Inside shed/barn
    - ~ Inside or near calf hutches
    - ~ Outside near shed/barn
    - ~ Within a fenced livestock area
    - ~ Within a fenced feed storage area
    - ~ Outside a fenced area
    - ~ Other, please specify:
    - ~ Have not seen deer feeding activity on my farm

24. What type of feed are deer eating? (mark all that apply)

Forages:
- ~ Hay
- ~ Silage: ~ Haylage
- ~ Corn silage
- ~ Other silage:

Grains:
- ~ Corn
- ~ Oats
- ~ Other grains:

Supplements: ~

25. Describe any other deer feeding activity (i.e. in orchard, landscape trees, etc.) on your farm:

26. Are there any cedar swamps, orchards, or croplands that might attract deer onto your property?

~ No
~ Yes If yes, please describe these

27. Have you observed any other wildlife species on your farm?

List:

Notes:

Appendix C

Bovine Tuberculosis: Identification of Environmental Reservoirs of *Mycobacterium bovis* in Michigan

On-Farm Environmental Sampling
Data Collection Form

1 **Farm #:** _____

Sampling Date: _____

Sampling Time: _____

Weather Conditions: _____

Sample #	Type	Location	Photo #	GPS mark	Notes
	Soil, hay, water, etc.	Description	Digital photo of sample site	Mark of exact site of sampling	Microclimate (damp, sunny, etc.)

LITERATURE CITED

Anderson, R.M., Trewhella, W., 1985, Population dynamics of the badger (Meles meles) and the epidemiology of bovine tuberculosis (Mycobacterium bovis). Philos Trans R Soc Lond B Biol Sci 310, 327-381.

Bruning-Fann, C.S., Schmitt, S.M., Fitzgerald, S.D., Fierke, J.S., Friedrich, P.D., Kaneene, J.B., Clarke, K.A., Butler, K.L., Payeur, J.B., Whipple, D.L., Cooley, T.M., Miller, J.M., Muzo, D.P., 2001, Bovine tuberculosis in free-ranging carnivores from Michigan. J Wildl Dis 37, 58-64.

Bruning-Fann, C.S., Schmitt, S.M., Fitzgerald, S.D., Payeur, J.B., Whipple, D.L., Cooley, T.M., Carlson, T., Friedrich, P., 1998, Mycobacterium bovis in coyotes from Michigan. J Wildl Dis 34, 632-636.

Butler, K.L., Fitzgerald, S.D., Berry, D.E., Church, S.V., Reed, W.M., Kaneene, J.B., 2001, Experimental inoculation of European starlings (Sturnus vulgaris) and American crows (Corvus brachyrhynchos) with Mycobacterium bovis. Avian Dis 45, 709-718.

Cooney, R., Kazda, J., Quinn, J., Cook, B., Muller, K., Monaghan, M., 1997, Environmental mycobacteria in Ireland as a source of non-specific sensitisation to tuberculins. Irish Veterinary Journal 50, 370-373.

Cornejo, B.J., Sahagun-Ruiz, A., Suarez-Guemes, F., Thornton, C.G., Ficht, T.A., Adams, L.G., 1998, Comparison of C18-carboxypropylbetaine and glass bead DNA extraction methods for detection of Mycobacterium bovis in bovine milk samples and analysis of samples by PCR. Appl Environ Microbiol 64, 3099-3101.

Corner, L.A., 2006, The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Vet Microbiol 112, 303-312.

DeLiberto, T.J., Vercauteren, K.C., Witmer, G.W., 2004. The Ecology of *Mycobacterium bovis* in Michigan. In: Activities Report and Conference Proceedings, Michigan Bovine Tuberculosis Eradication Project, Lansing, Michigan.

Diegel, K.L., Fitzgerald, S.D., Berry, D.E., Church, S.V., Reed, W.M., Sikarskie, J.G., Kaneene, J.B., 2002, Experimental inoculation of North American opossums (Didelphis virginiana) with Mycobacterium bovis. J Wildl Dis 38, 275-281.

Drobniewski, F., Strutt, M., Smith, G., Magee, J., Flanagan, P., 2003, Audit of scope and culture techniques applied to samples for the diagnosis of Mycobacterium bovis by hospital laboratories in England and Wales. Epidemiol Infect 130, 235-237.

Duffield, B.J., Young, D.A., 1985, Survival of Mycobacterium bovis in defined environmental conditions. Veterinary Microbiology 10, 193-197.

Dunn, J.R., Kaneene, J.B., Grooms, D.L., Bolin, S.R., Bolin, C.A., Bruning-Fann, C.S., 2005, Effects of positive results for Mycobacterium avium subsp paratuberculosis as determined by microbial culture of feces or antibody ELISA on results of caudal fold tuberculin test and interferon-gamma assay for tuberculosis in cattle. J Am Vet Med Assoc 226, 429-435.

Fitzgerald, S.D., Zwick, L.S., Diegel, K.L., Berry, D.E., Church, S.V., Sikarskie, J.G., Kaneene, J.B., Reed, W.M., 2003, Experimental aerosol inoculation of Mycobacterium bovis in North American opossums (Didelphis virginiana). J Wildl Dis 39, 418-423.

Frye, G., 1995, Bovine tuberculosis eradication: The program in the United States, In: Thoen, C.O., Steele, J.H. (Eds.) Mycobacterium bovis infection in animals and humans. Iowa State University Press, Ames, Iowa, pp. 119-129.

Garner, M. 2001. Movement patterns and behavior at winter feeding and fall baiting stations in a population of white-tailed deer infected with bovine tuberculosis in the northeastern Lower Peninsula of Michigan. Master Thesis, Michigan State University.

Goodchild, A.V., Clifton-Hadley, R.S., 2001, Cattle-to-cattle transmission of Mycobacterium bovis. Tuberculosis (Edinb) 81, 23-41.

Hickling, G.J. 2002. Dynamics of bovine tuberculosis in wild white-tailed deer in Michigan (Wildlife Division Report No. 3363. Michigan Department of Natural Resources), p. 34.

Holecek, D.F., Bristor, T.Y. 2003. The economic impact of bovine TB on the tourism industry in northeast Michigan (East Lansing, Michigan Travel, Tourism and Recreation Center, Michigan State University), pp. 1-6.

Hutchins, M.R., Harris, S., 1997, Effects of farm management practices on cattle grazing behavior and the potential for transmission of bovine tuberculosis from badgers to cattle. Veterinary Journal 153, 149-162.

Jackson, R., de Lisle, G.W., Morris, R.S., 1995, A study of the environmental survival of *Mycobacterium bovis* on a farm in New Zealand. New Zealand Veterinary Journal 43, 346-352.

Judge, L. 2006. The number of bovine tuberculosis (TB) positive farms identified in Michigan (1998-2006) by the United States Department of Agriculture, Animal Plant Health Inspection Service, Veterinary Services, Fine, A.E., ed. (East Lansing).

Kaneene, J.B., Bruning-Fann, C.S., Dunn, J., Mullaney, T.P., Berry, D., Massey, J.P., Thoen, C.O., Halstead, S., Schwartz, K., 2002a, Epidemiologic investigation of Mycobacterium bovis in a population of cats. Am J Vet Res 63, 1507-1511.

Kaneene, J.B., Bruning-Fann, C.S., Granger, L.M., Miller, R., Porter-Spalding, B.A., 2002b, Environmental and farm management factors associated with tuberculosis on cattle farms in northeastern Michigan. J Am Vet Med Assoc 221, 837-842.

Kent, P.T., Kubica, G.P. 1985. Public Health Mycobacteriology: A guide for level III laboratory (Atlanta, GA, Public Health Service, Centers for Disease Control, U.S. Department of Health and Human Services), pp. 31-70.

Laserson, K.F., Yen, N.T., Thornton, C.G., Mai, V.T., Jones, W., An, D.Q., Phuoc, N.H., Trinh, N.A., Nhung, D.T., Lien, T.X., Lan, N.T., Wells, C., Binkin, N., Cetron, M., Maloney, S.A., 2005, Improved sensitivity of sputum smear microscopy after processing specimens with C18-carboxypropylbetaine to detect acid-fast bacilli: a study of United States-bound immigrants from Vietnam. J Clin Microbiol 43, 3460-3462.

Little, T.W., Naylor, P.F., Wilesmith, J.W., 1982, Laboratory study of Mycobacterium bovis infection in badgers and calves. Veterinary Record 111, 550-557.

Livanainen, E., 1995, Isolation of mycobacteria from acidic forest soil samples: comparison of culture methods. J Appl Bacteriol 78, 663-668.

Livanainen, E., Martikainen, P.J., Vaananen, P., Katila, M.-L., 1999, Environmental factors affecting the occurrence of mycobacteria in brook sediments. Journal of Applied Microbiology 86, 673-681.

Maddock, E.C.G., 1933, Studies on the survival time of the bovine tubercle bacillus in soil, soil and dung, in dung and on grass, with experiments on the preliminary treatment of infected organic matter and the cultivation of the organism. Journal of Hygiene 33, 103-117.

Maddock, E.C.G., 1934, Further studies on the survival time of the bovine tubercle bacillus in soil, soil and dung, in dung and on grass, with experiments on feeding guinea-pigs and calves on grass artificially infected with bovine tubercle bacilli. Journal of Hygiene 34, 372-379.

Maddock, E.C.G., 1936, Experiments on the infectivity for healthy calves on bovine tubercle bacilli discharged in dung upon pasture. Part 1. From tubercular calves fed with emulsion of tubercle bacilli 1934-5. Part 2. From tubercular cows passing tubercle bacilli in their dung 1935-6. Journal of Hygiene 36, 594-601.

Manterola, J.M., Thornton, C.G., Padilla, E., Lonca, J., Corea, I., Martinez, E., Ausina, V., 2003, Comparison of the sodium dodecyl sulfate-sodium hydroxide specimen processing method with the C18-carboxypropylbetaine specimen processing method using the MB/BacT liquid culture system. Eur J Clin Microbiol Infect Dis 22, 35-42.

McCorry, T., Whelan, A.O., Welsh, M.D., McNair, J., Walton, E., Bryson, D.G., Hewinson, R.G., Vordermeier, H.M., Pollock, J.M., 2005, Shedding of Mycobacterium bovis in the nasal mucus of cattle infected experimentally with tuberculosis by the intranasal and intratracheal routes. Vet Rec 157, 613-618.

Menzies, F.D., Neill, S.D., 2000, Cattle-to-cattle transmission of bovine tuberculosis.[comment]. Veterinary Journal 160, 92-106.

Michigan, State.of. 2005. Chronology of bovine TB in Michigan since 1975.

Miller, R., Kaneene, J.B., Fitzgerald, S.D., Schmitt, S.M., 2003, Evaluation of the influence of supplemental feeding of white-tailed deer (Odocoileus virginianus) on the prevalence of bovine tuberculosis in the Michigan wild deer population. Journal of Wildlife Diseases 39, 84-95.

Mitscherlich, E., Marth, E.H., 1984, *Mycobacterium bovis*, In: Microbial survival in the environment: bacteria and rickettsiae important in human and animal health Springer-Verlag, Berlin ; New York, pp. 235-242.

Morris, R.S., Pfeiffer, D.U., Jackson, R., 1994, The epidemiology of Mycobacterium bovis infections. Vet Microbiol 40, 153-177.

Neill, S.D., Hanna, J., O'Brien, J.J., McCracken, R.M., 1988, Excretion of Mycobacterium bovis by experimentally infected cattle. Vet Rec 123, 340-343.

Neill, S.D., Pollock, J.M., Bryson, D.B., Hanna, J., 1994, Pathogenesis of Mycobacterium bovis infection in cattle. Vet Microbiol 40, 41-52.

Norby, B., Bartlett, P.C., Fitzgerald, S.D., Granger, L.M., Bruning-Fann, C.S., Whipple, D.L., Payeur, J.B., 2004, The sensitivity of gross necropsy, caudal fold and comparative cervical tests for the diagnosis of bovine tuberculosis. J Vet Diagn Invest 16, 126-131.

Norby, B., Bartlett, P.C., Grooms, D.L., Kaneene, J.B., Bruning-Fann, C.S., 2005, Use of simulation modeling to estimate herd-level sensitivity, specificity, and predictive values of diagnostic tests for detection of tuberculosis in cattle. Am J Vet Res 66, 1285-1291.

O'Brien, D.J., Fitzgerald, S.D., Lyon, T.J., Butler, K.L., Fierke, J.S., Clarke, K.R., Schmitt, S.M., Cooley, T.M., Derry, D.E., 2001, Tuberculous lesions in free-ranging white-tailed deer in Michigan. J Wildl Dis 37, 608-613.

O'Brien, D.J., Schmitt, S.M., Fierke, J.S., Hogle, S.A., Winterstein, S.R., Cooley, T.M., Moritz, W.E., Diegel, K.L., Fitzgerald, S.D., Berry, D.E., Kaneene, J.B., 2002, Epidemiology of Mycobacterium bovis in free-ranging white-tailed deer, Michigan, USA, 1995-2000. Prev Vet Med 54, 47-63.

O'Brien, D.J., Schmitt, S.M., Fitzgerald, S.D., Berry, D.E., Hickling, G.J., 2006, Managing the wildlife reservoir of Mycobacterium bovis: The Michigan, USA, experience. Vet Microbiol 112, 313-323.

O'Reilly, L.M., Daborn, C.J., 1995, The epidemiology of Mycobacterium bovis infections in animals and man: a review. Tuber Lung Dis 76 Suppl 1, 1-46.

Ozbek, A., Michel, F.C., Strother, M., Motiwala, A.S., Byrum, B.R., Shulaw, W.P., Thornton, C.G., Sreevatsan, S., 2003, Evaluation of two recovery methods for detection of Mycobacterium avium subsp. paratuberculosis by PCR: direct-dilution--centrifugation and C(18)-carboxypropylbetaine processing. FEMS Microbiol Lett 229, 145-151.

Padilla, E., Manterola, J.M., Gonzalez, V., Thornton, C.G., Quesada, M.D., Sanchez, M.D., Perez, M., Ausina, V., 2005, Comparison of the sodium hydroxide specimen processing method with the C18-carboxypropylbetaine specimen processing method using independent specimens with auramine smear, the MB/BacT liquid culture system, and the COBAS AMPLICOR MTB test. J Clin Microbiol 43, 6091-6097.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2002a, Lesion development in white-tailed deer (Odocoileus virginianus) experimentally infected with Mycobacterium bovis. Vet Pathol 39, 334-340.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2002b, Milk containing Mycobacterium bovis as a source of infection for white-tailed deer fawns (Odocoileus virginianus). Tuberculosis (Edinb) 82, 161-165.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2002c, Susceptibility of raccoons (Procyon lotor) to infection with Mycobacterium bovis. J Wildl Dis 38, 266-274.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2003, Aerosol exposure of white-tailed deer (Odocoileus virginianus) to Mycobacterium bovis. J Wildl Dis 39, 817-823.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2004a, Investigation of the transmission of Mycobacterium bovis from deer to cattle through indirect contact. Am J Vet Res 65, 1483-1489.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2004b, Shared feed as a means of deer-to-deer transmission of Mycobacterium bovis. J Wildl Dis 40, 87-91.

Palmer, M.V., Whipple, D.L., 2000. Transmission of *Mycobacterium bovis* from experimentally infected white-tailed deer to cattle through indirect contact. In: Proceedings of the Bovine Tuberculosis Conference, Lansing, Michigan, pp. 15-16.

Palmer, M.V., Whipple, D.L., Butler, K.L., Fitzgerald, S.D., Bruning-Fann, C.S., Schmitt, S.M., 2002d, Tonsillar lesions in white-tailed deer (Odocoileus virginianus) naturally infected with Mycobacterium bovis. Vet Rec 151, 149-150.

Palmer, M.V., Whipple, D.L., Olsen, S.C., 1999, Development of a model of natural infection with Mycobacterium bovis in white-tailed deer. J Wildl Dis 35, 450-457.

Palmer, M.V., Whipple, D.L., Payeur, J.B., Alt, D.P., Esch, K.J., Bruning-Fann, C.S., Kaneene, J.B., 2000, Naturally occurring tuberculosis in white-tailed deer. J Am Vet Med Assoc 216, 1921-1924.

Palmer, M.V., Whipple, D.L., Waters, W.R., 2001, Experimental deer-to-deer transmission of Mycobacterium bovis. Am J Vet Res 62, 692-696.

Payeur, J., Gray, M., Osorio, R. 2001. Procedure for processing tissue samples for mycobacteria isoloation (Ames, Iowa, National Veterinary Services Laboratories, Animal Plant Health Inspection Service, United States Department of Agriculture), pp. 1-10.

Pillai, S.D., Widmer, K.W., Ivey, L.J., Coker, K.C., Newman, E., Lingsweiler, S., Baca, D., Kelley, M., Davis, D.S., Silvy, N.J., Adams, L.G., 2000, Failure to identify non-bovine reservoirs of Mycobacterium bovis in a region with a history of infected dairy-cattle herds. Preventive Veterinary Medicine 43, 53-62.

Schmitt, S.M., Fitzgerald, S.D., Cooley, T.M., Bruning-Fann, C.S., Sullivan, L., Berry, D., Carlson, T., Minnis, R.B., Payeur, J.B., Sikarskie, J., 1997, Bovine tuberculosis in free-ranging white-tailed deer from Michigan. J Wildl Dis 33, 749-758.

Schmitt, S.M., O'Brien, D.J., Bruning-Fann, C.S., Fitzgerald, S.D., 2002, Bovine tuberculosis in Michigan wildlife and livestock. Ann N Y Acad Sci 969, 262-268.

Smith, B.L., 1972, Tuberculosis in the opossum. New Zealand Veterinary Journal 20, 199.

Tanner, M., Michel, A.L., 1999, Investigation of the viability of M. bovis under different environmental conditions in the Kruger National Park. Onderstepoort Journal of Veterinary Research 66, 185-190.

Technology, Integrated Research. Methods for the Use of the CB-18 TB Culture Kit with Lytic Decon II for Processing Specimens for the Detection of Mycobacteria by Culture, pp. 1-13.

Thornton, C.G., Cranfield, M.R., MacLellan, K.M., Brink, T.L., Jr., Strandberg, J.D., Carlin, E.A., Torrelles, J.B., Maslow, J.N., Hasson, J.L., Heyl, D.M., Sarro, S.J., Chatterjee, D., Passen, S., 1999, Processing postmortem specimens with C18-carboxypropylbetaine and analysis by PCR to develop an antemortem test for Mycobacterium avium infections in ducks. J Zoo Wildl Med 30, 11-24.

Thornton, C.G., MacLellan, K.M., Brink, T.L., Jr., Lockwood, D.E., Romagnoli, M., Turner, J., Merz, W.G., Schwalbe, R.S., Moody, M., Lue, Y., Passen, S., 1998a, Novel method for processing respiratory specimens for detection of mycobacteria by using C18-carboxypropylbetaine: blinded study. J Clin Microbiol 36, 1996-2003.

Thornton, C.G., MacLellan, K.M., Brink, T.L., Jr., Passen, S., 1998b, In vitro comparison of NALC-NaOH, tween 80, and C18-carboxypropylbetaine for processing of specimens for recovery of mycobacteria. J Clin Microbiol 36, 3558-3566.

Thornton, C.G., MacLellan, K.M., Brink, T.L., Jr., Wolfe, D.M., Llorin, O.J., Passen, S., 1998c, Processing respiratory specimens with C18-carboxypropylbetaine: development of a sediment resuspension buffer that contains lytic enzymes to reduce the contamination rate and lecithin to alleviate toxicity. J Clin Microbiol 36, 2004-2013.

USDA, Veterinary Services 2004. Bovine Tuberculosis Eradication: Uniform Methods and Rules Effective January 1, 2005 (Washington, DC, United States Department of Agriculture), pp. 1-27.

Van Donsel, D.J., Larkin, E.P., 1977, Persistence of *Mycobacterium bovis* BCG in Soil and on Vegetables Spray-Irrigated with Sewage Effluent and Sludge. Journal of Food Protection 40, 160-163.

Whipple, D.L., Bolin, C.A., Miller, J.M., 1996, Distribution of lesions in cattle infected with Mycobacterium bovis. J Vet Diagn Invest 8, 351-354.

Whipple, D.L., J.L., J., Payeur, J.B., 1999a. DNA fingerprinting of *Mycobacterium bovis* isolates from animals in northeast Michigan. In: Proceedings of the International Symposium of the World Association of Veterinary Laboratory Diagnosticians, p. 83.

Whipple, D.L., J.L., J., Payeur, J.B., 1999b. DNA fingerprinting of *Mycobacterium bovis* isolates from animals in northeast Michigan. In: Proceedings of the IX International Symposium of the World Association of Veterinary Laboratory Diagnosticians, World Association of Veterinary Laboratory Diagnosticians, College Station, Texas, p. 83.

Whipple, D.L., Jarnagin, J.L., Payeur, J.B., 1999c. DNA fingerprinting of *Mycobacterium bovis* isolates from animals in northeast Michigan. In: Proceedings of the IX International Symposium of the World Association of Veterinary Laboratory Diagnosticians, World Association of Veterinary Laboratory Diagnosticians, College Station, Texas, p. 83.

Whipple, D.L., Palmer, M.V., 2000. Survival of *Mycobacterium bovis* on feeds. In: Proceedings of the Bovine Tuberculosis Conference, Lansing, Michigan, p. 17.

Wilkins, M.J., Bartlett, P.C., Frawley, B., O'Brien, D.J., Miller, C.E., Boulton, M.L., 2003, Mycobacterium bovis (bovine TB) exposure as a recreational risk for hunters: results of a Michigan Hunter Survey, 2001. Int J Tuberc Lung Dis 7, 1001-1009.

Williams, S.R., Hoy, W.A., 1930, The viability of *B. tuberculosis* (*bovinus*) on pasture land, in stored faeces and in liquid manure. Journal of Hygiene 30, 413-419.

Wray, C., 1975, Survival and spread of pathogenic bacteria of veterinary importance within the environment. Veterinary Bulletin 45, 543-550.

Yajko, D.M., Wagner, C., Tevere, V.J., Kocagoz, T., Hadley, W.K., Chambers, H.F., 1995, Quantitative culture of Mycobacterium tuberculosis from clinical sputum specimens and dilution endpoint of its detection by the Amplicor PCR assay. J Clin Microbiol 33, 1944-1947.

Young, J.S., Gormley, E., Wellington, E.M., 2005, Molecular detection of Mycobacterium bovis and Mycobacterium bovis BCG (Pasteur) in soil. Appl Environ Microbiol 71, 1946-1952.