EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MICHIGAN AND A NOVEL STRATEGY FOR TARGETED SURVEILLANCE

By

Chika Chukwunonso Okafor

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Large Animal Clinical Sciences

2011

ABSTRACT

EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MICHIGAN AND A NOVEL STRATEGY FOR TARGETED SURVEILLANCE

By

Chika Chukwunonso Okafor

Despite ongoing eradication efforts, bovine tuberculosis (bTB) remains a challenge in Michigan livestock and wildlife. The overall objective of the studies described in this dissertation was to identify ways of improving eradication of bTB in Michigan and beyond. To first understand bTB in Michigan, a descriptive epidemiological study of livestock and wildlife was conducted using retrospective data. An important conclusion was that bTB has been contained to a focal area of Michigan; however eradication may require exploration of new surveillance strategies or diagnostic tests. To further understand current bTB surveillance strategies being used in Michigan and elsewhere, a review of the current literature was undertaken. A focus of the review was to identify gaps that should be addressed. An important conclusion from the review was that bTB screening at points-of-concentration (POC) of cattle is lacking and its adaptation could aid in the identification of high risk herds, thereby furthering bTB eradication efforts. Based on this, a study was conducted to explore the adaptation of the whole blood interferon-gamma (IFN-γ) assay for use at slaughter facilities, a POC of cattle. The goal of the study was to determine if blood obtained at exsanguination of cattle, experimentally sensitized with inactivated *Mycobacterium bovis* (*M. bovis*), can produce measureable IFN-γ to bTB. Results indicated that most sensitized cattle would retain their positive bTB IFN-γ assay result at exsanguination, despite a decline in IFN-γ production at exsanguination. To further

understand the decline in IFN-γ production, two trials were performed to evaluate the time of blood collection and total lymphocyte counts as potential factors affecting measurable IFN-γ production at slaughter. Results in both trials demonstrated that sensitized cattle were more likely to retain their positive bTB IFN-γ assay result at commencement of exsanguination than at successive time points. Therefore, it was concluded that time of blood collection is crucial to obtaining valid bTB IFN-γ assay result at exsanguination. A decline in IFN-γ production at exsanguination was associated with a decline in total lymphocyte counts, suggesting that reduced lymphocyte counts could be a responsible factor in the declined IFN-γ production. Therefore, to avoid false negative results, lymphocyte numbers should be standardized before antigen stimulation in blood collected at exsanguination or different IFN-γ assay classification criteria developed for such samples. Based on the results of the sensitization studies, cattle under field conditions were evaluated to determine if valid IFN-γ assay results can be obtained from blood collected at exsanguination. Cattle in this field study originated from herds belonging to one of three risk categories: bTB infected herds, a bTB exposed herd, and herds with no known history of bTB exposure. Cattle with and without bTB-like gross lesions were identified as bTB IFN-γ assay positive using blood collected at exsanguination and later one of the cattle was confirmed bTB positive by bacterial culture. All the IFN-γ assay positive cattle originated from either the infected or the exposed herds but none from the non-exposed herds. Therefore, blood collected at exsanguination could be used to identify bTB exposed herds or to monitor bTB status in bTB-free zones, providing an additional tool for the control and eradication of bTB. In conclusion, the entire project identified areas of prime focus in the Michigan bTB challenge and has provided evidence supporting the potential use of the bTB IFN-γ assay as a novel surveillance strategy that, if integrated at POC of cattle, could advance bTB control and eradication.

Copyright by
Chika Chukwunonso Okafor
2011

DEDICATION

To my wife, partner, and friend, Chisa and to our sons, Zika and Zife
Your prayers, support, encouragement, and sacrifice
Made this journey possible
I love you and I owe this work to you.

ACKNOWLEDGEMENTS

Special thanks to

- God for the Christ in me, through Christ's strength I did all things.
- My major advisor, Dr. Daniel Grooms, for the mentorship, guidance, and assistance in completing the project. I appreciate your patience and encouragement.
- Dr. John Kaneene, for the mentorship in Epidemiology and great advice.
- Dr. Steven Bolin, for the mentorship on bTB interferon gamma diagnostics.
- Dr. Joseph Gardiner, for the mentorship on biostatics.
- Dr. James Averill, for the mentorship in bTB surveillance.
- Dr. Colleen Bruning-Fann, Dr. Jean Ray, Dr. Greg Habing and Lee Anne Kroll, Tara Gravelyn, Rhyannon Moore, and Maria Lane for assistance with data collection.
- MSU Animal Science Meat laboratory and West Michigan Beef slaughter house in Grand Rapids Michigan, for permitting me to carry out this study within their facilities.
- The United States Department of Agriculture National Institute of Food and Agriculture Special TB Grant (agreement #: 2009-34427-19959) and the Edward and Roberta Sterner Fund for Bovine Health for funding this study.
- MSU Graduate School Dissertation Fellowship for funding the writing of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

A	IFN-γ response to stimulation with *M. avium* PPD
bTB	bovine tuberculosis
B	IFN-γ response to stimulation with *M. bovis* PPD
C	background IFN-γ response (unstimulated)
CCT	comparative cervical tuberculin test
CFT	caudal fold tuberculin test
DCPAH	Diagnostic Center for Population and Animal Health
ELISA	enzyme-linked immunosorbent assay
IFN-γ	interferon-gamma
M. avium	*Mycobacterium avium*
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MAPIA	multi-antigen printed immunoassay
M. bovis	*Mycobacterium bovis*
MSU	Michigan State University
NVSL	National Veterinary Services Laboratories
OD450 nm	Optical density measured at 450 nanometers
P	IFN-γ response to stimulation with Pokeweed mitogen
PPD	purified protein derivative
PBS	Phosphate buffered saline
POC	Points of concentration
Rcf	Relative centrifugal force
Se	sensitivity

Sp	specificity
UK	United Kingdom
USA	United States of America

INTRODUCTION

Problem statement

Bovine tuberculosis (bTB) is of significant economic importance due to livestock trade barriers, resources expended on bTB surveillance tests, payment of indemnities for the depopulation of bTB herds, and other measures of bTB eradication (Michel *et al.* 2010; Okafor *et al.* 2011b). The causative agent of bTB, *Mycobacterium bovis (M. bovis),* is a significant zoonotic pathogen and is one of the most important public health issues globally (de Kantor *et al.* 2010; Lobue *et al.* 2010; Michel *et al.* 2010; Thoen *et al.* 2009; Thoen *et al.* 2010). Although bTB has been eradicated from the majority of U.S. states, infected herds continue to be reported, and states may periodically lose their disease-free status. In Michigan, after fifteen years of implementing the Michigan Bovine Tuberculosis Eradication Project, bTB remains a challenge in Michigan livestock and wildlife. At least one bTB infected cattle herd has been identified in Michigan annually since 1998. Because of this ongoing bTB challenge, regulatory requirements for cattle movement have affected the cattle trade in Michigan. In addition, the state has spent approximately US$200 million on bTB eradication between 1994 and 2010 (Bridget Patrick 2010). Annually, over US$7 million is spent on bTB surveillance in cattle alone. Additional resources are spent on indemnity payment, cleaning and disinfection of the premises of bTB infected cattle herds, wildlife surveillance, and implementation of other eradication strategies. Therefore, it has become important to adapt strategies that could help in the eradication of bTB in a timely manner.

Overview of this dissertation

To correctly identify and address the challenges of bTB in Michigan, the studies presented in this dissertation were sequentially conducted. There are 5 studies presented in 5 chapters of this dissertation. The first study helped in the identification of the core areas of bTB challenges that need improvement and subsequent studies were conducted to address the identified challenge. Individually, the studies are complete manuscripts with abstract, introduction, materials and methods, result, discussion and conclusion. Collectively, the studies address a common challenge.

The objectives of the studies presented in this dissertation were to:

1. Describe the epidemiology of bTB in Michigan cattle, privately owned cervids, and wildlife between 1975 and 2010 and identify important lessons learned (Chapter 1).
2. Review the surveillance strategies for diagnosis of bTB in Michigan cattle, identify current gaps and recommend future prospects (Chapter 2).
3. Determine if interferon-gamma (IFN-γ) to bTB is measurable in blood collected during exsanguination of cattle experimentally sensitized with killed *Mycobacterium bovis* (Chapter 3).
4. Determine if time of blood collection and total lymphocyte count influence measurable reduction in IFN-γ production during slaughter of cattle (Chapter 4).
5. Determine if blood collected at exsanguination of cattle at slaughter, under field conditions, is practical in identifying cattle as IFN-γ positive for bTB (Chapter 5).

Presented at the end of this dissertation are a general discussion, closing thoughts, and directions for future research.

CHAPTER 1

DESCRIPTIVE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MICHIGAN (1975 – 2010): LESSONS LEARNED

ABSTRACT

Despite ongoing eradication efforts, bovine tuberculosis (bTB) remains a challenge in Michigan livestock and wildlife. The objectives of this study were to: 1) review the epidemiology of bTB in Michigan cattle, privately owned cervids, and wildlife between 1975 and 2010, and 2) identify important lessons learned from the review and eradication strategies. bTB information was accessed from the Michigan bTB Eradication Project agencies. Cattle herds (49), privately owned deer herds (4), and wild white-tailed deer (668) were found infected with bTB during the review period. bTB has occurred primarily in counties located at the northern portion of the state's Lower Peninsula. Currently used bTB eradication strategies have successfully controlled bTB spread. However additional changes in bTB surveillance, prevention, and eradication strategies could improve eradication efforts.

INTRODUCTION

After fifteen years of implementing the Michigan Bovine Tuberculosis Eradication Project, bovine tuberculosis (bTB) remains a challenge in Michigan livestock and wildlife. At least one bTB infected cattle herd has been identified in Michigan annually since 1998. Because of this ongoing bTB challenge, regulatory requirements for cattle movements have affected cattle trade in Michigan. In addition, the state has spent approximately US$200 million on bTB eradication between 1994 and 2010 (Bridget Patrick 2010). Annually, over US$7 million is spent on bTB surveillance in cattle alone. Additional resources are spent on indemnity payment, cleaning and disinfection of the premises of bTB infected cattle herds, wildlife surveillance, and implementation of other eradication strategies. Reviewing the epidemiology of the current bTB issues in Michigan could help advance bTB eradication strategies in Michigan, other regions of the country, or beyond.

Bovine TB is a chronic disease caused by *Mycobacterium bovis*. *M. bovis* is primarily a pathogen of cattle but can also infect other mammals including humans. Among domestic animals, cattle are the primary reservoir. However, other animal species including deer, monkeys, European badgers, brush tailed opossums, and elephants have become endemically infected, thus serving as reservoirs for pathogen transmission (Oreilly and Daborn 1995).

Bovine TB is mainly a respiratory disease and is transmitted primarily through aerosols (O'Brien *et al.* 2001; Oreilly and Daborn 1995). However, indirect transmission through ingestion of contaminated food items has been demonstrated in deer and cattle and is believed to be a major source of transmission in the current bTB outbreak in Michigan (Palmer *et al.* 2001; Palmer *et al.* 2002; Palmer *et al.* 2004; Palmer and Whipple 2006). Although wildlife and

domestic cattle commonly do not come in close physical contact with each other, transmission of *M. bovis* between domestic animals and wildlife has occurred over the years (Palmer 2007). Domestic animals as well as wildlife are significant reservoir hosts for human tuberculosis, caused by *M. bovis*. The most common means humans acquire *M. bovis* is via consumption of unpasteurized or insufficiently-cooked animal products from bTB animal (Grange 2001).

At the end of the twentieth century, tuberculosis was the leading killer of humans in the United States (US). During this time period, *M. bovis* was found to be distinct from *M. tuberculosis* and there was evidence that *M. bovis* could be passed between animals and humans, and that in humans, *M. bovis* produce symptoms that were clinically indistinguishable from *M. tuberculosis* (Olmstead and Rhode 2004). Due to the public health and economic relevance of bTB, the US bTB Eradication Program began in 1917. The program included comprehensive testing of imported and US bred cattle, improved animal tracking, destroying skin test positive animals ("reactors"), strengthening meat inspection for tuberculosis lesions, and commercialization of milk pasteurization. This program proved highly effective in controlling the disease and by the 1960's, the number of bTB-reactor cattle detected in the US had markedly declined (Olmstead and Rhode 2004).

In 1974, the last known bTB infected cattle herd in Michigan was depopulated; however a bTB positive wild white-tailed deer was harvested by a hunter in the following year (Schmitt *et al.* 1997). It was widely believed that the deer acquired the *M. bovis* infection as a spill-over from livestock. With no further identified cases of bTB in cattle, the state acquired bTB 'accredited free' status in 1979. At that time the extent of bTB in Michigan wildlife was unknown. A second bTB positive wild white-tailed deer was identified in 1994, nine miles from the location of the index case (Schmitt *et al.* 1997). With this occurrence, the Michigan Bovine

TB Eradication Project began in 1995. The project was charged with increasing bTB surveillance in wildlife as well as in cattle and privately owned cervid (captive or farmed deer) herds surrounding any identified bTB wild white-tailed deer. The project involves a multi-agency team of experts from the Michigan Department of Agriculture (MDA), the US Department of Agriculture Animal and Plant Health Inspection Services (USDA APHIS), Michigan Department of Natural Resources (MDNR), Michigan State University (MSU), and Michigan Department of Community Health (MDCH). Wildlife surveys conducted in the spring and fall of 1995 detected further cases among wild white-tailed deer. In 1998, cases of bTB infection began to re-emerge among cattle herds in the state. As a consequence, Michigan lost its bTB 'accredited-free' status in June 2000 and dropped to a 'modified accredited' status (Hickling 2002). Despite active eradication efforts, cases of bTB are still found in Michigan cattle, privately owned cervids, and wild white-tailed deer.

The objective of this study was to conduct a comprehensive descriptive epidemiological review of bTB in cattle, privately owned cervids, and wild white-tailed deer from 1975 to July 2010. Although previous epidemiological studies on bTB in Michigan have evaluated bTB challenge in cattle (Kaneene *et al.* 2002b), privately owned cervid herds (Kaneene *et al.* 2002c), wildlife (Bruning-Fann *et al.* 1998; Bruning-Fann *et al.* 2001; Kaneene *et al.* 2002a; O'Brien *et al.* 2006; O'Brien *et al.* 2002a; O'Brien *et al.* 2008; O'Brien *et al.* 2004), or both livestock and wildlife (Schmitt *et al.* 2002), none has provided extensive descriptive account of bTB challenge in both cattle and wildlife alongside with the lessons learned from over 15 years of eradication efforts. By reviewing and understanding the epidemiology of the current disease problem combined with a review of the strategies that have been implemented to eradicate the disease, important "lessons learned" can be identified, and new control strategies may emerge.

MATERIALS AND METHODS

Sources of data

Descriptive data on bTB in Michigan between 1995 and July 2010 were obtained from the partners of the Michigan bTB Eradication Project. Data on bTB infected cattle and privately owned cervid herds were obtained from MDA and USDA APHIS Veterinary Services (USDA APHIS VS). Data on bTB infected wild white-tailed deer and other wildlife was obtained from Michigan Department of Natural Resources (MDNR) and USDA APHIS Wildlife Services (USDA APHIS WS).

Type of data

The collected data comprised of host characteristics, geographical, and temporal distributions of the bTB infected animals/herds. The host characteristics included type of herd operations (cattle), type of deer, origin of the infected animals into the herds, and herd size. The geographical distribution was limited to the county level to protect confidentiality of herd owners. Annual records of bTB infection in animals/herds were used for temporal distribution. Additional information collected were the type of surveillance used to identify each bTB infected animal/herd, the type of bTB eradication used in each infected herd, the results of the epidemiological evaluation of the infected herds, and the various bTB eradication strategies/policies utilized by the Michigan bTB Eradication Project partners.

Data analyses

The occurrence of bTB in cattle herds, privately owned cervids, and wild white-tailed deer were expressed either as incidence count, percentage proportion, incidence rate, herd prevalence, sample prevalence, or prevalence odds. Incidence count represented the total number of bTB infected herds within the review period. Incidence rate was calculated as incidence count divided by the total population per year. In cattle herds, 12.5 years was used for the review time period (1998-July 2010). Captive cervid herd bTB prevalence was calculated as the number of bTB positive deer divided by the total number of deer in the herd. A bTB positive deer was classified as any deer that had gross lesions consistent with bTB and that tested positive for *M. bovis* on culture. Sample prevalence was calculated as the number of bTB positive wild white-tailed deer divided by the total number wild white-tailed deer tested. Prevalence odds of bTB infected wild white-tailed deer in an area were calculated as the probability that tested wild deer in the area were bTB positive [sample prevalence (p)] divided by the probability that the tested wild deer were not bTB positive (1-p).

RESULTS

Area description

The state of Michigan is located in the Upper Midwest region of the US. The state is made up of 83 counties and comprises two peninsulas: the Upper Peninsula (UP) and the Lower Peninsula (LP) (Figure 1.1). Michigan covers approximately 37 million acres. There are approximately 43,000 miles of rivers and streams, 11,000 inland lakes, and over 4,500 miles of shoreline along the Great Lakes. A variety of forest, wetland, and grasslands provides habitat to over 15,000 native species of insects, 1,815 native species of vascular plants, and 691 native species of animals. Among animal species, 68 different native wild mammals have been identified including white-tailed deer (*Odocoileus virginianus*), elk (C*ervus elaphus nelson*), black bear (*Ursus americanus*), coyotes (*Canis latrans*), opossum (*Didelphis virginiana*), bobcats (*Lynx rufus***)** and red fox (*Vulpes vulpes fulva*) (Kaneene *et al.* 2002c).

Livestock production is a significant part of the state's economy. Cattle are the most common livestock in the state, and include dairy, cow-calf (beef), and feedlot operations. The 2007 agricultural census reported approximately 14,500 cattle herds in Michigan (USDA Census of Agriculture 2007). The cattle herd types (dairy, beef, mixed, and feedlot) and the total number of herds in bTB affected counties is reported in Table 1.1. Other common domesticate livestock species include small ruminants such as sheep and goats, horses, swine, and poultry (USDA Census of Agriculture 2007).

Cattle

bTB surveillance in cattle

Surveillance for bTB is primarily done through live animal skin testing and through tuberculosis lesion detection at slaughter facilities (MDNR 2010). Common reasons for live animal testing include herd accreditation/reaccreditation, compliance with pasteurized milk ordinance (PMO) laws, herd surveillance in endemic areas of the state as required by Memorandums of Understanding with USDA ASPHIS VS, and tracing of any animal identified to have bTB-like lesions during slaughter inspection.

During herd surveillance, the caudal fold tuberculin (CFT) test is done on individual animals as a primary screening test (MDNR 2010). All respondents ("suspects") to the CFT and herd of origin are quarantined pending final classification as to whether any suspect animal is bTB infected or not. The suspects are subjected to a supplemental test, either the comparative cervical tuberculin (CCT) test or interferon-gamma (IFN-γ) assay. An animal classified as CCT or IFN-γ positive responder is designated as either a "reactor" or remains a "suspect". A "suspect" animal is retested with the IFN-γ assay within 30 days or the CCT after 60 days and if the animal is not "negative", it is automatically designated a "reactor". Reactors are purchased for diagnostic purposes, humanely euthanized, and necropsied by a veterinary pathologist (MDNR 2010).

At necropsy, the animal is visually inspected for possible bTB lesions. Samples are taken from lymph nodes of the head, thorax, and abdomen, as well as from all visible bTB-like lesions and submitted for further testing. Histopathological screening, acid-fast staining of tissues, polymerase chain reaction (PCR), and bacterial culture are tests that are routinely conducted on these samples. Either PCR or bacterial culture is the confirmatory test in bTB screening. If bTB

is confirmed in an animal, its herd of origin is declared bTB infected and will remain under quarantine; otherwise the herd is released from quarantine (MDNR 2010).

bTB eradication in cattle

In bTB infected herds, there are two disease eradication options for the herd. One is complete herd depopulation. The other is to develop a whole herd testing and removal plan with the cooperation of the owner and governmental agencies while the herd remains under quarantine (test and remove program). This plan includes the serial performance of bTB ante mortem screening tests over time and the subsequent removal of all test positive animals as outlined in the Bovine Tuberculosis Eradication: Uniform Methods and Rules (USDA APHIS ; USDA APHIS). The herd is released from quarantine when testing reveals a bTB negative herd after a minimum of 8 whole herd tests over approximately a 4 year period as outlined in the 1999 Uniform Methods and Rules (USDA APHIS). However, USDA APHIS VS typically did not utilize a test and remove program as outlined in the 1999 Uniform Methods and Rules until Michigan found an infected dairy herd in 2000. Most infected dairy herds prefer this program to depopulation as it allows for continuation of operations and cash flow. However, depopulation remains the disease eradication of choice in most infected beef herds.

bTB infected cattle herds

Bovine TB in cattle herds re-emerged in Michigan in 1998 after the last known case of bTB in the state was depopulated in 1974. Comprehensive details of` this bTB incident and key policy changes are detailed in Table 1.2. Between 1998 and July 2010, the USDA APHIS VS recorded a total of 49 bTB infected cattle herds in Michigan. At least one bTB cattle herd has

been identified yearly since 1998 with higher numbers of herds found during the period from 2000 through 2003, and the highest peak in 2001 with 8 bTB infected herds discovered (Figure 1.2). Since 2004, the number of infected herds has fluctuated between 1-4 herds/year. The average numbers of cattle per bTB infected beef and dairy herd were 84 and 147 respectively. The average size of bTB infected herds was larger than the median Michigan herd size of 14 and 130 cattle per beef and dairy herd respectively (USDA Census of Agriculture 2007). The number of cattle in bTB infected herds ranged from 6 to 495 (Figure 1.3). Included in these 49 infected herds are six premises which were removed from quarantine and then discovered to be re-infected at a later date. These re-infected herds either had completed the test and remove program successfully (n=1) or were depopulated (n=5).

Geographically, bTB in cattle has only been found in 7 counties located in the Michigan's LP. The number of bTB infected herds within the review period is represented in Figure 1.1. Within each county, the number of bTB infected herds based on management operations is presented in Figure 1.4. The 6 re-infected herds comprised 1 dairy herd and 5 beef herds. The re-infected dairy herd was located in Montmorency County, while 3 beef herds were located in Alpena County, and 2 beef herds in Alcona County. Eleven herds had a previously bTB infected herd located within a 3 mile radius: 6 were in Alcona county, 4 in Alpena, and 1 in Oscoda County. The bTB incidence rate per 1000 cattle herd-yrs ranged from 1.1 (Presque Isle) to 8.7 (Alcona) (Figure 1.5). The overall number of cattle herds in each county and those found infected with bTB is presented in Table 1.1.

Between 1995 and July 2010, the USDA and MDA have conducted approximately 35,000 whole herd tests implemented as a part of the Michigan Bovine TB Eradication Project. During these whole herd tests, 46 of the 49 bTB infected herds (94%) were identified. One herd

was identified through each of the following: movement testing, slaughter surveillance, and trace testing (investigation of herds which moved cattle to and from bTB infected herds).

Of the 49 bTB infected herds, 47 herds had a history of purchasing or moving cattle into their herd prior to being found bTB infected (open herds). The other two herds had no history of introducing new animals into the herd prior to being found bTB infected (closed herds). In regards to bTB infected animals, 147 cattle presented at necropsy with gross lesions compatible with bTB (mode = 1, median = 1, and range = 0-32), 149 cattle had histopathological lesions compatible with *M. bovis* infections (mode= 1, median = 1, and range = 0-30), 104 cattle were confirmed bTB positive using PCR (mode =1, median = 1, and range = 0-24), and 137 cattle were culture positive for *M. bovis* (mode = 1, median = 1, and range = 0-28). Out of 137 cattle confirmed through bacterial culture, 84% were raised on the farm, 7% were from unknown origin and 9% were moved onto the farms. From the moved animals, 2 were purchased from other states, one from Ohio and the other from Texas; all others were either intra state purchases or leasing/borrowing of bulls.

The 5 most heavily bTB infected herds (greater numbers of *M. bovis* culture positive cattle) were located in the following counties: Alpena (28 cattle), Emmet (27 cattle), Alpena (9 cattle), Montmorency (9 cattle), and Presque Isle (6 cattle). These herds were each located in different counties and epidemiological investigations indicated that wildlife exposure was the most likely source of bTB transmission into the herd. The likely explanation for the higher disease prevalence in these herds is differences in individual herd management. Management practices that lead to closer confinement of cattle or sharing of focused feed and water sources are more likely to lead to intra-herd transmission.

Cattle movement was believed to be the source bTB infection in the herd identified via trace testing. The source of bTB in the six infected herds located in Emmet (n = 3) and Antrim (n = 3) counties remains problematic. One herd brought no animals into the herd. Two herds brought in cattle from counties with a low prevalence of bTB in wild deer. The remaining three farms brought in cattle from counties with both high and low bTB prevalence in wild deer; however, on 1 farm the only bTB affected animal originated from a bTB free area. All cattle moving out of high prevalence areas were tested prior to movement for bTB with negative results. Regardless of location, all herds that were a source of livestock to bTB affected farms had whole herd tests with no additional bTB found. In addition, all cattle farms adjoining bTB affected farms were tested with no additional bTB affected herds found. Finally, there is a low sample prevalence of bTB in wild white-tailed deer in these two counties (Figure 1.5). In one area with high bTB prevalence in wild deer (Alcona County), five bTB infected herds had multiple possible routes of exposure. Each of the five herds shared fence-line contact with another bTB infected herd and moved animals between these herds, in addition to their location within an area with a high prevalence of bTB in wild white-tailed deer. The most probable source of bTB infection in each of these herds was not clear. Epidemiological reports on all other bTB affected herds (n = 37) suspected white-tailed deer to be the source of infection as all source farms and adjoining farms were tested for bTB with negative results. Wild white-tailed deer had access to all farms with bTB infected herds. All but one bTB infected cattle farm had attributes attractive to deer. These attributes included apple trees, accessible stored feed, water sources, and woodlands providing cover.

As a control measure, 43 of the bTB cattle herds (88%) were depopulated, from which 5 premises became re-infected with bTB. All animals repopulating these farms had a negative test

for bTB prior to entry onto the premises. Six (12%) herds entered a test-and-remove program, of which one dairy cattle herd became re-infected at two separate times. In this herd, the first re-infection occurred after the herd was removed from quarantine. This herd was again found infected on the final whole herd test of the test and remove program. The latest re-infection was not considered a separate infection because the herd was still quarantined at the time. Again, only bTB test negative animals were allowed onto these farms.

Privately owned cervids (captive or farmed deer)

The USDA AHPIS VS and the MDA identified 4 privately owned white-tailed deer herds infected with bTB between 1975 and July 2010. The first incidence was reported in Presque Isle County in 1997; all others were in Montmorency County in 2006, 2008, and 2009. The first (Palmer *et al.* 2000) and the second (O'Brien *et al.* 2009) infected herds were depopulated but due to inadequacy of indemnity funds, the disease control options were changed. Hence, the third, and the forth bTB infected privately owned white-tailed deer herds became hunting only operations, placed under long-term quarantine with no live animal movement off the operation.

The first infected herd had a herd prevalence of 5.3%. Out of the 262 deer in the herd, 9 had both gross and histopathological lesions compatible with bTB at depopulation while 14 deer were confirmed using bacterial culture. In the second infected herd, the herd prevalence was 1.2%. Out of the 330 deer in this herd, 9 and 5 deer had gross and histopathological bTB compatible lesions respectively, 1 tested positive by PCR and 4 tested positive by bacterial culture. There were 140 deer in the third infected herd at the time it was found infected. Beyond the one deer that made the herd positive, no other infected deer has been reported in this hunt-only herd. In the fourth (last) infected deer herd, out of the original 280 deer in the herd, 2 deer

were found with gross and histopathological bTB compatible lesions and later tested bTB positive by both PCR and bacterial culture. Subsequently, no further infected deer have been reported.

Wildlife

bTB surveillance in wild white-tailed deer

Starting in 1995, hunter-harvested, road-killed, and other dead wild white-tailed deer were tested for bTB infection. White-tailed deer have since been tested annually for bTB (MDNR 2010). Most bTB examinations occur during the fall deer hunting season. Hunters are requested to voluntarily turn in the heads of harvested wild white-tailed deer for bTB examination; in addition, carcasses bearing lesions considered suspicious by either hunters or the MDNR are collected (O'Brien *et al.* 2001). Hunter harvested deer accounted for 91% of all deer tested between 1975 and 2006 (O'Brien *et al.* 2001), and remains a significant source of bTB surveillance in wild white-tailed deer. The principal tissues examined are the parotid, mandibular, and medial and lateral retropharyngeal lymph nodes found in the head (Schmitt *et al.* 1997). Unlike in cattle, only lesioned tissue is subjected to mycobacterial culture.

bTB eradication in wild white-tailed deer

Reduction of both deer concentration and population has been the applied bTB eradication strategies in Michigan wild white-tailed deer. Restriction/ban of baiting and supplemental feeding in wild white-tailed deer was used to reduce deer concentration, while increased deer harvest was the approach aimed at reducing deer density (Table 1.2). These strategies were most intensively implemented in the area with the highest sample prevalence of

bTB in the wild deer [Deer Management Unit (DMU) 452]. This area contains portions of Alcona, Alpena, Montmorency, and Oscoda counties and has been the "core area" of bTB challenge in Michigan. Since 1995, there has been a 57% decline in bTB transmission rate among wild white-tailed deer located within DMU 452 (Schmitt 2010). The total number of statewide harvested wild white-tailed deer has increased annually from approximately 100,000 in 1975 to over 400,000 in 2009 (Frawley 2010). Consequently, since 1995 deer population has decreased over the years; in DMU 452, deer population dropped by 60,000 (40%) in 2009 (Schmitt 2010).

bTB infected wild white-tailed deer

Bovine TB in wild white-tailed deer was first reported in Michigan in 1975 with a second case in 1994 (Schmitt *et al.* 1997). Since then, more bTB cases has been found in white-tailed deer as well as other wildlife including elk, black bear, bobcat, coyote, opossum, raccoon, and red fox (Bruning-Fann *et al.* 1998; Bruning-Fann *et al.* 2001; Hickling 2002; O'Brien *et al.* 2006; O'Brien *et al.* 2002a; Schmitt *et al.* 1997; Schmitt *et al.* 2002). The increased identification of bTB in the wildlife, especially white-tailed deer, has led to numerous policy changes by the MDNR aimed at bTB eradication. Details of Michigan DNR policy changes have been reported (Hickling 2002; MDNR 2005) as have extensive treatment of policy implications (Conner *et al.* 2008; de Lisle *et al.* 2002; O'Brien *et al.* 2006). The key policy changes are presented in Table 1.2.

The total population of white-tailed deer tested between 1975 and the end of July 2010 and those infected with bTB is presented in Table 1.1. Out of 184,269 white-tailed deer tested, 668 were found infected with bTB. Among the bTB infected deer, 36% were from Alcona, 28%

from Alpena, 20% from Montmorency, 2% from Presque Isle, 11% from Oscoda and 3% came from all other counties. The county with the highest prevalence odds of finding bTB white-tailed deer was Alcona (0.0133); the prevalence odds in other counties were Alpena (0.01), Montmorency (0.0109), Oscoda (0.0077), Presque Isle (0.0014), Antrim (0.0002), Emmet (0.0006), and others (0.000 2) (Figure 1.5). Schmitt (Schmitt 2010) reported a higher annual sample prevalence of bTB in the DMU 452 than in the surrounding counties (Figure 1.6).

LESSONS LEARNED AND RECOMMENDATIONS

bTB remains an ongoing challenge

Despite ongoing control efforts, the continued identification of bTB in cattle and wildlife is strong evidence that bTB remains an ongoing challenge in Michigan. This is somewhat disheartening as significant resources have been expended to return Michigan to a bTB free status. However, with the strategies that have been implemented during the past 15 years, the disease appears to have been confined to a geographical region of the state as was observed by other studies (Hickling 2002; O'Brien *et al.* 2006). The initiation of statewide bTB testing of all cattle herds in 2000 could be responsible for the spike in the bTB incidence between 2000 and 2003, as this was the first time most of these herds were ever bTB tested. Since then, there has been a declining trend in incidence and sample prevalence of bTB in cattle and wildlife respectively; although recent evidence suggests that the downward trend has leveled off (Schmitt 2010). Earlier studies also observed a decline in sample bTB prevalence in Michigan's wild white-tailed deer (O'Brien *et al.* 2006; O'Brien *et al.* 2002a; Schmitt *et al.* 2002). These results suggest that progress has been made in the bTB eradication. However, it may be necessary to explore new and more aggressive control strategies in both cattle and wildlife that transcends political as well as social barriers, if complete eradication is to be accomplished.

Collaboration

Management of diseases that are transmissible between wildlife and domestic livestock can be a challenge and requires cooperation among their respective advocates in developing a reasonable and effective strategy that allows both to be maintained and prosper. Sharing of expertise is crucial in the eradication of a disease with many susceptible hosts as in bTB. All bTB cases in cattle and privately owned white-tailed deer herds as well as the majority of bTB infections in wildlife have been found in the northern portion of the Michigan's Lower Peninsula. The current containment of bTB to a geographical portion of Michigan is evidence of the successful collaborative efforts undertaken to eradicate bTB from Michigan. However, additional efforts and cooperation are needed to complete the eradication of bTB in Michigan. Increased cooperation between regulatory agencies, other stakeholders (for example hunters or local business owners), and livestock industry partners is needed. The development of a plan that is compatible with the long-term sustainability of both the livestock and wild life industries in Michigan should be targeted.

Surveillance

Whole herd testing of cattle farms has been crucial to identifying bTB infection, but this surveillance method is very expensive. Most bTB affected cattle herds were found through annual whole herd surveillance. The state annually spends millions of dollars towards bTB control and eradication. Between 1994 and 2010, the State of Michigan has spent approximately US$200 million on bTB eradication (Bridget Patrick 2010). Resources spent on whole herd testing of the livestock population contribute a significant part of the total expenses. Hidden costs rarely mentioned in the current bTB surveillance include such things such as injuries

among the livestock owners, veterinarians and technical staff that conduct bTB testing (Wilkins *et al.* 2009) and loss of production (e.g. temporary drop in milk production) that often occurs following restraint of cattle to administer and/or read ante mortem tests. Finally, there is an industry perception that the number of tests being done and the cost of surveillance in relation to the number of bTB herds found are excessive. This may lead to a decrease in long-term support of the current strategies from the cattle industry. Given these facts, there is a need for the exploration and subsequent adoption of less expensive, but just as reliable as surveillance methods such as targeted strategies using risk-based criteria. Examples where use of targeted risk-based criteria was successful for disease eradication/control include Bovine Spongiform Encephalopathy (BSE) program, Pseudo rabies in Pigs, and Brucellosis in cattle and swine. A reliable assay that could be used for bTB surveillance at points of cattle concentration, such as slaughter houses or livestock markets, would also be beneficial and could be used in conjunction with risk-based targeted strategies. Development of sophisticated targeted screening strategies would most likely produce a significant reduction in resource expenditure while at the same time maintaining the necessary rapid identification of bTB infected herds to eradicate the disease.

bTB transmission

The prevalence odds of bTB in wild white-tailed deer is highly correlated with the incidence rate of bTB in cattle herds ($r = 0.8$ and p value = 0.02). These prevalence odds estimates, calculated from sample prevalence, do not accurately represent the true odds of bTB infection in wild white-tailed deer. The impracticality of testing all wild deer and the imperfection of available screening tests makes the true prevalence of bTB in wild deer unknown in absolute numbers (O'Brien *et al.* 2002a; O'Brien *et al.* 2004) but a good approximation of the

extent to which the sample prevalence underestimates the true prevalence has been documented (O'Brien *et al.* 2002a; O'Brien *et al.* 2004). Since prevalence odds remain a measure of risk, this highly positive correlation result supports the theory of interspecies transmission. Furthermore, the majority of cattle herds infected with bTB shared common environmental and management features that are conducive to wild white-tailed deer-cattle interaction. These observations further support the claim that wildlife, and specifically wild white-tailed deer, are a reservoir of bTB infection for livestock in Michigan (Miller *et al.* 2003; Schmitt *et al.* 2002; Wilkins *et al.* 2008). Therefore, successfully mitigating such wildlife-cattle space interaction would be a great stride towards bTB eradication in Michigan.

With the advent of mandatory radio frequency identification (RFID) in Michigan and movement permits in the Northeast LP, it has become much easier to track cattle movement and rule in/out the possibility of cattle movement as a source of bTB transmission. This is a clear example of the utility of unique individual animal identification system in a disease control program. Using this available information in epidemiological investigations has helped in the understanding that cattle movement is not the most likely source of transmission in to most of the infected herds. However, it should be noted that inter herd spread has been linked to cattle movement in at least one herd and fence-line contact between infected herds has been identified as a potential mode of transmission in some of the infected Michigan herds. Therefore, these transmission modes should not be ignored and efforts to mitigate the risk of bTB spread through these transmission modes should be continued.

Wild white-tailed deer-cattle space interactions

Wild white-tailed deer-cattle space interactions includes wild white-tailed deer's access to cattle's feed and water sources, where *M. bovis* could be transmitted to cattle via ingestion of contaminated food and water (Palmer and Whipple 2006). This example of space interaction would explain the lower number of bTB infected dairy herds, where the animals are primarily kept inside and usually have limited close contact with wild deer, or where livestock producers report no contact of their cattle with wild-white tailed deer. Livestock owners should sustain practices that reduce wild white-tailed deer-cattle space interactions such as storage of feed in enclosures that protect it from deer access, limiting cattle access to stagnant water sources and areas of cover that are also attractive to wild white-tailed deer, and removing feedstuffs from cattle areas that are attractive to deer (e.g. wild apple trees).

Deer concentration and density reduction

Following the implementation of policies that reduced the population density and restricted practices which artificially increased wild white-tailed deer concentration, there has been a significant decreasing trend in the sample prevalence of bTB in wild white-tailed deer (O'Brien *et al.* 2006). Since 2006, the sample prevalence has leveled-off. The cause of the slight increase in the sample prevalence of bTB in the wild deer in 2006 is not clearly known and could be associated with the epidemiology of bTB in the wild deer, which remains to be fully understood.

With policies that have led to an increased harvest (Frawley 2010), wild white-tailed deer population has decreased (Schmitt 2010). Similarly, changes in deer management practices, including restriction/ban of baiting and supplemental feeding in wild white-tailed deer, has likely

helped reduce the transmission rate of bTB (Schmitt 2010). An increased harvest rate and a reduced transmission rate would cause a reduction in disease prevalence. Practices that have encouraged the reduction in deer concentration and deer density have likely contributed to the current containment of bTB. These practices will remain crucial for bTB eradication and should be supported.

Handling of bTB infected herds

Among cattle herds infected with bTB in Michigan, depopulation has been the major strategy aimed at eradication. Depopulation is a bTB eradication strategy that is effective in areas with limited reservoirs of bTB infection and the disease challenge is not ongoing. Given the herd sizes of bTB infected cattle herds as it relates to the indemnity paid in the depopulation, this strategy is expensive and can be disrupting to the herd owner. It is even more frustrating when the depopulation strategy fails to achieve its purpose of eradicating the disease. Of the 6 herds with bTB re-infection, 5 were previously depopulated. There were no observable differences in the epidemiological data that would explain any vulnerability in those 6 cattle herds with bTB re-infection. However, detailed study of these herds and their management practices could provide insight into their vulnerability to bTB infection. Depopulation appears to not be a guaranteed bTB eradication strategy in an area with a wildlife reservoir of bTB infection and where some wildlife-livestock space interaction occurs; an observation that may have influenced a 2010 policy change in UDSA APHIS VS plans for herd-specific bTB eradication. In determining how best to handle a bTB infected cattle herd in an area with a wildlife reservoir of bTB, measures must be taken to understand the exposure/transmission risks for bTB and then strive to mitigate those risks. In previous studies, restricting deer's access to cattle feed and

water was found to be associated with reduced odds of bTB infection in cattle herds while sharing of pastures, bulls, or fence-line contacts among cattle herds, especially those in close proximity to already infected herd, was associated with increased risk of acquiring bTB into the herd (Kaneene *et al.* 2002b). Therefore adopting herd-specific wildlife risk mitigation and other biosecurity practices need to be implemented and strictly enforced as part of the bTB eradication project.

Long-term quarantine of bTB infected privately owned white-tailed deer herds with no live animal movement off the operation is a strategy that has been implemented due to inadequacy of indemnity funds. The result of this confinement remains undetermined. There are reports of deer escaping from privately owned white-tailed deer facilities in Michigan as a result of damaged fences (O'Brien *et al.* 2005). Also, all of the bTB affected deer farms have fencing that could allow nose-to-nose contact between wild and captive deer. Although the true prevalence of bTB in these quarantined bTB infected privately owned white-tailed deer herds was not tested, given that the herd prevalence of bTB in the depopulated privately owned white-tailed deer herds is as high as the sample prevalence of bTB in the wild white-tailed deer in the area, any escape deer from the quarantined herds could pose a bTB risk. Therefore, depopulation of infected privately owned white-tailed deer herds would be a recommended choice if bTB eradication is to be achieved sooner rather than later.

Research

The bTB outbreak in Michigan has highlighted many knowledge gaps in our understanding about bTB. For successful eradication to occur in Michigan and in other regions of the world, significant research aimed at enhancing current eradication strategies as well as developing new eradication tools needs to be carried out. Areas of research which should be supported include:

- ***Vaccine Development*** – Extensive research on bTB vaccines for white-tailed deer is underway and the available results are promising (Nol *et al.* 2009; Nol *et al.* 2008; Palmer *et al.* 2007; Palmer *et al.* 2009; Palmer *et al.* 2010; Thacker *et al.* 2009; Waters *et al.* 2004). The development of vaccines that could be successfully deployed in either livestock or wildlife and as part of a disease eradication program would be extremely beneficial globally. Vaccine may be even more important in other regions of the world without the infrastructure to implement a bTB control program using currently available strategies. Successful vaccine deployment in wildlife could transcend any social and political challenges of the current strategies which target more deer harvest. Therefore, current and future research efforts should be supported.
- ***New Diagnostic Assays and Strategies*** – Currently available bTB diagnostic assays lack the desired sensitivity and specificity needed for effective bTB eradication in a timely manner. Development of inexpensive, accurate, and rapid diagnostic assays would be very valuable for the efficient identification of bTB infected animals/herds. Development and evaluation of new strategies to deploy diagnostic testing, such as at points of concentration, would be of further value. Newer *ante mortem* serological

(Fenton *et al.* 2010; Lyashchenko *et al.* 2008; Waters *et al.* 2006) and cell-mediated immune response (Okafor *et al.* 2011a) assays show potential improvements from the currently available assays and should be encouraged through research funding.

- ***Disease Transmission Risk Factors*** – Studies that evaluate bTB transmission risk factors have been conducted (Kaneene *et al.* 2002b; Palmer *et al.* 2002; Palmer *et al.* 2004; Palmer and Whipple 2006). However, inadequate research has been done on quantifying how much each known risk factor contributes to disease transmission. Understanding and quantifying risk factors important for bTB transmission within and between species would be very valuable for the successful deployment of targeted surveillance strategies and for implementing herd control programs.
- ***Ecology and Epidemiology of bTB in Non-cattle Species*** – With the emergence of bTB reservoirs in wildlife, numerous studies that strive to understand bTB epidemiology have been conducted (de Lisle *et al.* 2002; Kaneene *et al.* 2002a; O'Brien *et al.* 2002a). The results of these studies have advanced bTB eradication efforts. However, bTB epidemiology, especially in non-cattle species, is still not well understood. Better understanding of how the disease is maintained and transmitted in these "new" hosts is necessary for successful control and eradication.
- ***Sociological Aspects of Disease Eradication Programs –*** Effective disease control programs need commitment from all parties affected, whether directly or peripherally. As can be seen from a previous study (Dorn and Mertig 2005), stakeholders attitudes have influenced the progress of bTB eradication in Michigan. Understanding societal concerns and developing strategies to mitigate these concerns is extremely important for successful deployment of a disease control program.

CONCLUSION

Despite ongoing eradication efforts, bTB remains a major challenge for Michigan. Policies and strategies implemented since 1994 have appeared to contain cases of bTB in cattle and privately owned deer herds as well as approximately 99.6% of the bTB infected wild white-tailed deer to the northern portions of the Lower Peninsula of Michigan, particularly counties in the North East portion. Active collaboration among the bTB Eradication Project partners, funding agencies, and the various stakeholders have contributed to the current progress and should be encouraged further for onward bTB eradication. Wild white-tailed deer remains the significant source of transmission of bTB to the livestock, most likely through indirect transmission. Mitigation strategies that decrease interactions and indirect transmission as well as supporting actions aimed at reducing the disease prevalence in wildlife should continue and be enhanced. These actions include decreasing wild white-tailed deer population density, decreasing opportunities for close congregation of wild deer, and developing novel strategies for increasing resistance to bTB such as vaccines. Bovine TB surveillance strategies associated with the current eradication program has been effective but expensive. Therefore, development of effective but less expensive disease surveillance system would be beneficial. Finally, additional research is needed to improve our understanding about bTB epidemiology as well as disease eradication techniques that would transcend social and political issues. Supporting, conducting, and implementing the results of such research would greatly improve bTB eradication efforts.

Table 1.1: Descriptive epidemiology of bTB in MI cattle, privately owned and wild white-tailed deer (1975-July 2010)

County	bTB (+) cattle herds[a]	Total cattle herds[b]	bTB Incidence rate/1000 cattle herd-yrs[a]	bTB (+) privately owned cervid herds[a]	bTB (+) wild white-tailed deer[c]	Total wild white-tailed deer tested[c]	Prevalence odds of bTB (+) wild white-tailed deer[c]
Alcona	13	119	8.7	0	240	18,451	0.0132
Alpena	21	231	7.3	0	186	18,776	0.0100
Montmorency	4	87	3.7	3	130	12,027	0.0109
Oscoda	3	80	3.0	0	74	9,624	0.0077
Presque Isle	2	140	1.1	1	13	9,404	0.0014
Antrim	3	98	2.4	0	1	5,133	0.0002
Emmet	3	135	1.8	0	2	3,413	0.0006
Others[d]	0	13,564	0	0	22	107,441	0.0002

[a] Data 1998-July 2010, [b] Data from the 2007 Michigan Agricultural census (USDA Census of Agriculture 2007), [c] Data 1975- July 2010, [d] Other counties in Michigan

Table 1.2: Timeline of bTB in Michigan (1975-July 20010)

Time	Event
1974	• Last known bTB infected cattle herd in Michigan depopulated.
1975	• bTB infected wild white-tailed deer harvested by a hunter in Alcona County.
1979	• State of Michigan designated as bTB accredited free.
1994	• bTB infected wild white-tailed deer harvested by a hunter in Alpena County.
1995	• bTB surveillance of hunter killed wild white-tailed deer, cattle, and privately owned cervid herds in 16 km radius around location of 1994 bTB infected wild white-tailed deer was initiated. • MDNR conducted bTB surveillance in wild white-tailed deer within portions of Alcona, Alpena, Montmorency, and Oscoda counties [Deer Management Unit (DMU) 452]. • 18 of 403 (4.47%) wild white-tailed deer found infected with bTB. • Testing of all cattle and privately owned cervid herds located within 5 miles of any bTB positive wild white-tailed deer initiated.
1996	• Statewide bTB surveillance in wild white-tailed deer & other wildlife began. • MDNR expanded bTB surveillance in wild white-tailed deer beyond DMU 452 to include all of Alcona, Alpena, Montmorency, and Oscoda counties. • Disease Control Permits issued. • 56 of 4,966 (1.13%) wild white-tailed deer found infected with bTB. • 1 coyote found infected with bTB.
1997	• The 1st privately owned white-tailed deer herd found infected with bTB. • 73 of 3,720 (1.96%) wild white-tailed deer found infected with bTB. • 2 coyotes found infected with bTB.
1998	• 3 beef cattle herds found infected with bTB. • State of Michigan's bTB-free status suspended. • bTB testing of all cattle and cervid herds in 5-county area initiated. • Deer feeding banned, baiting restricted, and doe harvest increased in an Enforced Restricted Area (ERA) bordered by interstate road (I-75), state road (M-55), and shoreline of Lake Huron. • DMU 452 expanded to encompass 5-county area (Alcona, Alpena, Montmorency, Oscoda, Presque Isle counties). • Antlerless hunting permits issued liberally (1 per day) in the DMU 452. • 78 of 9,057 (0.86%) wild white-tailed deer found infected with bTB. • 1 bear, 2 raccoons, and 2 coyotes found infected with bTB.

Table 1.2 (cont'd)

Time	Event
1999	• Baiting regulation initiated in the northeastern Lower Peninsula of the state. • Unlimited antlerless hunting permits made available in the DMU 452. • bTB testing for movement from any cattle herds East of I-75 and North of M-55 initiated. • 1 beef cattle herd found infected with bTB. • 58 of 19,496 (0.3%) wild white-tailed deer found infected with bTB.
2000	• Baiting/feeding of Deer and Elk banned in counties with bTB positive wild white-tailed deer. • Statewide official ear tag identification of cattle initiated. • Statewide bTB testing of all cattle herds by the end of 2003 initiated. • 2 dairy and 5 beef cattle herds found infected with bTB. • State status dropped to Modified Accredited. • 53 of 25,858 (0.2%) wild white-tailed deer found infected with bTB.
2001	• DMU 452 redefined to what it was in 1996 but the area shifted slightly east from the original 1996 DMU 452. • USDA APHIS began fencing project on bTB high risked cattle farms. • 8 beef cattle herds found infected with bTB. • 61 of 24,278 (0.25%) wild white-tailed deer found infected with bTB.
2002	• bTB program changed: a. Alcona, Alpena, Montmorency, Presque Isle counties – annual whole herd test (WHT) of all cattle herds except feedlots was initiated. A negative bTB test required for movement of sexually active cattle if > 6 months from WHT. b. Cheboygan, Crawford, Iosco, Ogemaw, Oscoda, Otsego counties – Biennial WHT of all cattle herds except feedlots was initiated. A negative bTB test required for movement of sexually active cattle if > 6 months from WHT. c. Antrim, Arenac, Charlevoix, Emmet, Gladwin, Kalkaska, Roscommon counties - 2 WHT to be completed between 2000 –2003. A negative bTB test required for movement of sexually active cattle if > 6 months from WHT. *Emmet County began annual WHT of all cattle herds except feedlots. • Antlerless hunting permits were increased for the northeast of the state. • 1 mixed, 2 dairy, and 4 beef cattle herds found infected with bTB. • 51 of 18,100 (0.28%) wild white-tailed deer found infected with bTB
2003	• Statewide WHT completed. • 2 dairy and 4 beef cattle herds found infected with bTB. • 32 of 17,302 (0.18%) wild white-tailed deer found infected with of bTB.

Table 1.2 (cont'd)

Time	Event
2004	• State of Michigan acquired split state status: Modified Accredited zone (MAZ) and Modified Accredited Advanced zone (MAAZ). • The gamma interferon assay approved for follow-up testing of caudal fold test suspects. • bTB program changed: a. Annual WHT of all cattle herds in MAZ except feedlots initiated. Negative TB test for movement of sexually intact cattle if > 60 days from WHT. b. Rest of Michigan – stratified random surveillance of 1500 herds every two years was initiated. • 2 dairy cattle herds found infected with bTB. • 28 of 15,131 (0.19%) wild white-tailed deer found infected with bTB.
2005	• Upper Peninsula part of the state elevated to bTB-Free status. • 3 beef cattle herds found infected with bTB. • 16 of 7,364 (0.22%) wild white-tailed deer found infected with bTB.
2006	• The 2nd privately owned deer herd found infected with bTB. • 2 dairy, 1 mixed, and 1 beef cattle herd found infected with bTB. • 41 of 7,914 (0.52%) wild white-tailed deer found infected with bTB.
2007	• Implementation of official electronic identification ear tags mandatory for all cattle within the state. • Annual WHT of feedlots within the MAZ implemented. • 1 dairy and 2 beef cattle herds found infected with bTB. • 27 of 8,316 (0.32%) wild white-tailed deer found infected with bTB.
2008	• One time WHT of all cattle herds located in Arenac, Clare, Gladwin, Grand Traverse, Iosco, Kalkaska, Missaukee, Ogemaw, Osceola, Roscommon, and Wexford counties within 3 years initiated. • A beef, and a mixed cattle herd infected with bTB. • The 3rd privately owned deer herd found infected with bTB. • 37 of 16,309 (0.23%) wild white-tailed deer found infected with bTB.
2009	• 1 beef cattle herd found infected with bTB. • The 4th privately owned deer herd found infected with bTB. • 31 of 5,722 (0.54%) wild white-tailed deer found infected with bTB.
July 2010	• 3 beef cattle herds found infected with bTB. • 6 of 306 (1.96%) wild white-tailed deer found infected with bTB.

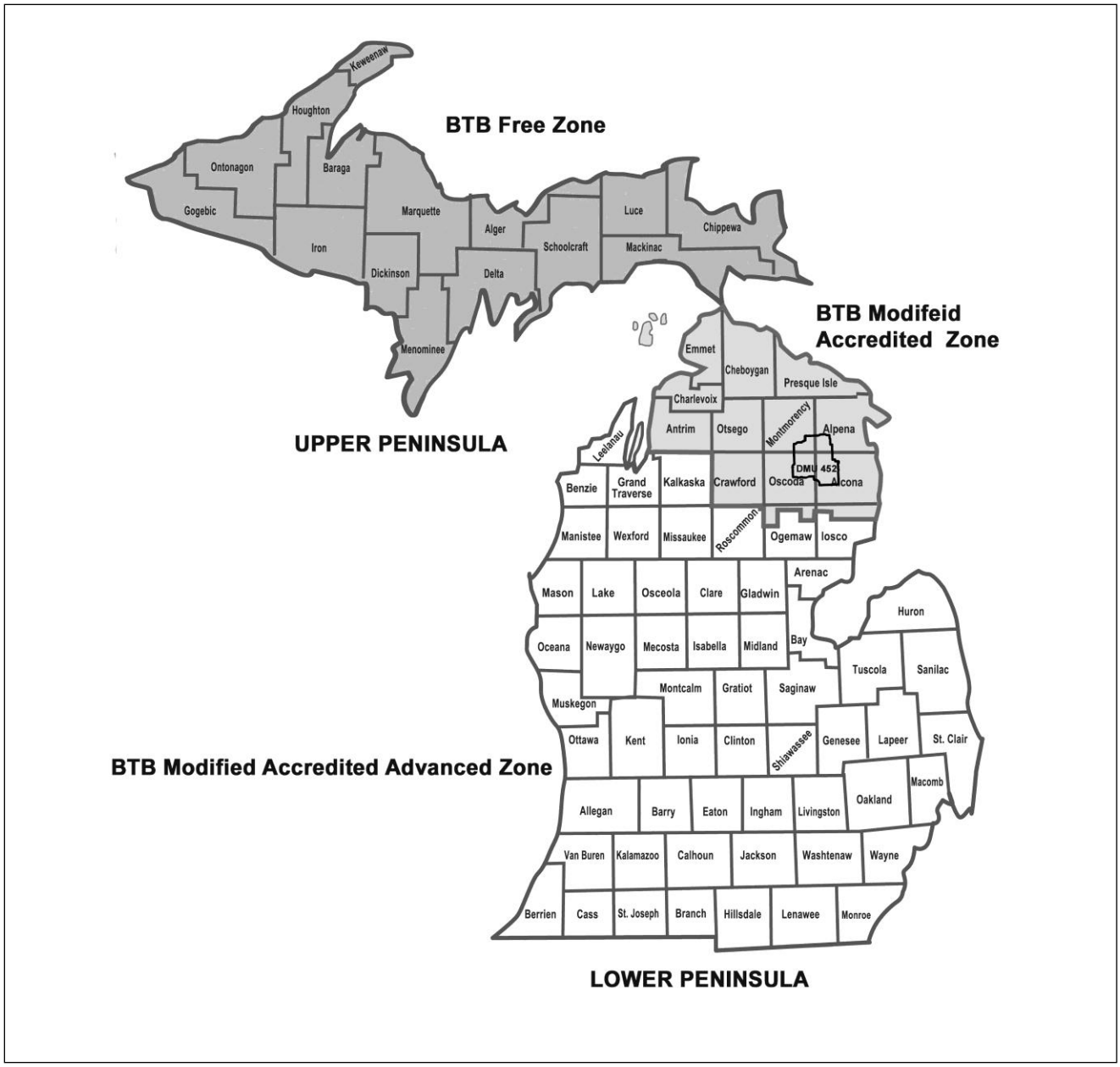


Figure 1.1: State of Michigan map showing the Upper and Lower Peninsula, counties and bTB zones as of December 2009

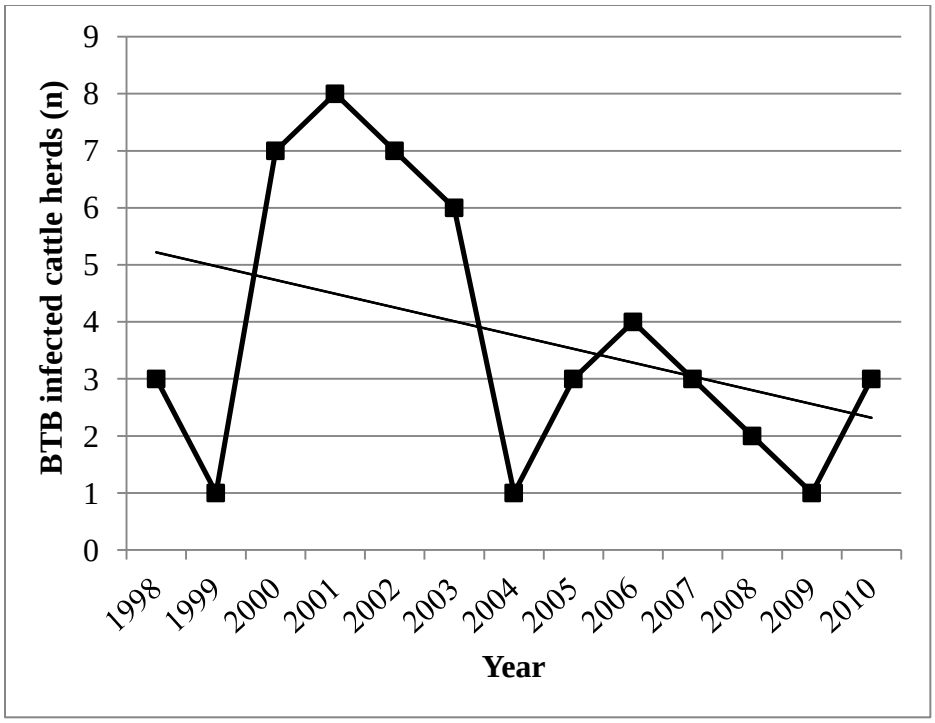


Figure 1.2: Annual incidence count of bTB cattle herds in Michigan (with linear trend line)

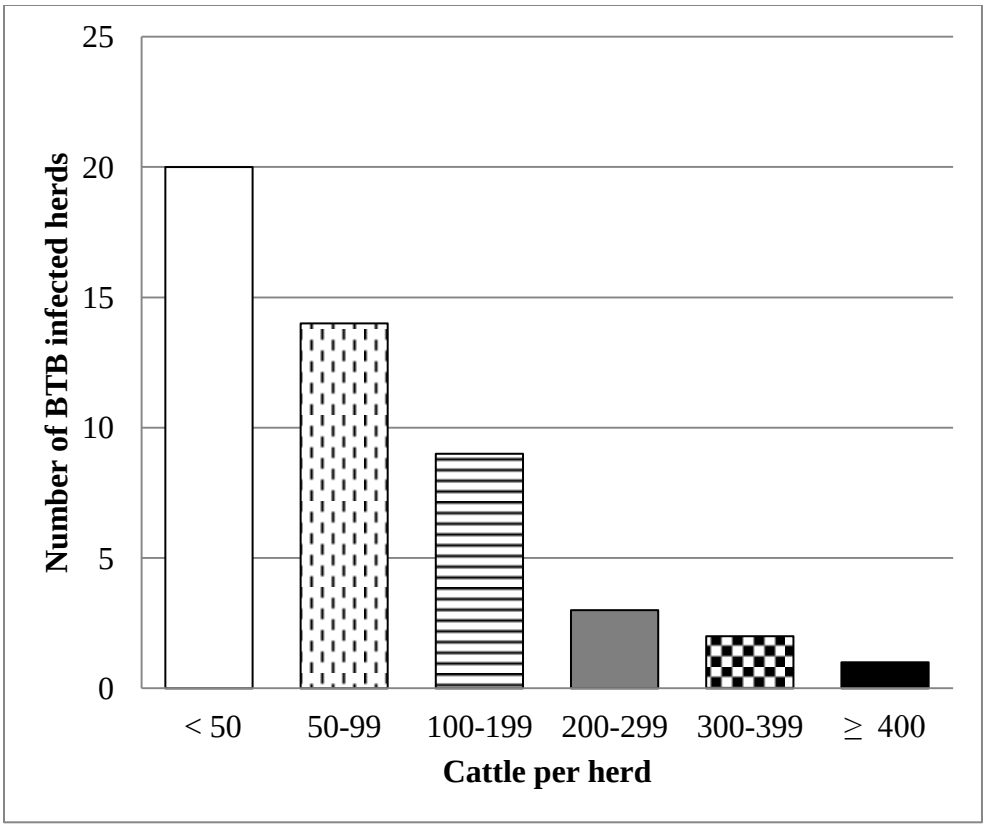


Figure 1.3: Herd sizes of bTB infected cattle herds in Michigan, 1998-July 2010

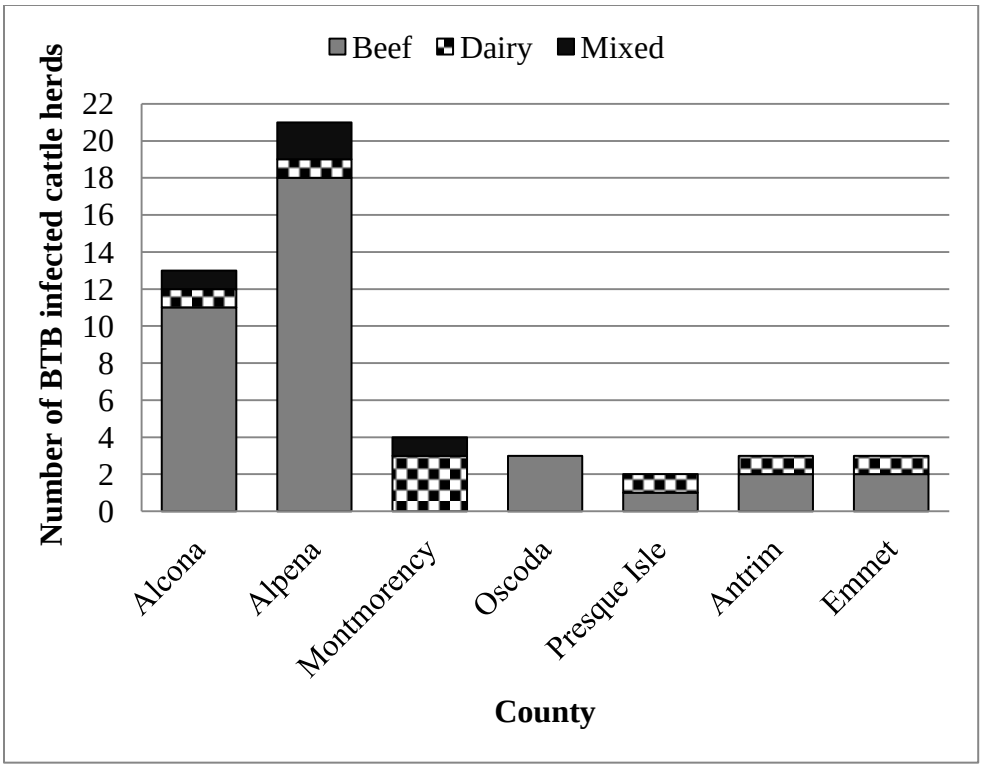


Figure 1.4: bTB incidence count in Michigan cattle herds by type, 1998-July 2010

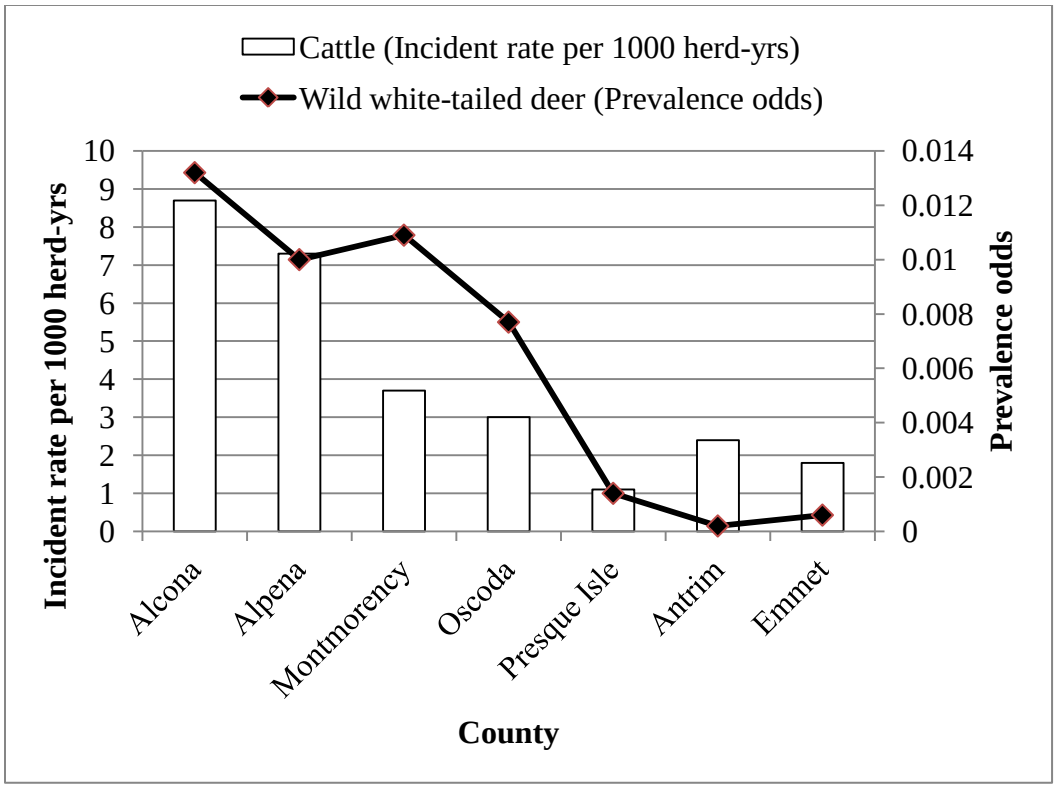


Figure 1.5: Prevalence odds and incident rate of bTB in Michigan wild white-tailed deer and cattle herds respectively, 1975-July2010

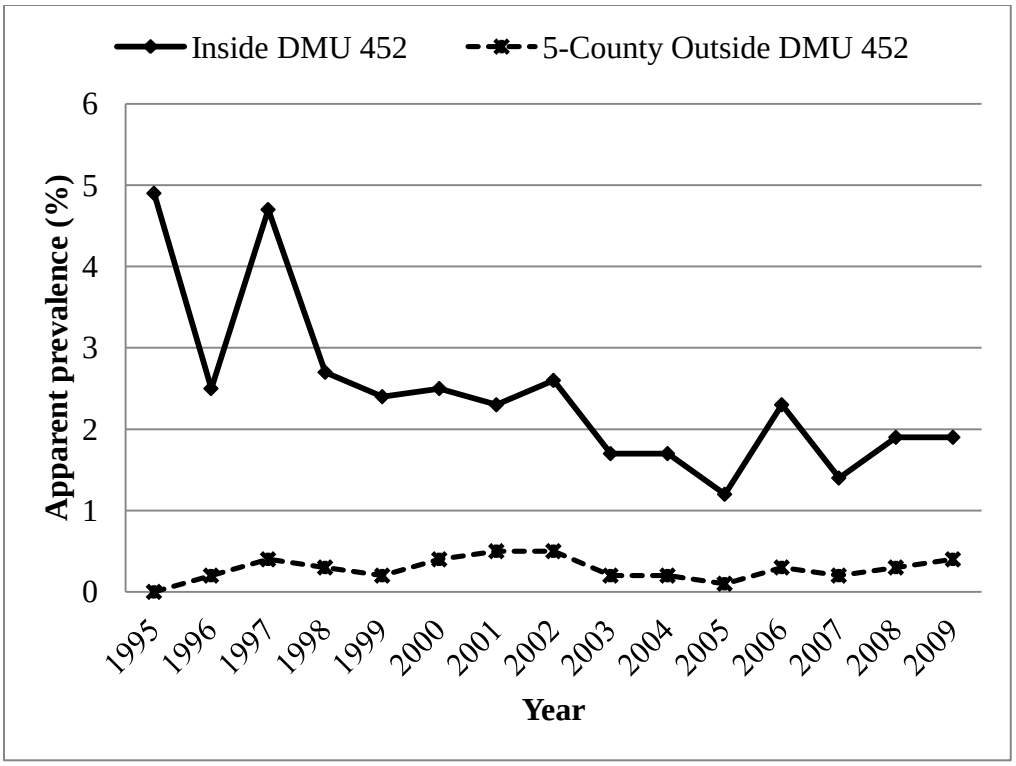


Figure 1.6: Sample prevalence of bTB in Michigan wild white-tailed deer

REFERENCES

REFERENCES

Bridget Patrick. (personal communications 2010). Animal Industry Division, Michigan Department of Agriculture, P O Box 30017 Lansing MI 48909.

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., Cooley, T. M., Miller, J. M., and Muzo, D. P. (2001). Bovine tuberculosis in free-ranging carnivores from Michigan. *J Wildlife Dis* **37**(1), 58-64.

Bruning-Fann, C. S., Schmitt, S. M., Fitzgerald, S. D., Payeur, J. B., Whipple, D. L., Cooley, T. M., Carlson, T., and Friedrich, P. (1998). *Mycobacterium bovis* in coyotes from Michigan. *J Wildlife Dis* **34**(3), 632-636.

Conner, M. M., Ebinger, M. R., Blanchong, J. A., and Cross, P. C. (2008). Infectious disease in cervids of north America - Data, models, and management challenges. *Ann NY Acad Sci* **1134**, 146-172.

de Lisle, G. W., Bengis, R. G., Schmitt, S. M., and O'Brien, D. J. (2002). Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Rev Sci Tech OIE* **21**(2), 317-334.

Dorn, M. L., and Mertig, A. G. (2005). Bovine Tuberculosis in Michigan: Stakeholder Attitudes and Implications for Eradication Efforts. *Wildlife Soc B* **33**(2), 539-552.

Fenton, K. A., Fitzgerald, S. D., Kaneene, J. B., Kruger, J. M., Greenwald, R., and Lyashchenko, K. P. (2010). Comparison of three immunodiagnostic assays for antemortem detection of *Mycobacterium bovis* stimulation in domestic cats. *J Vet Diagn Invest* **22**(5), 724-729.

Frawley, B. J. (2010). Michigan deer harvest survey report 2009 seasons. http://www.michigan.gov/documents/dnr/report3513_327318_7.pdf (MDNR Wildlife Report No. 3513). *Accessed March 15, 2011.*

Grange, J. M. (2001). *Mycobacterium bovis* infection in human beings. *Tuberculosis* **81**(1-2), 71-77.

Hickling, G. J. (2002). Dynamics of bovine tuberculosis in wild white-tailed deer in Michigan. http://ww2.dnr.state.mi.us/publications/pdfs/HuntingWildlifeHabitat/Reports/WLD-library/3301-3399/3363.pdf MDNR Wildlife Division Report(3363). *Accessed August 10, 2011.*

Kaneene, J. B., Bruning-Fann, C. S., Dunn, J., Mullaney, T. P., Berry, D., Massey, J. P., Thoen, C. O., Halstead, S., and Schwartz, K. (2002a). Epidemiologic investigation of *Mycobacterium bovis* in a population of cats. *Am J Vet Res* **63**(11), 1507-1511.

Kaneene, J. B., Bruning-Fann, C. S., Granger, L. M., Miller, R. A., and Porter-Spalding, B. A. (2002b). Environmental and farm management factors associated with tuberculosis on cattle farms in northeastern Michigan. *JAVMA* **221**(6), 837-842.

Kaneene, J. B., VanderKlok, M., Bruning-Fann, C. S., Palmer, M. V., Whipple, D. L., Schmitt, S. M., and Miller, R. (2002c). Prevalence of *Mycobacterium bovis* infection in cervids on privately owned ranches. *JAVMA* **220**(5), 656-659.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Chambers, M. A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B. M., Jackson, R., O'Brien, D. J., Schmitt, S., Palmer, M. V., Delahay, R. J., and Waters, W. R. (2008). Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. *Vet Microbiol* **132**(3-4), 283-292.

MDNR. History of legislation and regulations for bovine tuberculosis in Michigan wildlife. http://www.michigan.gov/documents/Bait_Feed_History_138339_7.pdf . *Accessed Novemeber 14, 2010.*

MDNR. Bovine tuberculosis. http://www.michigan.gov/emergingdiseases . *Accessed Novemeber 14, 2010.*

Miller, R., Kaneene, J. B., Fitzgerald, S. D., and 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. *J Wildlife Dis* **39**(1), 84-95.

Nol, P., Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Waters, W. R., Palmer, M. V., Nonnecke, B. J., Keefe, T. J., Thacker, T. C., Rhyan, J. C., Aldwell, F. E., and Salman, M. D.

(2009). Humoral immune responses of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG vaccination and experimental challenge with *M. bovis. Clin Vaccine Immunol* **16**(3), 323-329.

Nol, P., Palmer, M. V., Waters, W. R., Aldwell, F. E., Buddle, B. M., Triantis, J. M., Linke, L. M., Phillips, G. E., Thacker, T. C., Rhyan, J. C., Dunbar, M. R., and Salman, M. D. (2008). Efficacy of oral and parenteral routes of *Mycobacterium bovis* bacille Calmette-Guerin vaccination against experimental bovine tuberculosis in white-tailed deer (*Odocoileus virginianus*): A feasibility study. *J Wildlife Dis* **44**(2), 247-259.

O'Brien, D. J., Bernardi, P., Dubay, S., Mayhew, S., Moritz, W. E., and Purol, D. (2005). A Risk-based audit of the captive/privately owned cervid industry in Michigan. http://www.michigan.gov/documents/CPOCAuditReport_Final_118651_7.pdf . *Accessed August 10, 2010.*

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., and Berry, D. E. (2001). Tuberculous lesions in free-ranging white-tailed deer in Michigan. *J Wildlife Dis* **37**(3), 608-613.

O'Brien, D. J., Schmitt, S. M., Berry, D. E., Fitzgerald, S. D., Lyon, T. J., Vanneste, J. R., Cooley, T. M., Hogle, S. A., and Fierke, J. S. (2008). Estimating the true prevalence of *Mycobacterium bovis* in free-ranging elk in Michigan. *J Wildlife Dis* **44**(4), 802-810.

O'Brien, D. J., Schmitt, S. M., Berry, D. E., Fitzgerald, S. D., Vanneste, J. R., Lyon, T. J., Magsig, D., Fierke, J. S., Cooley, T. M., Zwick, L. S., and Thomsen, B. V. (2004). Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan. *J Wildlife Dis* **40**(1), 42-52.

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., and Kaneene, J. B. (2002). Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995-2000. *Prev Vet Med* **54**(1), 47-63.

O'Brien, D. J., Schmitt, S. M., Fitzgerald, S. D., Berry, D. E., and Hickling, G. J. (2006). Managing the wildlife reservoir of *Mycobacterium bovis*: The Michigan, USA, experience. *Vet Microbiol* **112**(2-4), 313-323.

O'Brien, D. J., Schmitt, S. M., Lyashchenko, K. P., Waters, W. R., Berry, D. E., Palmer, M. V., McNair, J., Greenwald, R., Esfandiari, J., and Cosgrove, M. K. (2009). Evaluation of blood assays for detection of *Mycobacterium bovis* in white-tailed deer (*Odocoileus virginianus*) in Michigan. *J Wildlife Dis* **45**(1), 153-164.

Okafor, C. C., Grooms, D. L., Bolin, S. R., and Kaneene, J. B. (2011). Detection of bovine interferon-γ response in blood collected during exsanguination of cattle sensitized with *Mycobacterium bovis*. *Am J Vet Res. Accepted June, 2011.*

Olmstead, A. L., and Rhode, P. W. (2004). An impossible undertaking: The eradication of bovine tuberculosis in the United States. *Journal of Economic History* **64**(3), 734-772.

Oreilly, L. M., and Daborn, C. J. (1995). The epidemiology of *Mycobacterium bovis* infections in animals and man - A Review. *Tubercle and Lung Disease* **76**, 1-46.

Palmer, M. V. (2007). Tuberculosis: A reemerging disease at the interface of domestic animals and wildlife. *Wildlife and Emerging Zoonotic Diseases: the Biology, Circumstances and Consequences of Cross-Species Transmission* **315**, 195-215.

Palmer, M. V., Thacker, T. C., and Waters, W. R. (2007). Vaccination of white-tailed deer (*Odocoileus virginianus*) with *Mycobacterium bovis* bacillus Calmette Guerin. *Vaccine* **25**(36), 6589-6597.

Palmer, M. V., Thacker, T. C., and Waters, W. R. (2009). Vaccination with *Mycobacterium bovis* BCG strains Danish and Pasteur in *white-tailed deer* (*Odocoileus virginianus*) experimentally challenged with *Mycobacterium bovis*. *Zoonoses Public Hlth* **56**(5), 243-251.

Palmer, M. V., Thacker, T. C., Waters, W. R., Robbe-Austerman, S., Lebepe-Mazur, S. M., and Harris, N. B. (2010). Persistence of *Mycobacterium bovis* Bacillus Calmette-Guerin in white-tailed deer (*Odocoileus virginianus*) after oral or parenteral vaccination. *Zoonoses Public Hlth* **57**(7-8), E206-E212.

Palmer, M. V., Waters, W. R., and Whipple, D. L. (2004). Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact. *Am J Vet Res* **65**(11), 1483-1489.

Palmer, M. V., Waters, W. R., and Whipple, D. L. (2002). Milk containing *Mycobacterium bovis* as a source of infection for white-tailed deer fawns (*Odocoileus virginianus*). *Tuberculosis* **82**(4-5), 161-165.

Palmer, M. V., and Whipple, D. L. (2006). Survival of *Mycobacterium bovis* on feedstuffs commonly used as supplemental feed for white-tailed deer (*Odocoileus virginianus*). *J Wildlife Dis* **42**(4), 853-858.

Palmer, M. V., Whipple, D. L., Payeur, J. B., Alt, D. P., Esch, K. J., Bruning-Fann, C. S., and Kaneene, J. B. (2000). Naturally occurring tuberculosis in white-tailed deer. *JAVMA* **216**(12), 1921-1924.

Palmer, M. V., Whipple, D. L., and Waters, W. R. (2001). Experimental deer-to-deer transmission of *Mycobacterium bovis*. *Am J Vet Res* **62**(5), 692-696.

Schmitt, S. M. (2010). Bovine tuberculosis in wildlife. http://www.michigan.gov/documents/emergingdiseases/DNRESchmittPublicMeeting062210_325662_7.pdf (MDA-DNRE TB Informational Meetings June 22-23,2010). *Accessed May 2, 2011.*

Schmitt, S. M., Fitzgerald, S. D., Cooley, T. M., BruningFann, C. S., Sullivan, L., Berry, D., Carlson, T., Minnis, R. B., Payeur, J. B., and Sikarskie, J. (1997). Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *J Wildlife Dis* **33**(4), 749-758.

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

Thacker, T. C., Palmer, M. V., and Waters, W. R. (2009). T-Cell mRNA expression in response to *Mycobacterium bovis* BCG vaccination and *Mycobacterium bovis* infection of white-tailed deer. *Clin Vaccine Immunol* **16**(8), 1139-1145.

USDA APHIS. Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective January 1, 2005.

USDA APHIS. Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective January 22, 1999.

USDA Census of Agriculture. (2007). http://www.agcensus.usda.gov/Publications/2007/Full_Report/Volume_1,_Chapter_2_US_State_Level/st99_2_011_011.pdf . *Accessed May 15, 2009.*

Waters, W. R., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Vordermeier, H. M., Hewinson, R. G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J. M., Andersen, P., and Lyashchenko, K. P. (2006). Early Antibody Responses to Experimental *Mycobacterium bovis* Infection of Cattle. *Clin Vaccine Immunol* **13**(6), 648-654.

Waters, W. R., Palmer, M. V., Whipple, D. L., Slaughter, R. E., and Jones, S. L. (2004). Immune responses of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG vaccination. *J Wildlife Dis* **40**(1), 66-78.

Wilkins, M. J., Meyerson, J., Bartlett, P. C., Spieldenner, S. L., Berry, D. E., Mosher, L. B., Kaneene, J. B., Robinson-Dunn, B., Stobierski, M. G., and Boulton, M. L. (2008). Human *Mycobacterium bovis* infection and bovine tuberculosis. outbreak, Michigan, 1994-2007. *Emerg Infect Dis* **14**(4), 657-660.

Wilkins, M. J., Bartlett, P. C., Judge, L. J., Erskine, R. J., Boulton, M. L., and Kaneene, J. B. (2009). Veterinarian injuries associated with bovine TB testing livestock in Michigan, 2001. *Prev Vet Med* **89**(3-4), 185-190.

CHAPTER 2

SURVEILLANCE STRATEGIES FOR DIAGNOSIS OF BOVINE TUBERCULOSIS IN MICHIGAN CATTLE: CURRENT GAPS AND FUTURE PROSPECTS

ABSTRACT

Surveillance strategies that target susceptible animals at higher risk of bovine tuberculosis (bTB) are important in the control or eradication of bTB. Although surveillance strategies for diagnosis of bTB currently used in the state of Michigan in the United States (US) have been helpful, improvements are needed. This study reviews the currently used surveillance strategies for diagnosis of bTB, emphasizing the strengths, limitations, and suggestions for the future. In Michigan, serial testing of cattle for bTB on the farm, using either two separate and successive intradermal tests or a combination of the caudal fold intradermal test and the interferon-gamma assay, has been most common. At slaughter, bTB surveillance in cattle has mostly relied on gross physical examination of lymph nodes and subsequent further examination of tissues. Improving the detection of cattle herds exposed to or at early stages of bTB infection could be achieved using the current or emerging assays adapted for use at points of concentration of cattle. Such a prospective strategy could lead to targeted surveillance for bTB in the animal's herd of origin. This strategy has potentials for improving bTB eradication efforts.

INTRODUCTION

A better bovine tuberculosis (bTB) surveillance approach is needed in Michigan cattle. Despite over a decade of efforts in implementing the Michigan Bovine Tuberculosis Eradication Project, bovine tuberculosis (bTB) remains a challenge in Michigan livestock and wildlife (Okafor *et al.* 2011b). Due to this ongoing bTB challenge, regulatory requirements for cattle movement have affected cattle trade in Michigan (Okafor *et al.* 2011b), and Michigan has spent approximately US$200 million on bTB eradication between 1994 and 2010 (Bridget Patrick 2010). Annually, over US$7 million is spent on bTB surveillance in cattle alone (Okafor *et al.* 2011b). Recent challenges to Michigan's economy could result in decreased budget allocation for bTB surveillance and would hinder bTB eradication efforts. In such economic situations, it is important to set priorities in the protection of both animal and public health and to allocate resources effectively and efficiently (Stark *et al.* 2006). Reviewing the current bTB surveillance strategies in Michigan cattle could help identify the possibilities for more targeted surveillance.

'Surveillance' and 'targeted surveillance' are similar in their objectives but their approach differs. Disease surveillance is an active, systematic, and ongoing investigation aimed at early disease detection, disease estimation, or providing evidence of disease absence within a population, with planned strategies of disease eradication if detected. The entire population can be used in disease surveillance. One example is the visual inspection of slaughtered cattle for bTB in the United States of America (US). In the field of veterinary medicine or veterinary public health, the term 'targeted surveillance', sometimes referred to as 'risk-based surveillance', is a surveillance method that focus on sampling high-risk populations in which commonly known risk factors exist, under the assumption that the disease/condition of interest would be

more common in the targeted population than in the general population (Salman *et al.* 2003; Salman 2008). This surveillance strategy often results in a substantial reduction in the sample size required in either disease detection or prevalence estimation (Williams *et al.* 2009b). Targeted surveillance is an important approach aimed at achieving a higher benefit-cost ratio with existing or reduced resources (Stark *et al.* 2006). The considerations from this approach are useful in supporting both strategic and operational decision making in disease prevention/eradication (Stark *et al.* 2006).

EXAMPLES OF TARGETED SURVEILLANCE IN VETERINARY MEDICINE

The concept of targeted surveillance has been used in many diseases of veterinary importance and has become increasingly popular surveillance approach where the prevalence of disease is low (Christensen and Gardner 2000; OIE 2004; OIE 2011; Prattley *et al.* 2007a; Prattley *et al.* 2007b; Tavornpanich *et al.* 2006; Tavornpanich *et al.* 2008; Williams *et al.* 2009b). This surveillance approach can either be external to the population of interest (preemptive) or operate within the population (post facto). Preemptive targeted surveillance intends to protect a naïve susceptible populations by isolating the disease agent before its intrusion (Thurmond 2003). An example of preemptive targeted surveillance is a 30-day quarantine requirement for imported ruminants into the US by the United States Department of Agriculture, except from Canada and Mexico, due to concerns of importing foreign animal disease such as Bovine Spongiform Encephalopathy (BSE) or Foot-and Mouth Disease (FMD) (USDA APHIS VS 2011). Post facto targeted surveillance aims at detecting disease after it has entered the population, preferably before transmission occurs among susceptible hosts (Thurmond 2003). An example of this latest approach is the examination of culled cattle in the United Kingdom for evidence of BSE (Doherr *et al.* 2001; Doherr *et al.* 2002; Morignat *et al.* 2002). Factors used for post facto targeted sampling in BSE were age and clinical presentation at slaughter or at the farm (if emergency death occurred) (Doherr and Audige 2001). Surveillance samples collected at slaughter facilities in the US for Brucellosis is another example of post facto targeted samples because breeding cows that abort have a higher probability of being culled or sent to slaughter (Williams *et al.* 2009b).

IMPORTANT CONSIDERABLE FACTORS IN THE APPLICATION OF TARGETED SURVEILLANCE

Epidemiology of disease: Targeted surveillance approach differs with disease of concern. Because risk-factors of a disease are important aspects of conducting targeted surveillance, knowledge of disease epidemiology is crucial to a successful targeted surveillance program. Drawbacks associated with targeted surveillance include the acquisition of accurate epidemiological information, especially in rare diseases. Other drawbacks include the determination of the number and most appropriate subjects to sample (Williams *et al.* 2009b). Sample size determination, among the subpopulation with an increased prevalence of a disease, is a critical step in targeted surveillance. Otherwise, the cost-benefit of targeted surveillance is defeated. To address the challenges of determining the appropriate subjects to sample within the population, a point-based system for demonstrating disease freedom has been used for target sampling (Cannon 2002). This concept assigns a relative value to each sample based on risk factors. Hence, sample subjects drawn from a subpopulation with higher disease prevalence receive higher points than subjects drawn from the non-targeted or general population. After the appropriate sample subjects are identified, there is yet another challenge of collecting the appropriate biological samples from the subjects. Disease detection relies on the appropriateness and quality of the biological samples collected, on attributes of screening/diagnostic tests, and on the experience of the diagnostic laboratory personnel (Doherr and Audige 2001). The series of events that should occur before a diseased animal is identified using targeted surveillance ultimately depends on knowledge of the epidemiology of the disease of concern.

Cost: Cost saving is one of the tenets of targeted surveillance. A surveillance system would be evaluated on its overall net value in maintaining or protecting animal health, given projected costs associated with disease in the absence of a surveillance system (Thurmond 2003). The decision to embark on disease surveillance or on a specific targeted surveillance system relies on many attributes including the direct economic, political, and social benefits of not having the disease. However, these benefits must be balanced against the cost of the surveillance system (Thurmond 2003). Evaluations of the cost of a targeted surveillance system is complex and relies on factors such as monetary value of the diagnostics tests used, labor costs, and sampling logistics involved in achieving a predefined goal. In preemptive targeted surveillance, evaluations of surveillance costs can be very challenging because the surveillance benefits many not be accurately assessed until a disease outbreak occurs. Similarly, it is difficult to evaluate the cost benefits of post factor targeted surveillance that involves rare diseases, especially when disease eradication takes longer than previously expected. For example, Michigan spends over US$7 million annually on bTB surveillance in cattle alone, but bTB in the state has been contained but not yet eradicated after 15 years (Okafor *et al.* 2011b). Given that the predefined goal of Michigan was bTB eradication, the cost benefits of the current surveillance may not be realized. Due to the challenges of cost-benefit estimations, stochastic bio-economic modeling has been useful in determining the optimal cost efficiency of various targeted surveillance programs in veterinary medicine (Audige and Beckett 1999; Prattley *et al.* 2007a; Prattley *et al.* 2007b; Tavornpanich *et al.* 2008; van Asseldonk *et al.* 2005; Webb *et al.* 2001; Williams *et al.* 2009a).

Attributes of screening/diagnostic tests: The success of a targeted surveillance system is dependent on the accuracy of the employed screening or diagnostic tests as well as the temporal efficiency of the tests. Accuracy of a test relates to its ability to give a true measure of the substance being measured (Dohoo *et al.* 2003). This accuracy is measured by the test's sensitivity (*Se*) as well as its specificity (*Sp*). Test *Se* is the probability that a test will correctly identify an infected animal when the disease is truly present, and test *Sp* is the probability that the test will correctly note the absence of a disease in an animal when the disease is truly not present. In preemptive targeted surveillance, where the objective is to rule out a disease, a test with high *Se* is preferred, to reduce the risk of not detecting infected animals. Conversely, in post facto targeted surveillance, where the goal is mainly to confirm a disease, a test with high *Sp* is desirable to reduce the risk of false positive results. Because no test has a perfect accuracy, multiple tests are often used in series or in parallel to improve the desired outcome of interest. For example, the use of multiple tests in series increases *Sp* but decreases *Se;* whereas parallel testing increases *Se* and decreases *Sp*. Another desired attribute of a screening/diagnostic test used in targeted surveillance is timeliness of detection (Thurmond 2003). This is crucial because a goal of targeted surveillance is to capture the disease and take actions in a timely manner such that would minimize disease transmission.

Social issues: Because identification of animal disease within a subpopulation often affects livestock trade, especially in the affected subpopulation, inclusion or exclusion of an area of the population for targeted surveillance is often associated with social and political challenges. These social challenges have been observed among livestock and wildlife stakeholders in both the expansion and subsequent reduction of bTB core areas in Michigan due to the stigmatization associated with labeling an area as harboring bTB infected animals (O'Brien *et al.* 2002b; O'Brien *et al.* 2002a). Experts have identified social issues as a major hindrance to a successful bTB management in Michigan as well as in Minnesota (Riley *et al.* 2011). The manifestation of social issues could be explained by the stress often experienced by bTB affected farm families due to the rigors of repeated bTB testing on their premises; as such, these families have been described as recipients of environmental injustice (Griffore and Phenice 2008). One way to improve social acceptance in targeted surveillance is to improve communication of information by the policy makers to the people concerned on the overall benefits of disease eradication.

GLOBAL SURVEILLANCE STRATEGIES FOR BTB

Although bTB was once found worldwide, control programs have eliminated or nearly eliminated this disease from domesticated animals in many countries (The Center for Food Security & Public Health 2009). Global distribution of bTB is highly variable with herd prevalence of bTB as low as <0.001 in the United States and as high as 4.37% in Ireland, with several countries in between (Schiller *et al.* 2010). Countries currently recognized as officially bTB-free (OTF) include Australia, Iceland, Denmark, Sweden, Norway, Germany, France, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Scotland, Canada, Singapore, Jamaica, Barbados and Israel (Schiller *et al.* 2010; Schiller *et al.* 2011; The Center for Food Security & Public Health 2009). Eradication programs are in progress in the United Kingdom (U.K), Ireland and other European countries, Japan, New Zealand, the United States, Mexico, and some countries of Central and South America (The Center for Food Security & Public Health 2009). Bovine tuberculosis is still widespread in Africa, parts of Asia and some Middle Eastern countries (The Center for Food Security & Public Health 2009). Although bTB has been eradicated from the majority of U.S. states, a few infected herds continue to be reported, and a few states may periodically lose their disease-free status. Since 2005, states where bTB infected animals have been isolated include Michigan, Minnesota, California, Texas, Ohio, New Mexico, Colorado, Nebraska, South Dakota, Kentucky, and Indiana . Trade and wildlife reservoirs both in non-OTF and OTF countries are major factors for re-infections of and spillover to livestock (Schiller *et al.* 2011). Situations where wildlife reservoirs have complicated eradication efforts in many countries and regions include bTB

infected badgers in the U.K. and Ireland, infected brush-tailed opossums in New Zealand, and infected wild white-tailed deer in Michigan and in Minnesota.

Commonly used tests for bTB surveillance in live animals include the caudal fold tuberculin (CFT) test, mid-cervical intradermal tuberculin test (CIT) [referred to as single cervical test (SCT) in the United States], comparative cervical tuberculin (CCT) test, and bTB interferon-gamma (IFN-γ) assay. Primary screening for bTB in live cattle is commonly conducted using any of the tuberculin tests and the IFN-γ assay as an ancillary test. CFT is the primary screening test in the United States and New Zealand, CCT in the U.K, and Ireland, and either CIT or CCT in Spain, Italy, and France. In both Germany and Austria, live animal screening is routinely not performed (Schiller *et al.* 2010). In many developed countries, such as the Unites States, the U.K, Spain, Italy, France and New Zealand, both a form of the tuberculin test (skin testing) and the IFN-γ assay (blood based assay) are used for live animal screening for bTB. Due to practice of nomadic livestock farming in most countries in Africa, live animal screening has been impractical and bTB surveillance has relied mainly on slaughter examination.

During commercial slaughter, examination of tissues for bTB-compatible lesions is used as the cost-efficient method for passive surveillance of bTB both in OTF and non-OTF countries. In non-OTF countries, slaughter surveillance is used to supplement live cattle testing (Schiller *et al.* 2010). Identification of bTB animal at slaughter initiates a targeted investigation of the herd of origin and any other potentially exposed animals (Okafor *et al.* 2011b; Olea-Popelka *et al.* 2008; Whipple *et al.* 1996). The success of such investigations is variable. In Australia, which has successfully eradicated bTB, meticulous animal identification was credited as a major factor in successful eradication (Corner 2007; Cousins and Roberts 2001; Radunz 2006; Tweddle and Livingstone 1994). However, in the USA, because of the lack of uniform animal identification

regulations combined with inconsistent record-keeping, only 50–70% of such investigations result in identification of the herd of origin and in finding all exposed animals (Kaneene *et al.* 2006). The herds of origin of such animals are placed under precautionary movement restrictions pending confirmation of bTB in slaughter lesions by histopathology, culture and/or PCR (Schiller *et al.* 2010). Within the herd of origin, either only bTB identified animals are removed or the entire herd is depopulated, depending on the bTB eradication procedure of a given county or region.

CURRENTLY USED BTB SURVEILLANCE STRATEGIES IN MICHIGAN

Michigan has moved from a bTB 'accredited free' status in 1979 to a 'modified accredited' status in 2000 (Hickling 2002), and to a split-state status: modified accredited zone (MAZ) and modified accredited advanced zone (MAAZ) in 2004 (Okafor *et al.* 2011b). In 2005, the upper-peninsular portion of Michigan was declared a bTB-free zone (BFZ), such that Michigan's split-state status comprised of three zones: MAZ, MAAZ, and BFZ (Okafor *et al.* 2011b). The numbers of counties within these zones have varied over the years. In 2010, the MAAZ was subdivided into three subzones. Subzone1, subzone 2, and subzone 3 are comprised of counties with the greatest, moderate, and lowest risks of bTB exposure respectively. The focus of this review will be the zones and subzones, because bTB surveillance strategies have been variable between these zones.

Surveillance for bTB is primarily done through live animal skin testing and through tuberculosis lesion detection at slaughter facilities (MDNR 2010). The CFT, CCT, and IFN-γ assay are the herd surveillance tests used in live animals. The procedures for these tests have been described previously (Okafor *et al.* 2011a). With the implementation of mandatory official electronic identification ear tags for all cattle within the state in 2007, all Michigan cattle are officially identified with a Radio Frequency Identification (RFID) tag prior to movement from the farm of origin. Michigan RFID tags are tied to the herd of origin by the premises identification number. This information helps quickly identify the animal's herd of origin in a disease investigation.

Live animal bTB surveillance testing involves whole herd bTB testing and tests are performed within variable intervals in the different zones. Within each zone, testing depends on

the class, purpose, and age of cattle. In the MAZ, all cattle herds, other than freezer beef herds, undergo annual whole herd bTB testing. In subzone 1 of MAAZ, all cattle owners who sell or move breeding cattle are required to have an annual whole herd bTB test conducted on their herd. The whole herd test includes all cattle 12 months and older, all purchased additions of any age, and all goats, bison, and privately owned cervids in contact with the herd. Farmers that move cattle, but do not move breeding stock, must have cattle tested every two years. Farmers that sell directly to slaughter must test their cattle every three years. In subzone 2 of MAAZ, a multi-year testing program began in 2007 to test every herd at least one time. This program was completed in 2010, and a new surveillance plan that is consistent with the risk of bTB transmission in that area is expected. Within subzone 3 of MAAZ and the BFZ, testing is performed in herds designated as high risk by epidemiologists (such as traces and circle tests around positive deer) (MDNR 2010). In addition to scheduled surveillance testing, bTB testing may be required for movement of cattle within and out of the state. The testing requirements vary upon where the cattle originate, where they are going, their age, gender, and purpose; and for cattle in the MAAZ subzone 1, if they have a Verified Wildlife Risk Mitigation Action Plan (WRMAP) (MDNR 2010). Live cattle screening has been the most efficient method of identifying bTB infected herds in Michigan, but at an enormous cost (Okafor *et al.* 2011b).

Although current surveillance strategy places greater bTB testing emphasis on zones/subzones with higher bTB prevalence or higher perceived bTB risk, the risk of infection is not homogeneous across all cattle herds within those zones/subzones. There is an additional cost-benefit if the sampling occurs less frequently, or on a smaller subpopulation of the MAZ. Recent challenges to Michigan's economy could result in decreased budget allocation to bTB surveillance, and would hinder bTB eradication efforts. In such economic situations, it is

important to set additional priorities in the eradication efforts of bTB and to allocate resources effectively and efficiently.

Slaughter surveillance for bTB involves gross examination of organs and tissues during carcass evisceration. Lymph nodes from the head (sub mandibular, parotid, and medial and lateral retropharyngeal), thorax, and abdomen are examined for gross lesions compatible with bTB by USDA Food Safety and Inspection Service personnel, using standard operating protocols (USDA APHIS VS 2005). If any compatible lesion is found, such samples are sent to the National Veterinary Services Laboratory (NVSL) at Ames, Iowa for further histopathological screening, acid-fast staining of tissues, polymerase chain reaction (PCR), and bacterial culture. Either PCR or bacterial culture is the confirmatory test in bTB screening. If *M. bovis* is cultured from the tissues of an animal, its herd of origin is declared bTB infected and will remain under quarantine until whole herd bTB screening is performed (MDNR 2010). Trace-back to herds of animal origin was a challenge to slaughter surveillance in the Unites States due to the then lack of uniform animal identification regulations combined with inconsistent record-keeping (Kaneene *et al.* 2006). Since 2007, traceability within Michigan has improved due to the implementation of mandatory RFID for all cattle prior to movement. However, overall slaughter surveillance has been the least effective method of identifying bTB infected cattle herds in Michigan, contributing 2% of infected cattle herds found between 1998 and 2010 (Okafor *et al.* 2011b).

FUTURE PROSPECTS IN MICHIGAN BTB SURVEILLANCE STRATEGIES

Because of decreased funding for bTB eradication is an impending concern, it is therefore crucial to adapt bTB surveillance at points of concentration (POC) of cattle such as slaughter houses or livestock markets. Development of targeted screening strategies at POC of cattle would most likely produce a significant reduction in resource expenditures while at the same time maintaining the necessary rapid identification of bTB infected herds to eradicate bTB. Such strategy would require an assay that involves a single contact with an animal or a cow-side assay.

In targeted surveillance, it is important to identify the subpopulation with the greatest risk of interest. In a disease such as bTB, the identification of the most appropriate cattle herds and the specific number of animals to be sampled from each of the chosen herds can be challenging. Given that the epidemiology of bTB has been described (Okafor *et al.* 2011b), Michigan RFID tags on cattle would be valuable in identifying cattle from zones of higher interest. Age of cattle is another factor that would be used in the determination of the specific cattle to be sampled from a herd located in the zones where risk of bTB is higher. Age has been reported as a predictor of bTB in cattle (Munyeme *et al.* 2009). Although cattle of all ages are susceptible to *M. bovis*, bTB is found mostly in older animals (Zhu and Lin 2011). An increased probability of becoming exposed to *M. bovis* over a longer period of time likely explains why age is a risk factor in older animals (Zhu and Lin 2011). Slaughter surveillance for bTB is best positioned to identify cattle exposed to or infected with *M. bovis* because cattle presented at slaughter are older relative to other cattle at each respective farm.

Success of a targeted surveillance is partly dependent on the diagnostic performance of the screening test and on the surveillance strategy. The diagnostic performance includes the

accuracy, precision, rapidity, and efficiency with which the screening assay is able to isolate disease or risk in the population (Thurmond 2003). Because most screening tests are absolutely imperfect in accuracy, these shortfalls are often addressed by strategically applying a combination of screening tests towards high risk groups.

Future surveillance strategies should combine the advantages of the uniqueness of slaughter facilities, RFID, the age distribution of cattle presented at slaughter to identify bTB exposed animals. Because current slaughter surveillance, which mostly relies on gross physical inspection of cattle tissues as a primary screening test, lacks the sensitivity to identify pre clinical stages of bTB, complementary bTB screening tests should be performed at slaughter. Blood based assays would be good complements to slaughter surveillance. Among the currently used bTB tests, IFN-γ assay is a blood based cell-mediated assay and possesses potentials for easier adaptability at slaughter (Okafor *et al.* 2011b). Other recent promising serological assays include a rapid test based on lateral-flow technology known as multiple antigen print immunoassay (MAPIA)(Fenton *et al.* 2010; Waters *et al.* 2006), a florescence polarization assay (Jolley *et al.* 2007), a rapid immunochromatographic test (Lyashchenko *et al.* 2008), a dual path platform assay (Greenwald *et al.* 2009), a chemiluminescent platform (Green *et al.* 2009), and an improved ELISA (IDEXX 2011).

In summary, the use of blood-based bTB assay in parallel with current slaughter surveillance for bTB could be very valuable. Such a strategy could improve the detection of cattle herds at early stages of bTB infection, leading to targeted surveillance of bTB to the originating herds. This strategy has potential of improving bTB eradication efforts at a lesser cost. For example, if the strategy is successful in identifying earlier bTB infections in cattle before the routine whole herd surveillance, the frequency of performing whole herd surveillance

may be reduced in the future. This proposed surveillance strategy may increase cost of surveillance in a short-term but decrease surveillance cost over a long-term. A cost-effective test does not generally mean the cheapest test (Schiller *et al.* 2010). In the estimation of cost-effectiveness, midterm and long-term overall cost reductions on eradication of bTB should be considered. Review of the current and emerging bTB blood-based tests that could be adapted for a POC surveillance, their accuracy, reliability, long-term cost benefits would aid identify strategies that provide the greatest impact at a lowest cost in the eradication of bTB in Michigan.

REFERENCES

REFERENCES

Audige, L., and Beckett, S. (1999). A quantitative assessment of the validity of animal-health surveys using stochastic modelling. *Prev Vet Med* **38**, 259-276.

Bridget Patrick. (personal communications 2010). Animal Industry Division, Michigan Department of Agriculture, P O Box 30017 Lansing MI 48909.

Cannon, R. M. (2002). Demonstrating disease freedom - combining confidence levels. *Prev Vet Med* **52**(3-4), 227-249.

Christensen, J., and Gardner, I. A. (2000). Herd-level interpretation of test results for epidemiologic studies of animal diseases. *Prev Vet Med* **45**(1-2), 83-106.

Corner, L. A. L. (2007). Bovine tuberculosis control in Australia, New Zealand and Ireland: Wild animals and the epidemiology of tuberculosis in domestic animals. *Cattle Practice* **15**, 3-12.

Cousins, D. V., and Roberts, J. L. (2001). Australia's campaign to eradicate bovine tuberculosis: the battle for freedom and beyond. *Tuberculosis* **81**(1-2), 5-15.

Doherr, M. G., and Audige, L. (2001). Monitoring and surveillance for rare health-related events: a review from the veterinary perspective. *Philos Trans R Soc Lond B Biol Sci* **356**, 1097-1106.

Doherr, M. G., Heim, D., Fatzer, R., Cohen, C. H., Vandevelde, M., and Zurbriggen, A. (2001). Targeted screening of high-risk cattle populations for BSE to augment mandatory reporting of clinical suspects. *Prev Vet Med* **51**, 3-16.

Doherr, M. G., Hett, A. R., Cohen, C. H., Fatzer, R., Rufenacht, J., Zurbriggen, A., and Heim, D. (2002). Trends in prevalence of BSE in Switzerland based on fallen stock and slaughter surveillance. *Vet Rec* **150**, 347-348.

Dohoo, I., Martin, W., and Stryhn, H. (2003). Veterinary Epidemiologic Research, pp. 47-48. AVC Inc. Charlottetown, Prince Edward Island, Canada.

Fenton, K. A., Fitzgerald, S. D., Kaneene, J. B., Kruger, J. M., Greenwald, R., and Lyashchenko, K. P. (2010). Comparison of three immunodiagnostic assays for antemortem detection of *Mycobacterium bovis* stimulation in domestic cats. *J Vet Diagn Invest* **22**(5), 724-729.

Green, L. R., Jones, C. C., Sherwood, A. L., Garkavi, I. V., Cangelosi, G. A., Thacker, T. C., Palmer, M. V., Waters, W. R., and Rathe, C. V. (2009). Single-antigen serological testing for bovine tuberculosis. *Clin Vaccine Immunol* **16**(9), 1309-1313.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J. B., Harris, B., Sofranko, D., Waters, W. R., and Lyashchenko, K. P. (2009). Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clin Vaccine Immunol* **16**(5), 605-612.

Griffore, R. J., and Phenice, L. A. (2008). Farm families and bovine tuberculosis: a critical perspective. *Critical Social Work* **9**(1). *Accessed August 24, 2011.*

Hickling, G. J. (2002). Dynamics of bovine tuberculosis in wild white-tailed deer in Michigan. http://ww2.dnr.state.mi.us/publications/pdfs/HuntingWildlifeHabitat/Reports/WLD-library/3301-3399/3363.pdf MDNR Wildlife Division Report(3363). *Accessed August 10, 2011.*

IDEXX. IDEXX *M. bovis* Ab Test. http://www.idexx.com/view/xhtml/en_us/livestock-poultry/ruminant/bovine-tuberculosis.jsf?SSOTOKEN=0 . *Accessed September 1, 2011.*

Jolley, M. E., Nasir, M. S., Surujballi, O. P., Romanowska, A., Renteria, T. B., De la Mora, A., Lim, A., Bolin, S. R., Michel, A. L., Kostovic, M., and Corrigan, E. C. (2007). Fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in bovine sera. *Vet Microbiol* **120**(1-2), 113-121.

Kaneene, J. B., Miller, R., and Meyer, R. M. (2006). Abattoir surveillance: The U.S. experience. *Vet Microbiol* **112**(2-4), 273-282.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Chambers, M. A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B. M., Jackson, R., O'Brien, D. J., Schmitt, S., Palmer, M. V., Delahay, R. J., and Waters, W. R. (2008). Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. *Vet Microbiol* **132**(3-4), 283-292.

MDNR. Bovine tuberculosis. http://www.michigan.gov/emergingdiseases. *Accessed November 14, 2010.*

Morignat, E., Ducrot, C., Roy, R., Baron, T., Vinard, J. L., Biacabe, A. G., Madec, J. Y., Bencsik, A., Debeer, S., Eliazsewicz, M., and Calavas, D. (2002). Targeted surveillance to assess the prevalence of BSE in high-risk populations in western France and the associated risk factors. *Vet Rec* **151**, 73-77.

Munyeme, M., Muma, J. B., Samui, K. L., Skjerve, E., Nambota, A. M., Phiri, I. G. K., Rigouts, L., and Tryland, M. (2009). Prevalence of bovine tuberculosis and animal level risk factors for indigenous cattle under different grazing strategies in the livestock/wildlife interface areas of Zambia. *Trop Anim Health Pro* **41**(3), 345-352.

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., and Kaneene, J. B. (2002a). Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995-2000. *Prev Vet Med* **54**(1), 47-63.

O'Brien, D. J., Schmitt, S. M., and Hickling, G. J. (2002b). Comments offered on Michigan bovine TB study. *JAVMA* **221**(10), 1380-1381.

OIE. (2004). FAO/OIE/WHO call for targeted strategy including poultry vaccination to help curb avian flu - Experts issue recommendations to manage crisis. http://www.oie.int/en/for-the-media/press-releases/detail/article/faooiewho-call-for-targeted-strategy-including-poultry-vaccination-to-help-curb-avian-flu-expert/ . *Accessed August 23, 2011.*

OIE. Bovine Spongiform Encephalopathy. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.11.5.htm#surveillance . *Accessed August 23, 2011.*

Okafor, C. C., Grooms, D. L., Bolin, S. R., and Kaneene, J. B. (2011a). Detection of bovine interferon-γ response in blood collected during exsanguination of cattle sensitized with *Mycobacterium bovis*. *Am J Vet Res. Accepted June, 2011.*

Okafor, C. C., Grooms, D. L., Bruning-Fann, C. S., Averill, J. J., and Kaneene, J. B. (2011b). Descriptive epidemiology of bovine Tuberculosis in Michigan (1975-2010); Lessons Learned. *Veterinary Medicine International* 2011:874924.

Olea-Popelka, F. J., Costello, E., White, P., McGrath, G., Collins, J. D., O'Keeffe, J., Kelton, D. F., Berke, O., More, S., and Martin, S. W. (2008). Risk factors for disclosure of additional tuberculous cattle in attested-clear herds that had one animal with a confirmed lesion of tuberculosis at slaughter during 2003 in Ireland. *Prev Vet Med* **85**(1-2), 81-91.

Prattley, D. J., Cannon, R. M., Wilesmith, J. W., Morris, R. S., and Stevenson, M. A. (2007a). A model (BSurvE) for estimating the prevalence of bovine spongiform encephalopathy in a national herd. *Prev Vet Med* **80**(4), 330-343.

Prattley, D. J., Morris, R. S., Cannon, R. M., Wilesmith, J. W., and Stevenson, M. A. (2007b). A model (BSurvE) for evaluating national surveillance programs for bovine spongiform encephalopathy. *Prev Vet Med* **81**(4), 225-235.

Radunz, B. (2006). Surveillance and risk management during the latter stages of eradication: Experiences from Australia. *Vet Microbiol* **112**(2-4), 283-290.

Riley, S. J., Muter, B. A., and Gore, M. L. (2011). Expert perspectives on bovine tuberculosis management policies in Michigan and Minnesota. *Accessed May 5, 2011*.

Salman, M. D. (2008). Surveillance and monitoring systems for animal health programs and disease surveys, pp. 3-13. Iowa State Press.

Salman, M. D., Stark, K. D. C., and Zepeda, C. (2003). Quality assurance applied to animal disease surveillance systems. *Rev Sci Tech OIE* **22**(2), 689-696.

Schiller, I., Oesch, B., Vordermeier, H. M., Palmer, M. V., Harris, B. N., Orloski, K. A., Buddle, B. M., Thacker, T. C., Lyashchenko, K. P., and Waters, W. R. (2010). Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transbound Emerg Dis* **57**(4), 205-220.

Schiller, I., RayWaters, W., Vordermeier, H. M., Jemmi, T., Welsh, M., Keck, N., Whelan, A., Gormley, E., Boschiroli, M. L., Moyen, J. L., Vela, C., Cagiola, M., Buddle, B. M., Palmer, M., Thacker, T., and Oesch, B. (2011). Bovine tuberculosis in Europe from the perspective of an officially tuberculosis free country: Trade, surveillance and diagnostics. *Vet Microbiol* **151**(1-2), 153-159.

Stark, K., Regula, G., Hernandez, J., Knopf, L., Fuchs, K., Morris, R., and Davies, P. (2006). Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: Review of current approaches. *BMC Health Serv Res* **6**(1), 20.

Tavornpanich, S., Gardner, I. A., Carpenter, T. E., Johnson, W. O., and Anderson, R. J. (2006). Evaluation of cost-effectiveness of targeted sampling methods for detection of *Mycobacterium avium* subsp paratuberculosis infection in dairy herds. *Am J Vet Res* **67**(5), 821-828.

Tavornpanich, S., Munoz-Zanzi, C. A., Wells, S. J., Raizman, E. A., Carpenter, T. E., Johnson, W. O., and Gardner, I. A. (2008). Simulation model for evaluation of testing strategies for detection of paratuberculosis in Midwestern US dairy herds. *Prev Vet Med* **83**(1), 65-82.

The Center for Food Security & Public Health. (2009). Bovine tuberculosis. Iowa State University. *Accessed July 29, 2011*.

Thurmond, M. C. (2003). Conceptual foundations for infectious disease surveillance. *J Vet Diagn Invest* **15**, 501-514.

Tweddle, N. E., and Livingstone, P. (1994). Bovine tuberculosis-control and eradication programs in Australia and New-Zealand. *Vet Microbiol* **40**(1-2), 23-39.

USDA APHIS VS. (2005) Tuberculosis sample submission manual for meat inspection personnel. http://www.aphis.usda.gov/animal_health/animal_diseases/tuberculosis/downloads/tb_guidebook.pdf . *Accessed June 12, 2011.*

USDA APHIS VS. Guidelines for the importation of cattle (ruminants) into the United States (except from Canada and Mexico). http://www.aphis.usda.gov/import_export/animals/live_animals.shtml . *Accessed September 8, 2011*

van Asseldonk, M. A. P. M., van Roermund, H. J. W., Fischer, E. A. J., de Jong, M. C. M., and Huirne, R. B. M. (2005). Stochastic efficiency analysis of bovine tuberculosis-surveillance programs in the Netherlands. *Prev Vet Med* **69**(1-2), 39-52.

Waters, W. R., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Vordermeier, H. M., Hewinson, R. G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J. M., Andersen, P., and Lyashchenko, K. P. (2006). Early antibody responses to experimental *Mycobacterium bovis* infection of cattle. *Clin Vaccine Immunol* **13**(6), 648-654.

Webb, C. R., Wilesmith, J. W., Simmons, M. M., and Hoinville, L. J. (2001). A stochastic model to estimate the prevalence of scrapie in Great Britain using the results of an abattoir-based survey. *Prev Vet Med* **51**, 269-287.

Whipple, D. L., Bolin, C. A., and Miller, J. M. (1996). Distribution of lesions in cattle infected with *Mycobacterium bovis*. *J Vet Diagn Invest* **8**(3), 351-354.

Williams, M. S., Ebel, E. D., and Wells, S. J. (2009a). Poisson sampling: A sampling strategy for concurrently establishing freedom from disease and estimating population characteristics. *Prev Vet Med* **89**(1-2), 34-42.

Williams, M. S., Ebel, E. D., and Wells, S. J. (2009b). Population inferences from targeted sampling with uncertain epidemiologic information. *Prev Vet Med* **89**(1-2), 25-33.

Zhu, J. G., and Lin, Y. A. (2011). Surveillance of infection by *Mycobacterium bovis*. *Rev Med Microbiol* **22**(2), 17-21.

CHAPTER 3

DETECTION OF BOVINE TUBERCULOSIS INTERFERON-GAMMA RESPONSE IN BLOOD COLLECTED DURING EXSANGUINATION OF CATTLE SENSITIZED WITH *MYCOBACTERIUM BOVIS*

ABSTRACT

The objective of this study was to determine if an interferon-gamma (IFN-γ) response to bovine tuberculosis (bTB) can be detected in blood collected during exsanguination of cattle at slaughter. Twelve cows were experimentally sensitized with inactivated *Mycobacterium bovis* (*M. bovis)* in mineral oil [Sensitinogen®, USDA, APHIS, NVSL], creating an immune response that mimics a natural *M. bovis* infection. Three control cows were injected with mineral oil only. By 5 weeks after sensitization, all 12 cows showed positive reactions in the whole blood IFN-γ assay (BOVIGAM®, Prionics AG, Zurich Switzerland). At that time, all 15 cows were scheduled for slaughter. At slaughter, samples of blood were collected from each cow immediately before stunning and again at exsanguination (within 90 s post stunning). The blood samples were delivered to the laboratory within an hour of collection and the whole blood IFN-γ assay was performed. Conditional probability and paired t-test were used to analyze changes in the categorical test interpretation and qualitative bovine IFN-γ production respectively. All 12 sensitized cows were IFN-γ positive for bTB immediately before stunning and nine (75%) remained positive at exsanguination. There was a significant decrease in the mean background-corrected IFN-γ ELISA optical density values at exsanguination ($P < 0.05$).

This study demonstrates that an IFN-γ response to bTB present before slaughter in cattle can be detected at exsanguination. These findings support further development and use of the IFN-γ assay on blood collected during exsanguination as part of a bTB surveillance program.

INTRODUCTION

Bovine tuberculosis (bTB) is of significant economic importance due to livestock trade barriers, resources expended on bTB surveillance tests, payment of indemnity for the depopulation of bTB herds, and other measures of bTB eradication (Michel *et al.* 2010; Okafor *et al.* 2011b). The causative agent of bTB, *Mycobacterium bovis (M. bovis),* is a significant zoonotic pathogen and is one of the most important public health issues globally (de Kantor *et al.* 2010; Lobue *et al.* 2010; Michel *et al.* 2010; Thoen *et al.* 2009; Thoen *et al.* 2010). As the global economy is challenged, there are concerns that budget cuts to bTB surveillance could hinder bTB eradication efforts. Therefore, it has become important to develop bTB surveillance strategies that are less expensive. A cost saving approach would be to adapt bTB surveillance at points of concentration (POC) of cattle, such as slaughter houses or livestock markets, with the goal of conducting regional surveillance at a single location instead of making trips to numerous farms. This approach would save considerable time and money.

Intradermal tuberculin testing and the interferon-gamma (IFN-γ) assay are the most commonly used bTB surveillance tests (de la Rua-Domenech *et al.* 2006). Intradermal tuberculin testing requires that two contacts are made with each animal by a veterinarian before a presumptive diagnosis is made, which makes this a costly screening method and not suitable for POC screening (Okafor *et al.* 2011b). On the contrary, the IFN-γ assay requires only one contact with an animal, making it adaptable for POC screening.

The IFN-γ assay is an internationally approved ante mortem *in vitro* screening test for bTB that measures bovine IFN-γ released by lymphocytes in response to antigen stimulation (Rothel *et al.* 1990). Besides being adaptable to POC screening, the IFN-γ assay has some

additional advantages. Single contact testing with the IFN-γ assay can reduce the cost of labor and other resources involved in bTB screening(de la Rua-Domenech *et al.* 2006) and several reports indicate the IFN-γ assay is equivalent to or better than intradermal skin tests for detection of early stage infection with bTB (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006; Monaghan *et al.* 1997; Neill *et al.* 1994; Pollock *et al.* 2005; Schiller *et al.* 2010; Wood *et al.* 1991; Wood *et al.* 1992; Wood and Jones 2001; Wood and Rothel 1994).

A convenient place for POC disease screening in livestock is at slaughter. As an example, in the United States, bTB screening is conducted at slaughter by gross examination of the carcass and blood is collected at exsanguination for Brucellosis screening (Ebel *et al.* 2008). Use of the IFN-γ assay on blood collected at slaughter might facilitate bTB screening. It has been reported that bovine IFN-γ response to bTB antigen stimulation decreases by at least 50% when blood is collected 5 minutes or longer from the exposed jugular vein after slaughter when compared with blood collected before slaughter (Rothel *et al.* 1992). However, it is unclear from this study at what specific point after slaughter the decreased IFN-γ response was observed, if the decrease affected the subsequent characterization of the animal as either bTB positive or negative, and if the slaughtering process followed the standard operating protocol of slaughter facilities. Filling these knowledge gaps would help our understanding of any usefulness of the IFN-γ assay for POC screening of cattle for bTB at slaughter.

We hypothesized that in cattle immunologically challenged with inactivated *M. bovis*, the bovine IFN-γ response following antigen stimulation would not differ significantly between blood collected before stunning and during exsanguination. The objective of this study was to determine if bovine IFN-γ response to bTB could be detected in blood collected during exsanguination. The specific aims were to: (1) determine if categorical change in interpretation

of the IFN-γ assay occurs between samples of blood collected before stunning or at exsanguination; (2) determine if there is a significant difference between the mean corrected optical density (OD450 nm) values of the IFN-γ assay conducted on blood collected at the aforementioned time points.

MATERIALS AND METHODS

Sample size

Sample size calculation for paired categorical attributes (McNemar Test) was used (StatTools Web site 2011). Based on the report of a previous study (Rothel *et al.* 1992), the following conditions were set for the calculation: probability of type 1 error (α) = 5%; power (1-β) = 80%; expected proportion to switch from being bTB IFN-γ positive to bTB IFN-γ negative = 60%; and expected proportion to switch from being bTB IFN-γ negative to bTB IFN-γ positive = 1%. The current study focused on determining if the results of the IFN-γ assay would switch from positive before slaughter to negative after stunning; therefore, 12 cattle were required. In addition, we added 3 cattle to serve as environmental (negative) controls.

Animals

The study protocol was approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC) [AUF # 08/09-125-00]. Fifteen clinically healthy, non-pregnant adult Holstein cull cows (ages 3-5 yrs) were used. All study subjects were caudal fold tuberculin test negative for bTB in the previous year and bTB IFN-γ assay negative immediately before the study started. In addition, all cattle were test negative for *Mycobacterium avium* subsp. *paratuberculosis* as determined by serum ELISA and fecal culture. The cows were housed at the Michigan State University (MSU) Beef Cattle Research and Teaching Center for the duration of the study.

Study design

Following acquisition, cattle were randomly divided into three groups of five cows each and housed in adjacent pens. After a one week acclimation period, four cows from each group were randomly selected to be immunologically challenged with inactivated *M. bovis*. The remaining cow in each group served as a non-sensitized control. Each cow in the group selected for sensitization was injected with 0.1 ml of inactivated *M. bovis* in mineral oil[a] subcutaneously in the ventral brisket. Non-sensitized cows were injected with 0.1 ml of mineral oil administered subcutaneously in the ventral brisket. At 45 days post sensitization, blood was drawn from the jugular vein and assayed for a bTB IFN-γ response. It was determined that all sensitized cows were IFN-γ positive for bTB using the cut-off established by the USDA; non-sensitized cows were IFN-γ negative for bTB. Afterwards, all cows were scheduled for slaughter at the MSU Department of Animal Science Meats Laboratory. Blood samples for bTB IFN-γ assay were collected from each animal immediately before stunning and at exsanguination. Prior to stunning, whole blood (6-8 ml) was collected from the coccygeal vein/artery of each animal with a 20g vacutainer needle into a 10 ml vacutainer tube containing sodium heparin. Cows were then stunned by captive bolt and exsanguinated using the slaughter facility's standard operating protocols. Blood was collected immediately after the jugular and carotid arteries were severed and blood was free flowing from the cervical cut. The time from stunning to blood collection ranged from 60 to 90 s. Exsanguination blood was collected into a 10 ml syringe with the plunger removed. The syringe plunger was then replaced and blood immediately transferred with an 18g needle into two 10 ml vacutainer tubes containing sodium heparin. Blood samples were placed in a cooler and delivered to the Michigan State University Diagnostic Center for Population and Animal Health (DCPAH) within one hour of collection.

Interferon-gamma testing

The DCPAH is approved by the USDA to conduct IFN-γ testing in support of bTB eradication. All assays for IFN-γ were conducted at the DCPAH using a commercially available antigen-capture ELISA[b], according to the manufacturers' recommended protocol. In brief, aliquots of each blood sample (1.5 mL) were dispensed to each of 4 wells of a 24-well tissue culture plate and labeled as C (no antigen control), A (*M. avium*), B (*M. bovis*), and P (positive antigen control). In the order the labels are listed above, each aliquot of blood was mixed with 100µl sterile phosphate buffered saline, avian PPD (purified protein derivative), bovine PPD, and pokeweed mitogen. The plates were incubated for 16-24 h at 38^0C (100.4^0F) in a humidified atmosphere. Following incubation, plates were centrifuged at 1730 rcf for 10 min and the plasma harvested. Each plasma sample was then assayed in duplicate for IFN-γ production by ELISA. Optical density measured at 450 nm (OD450 nm) was used to detect IFN-γ production in each culture plate.

Results were expressed as the background-corrected OD450 nm with the no-antigen control (C) subtracted from both *M. bovis* (B*)* and *M. avium* (A) responses. Production of IFN-γ in response to pokeweed mitogen (P), a non-specific mitogen, was used to confirm lymphocyte viability. For an assay to be considered valid this condition, $P - C \geq 0.1$, must be met. An animal was considered IFN-γ positive for bTB if the difference between OD450 nm readings of the B and the C (B-C) as well as the difference between the B and the A (B-A) were both ≥ 0.1; if either difference (B-C or B-A) was < 0.1, the animal was considered IFN-γ negative for bTB.

Statistical analysis

Independent *t*-test[c] was used to analyze if age and weight differed significantly ($p<0.05$) between the sensitized and non-sensitized cattle. Pearson's correlation[d] was used to analyze any relationship between the weight of the animals and the OD450 nm IFN-γ response. Conditional probability[e] calculation was done on all sensitized cattle. Paired *t*-test[c] analysis was used to test significance ($p<0.05$) of the IFN-γ responses at the two measured time points for the following: C, B-C, B-A, A-C, and P-C. Changes in the IFN-γ responses were represented diagrammatically using boxplot[f]. All data analysis for this project was generated using SAS software (SAS Institute Inc 2009).

RESULTS

The study animals were aged 3-5 years and their weights ranged from 611 to 914 kg (1345 to 2010 lbs). Neither age nor weight differed significantly between the sensitized and non-sensitized cows. There was no significant correlation ($p=0.127$) between weight of the animals and IFN-γ response to bovine PPD (B – C). All assayed blood samples met the assay validity condition: $P - C \geq 0.1$.

Categorically, all 12 sensitized cows were IFN-γ positive for bTB (B-C and B-A ≥ 0.1) before stunning and 9 of them remained bTB IFN-γ positive at exsanguination. The probability that cattle with IFN-γ positive result for bTB before stunning would remain positive at exsanguination was 75% (95% CI: 47-91%); all 3 non-sensitized cows were bTB IFN-γ negative both before stunning and at exsanguination (Table 3.1).

There was a reduction in the corrected bTB IFN-γ optical density reading between samples of blood collected before stunning and comparable samples of blood collected at exsanguination for most cows. Among the sensitized cattle, there was a significant decrease in the mean corrected bTB IFN-γ optical density readings for B-C, B-A, A-C, and P-C (Table 3.2, Figures 3.1 & 3.2). A significant decrease or increase was not found in the mean corrected bTB IFN-γ optical density readings among the non-sensitized cows (Table 3.3, Figures 3.1 & 3.2).

In this study, the reduction in the qualitative IFN-γ response at exsanguination decreased the sensitivity of the assay. At exsanguination, the background-corrected OD450 nm (B-C) of all the sensitized cattle were ≥ 0.1 (Figure 3.3), but 3 cows (E, J, and K) had their B-A IFN-γ optical density readings dropped below 0.1 (Figure 3.4). These 3 cows (E, J, and K) were classified as

bTB negative at exsanguination, because each failed to satisfy both conditions for classifying an animal as bTB positive. One cow (D) had an increased background-corrected IFN-γ optical density readings at exsanguination for B-A when compared to the IFN-γ optical density readings produced before stunning (Figures 3.1& 3.2) while two cows (G and H) showed similar increased IFN-γ optical density readings for (B-C and B-A) [Figures 3.1& 3.2].

DISCUSSION

To our knowledge, this study is the first to demonstrate the possibility of obtaining a valid bTB IFN-γ assay interpretation using blood collected during exsanguination at slaughter. Nine (75%) of the bTB sensitized cattle retained their positive classification at exsanguination. This finding supports further development of the IFN-γ assay for POC screening of cattle at slaughter as part of a regional or national bTB eradication program. A potential application of this assay as a POC screening strategy would be to enhance already in place visual slaughter surveillance as a tool to identify bTB suspect herds for subsequent targeted whole herd testing. This strategy would be similar to the one currently used for slaughter surveillance for brucellosis in the United States. Collection of exsanguination blood for the at-slaughter IFN-γ assay screening would be unlikely to introduce a delay in slaughter process as exsanguination blood would be collected alongside blood collection for brucellosis surveillance. If used in parallel with routine visual slaughter surveillance, the at-slaughter IFN-γ assay could enhance detection of bTB infected cattle herds because the assay is capable of detecting bTB infection before the onset of gross lesions (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006; Monaghan *et al.* 1997; Neill *et al.* 1994; Pollock *et al.* 2005; Schiller *et al.* 2010; Wood *et al.* 1991; Wood *et al.* 1992; Wood and Jones 2001; Wood and Rothel 1994). This strategic application of the IFN-γ assay for bTB surveillance would be economical because it could be used to reduce the amount of whole herd testing done using individual animal screening tests (e.g. CFT), thus potentially reducing surveillance costs and likely facilitate bTB control/eradication efforts.

Although blood collected at exsanguination was found capable of producing bovine IFN-γ in response to antigen stimulation, there was a statistically significant decrease in the optical density reading for each stimulant (avian PPD, bovine PPD, and pokeweed mitogen) from before to after stunning. Test interpretation for animals that had comparatively higher IFN-γ assay optical density readings before slaughter was not affected by the decline in optical density readings observed after stunning. In animals that were borderline positive for bTB before slaughter (E, J, and K), the decrease in their IFN-γ assay optical density readings after stunning resulted in their bTB classification changing to negative. For those cattle, the B-A calculation in IFN-γ assay fell below the 0.1 cut off value (Figure 3.4). The B-C calculation also produced a smaller number but the assay remained above the 0.1 value (Figure 3.3). Given the observed drop in IFN-γ response using blood collected at exsanguination, one approach to correct this problem and increase the sensitivity of the IFN-γ assay would be to lower the cut off value to a number that does not compromise the specificity of the assay. As an example, in this study, dropping the cut off value for B-C and B-A to 0.04 would have resulted in the detection of 92% of the sensitized cattle without any false detection of the non-sensitized cows. Prospectively, such reduction in cut off value could decrease the specificity of the assay and would need to be evaluated in future studies.

The observed decline in the IFN-γ response at exsanguination is in agreement with results of a previous study in which samples of blood collected from experimentally infected cattle at 5 minutes following exsanguination showed decreased production of bovine IFN-γ (Rothel *et al.* 1992). Although it was unclear from the report of that study if the drop in IFN-γ production affected the diagnostic classification of the cattle, the authors considered the drop was significant and expressed concern that blood collected post mortem would not be useful for conducting the

bTB IFN-γ assay. However, in that study, blood was collected at 5 or more minutes after the animals were slaughtered compared with 1 to 2 minutes after stunning in the current study. Although the difference between studies in time of blood collection after stunning was small, a few minutes may be critical to the assay. Therefore, it can be inferred that bovine IFN-γ response declines after stunning and time lapse between stunning and blood collection is a critical factor for the IFN-γ assay.

There may be many reasons why the reduction in the IFN-γ response occurred at exsanguination, including a reduction in number of lymphocytes in the blood, or a loss in the lymphocyte function. Previous studies have demonstrated that acute stress results in a significant decrease in absolute number of T cells, B cells, and other cells in the blood (Dhabhar *et al.* 1995; Dhabhar *et al.* 1994; Dhabhar and Mcewen 1997). This decrease in blood leukocyte numbers represents a redistribution of leukocytes from the blood to organs such as the bone marrow and skin (Dhabhar 1998; Dhabhar and Mcewen 1996). The act of stunning, which renders an animal unconscious, could result in a physiological state that mimics acute stress. Since IFN-γ is released by lymphocytes, any decrease in absolute number of lymphocytes would decrease IFN-γ responses as well. It is also possible that stunning could have affected lymphocyte function. Some level of stress may inhibit T cell activation, antigen presentation, and suppress effector cell function (Dhabhar and Mcewen 1997). Another possible factor in the observed decline in the IFN-γ response could be the difference in methods of blood collection. The pre-stunning blood was collected via vacutainer tube from the coccygeal vein/artery while exsanguination blood was free flowing originating from the jugular vein/carotid arteries. Statistically significant differences in white blood cell counts were not found when cattle blood was collected directly into blood tubes from the tail or jugular veins (Sears *et al.* 1978). However, reports were not

found that indicated free flowing blood would affect the blood cell numbers. Understanding the reasons for decreased IFN-γ production in exsanguination blood was outside the scope of this study, but would be useful in developing strategies to improve accuracy if used in slaughtered animals.

Two concerns were identified with the future application of this assay. First, the implication of the decline in IFN-γ response at exsanguination is that a few animals may be missed. The limitation could be addressed by reducing the assay's cut off value for blood collected at exsanguination or by the complementary visual slaughter surveillance which is expected to be used in parallel. Second, the implementation of the at-slaughter IFN-γ assay surveillance could increase the burden on diagnostic laboratories and personnel at harvest facilities. The added burden would need to be offset by decreased costs in other areas of the bTB control program while maintaining or even improving the accuracy of the surveillance program.

The use of sensitized cattle, as opposed to a natural bTB infected cattle, is a limitation of this study. The extent to which the results obtained in this study applies to a natural bTB infected cattle remains to be verified. Cattle challenged with Sensitinogen® and cattle infected naturally with *M. bovis* are known to produce a similar immune responses, which has been exploited in many experiments where a bTB immune response is desired (Buddle *et al.* 1995; Goff 1996; Monaghan *et al.* 1997; Neill *et al.* 1994; Rothel *et al.* 1990; Rothel *et al.* 1992; Waters *et al.* 2006; Wood and Jones 2001).

Future studies should explore factors responsible for the reduction in IFN-γ response at exsanguination, impact of the duration between stunning and exsanguination on IFN-γ response, and the effect of time of blood collection following exsanguination on the IFN-γ response. In addition, it would be useful to understand how the assay would perform with samples of blood

obtained from cattle naturally infected with bTB, and at different stages of the disease progression. Furthermore, it is necessary to conduct a cost analysis of the proposed strategy or alternative strategies in order to determine if there are economic advantages/disadvantages. Filling these knowledge gaps would further determine the usefulness and practicality of the bTB IFN-γ assay at slaughter.

CONCLUSION

Bovine IFN-γ response to bTB present before slaughter in cattle can be detected at exsanguination, when blood is collected within 90 s post stunning. This study supports further development of the IFN-γ assay for POC screening of cattle at slaughter as part of a regional or national bTB eradication. Future application of the IFN-γ assay at slaughter for bTB surveillance could improve upon current bTB surveillance strategies and help support continued eradications efforts in the United States.

FOOTNOTE

[a] Sensitinogen®, USDA APHIS, NVSL, Ames, Iowa

[b] BOVIGAM®, Prionics AG, Zurich Switzerland

[c] PROC TTEST, SAS Institute, Cary, North Carolina

[d] PROC PCORR, SAS Institute, Cary, North Carolina

[e] PROC FREQ AGREE, SAS Institute, Cary, North Carolina

[f] PROC BOXPLOT, SAS Institute, Cary, North Carolina

Table 3.1: Before stunning and at exsanguination bTB IFN-γ categorical results of cattle groups (sensitized with *Mycobacterium bovis* and non-sensitized cows)

		At exsanguination		
		bTB +	bTB -	Total
Before stunning	bTB +	9	3	12
	bTB -	0	3	3
Total		9	6	15

Table 3.2: Corrected bTB IFN-γ response obtained before stunning and at exsanguination of cattle sensitized with *Mycobacterium bovis*

	Before stunning		At exsanguination		
Response	Mean OD450 nm	Std dev	Mean OD450 nm	Std dev	P value
C	0.037	0.012	0.040	0.035	0.831
B-C	1.260	0.822	0.566	0.391	0.015
B-A	0.801	0.543	0.397	0.317	0.022
A-C	0.459	0.308	0.168	0.101	0.009
P-C	2.955	0.914	2.253	0.711	0.010

Table 3.3: Corrected bTB IFN-γ response obtained before stunning and at exsanguination of the non-sensitized cattle

	Before stunning		At exsanguination		
Response	Mean OD450 nm	Std dev	Mean OD450 nm	Std dev	P value
C	0.055	0.033	0.031	0.005	0.361
B-C	0.011	0.031	0.016	0.025	0.734
B-A	-0.146	0.184	-0.043	0.035	0.381
A-C	0.157	0.212	0.059	0.055	0.401
P-C	2.955	0.516	2.193	0.914	0.232

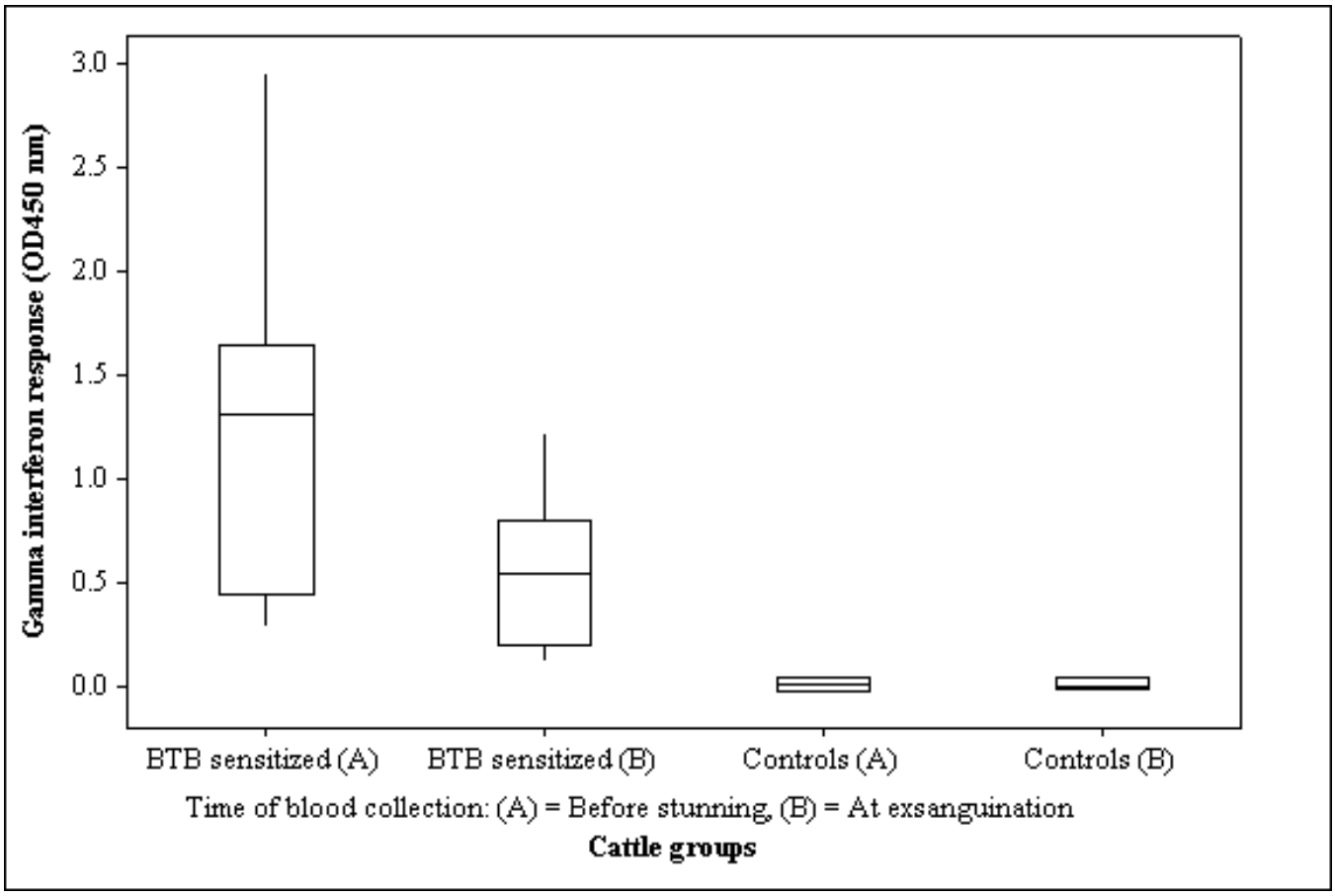


Figure 3.1: Boxplot displaying background-corrected IFN-γ response produced by the *M. bovis* PPD (B-C).

The length of the box represents the interquartile range (the distance between the 25th and 75th percentiles). The horizontal line in the box interior represents the group median. The vertical lines issuing from the box extend to the group minimum and maximum values.

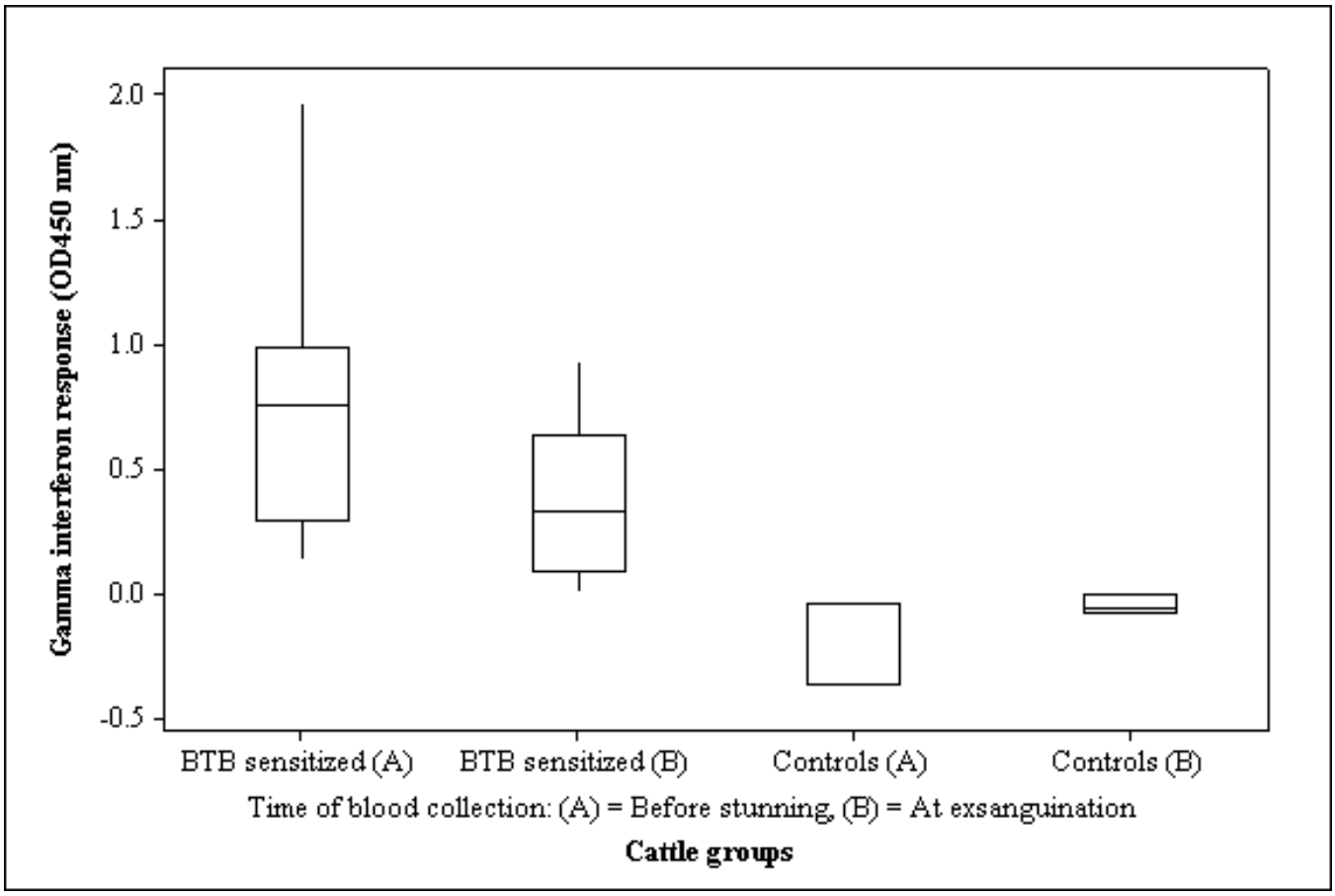


Figure 3.2: Boxplot displaying IFN-γ response of *M. avium* PPD subtracted from *M. bovis* PPD (B-A)

The length of the box represents the interquartile range (the distance between the 25th and 75th percentiles). The horizontal line in the box interior represents the group median. The vertical lines issuing from the box extend to the group minimum and maximum values.

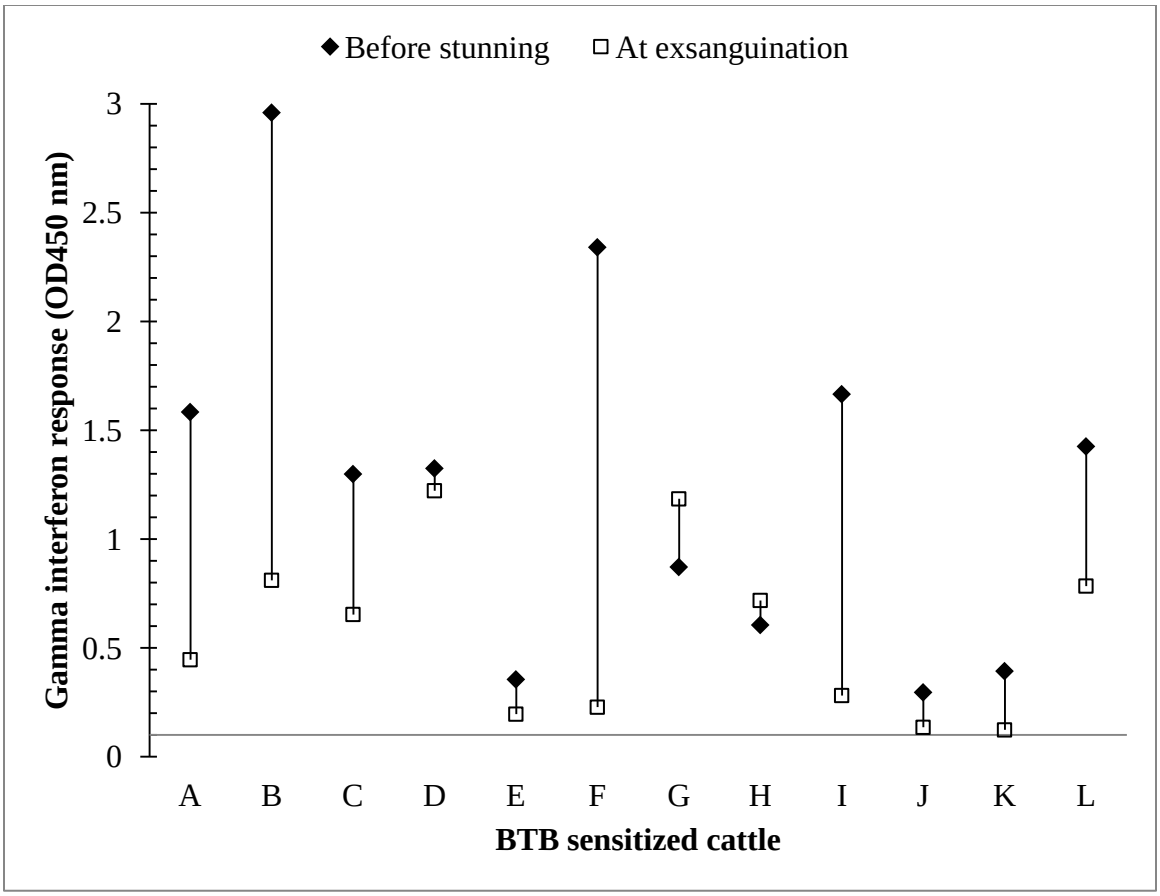


Figure 3.3: *M. bovis* PPD IFN-γ response (B-C) in bTB sensitized cattle

The horizontal line (0.1) represents the cutoff line of the IFN-γ assay. Cattle below this line are classified as negative for bTB.

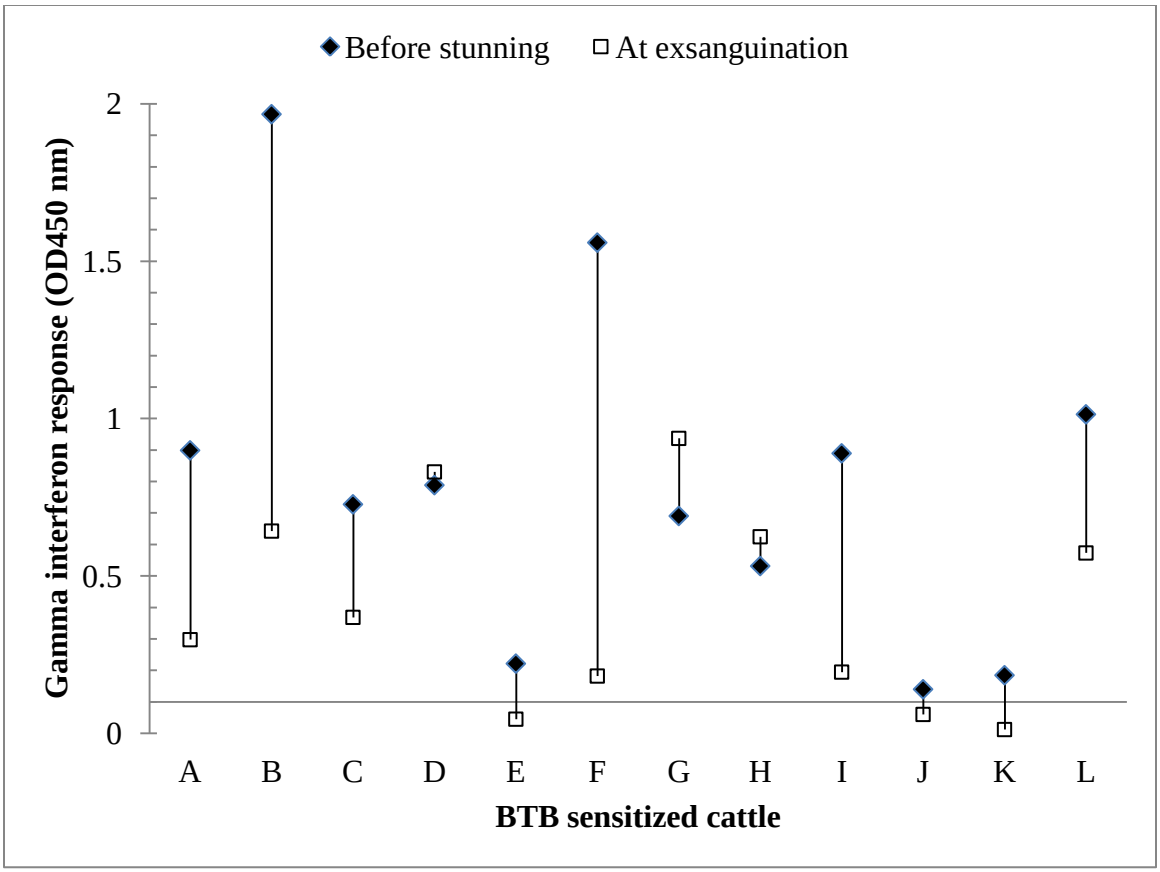


Figure 3.4: Difference of PPD IFN-γ response (B-A) in bTB sensitized cattle

The horizontal line (0.1) represents the cutoff line of the IFN-γ assay. Cattle below this line are classified as negative for bTB.

REFERENCES

REFERENCES

Buddle, B. M., Delisle, G. W., Pfeffer, A., and Aldwell, F. E. (1995). Immunological responses and protection against *Mycobaterium bovis* in calves vaccinated with a low-dose of BCG. *Vaccine* **13**(12), 1123-1130.

Buddle, B. M., Livingstone, P. G., and de Lisle, G. W. (2009). Advances in ante-mortem diagnosis of tuberculosis in cattle. *New Zeal Vet J* **57**(4), 173-180.

de Kantor, I. N., Lobue, P. A., and Thoen, C. O. (2010). Human tuberculosis caused by *Mycobacterium bovis* in the United States, Latin America and the Caribbean. *Int J Tuberc Lung D* **14**(11), 1369-1373.

de la Rua-Domenech, R., Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G., Christiansen, K. H., and Clifton-Hadley, R. S. (2006). Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, [gamma]-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci* **81**(2), 190-210.

Dhabhar, F. S. (1998). Stress-induced enhancement of cell-mediated immunity. *Neuroimmunomodulat* 840, 359-372.

Dhabhar, F. S., and Mcewen, B. S. (1996). Stress-induced enhancement of antigen-specific cell-mediated immunity. *J Immunol* **156**(7), 2608-2615.

Dhabhar, F. S., and Mcewen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. *Brain Behav Immun* **11**(4), 286-306.

Dhabhar, F. S., Miller, A. H., Mcewen, B. S., and Spencer, R. L. (1995). Effects of Stress on Immune Cell Distribution - Dynamics and Hormonal Mechanisms. *J Immunol* **154**(10), 5511-5527.

Dhabhar, F. S., Miller, A. H., Stein, M., Mcewen, B. S., and Spencer, R. L. (1994). Diurnal and Acute Stress-Induced Changes in Distribution of Peripheral-Blood Leukocyte Subpopulations. *Brain Behav Immun* **8**(1), 66-79.

Ebel, E. D., Williams, M. S., and Tomlinson, S. M. (2008). Estimating herd prevalence of bovine brucellosis in 46 U.S.A. states using slaughter surveillance. *Prev Vet Med* **85**(3-4), 295-316.

Goff, B. S. L. (1996). Effect of dexamethasone treatment of tuberculous cattle on results of the gamma-interferon test for *Mycobacterium bovis*. *Veterinary Immunol Immunop* **53**(1-2), 39-47.

Lobue, P. A., Enarson, D. A., and Thoen, C. O. (2010). Tuberculosis in humans and animals: an overview. *Int J Tuberc Lung D* **14**(9), 1075-1078.

Michel, A. L., Mnller, B., and van Helden, P. D. (2010). *Mycobacterium bovis* at the animal-human interface: A problem, or not? *Vet Microbiol* **140**(3-4), 371-381.

Monaghan, M., Quinn, P. J., Kelly, A. P., McGill, K., McMurray, C., OCrowley, K., Bassett, H. F., Costello, E., Quigley, F., Rothel, J. S., Wood, P. R., and Collins, J. D. (1997). A pilot trial to evaluate the gamma-interferon assay for the detection of *Mycobacterium bovis* infected cattle under Irish conditions. *Irish Vet J* **50**(4), 229-232.

Neill, S. D., Cassidy, J., Hanna, J., Mackie, D. P., Pollock, J. M., Clements, A., Walton, E., and Bryson, D. G. (1994). Detection of *Mycobaterium bovis*Infection in Skin Test-Negative Cattle with An Assay for Bovine Interferon-Gamma. *Vet Rec* **135**(6), 134-135.

Okafor, C. C., Grooms, D. L., Bruning-Fann, C. S., Averill, J. J., and Kaneene, J. B. (2011). Descriptive Epidemiology of Bovine Tuberculosis in Michigan (1975-2010); Lessons Learned. *Veterinary Medicine International* 2011:874924.

Pollock, J. M., Welsh, M. D., and McNair, J. (2005). Immune responses in bovine tuberculosis: Towards new strategies for the diagnosis and control of disease. *Veterinary Immunol Immunop* **108**(1-2), 37-43.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1990). A Sandwich Enzyme-Immunoassay for Bovine Interferon-Gamma and Its Use for the Detection of Tuberculosis in Cattle. *Aust Vet J* **67**(4), 134-137.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1992). The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle - Conditions affecting the production of gamma-interferon in whole-blood culture. *Aust Vet J* **69**(1), 1-4.

SAS Institute Inc. SAS/STAT user's guide: statistics, version 9.2 edition. Cary, North Carolina. 2009.

Schiller, I., Oesch, B., Vordermeier, H. M., Palmer, M. V., Harris, B. N., Orloski, K. A., Buddle, B. M., Thacker, T. C., Lyashchenko, K. P., and Waters, W. R. (2010). Bovine Tuberculosis: A

Review of Current and Emerging Diagnostic Techniques in View of their Relevance for Disease Control and Eradication. *Transbound Emerg Dis* **57**(4), 205-220.

Sears, P. M., Paape, M. J., Pearson, R. E., and Gwazdauskas, F. C. (1978). Comparison Between Tail Vein and Jugular Vein Cannulation in Cattle. *Journal of Dairy Science* **61**(7), 974-979.

StatTools Web site. Programs for sample size estimation, McNemar test. http://www.stattools.net/SSizMcNemar_Pgm.php . *Accessed January10, 2011*.

Thoen, C. O., Lobue, P. A., and de Kantor, I. (2010). Why has zoonotic tuberculosis not received much attention? *Int J Tuberc Lung D* **14**(9), 1073-1074.

Thoen, C. O., Lobue, P. A., Enarson, D. A., Kaneene, J. B., and de Kantor, I. N. (2009). Tuberculosis: a re-emerging disease in animals and humans. *Veter Ital Ser* **45**(1), 135-181.

Waters, W. R., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Vordermeier, H. M., Hewinson, R. G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J. M., Andersen, P., and Lyashchenko, K. P. (2006). Early Antibody Responses to Experimental *Mycobacterium bovis* Infection of Cattle. *Clin Vaccine Immunol* **13**(6), 648-654.

Wood, P. R., Corner, L. A., Rothel, J. S., Baldock, C., Jones, S. L., Cousins, D. B., Mccormick, B. S., Francis, B. R., Creeper, J., and Tweddle, N. E. (1991). Field Comparison of the Interferon-Gamma Assay and the Intradermal Tuberculin Test for the Diagnosis of Bovine Tuberculosis. *Aust Vet J* **68**(9), 286-290.

Wood, P. R., Corner, L. A., Rothel, J. S., Ripper, J. L., Fifis, T., Mccormick, B. S., Francis, B., Melville, L., Small, K., De Witte, K., Tolson, J., Ryan, T. J., de Lisle, G. W., Cox, J. C., and Jones, S. L. (1992). A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. *Vet Microbiol* **31**(1), 71-79.

Wood, P. R., and Jones, S. L. (2001). BOVIGAM (TM): an in vitro cellular diagnostic test for bovine tuberculosis. *Tuberculosis* **81**(1-2), 147-155.

Wood, P. R., and Rothel, J. S. (1994). In vitro immunodiagnostic assays for bovine tuberculosis. *Vet Microbiol* **40**(1-2), 125-135.

CHAPTER 4

FACTORS THAT CAN AFFECT MEASURABLE INTERFERON-GAMMA PRODUCTION IN CATTLE AT TIME OF SLAUGHTER

ABSTRACT

The objective of this study was to determine if time of blood collection and total lymphocyte count influence measurable reduction in interferon-gamma (IFN-γ) production during exsanguination of cattle at slaughter. Five adult cull Holstein cows and five Angus steers were used in two separate trials. In trial 1, four Holstein cows were experimentally sensitized to bTB with inactivated *Mycobacterium bovis* (*M. bovis)* in mineral oil [Sensitinogen®, USDA, APHIS, NVSL], creating an immune response that mimics a natural *M. bovis* infection. One Holstein cow was injected with mineral oil only and served as a negative control. After blood from the sensitized cows became reactive against tuberculin using bTB IFN-γ assay (BOVIGAM®, Prionics AG, Zurich Switzerland), all five cows were scheduled for slaughter. The bTB IFN-γ assay was performed on blood collected at the housing facility of the cattle, after a 30 minute truck ride to the slaughter facility, immediately before stunning, at commencement of exsanguination, and at 5 minutes after exsanguination commenced. In trial 2, five Angus steers were treated as in Trial 1 and initial sensitization protocol was identical to trial 1. IFN-γ assay responses to *M. bovis* and *Mycobacterium avium (M. avium),* and total lymphocyte count were evaluated on blood collected at the housing facility of the cattle, at commencement of exsanguination, and at 2 successive one minute intervals. Results indicated that the decline in IFN-γ production observed following exsanguination was in parallel with the corresponding

decline in total lymphocyte count. At 5 minutes post commencement of exsanguination, all previously positive cattle were negative in the IFN-γ assay. This study demonstrates that production of bTB IFN-γ response declines in blood collected at later time points following exsanguination, emphasizing the importance of immediate blood collection to reduce the risk of false negative results. The decline in lymphocyte count following exsanguination could be responsible for the corresponding decline in IFN-γ production. These findings provide useful information necessary for making changes to either the stimulation process or the interpretation of bTB IFN-γ assay on blood collected during exsanguination.

INTRODUCTION

Mycobacterium bovis (M. bovis), the causative agent of bovine tuberculosis (bTB), is one of the most important zoonotic pathogen causing public health issues globally (de Kantor *et al.* 2010; Lobue *et al.* 2010; Michel *et al.* 2010; Thoen *et al.* 2009; Thoen *et al.* 2010). bTB remains a disease of significant economic importance in affected countries due to its impact on livestock trade and resources expended on its eradication (Michel *et al.* 2010; Okafor *et al.* 2011b). Recent challenges to the global economy could result in a decreased budget allocation to bTB surveillance and would hinder its eradication efforts (Okafor *et al.* 2011a). In such economic situations, it is important to set priorities in the protection of both animal and public health and to allocate resources effectively and efficiently (Stark *et al.* 2006). Surveillance of cattle for bTB at point-of-concentration (POC), such as slaughter facilities, would be less costly than on-farm testing. In addition, a surveillance strategy focused on POC testing could aid in identification of early stage infections before infection spreads between animals.

The bTB interferon-gamma (IFN-γ) assay is an internationally approved screening assay which measures IFN-γ released by lymphocytes in response to antigen stimulation (Rothel *et al.* 1990). In a previous study, Okafor et al (Okafor *et al.* 2011a) demonstrated that cattle that were bTB IFN-γ assay positive before slaughter remained positive at exsanguination. However, blood collected during exsanguination has been reported to have a decreased bTB IFN-γ production upon antigen stimulation (Okafor *et al.* 2011a; Rothel *et al.* 1992). The factors associated with this measurable decline in IFN-γ production from whole blood are not known. Furthermore, the rate of measurable decline in IFN-γ production from whole blood after exsanguination is not

known. One critical factor could be a decrease in total lymphocyte count in blood at the time of exsanguination, which would result in decreased bTB IFN-γ production.

The objective of this study was to determine if time of blood collection and reduction in total lymphocyte count influence the reduction in IFN-γ production during exsanguination of cattle at slaughter. We hypothesized that in cattle immunologically challenged with inactivated *M. bovis*, (1) the bTB IFN-γ production following antigen stimulation would decline with time after the commencement of exsanguination; (2) that the total lymphocyte count correlates with the IFN-γ response produced. An understanding of these factors would support the development of an optimal use of bTB IFN-γ assay for POC screening of cattle for bTB at slaughter.

MATERIALS AND METHODS

Animals

Five clinically healthy, non-pregnant adult Holstein cows (ages 2-3 yrs) and five clinically healthy Angus steers (ages 1.5 yrs) were used in trial 1 and trial 2 respectively. All study subjects were negative in the caudal fold tuberculin test for bTB within the previous year and were negative in the bTB IFN-γ assay immediately before the study started. With the exception of one animal, all cattle were negative for antibody against *Mycobacterium avium* subsp. *paratuberculosis* (MAP) as determined by serum ELISA, and negative for the presence of culturable MAP in feces. One animal was ELISA positive for antibody against MAP and fecal culture negative for MAP. That animal was used as a control in trial 2. The cattle were housed at the Michigan State University (MSU) Beef Cattle Teaching and Research Center for the duration of the study.

Study design

This study was approved by the Michigan State University Institutional Animal Care and Use Committee (AUF # 08/09-125-00). Two separate trials were conducted. In trial 1, after a one week acclimation period, four of the Holstein cows were randomly selected and injected subcutaneously in the ventral brisket with 0.1 ml of inactivated *M. bovis* in mineral oil[a]. The remaining Holstein cow served as a non-sensitized control and was injected with 0.1 ml of mineral oil administered subcutaneously in the ventral brisket. At 45 days post sensitization, blood was drawn from the jugular vein and assayed for a bTB IFN-γ production. It was determined that all sensitized cows were bTB IFN-γ assay positive using the cut-off established

by the USDA; the non-sensitized cow remained bTB IFN-γ assay negative. Afterwards, all cows were scheduled for slaughter at the MSU Department of Animal Science Meat Science Laboratory. Blood samples for bTB IFN-γ assay were collected from each animal at the housing facility (W), after 30 minutes of trucking the cows to the slaughter facility (X), at approximately 15 hrs post trucking and immediately before stunning (Y), at commencement of exsanguination(Z0), and at 5 minutes post exsanguination commencement (Z5). All blood samples (6-8 ml) were collected from live cattle with a 20g vacutainer needle into a 10 ml vacutainer tube containing sodium heparin. At the housing facility, blood was collected from the jugular vein. At the slaughter facility, samples of blood were collected from the coccygeal vein/artery of live cattle. Cows were stunned by captive bolt and exsanguinated, using the slaughter facility's standard operating protocols. Free flowing blood was collected immediately after the jugular and carotid arteries were severed. The time from stunning to the first blood collection at exsanguination ranged from 60 to 90 s. The time from stunning to collection of blood ranged from 60 to 90 s. A second sample of blood was collected 5 minutes after exsanguination commenced. Exsanguination blood samples were collected into a 10 ml syringe with the plunger removed. The syringe plunger was then replaced and blood immediately transferred with an 18g needle into two 10 ml vacutainer tubes containing sodium heparin. All blood samples were placed in a cooler and delivered to the Michigan State University Diagnostic Center for Population and Animal Health (DCPAH) within one hour of collection.

Five Angus steers were used in trial 2. The sensitization protocol described for trial 1 was used. After the steers were scheduled for slaughter, blood was collected at the following time points: at the housing facility (W), at commencement of exsanguination (Z0), at 1 minute post commencement of exsanguination (Z1), and at 2 minutes post commencement of

exsanguination (Z2). In addition to performing bTB IFN-γ assay on the blood samples, total lymphocyte count was conducted. The blood sample (3-5 ml) for the blood count was collected into a 7 ml vacutainer tube containing ethylenediaminetetraacetic acid (EDTA). Within one hour of sample collection, the IFN- γ assay was started and total lymphocyte count was performed at the DCPAH.

Sample testing

The IFN-γ assay was conducted at DCPAH using a commercially available antigen-capture ELISA[b], according to the manufacturer's recommended protocol was detailed in a previous study (Okafor *et al.* 2011a). Briefly, 1.5 ml aliquots of each blood sample were dispensed to each of 4 wells of a 24-well tissue culture plate. The wells contained 100µl of sterile phosphate buffered saline (negative antigen control), 100µl *Mycobacterium avium* purified protein derivative (avian PPD stimulation antigen, Prionics USA Inc., La Vista, NE), 100µl *Mycobacterium bovis* purified protein derivative (bovine PPD stimulation antigen, Prionics USA Inc., La Vista NE), or 16 µg pokeweed mitogen as a positive antigen stimulant (Lectin from *Phytolacca americana*, Sigma Life Science, St Louis, MO). Following incubation for 16-24 h at 38^0C (100.4^0F) in a humidified atmosphere, plates were centrifuged at 1730 rcf for 10 minutes and the plasma harvested. Each plasma sample was then assayed in duplicate for IFN-γ production by ELISA. Optical density measured at 450 nm (OD450 nm) was used to detect IFN-γ production in each culture plate. Results were expressed as the background-corrected OD450 nm with the no-antigen control (C) subtracted from both bovine PPD (B*)* and avian PPD (A) IFN-γ responses. Pokeweed mitogen (P) was used to confirm lymphocyte functionality for production of an IFN-γ response. Before an assay result is considered valid, P –

C ≥ 0.1 must be met, otherwise the result is reported as non viable. An animal was considered positive in the bTB IFN-γ assay if the difference between OD450 nm readings of the B and the C wells (B-C), and the difference between the B and the A wells (B-A) were both ≥ 0.1; if either difference (B-C) or (B-A) was < 0.1, the animal was considered negative in the bTB IFN-γ assay.

Total lymphocyte count was measured by an ADVIA® 120 hematology system[c]. Lymphocyte count was derived from the automated differential values when routine acceptance criteria were met; otherwise 100-cell microscopic differential count was performed manually by board certified clinical pathologists.

Statistical analyses

In both trials 1 and 2, descriptive statistics was performed on the data. On each animal in trial 1, both the categorical interpretation of the IFN-γ assay and the background-corrected ELISA OD values were reported for the 5 measured points: W, X, Y, Z0, and Z5. At the 4 measured points in trial 2 (W, Z0, Z1, and Z2), categorical interpretation of the IFN-γ assay, the background-corrected ELISA OD values, and the total lymphocyte count were reported on each animal. Changes in the IFN-γ productions and the hematologic evaluations were represented diagrammatically using gplot[d] procedure as contained in SAS software (SAS Institute Inc 2009).

RESULTS

In trial 1, the interpretation of bTB IFN-γ assay results at each time point were as follows: (W) all 4 sensitized cows were bTB IFN-γ assay positive; (X) 3 cows (75%) remained bTB IFN-γ assay positive; (Y) all 4 sensitized cows were again bTB IFN-γ assay positive; (Z0) 2 cows (50%) remained bTB IFN-γ assay positive; and (Z5) all 4 sensitized cows were bTB IFN-γ assay negative. The non-sensitized cow remained bTB IFN-γ assay negative throughout the trial. These results are summarized in Table 4.1. The values of B-C and B-A started high at time point W, declined at X, increased at Y, declined again at Z0 and the values fell below cut-off line for the assay at Z5 (Table 4.2). Graphical representations of the bovine and avian IFN-γ productions are presented in Figures 4.1a and 4.1b respectively.

In trial 2, the interpretation of bTB IFN-γ assay results at each time point were as follows: (W) all 4 sensitized animals were bTB IFN-γ assay positive. Subsequently 2 animals (50%) remained bTB IFN-γ assay positive at (Z0), (Z1), and (Z2). The non-sensitized cow remained bTB IFN-γ assay negative throughout the trial. These results are summarized in Table 4.3. In comparison to the commencement of exsanguination (Z0), the values of B-C, total lymphocyte count declined at Z1 and further declined at Z2 (Figures 4.2a-d). The values of B-C, B-A, and total lymphocyte count are reported in Table 4 4.

DISCUSSION

This study demonstrates that time of blood collection after exsanguination, is critical to obtaining reliable results from the bTB IFN-γ assay. Based on our results, IFN-γ production declines with time following exsanguination. At 5 minutes post commencement of exsanguination, all previously positive cattle were negative in the IFN-γ assay, using established interpretation criteria. The decline in measurable IFN-γ production in response to antigen stimulation following exsanguination is in agreement with the results of previous similar studies (Okafor *et al.* 2011a; Rothel *et al.* 1992). The findings of this study highlight the need for timely collection of blood post exsanguination if bTB IFN-γ assay is to be implemented as part of a bTB surveillance program at slaughter.

Trucking of cattle for 30 minutes resulted in a transient drop in the IFN-γ productions. However, after an overnight rest, the corrected ELISA values for measurable IFN-γ were similar with those obtained on the housing facility. The decline in IFN-γ production following trucking could be due to a transport-associated transient suppression of lymphocyte count and functions. Although not measured in this trial, lymphocyte count and functions have been shown to be transiently suppressed after transport of cattle (Stanger *et al.* 2005). Additionally, short-term transportation (approximately 45 min) of cattle has been reported to induce reduction in lymphocyte count (Odore *et al.* 2011).

Decline in total lymphocyte count could be responsible for a corresponding decline in the IFN-γ responses to bTB assay. It was observed that between collection of blood before stunning and at exsanguination as well as with each passing minute post exsanguination, there was a parallel decline in total lymphocyte count. Since IFN-γ is released by lymphocytes (Odore *et al.*

2011), it is likely that a decrease in absolute lymphocyte count would cause a decline in a specific stimulated IFN-γ production as well. The factors responsible for the declining lymphocyte counts were not studied. One possible explanation is the accumulation and adhesion of leukocytes to the epithelial cells of blood vessel and other tissues at the site of injury in the early phases of inflammation, also called 'margination'. Another possible explanation for declining lymphocyte count at exsanguination could be stress. Stress associated with a 45 minute transportation of cattle has been found to induce reduction in lymphocytes (Odore *et al.* 2011). Similarly, an hour of acute stress has been demonstrated to result in a significant decrease in absolute number of leukocytes in rats (Dhabhar *et al.* 1995; Dhabhar *et al.* 1994; Dhabhar and Mcewen 1997). This decrease in blood leukocyte numbers represents a redistribution of leukocytes from the blood to organs such as the bone marrow and skin of rats (Dhabhar 1998; Dhabhar and Mcewen 1996). Assuming stunning causes similar lymphocyte redistribution as seen in short-term transported cattle or in acutely stressed rats, any decrease in absolute number of lymphocytes would decrease IFN-γ productions as well since IFN-γ is released by lymphocytes. It is also possible that stunning could have affected lymphocyte function as was observed after 72 hour transportation of cattle (Stanger *et al.* 2005).

The criteria for classification of an animal as positive or negative for bTB as used in this study contributed to the number of animals reported as negative for bTB at the various time points in the trials. In a few animals whose IFN-γ assay results were thought to be out of sync with the trend observed in most animals in this study, repeat tests on saved frozen samples were performed 16 months after the initial test and the values of the IFN-γ productions were similar. Although the goal of this study was not to measure sensitivity or specificity, a change in the cut-off value for B-A would increase the number of sensitized animals reported as positive for IFN-γ

at the measured points. As an example, if the criteria for interpretation of an animal as IFN-γ positive for bTB was that B-C be ≥ 0.1 and B-A be ≥ 0.05, animal 1b would be IFN-γ positive for bTB at both X and Z0. With this criterion, animals 1d, 2b, and 2c would be IFN-γ positive for bTB at Z5, Z1, and Z0 respectively. In all of these, the non-sensitized cattle would never be classified as IFN-γ positive for bTB.

Useful data was obtained in this study, although the study was limited in terms of number of cattle used and time points chosen for collection of samples of blood for testing. First, it was shown that under experimental conditions the interpretation of the IFN-γ likely would not change from before slaughter for most cattle if blood used for the IFN-γ assay is obtained immediately after commencement of exsanguination. Second, the number of total lymphocytes in flowing blood drops quickly after the exsanguination process commences, which likely contributes to the drop in IFN-γ production observed with blood obtained at various time points during exsanguination. Finally, a relatively short period of transportation was sufficient to cause a reduction in IFN-γ production when blood obtained after transport was compared with blood obtained immediately before transport. This last finding is important should the IFN-γ assay be used to test cattle for bTB at POC. Based on these findings, specific protocols of the assay could be reconsidered and tested to improve the reliability of bTB IFN-γ assay for adoption as a surveillance tool at slaughter. For example, to compensate for the decline in lymphocyte count, an adjusted ELISA OD values cut-off limit for categorizing animals as either bTB positive or negative could be developed for blood collected at exsanguination. To understand the most appropriate cut-off limit, more number of animals would be useful and the use of cattle under natural conditions of *M. bovis* exposure would be valuable.

CONCLUSION

This study provides valuable information necessary for adaption bTB IFN-γ assay to bTB slaughter surveillance. It demonstrates that production of IFN-γ declines in blood collected at later time points following exsanguination emphasizing the importance of immediate blood collection to reduce the risk of false negative results. The decline in total lymphocyte count following exsanguination could be responsible for the corresponding decline in IFN-γ production. Developing specific sample collection protocols to compensate for the diminished IFN-γ production, and further evaluation of the cut off points used to interpret the IFN-γ assay, would facilitate the integration of the IFN-γ assay into bTB slaughter surveillance.

Table 4.1: bTB IFN- γ assay categorical results of cattle at different time points

		Time of blood collection				
Animal Id	**Condition**	**W**	**X**	**Y**	**Z0**	**Z5**
1a	Sensitized	+	+	+	+	-
1b	Sensitized	+	-	+	-	-
1c	Sensitized	+	+	+	-	-
1d	Sensitized	+	+	+	+	-
1e	Non-sensitized	-	-	-	-	-

W = at the hosing facility of the cattle, X = after 30-minute trucking, Y = immediately before stunning (~15 hrs post trucking), Z0 = at commencement of exsanguination, Z5 = 5 minutes post commencement of exsanguination.

Table 4.2: IFN-γ production results in cattle at different time points

Animal Id	Condition	Time of blood collection				
		W	X	Y	Z0	Z5
B-C						
1a	Sensitized	0.840	0.535	1.666	0.282	0.123
1b	Sensitized	0.409	0.129	0.296	0.136	0.091
1c	Sensitized	0.563	0.638	0.394	0.124	0.015
1d	Sensitized	1.119	1.265	1.426	0.785	0.122
1e	Non-sensitized	0.009	0.000	-0.012	0.006	*
B-A						
1a	Sensitized	0.537	0.336	0.890	0.195	0.006
1b	Sensitized	0.211	0.084	0.140	0.060	0.050
1c	Sensitized	0.324	0.387	0.185	0.012	0.005
1d	Sensitized	0.852	1.015	1.014	0.573	0.084
1e	Non-sensitized	-0.036	-0.012	-0.038	-0.005	*

W = at the hosing facility of the cattle, X = after 30-minute trucking, Y = immediately before stunning (~15 hrs post trucking), Z0 = at commencement of exsanguination, Z5 = 5 minutes post commencement of exsanguination, * (value was not collected).

Table 4.3: bTB IFN-γ assay categorical results of cattle at different time points

		Time of blood collection			
Animal Id	**Condition**	**W**	**Z0**	**Z1**	**Z2**
2a	Sensitized	+	+	+	+
2b	Sensitized	+	+	-	-
2c	Sensitized	+	-	-	-
2d	Sensitized	+	-	+	+
2e	Non-sensitized	-	-	-	-

W = at the housing facility of cattle, Z0 = at commencement of exsanguination, Z1 = 1 minute post commencement of exsanguination, Z2 = 2 minutes post commencement of exsanguination.

Table 4.4: IFN-γ productions and total lymphocyte count in cattle at different time points

Animal Id	Condition	Time of blood collection			
		W	**Z0**	**Z1**	**Z2**
		B-C			
2a	Sensitized	0.207	0.924	0.823	0.543
2b	Sensitized	0.262	0.322	0.141	0.056
2c	Sensitized	0.628	0.184	0.016	0.061
2d	Sensitized	0.142	0.990	0.802	0.510
2e	Non-sensitized	0.366	0.285	0.015	0.026
		B-A			
2a	Sensitized	0.070	0.244	0.538	0.253
2b	Sensitized	0.121	0.133	0.061	0.046
2c	Sensitized	0.154	0.077	-0.013	-0.083
2d	Sensitized	0.102	-0.133	0.462	0.207
2e	Non-sensitized	-1.194	-0.205	-0.052	-0.127
		Total lymphocyte count[†]			
2a	Sensitized	4.45	4.46	3.64	3.21
2b	Sensitized	7.23	6.24	4.33	2.94
2c	Sensitized	4.53	3.93	*	1.94
2d	Sensitized	4.45	3.69	3.34	3.31
2e	Non-sensitized	4.58	4.00	2.29	2.56

W = at the housing facility of cattle, Z0 = at commencement of exsanguination, Z1 = 1 minute post commencement of exsanguination, Z2 = 2 minutes post commencement of exsanguination, * (value was not collected), [†] (n x 10^3/uL), normal reference range for lymphocyte is [1.13-4.44] x 10^3/uL.

(a)

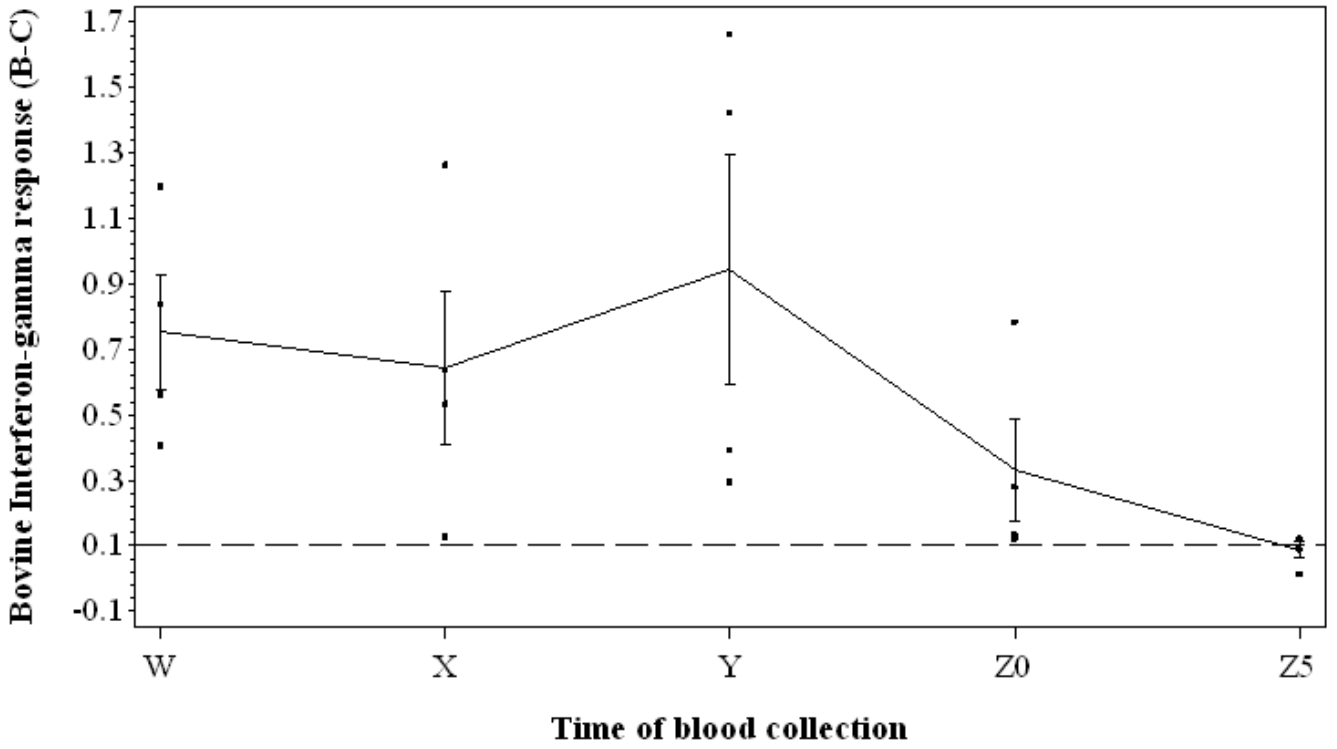
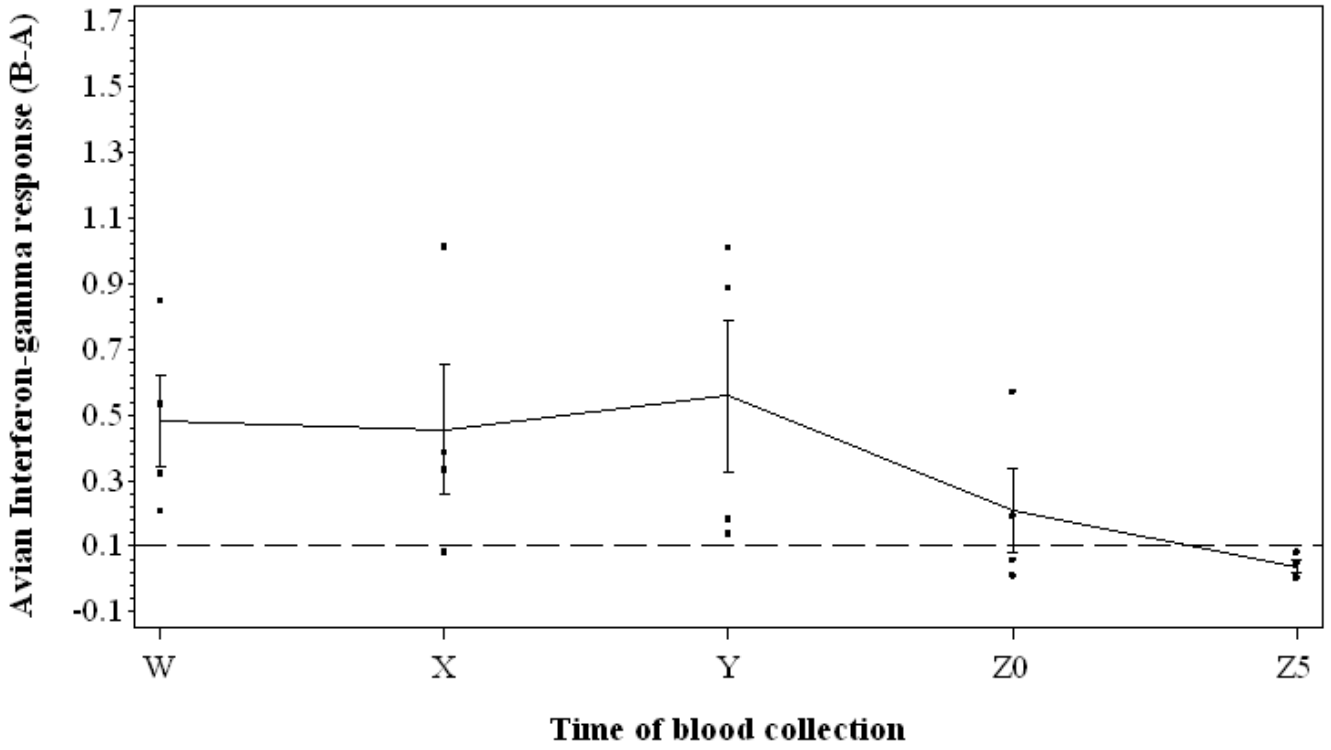


Figure 4.1: IFN-γ response to *M. bovis* (a) and to *M. avium* (b) PPD stimulation in 4 cattle sensitized with killed *M. bovis*

W = at the farm, X = after 30-minute trucking, Y = immediately before stunning (~15 hrs post trucking), Z0 = at commencement of exsanguination, Z5 = 5 minutes post commencement of exsanguination. Dot = each animal's IFN-γ production. Line crosses each bar at the group mean. Bar = 1 standard deviation from the group mean. Broken line = 0.1 cut-off line above which an animal is classification as positive for bTB and negative if below

Figure 4.1 (cont'd)

(b)



(c)

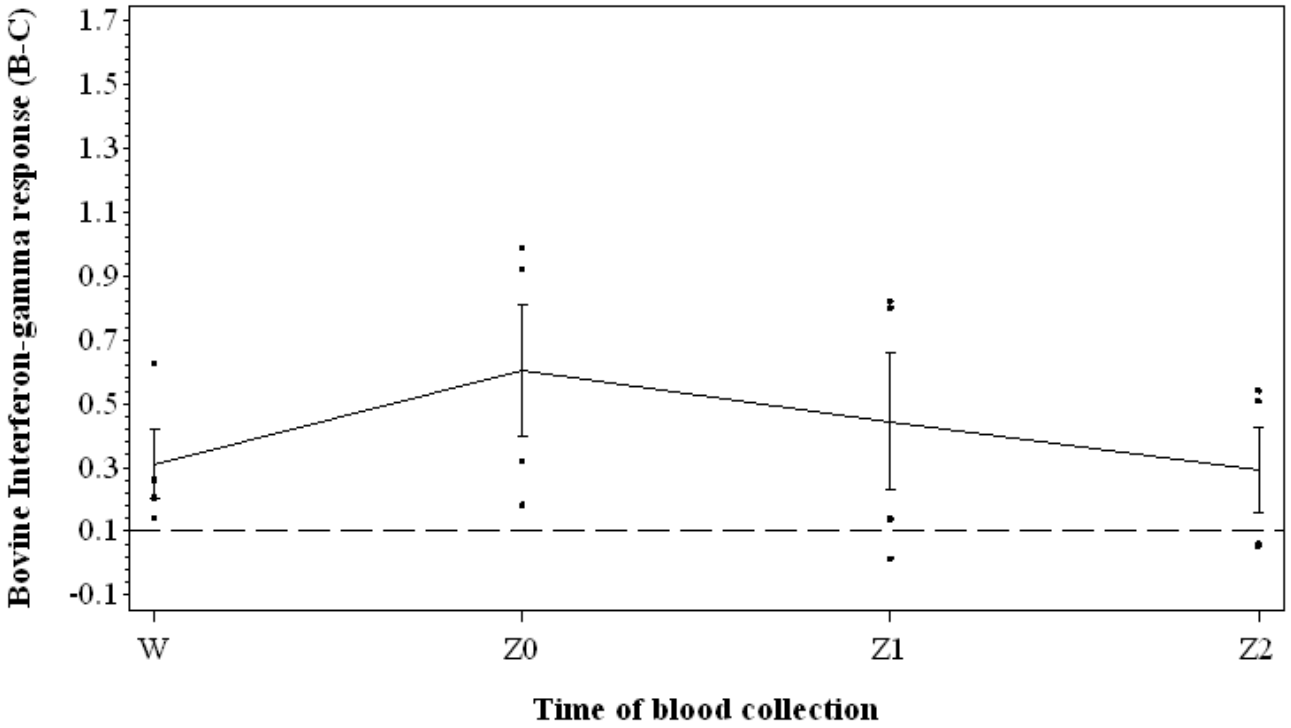


Figure 4.2: IFN- γ response to *M. bovis* (c) and to *M. avium* (d) PPD stimulation, and total lymphocyte count (e) in 4 cattle sensitized with killed *M. bovis*
W = at the farm, Z0 = at commencement of exsanguination, Z1 = 1 minute post commencement of exsanguination, Z2 = 2 minutes post commencement of exsanguination, Dot = each animal's value.
Line crosses each bar at the group mean. Bar = 1 standard deviation from the group mean.
Broken line = 0.1 cut-off line above which an animal is classification as positive and classified as negative if otherwise.

Figure 4.2 (cont'd)

(d)

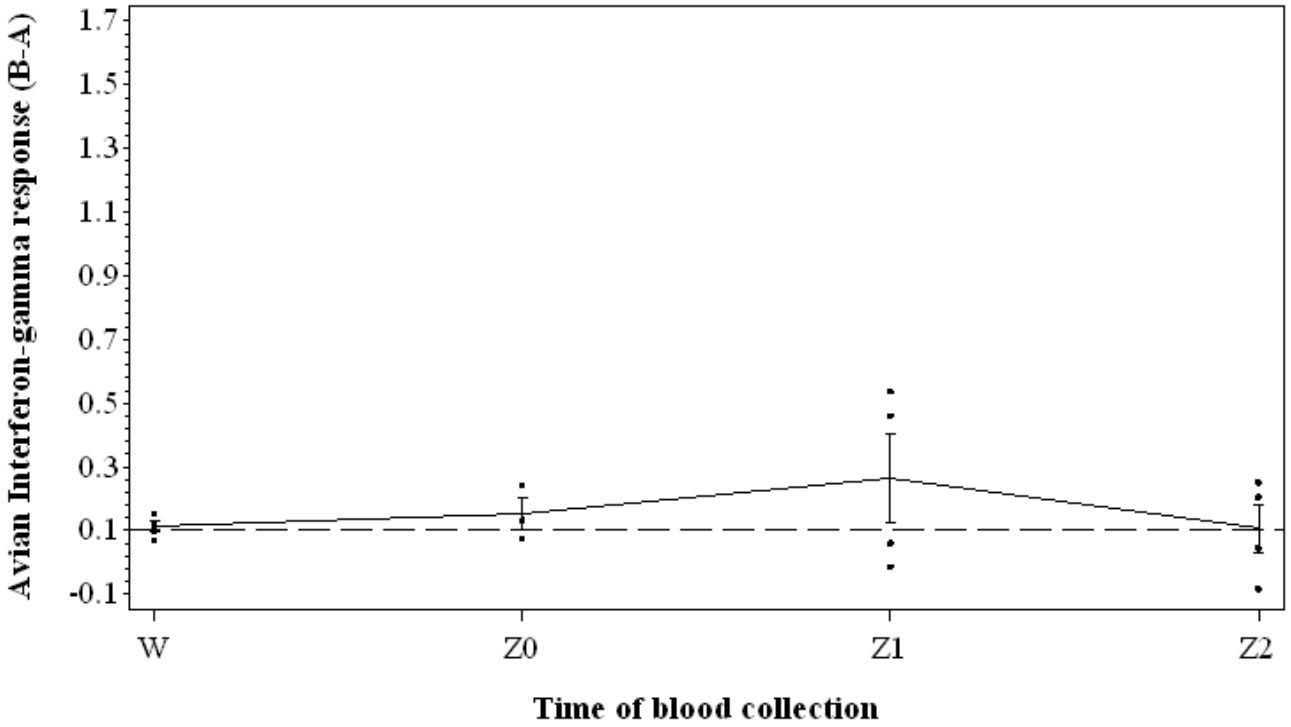
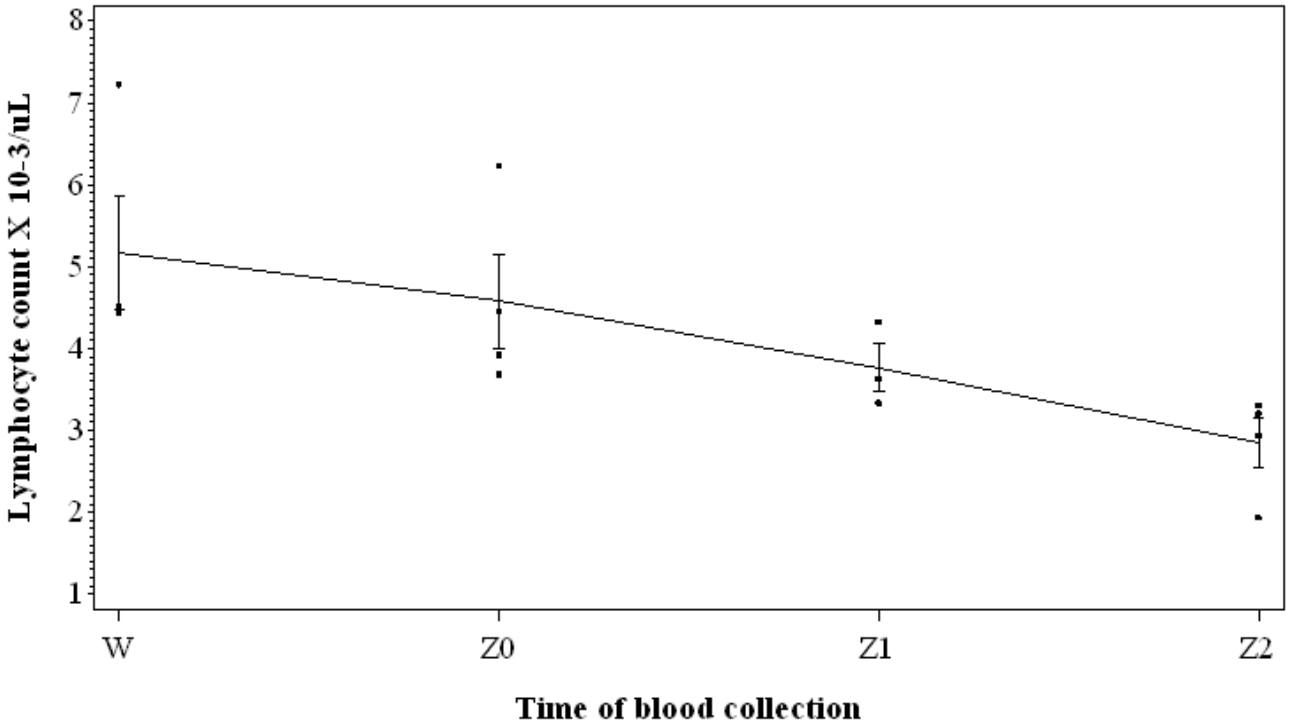


Figure 4.2 (cont'd)

(e)



FOOTNOTES

[a] Sensitinogen®, USDA APHIS, NVSL, Ames, Iowa

[b] BOVIGAM®, Prionics AG, Zurich Switzerland

[c] ADVIA®, Siemens Healthcare Diagnostics Inc., Tarrytown, New York

[d] PROC GPLOT, SAS Institute, Cary, North Carolina

REFERENCES

REFERENCES

de Kantor, I. N., Lobue, P. A., and Thoen, C. O. (2010). Human tuberculosis caused by *Mycobacterium bovis* in the United States, Latin America and the Caribbean. *Int J Tuberc Lung D* **14**(11), 1369-1373.

Dhabhar, F. S. (1998). Stress-induced enhancement of cell-mediated immunity. *Neuroimmunomodulat* **840**, 359-372.

Dhabhar, F. S., and Mcewen, B. S. (1996). Stress-induced enhancement of antigen-specific cell-mediated immunity. *J Immunol* **156**(7), 2608-2615.

Dhabhar, F. S., and Mcewen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. *Brain Behav Immun* **11**(4), 286-306.

Dhabhar, F. S., Miller, A. H., Mcewen, B. S., and Spencer, R. L. (1995). Effects of Stress on Immune Cell Distribution - Dynamics and Hormonal Mechanisms. *J Immunol* **154**(10), 5511-5527.

Dhabhar, F. S., Miller, A. H., Stein, M., Mcewen, B. S., and Spencer, R. L. (1994). Diurnal and Acute Stress-Induced Changes in Distribution of Peripheral-Blood Leukocyte Subpopulations. *Brain Behav Immun* **8**(1), 66-79.

Lobue, P. A., Enarson, D. A., and Thoen, C. O. (2010). Tuberculosis in humans and animals: an overview. *Int J Tuberc Lung D* **14**(9), 1075-1078.

Michel, A. L., Mnller, B., and van Helden, P. D. (2010). *Mycobacterium bovis* at the animal-human interface: A problem, or not? *Vet Microbiol* **140**(3-4), 371-381.

Odore, R., Badino, P., Re, G., Barbero, R., Cuniberti, B., D'Angelo, A., Girardi, C., Fraccaro, E., and Tarantola, M. (2011). Effects of housing and short-term transportation on hormone and lymphocyte receptor concentrations in beef cattle. *Res Vet Sci* **90**(2), 341-345.

Okafor, C. C., Grooms, D. L., Bolin, S. R., and Kaneene, J. B. (2011a). Detection of bovine interferon-γ response in blood collected during exsanguination of cattle sensitized with *Mycobacterium bovis*. *Am J Vet Res. Accepted June, 2011.*

Okafor, C. C., Grooms, D. L., Bruning-Fann, C. S., Averill, J. J., and Kaneene, J. B. (2011b). Descriptive epidemiology of bovine tuberculosis in Michigan (1975-2010); lessons learned. *Veterinary Medicine International* **2011:874924**.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1990). A sandwich enzyme-immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. *Aust Vet J* **67**(4), 134-137.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1992). The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle - Conditions affecting the production of gamma-interferon in whole-blood culture. *Aust Vet J* **69**(1), 1-4.

SAS Institute Inc. SAS/STAT user's guide: statistics, version 9.2 edition. Cary, North Carolina. 2009.

Stanger, K. J., Ketheesan, N., Parker, A. J., Coleman, C. J., Lazzaroni, S. M., and Fitzpatrick, L. A. (2005). The effect of transportation on the immune status of Bos indicus steers. *J Anim Sci* **83**(11), 2632-2636.

Stark, K., Regula, G., Hernandez, J., Knopf, L., Fuchs, K., Morris, R., and Davies, P. (2006). Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: Review of current approaches. *BMC Health Serv Res* **6**(1), 20.

Thoen, C. O., Lobue, P. A., and de Kantor, I. (2010). Why has zoonotic tuberculosis not received much attention? *Int J Tuberc Lung D* **14**(9), 1073-1074.

Thoen, C. O., Lobue, P. A., Enarson, D. A., Kaneene, J. B., and de Kantor, I. N. (2009). Tuberculosis: a re-emerging disease in animals and humans. *Veter Ital Ser* **45**(1), 135-181.

CHAPTER 5

INTERFERON-GAMMA ASSAY ON BLOOD COLLECTED DURING EXSANGUINATION OF CATTLE: A SURVEILLANCE TOOL FOR BOVINE TUBERCULOSIS

ABSTRACT

Improving bovine tuberculosis (bTB) surveillance is imperative for continued progress in global bTB eradication in a timely and cost effective manner. The development of point-of-concentration (POC) surveillance strategies would be beneficial to bTB eradication. The interferon-gamma (IFN-γ) assay has been shown to detect an IFN-γ response to bTB in blood collected during exsanguination of cattle experimentally exposed to killed *M. bovis* and has been suggested as a potential POC surveillance tool. However, this assay has not been performed on blood collected at exsanguination from cattle naturally exposed to *M. bovis* under field conditions. The aim of this study were to determine (1) if blood collected at exsanguination of cattle at slaughter, under field conditions, is practical in identifying cattle as IFN-γ positive for bTB and (2) if the bTB IFN-γ assay result obtained at exsanguination is as reliable as results obtained from live animals. Cattle from three risk groups were used in this study: the highest risk group consisted of 3 herds (149 cattle) from which bTB infected animal(s) had been found (Group1); the second highest risk group consisted of 24 cattle from a potentially exposed herd that purchased cattle from a bTB infected herd (Group 2); and the lowest risk group consisted of 60 cattle from herds with no known history of bTB infection or exposure (Group 3). The IFN-γ assay was performed on blood collected both before stunning and during exsanguination of cattle

at slaughter. An enhanced slaughter inspection for bTB-like gross lesions was performed on all cattle. In addition, lymph nodes were cultured for *Mycobacterium bovis* for all cattle positive on the bTB IFN-γ assay and for most cattle negative on the bTB IFN-γ assay. Cattle, both with and without bTB-like gross lesions, were identified as bTB IFN-γ assay positive using blood collected at exsanguination. No bTB IFN-γ assay positive cattle originated from herds with low risks/no history of bTB exposure. This study demonstrates that blood collected at exsanguination of cattle is both a practical and moderately reliable sample for accessing bTB infection using the IFN-γ assay. Integration of bTB IFN-γ assay as a POC surveillance tool would help identify bTB exposed herds and could be advantageous in the control and eradication of bTB.

INTRODUCTION

Bovine tuberculosis (bTB) is among the most important global livestock diseases due to its economic and public health importance. Improving bTB surveillance is imperative for continued progress in global bTB eradication. The whole blood interferon-gamma (IFN-γ) assay is gaining acceptance internationally as an *in vitro* screening test for bTB that measures IFN-γ released by lymphocytes in response to antigen stimulation (Rothel *et al.* 1990). This assay is suitable for screening of cattle at a point-of-concentration (POC) because only a single contact with an animal is required to perform the test, where as intradermal tuberculin testing for bTB requires two contacts with each animal at approximately 72 hours intervals (Okafor *et al.* 2011a). In addition, the IFN-γ assay performs as well or better than intradermal skin tests in detection of early stage infection with bTB (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006; Monaghan *et al.* 1997; Neill *et al.* 1994; Pollock *et al.* 2005; Schiller *et al.* 2010; Wood *et al.* 1991; Wood *et al.* 1992; Wood and Jones 2001; Wood and Rothel 1994). Single contact testing with the whole blood IFN-γ assay can reduce the cost of labor and other resources involved in bTB screening (de la Rua-Domenech *et al.* 2006).

A diagnostic assay for bTB that can be adapted for use at a POC, such as slaughter facilities or sale barns, would be useful in bTB surveillance. The adaptability of the bTB IFN-γ assay for a POC screening has not been fully evaluated. A previous study suggested that post mortem blood samples are inadequate for use in bTB IFN-γ assay due to a rapid decline in reactivity of the IFN-γ production by whole blood at slaughter, which changed the interpretation of the test from positive to negative (Rothel *et al.* 1992). In a recent study, however, it was demonstrated that whole blood from cattle sensitized with *Mycobacterium bovis* continued to

produce sufficient bTB IFN-γ to retain a positive test interpretation when collected soon after stunning of the cattle at slaughter (Okafor *et al.* 2011a). In that study, 9 of 12 (75%) sensitized cattle remained positive, using IFN-γ assay, when blood was collected within 2 minutes of stunning. This observation suggests that assay is reliable when exsanguination blood sample is used. However, the performance of IFN-γ assay at a slaughter facility POC was not evaluated using cattle positive on the IFN-γ assay due to field exposure with *M. bovis* or some other immunostimulant.

The objective of this study was to determine if bTB IFN-γ assay performed on blood collected during exsanguination of cattle could be used for bTB surveillance at a POC of cattle, specifically in slaughter facilities. We hypothesized that the interpretation of the IFN-γ assay performed on blood collected during exsanguination of cattle would remain unchanged from the interpretation obtained using blood obtained antemortem.

MATERIALS AND METHODS

Study animals

This study protocol was approved by the Michigan State University Institutional Animal Care and Use Committee (AUF # 08/09-125-00). Cattle from 3 risk categories of bTB exposure were used in this field study. Group1, the highest risk group, comprised 149 cattle from 3 herds from which bTB infected animal(s) had been found. Group 2, the second highest risk group, consisted of 24 cattle from a potentially exposed herd. Group 3, the lowest risk group, comprised 60 cattle from herds with no known history of bTB infection or exposure.

The 3 herds in Group 1 were cow-calf herds that originated from the bTB modified accredited zone (MAZ) in Michigan (Okafor *et al*. 2011b) and were scheduled for depopulation because bTB had been confirmed in each herd. These herds were designated as A, B, and C. At the time herd A was found infected, of the 48 cattle that were screened with tuberculin tests, 16 were later found positive for bTB by bacterial culture of lymphoid tissues. Herd A was depopulated 8 weeks after it was confirmed infected with bTB. At depopulation, 55 cattle ranging in age from 9 months (m) to 14 years (yrs) were scheduled for slaughter and all of those cattle were tested in the current study. In herd B, of the initial 17 cattle that were screened with tuberculin tests, one animal was later confirmed positive for bTB by bacterial culture done using lymphoid tissue harvested at post mortem examination. Herd B was depopulated 7 weeks after it was confirmed infected with bTB. At depopulation of herd B, samples were collected at slaughter from 16 cattle, ages 2 through 11 yrs. In herd C, 89 cattle underwent initial screening with tuberculin test; one animal was later confirmed positive for bTB by bacterial culture of lymphoid tissue collected at post mortem examination. Depopulation of herd C occurred one

year after it was confirmed infected with bTB and samples were collected at slaughter from 78 cattle ranging in age from 6 m to adult.

Cattle in Group 2 originated from a potentially bTB exposed herd in Ohio that had purchased 64 cattle from a bTB infected herd. Approximately 4800 cows in herd D were tested and 353 were caudal fold tuberculin test responders. From these responders, 52 cows were IFN-γ assay positive. Those 52 cows were retested after 7 days and 24 remained IFN-γ assay positive. Those 24 adult cows (>2 yrs) were scheduled for slaughter approximately 2 months following the last round of testing using the IFN-γ assay.

Cattle in Group 3 (n=60) originated from herds that had tested negative for *M. bovis* exposure when Michigan completed statewide bTB surveillance in 2003 and were located in the bTB modified accredited advanced zone Michigan (MAAZ) of Michigan (Okafor *et al.* 2011b). Despite extensive surveillance, no cases of bTB in cattle has been reported from this zone since 1979 (Okafor *et al.* 2011b). The number of cattle used in this group was determined based on available information on the specificity of the bTB IFN-γ assay. The median specificity of the assay has been reported as 96.6%, with a range of 85.0-99.6% (de la Rua-Domenech *et al.* 2006). Based on an assumed specificity of 96.6%, we expected that at least one IFN-γ assay positive animal would be identified from the 60 cattle sampled.

Sample collection protocol

Blood was collected from each animal either at the farm in herds A and B before transport to a slaughter facility for reasons of convenience or immediately before stunning at the slaughter facility for all other cattle. Blood was collected again from the cattle during exsanguination. The whole blood (6-8 ml) obtained antemortem was collected from the

coccygeal vein/artery using a 20g vacutainer needle and in a 10 ml vacutainer tube containing sodium heparin.

At the slaughter facility, cattle were stunned by captive bolt and exsanguinated using the facility's standard operating protocols. Free flowing blood was collected immediately after the jugular and carotid arteries were severed. The time from stunning to blood collection ranged from 60 to 150 s. Exsanguination blood was collected into a 10 ml syringe with the plunger removed. The syringe plunger was then replaced and blood immediately transferred with an 18g needle into a 10 ml vacutainer tube containing sodium heparin. Blood samples were transported chilled to the Michigan State University Diagnostic Center for Population and Animal Health (DCPAH).

During carcass evisceration, lymph nodes from the head (sub mandibular, parotid, and medial and lateral retropharyngeal) thorax, and abdomen were examined for gross lesions compatible with bTB by USDA Food Safety and Inspection Service personnel, using standard operating protocols (USDA APHIS VS 2005). In addition, lymph nodes from all slaughtered cattle from herd A in Group 1 and from Group 2 were submitted to the National Veterinary Services Laboratory for culture of *M. bovis*, regardless of presence of lesions compatible with bTB. For cattle from herds B and C in Group 1 and from Group 3, only lymph nodes from IFN-γ assay positive animals were submitted for culture of *M. bovis*. Also, lymph nodes were submitted for culture for *M. bovis* from 2 age-matched controls from herds B and C in Group 1 and from Group 3. The age matched controls were negative on the IFN-γ assay and lacked visible gross lesions compatible with bTB during slaughter inspection. Portions of the distal ileum and ileocecal lymph node were collected from all cattle that positive on the IFN-γ assay

and from 2 age-matched controls within the same group for Johne's disease screening by culturing collected tissues for *Mycobacterium avium subsp*. paratuberculosis (MAP).

Blood screening (Interferon-gamma assay)

The DCPAH is approved by the United States Department of Agriculture (USDA) to conduct bTB IFN-γ assay in support of bTB eradication. The IFN-γ assay was performed using a commercially available antigen-capture ELISA in accordance with the manufacturers' recommended protocol and was detailed in a previous study (Okafor *et al.* 2011a). Briefly, 1.5 ml aliquots of each blood sample were dispensed to each of 4 wells of a 24-well tissue culture plate. The wells contained 100µl of sterile phosphate buffered saline (negative antigen control), 100µl *Mycobacterium avium* purified protein derivative (avian PPD stimulation antigen, Prionics USA Inc., La Vista, NE), 100µl *Mycobacterium bovis* purified protein derivative (bovine PPD stimulation antigen, Prionics USA Inc., La Vista NE), or 16 µg pokeweed mitogen as a positive antigen stimulant (Lectin from *Phytolacca americana*, Sigma Life Science, St Louis, MO). Following incubation for 16-24 h at 38^0C (100.4^0F) in a humidified atmosphere, plates were centrifuged at 1730 rcf for 10 minutes and the plasma harvested. Each plasma sample was then assayed in duplicate for IFN-γ production by ELISA. Optical density measured at 450 nm (OD450 nm).

Results were expressed as the background-corrected OD450 nm with the no-antigen control (C) subtracted from both bovine PPD (B*)* and avian PPD (A) IFN-γ ELISA wells. Pokeweed mitogen (P) was used to confirm lymphocyte functionality for production of an IFN-γ response. Before an assay result is considered valid, $P - C \geq 0.1$ must be met, otherwise the result is reported as non viable. An animal was considered positive in the bTB IFN-γ assay if the

difference between OD450 nm readings of the B and the C wells (B-C), and the difference between the B and the A wells (B-A) were both ≥ 0.1; if either difference (B-C) or (B-A) was < 0.1, the animal was considered negative in the bTB IFN-γ assay.

Statistical analyses

Descriptive statistics was used to assess the results of the IFN-γ assay results, gross examination, *M. bovis* and MAP culture on the cattle. Kappa coefficient (r) was calculated to measure the agreement in the bTB IFN-γ assay results between blood samples collected from live animals versus those collected at exsanguination. Analyses were done using Statistical Analysis Software (SAS) (SAS Institute Inc 2009).

RESULTS

For blood collected from live animals, 12 of the 233 cattle screened (5%) were positive in the IFN-γ assay, 217 cattle (93%) were negative in the IFN-γ assay, and the blood samples from 4 cattle (2%) were non viable in the IFN-γ assay. During exsanguination, 5 of the 233 cattle (2%) were positive in the IFN-γ assay, 215 cattle (92%) were negative in the IFN-γ assay and 13 cattle (6%) had non-viable results (Table 1). There was a significant ($p < 0.0001$) moderate agreement ($r = 0.453$) in the bTB IFN-γ assay results obtained between blood samples collected from live cattle and those collected at exsanguination. The value of r was between 95% confidence limits (0.157, 0.749). All bTB IFN-γ assay positive cattle originated from either known bTB infected or potentially bTB exposed herds; none of the cattle from the bTB non-exposed herds was IFN-γ assay positive.

Within Group 1 (the highest risk group that consisted of 3 herds from which bTB infected animal(s) had been found), IFN-γ assay positive cattle originated from herds A and B only. In herd A, 3 of the 55 slaughtered animals (5%) were IFN-γ assay positive, 40 cattle (73%) were IFN-γ assay negative, and 12 cattle (22%) had samples of blood that were non viable in the IFN-γ assay at exsanguination. All 3 cattle that were IFN-γ assay positive at exsanguination were positive before stunning. Among the 3 bTB IFN-γ assay positive cattle, only 2 animals had gross lesions compatible with bTB at slaughter. Subsequently, *M. bovis* was cultured from all 3 IFN-γ assay positive cattle, from 7 of the 55 IFN-γ assay negative cattle, and from 2 of the 12 cattle that had non-viable results in the IFN-γ assay. Of these 7 bTB infected cattle that were IFN-γ assay negative, only 5 animals had gross lesion compatible with bTB at necropsy (Table 2).

In herd B, none of the cattle that were IFN-γ assay positive before stunning but one animal was positive in the IFN-γ at exsanguination. None of the cattle from herd B had gross lesions compatible with bTB on slaughter inspection. Additionally, *M. bovis* was not cultured from lymphoid tissue collected from the cow that was IFN-γ assay positive at exsanguination or from the age-matched controls. In herd C, all 78 cattle were IFN-γ assay negative both before stunning and at exsanguination. None of the cattle from herd C had lesions compatible with bTB at slaughter inspection.

In Group 2, the potentially bTB exposed cattle, 1 cow was IFN-γ assay positive both before stunning and at exsanguination. Seven other animals were IFN-γ assay positive before stunning but IFN-γ assay negative at exsanguination. None of the cattle in Group has lesions compatible with bTB at slaughter inspection and *M. bovis* was not cultured from lymphoid tissues from any of the 24 cattle. In Group 3, all 60 cattle that originated from bTB non-exposed herds were IFN-γ assay negative both before stunning and at exsanguination. Additionally, none of the cattle had lesions compatible with bTB at slaughter inspection. The MAP organism was not cultured from the ileum or ileocecal lymph nodes collected from the cattle that were IFN-γ assay positive at exsanguination. However, MAP was cultured from the tissues of one of the age-matched controls in herd A.

DISCUSSION

This study is the first to demonstrate that under field conditions, the IFN-γ assay can detect bTB positive cattle when blood used in the assay is collected at exsanguination. Those results agree with results from a similar study that used experimentally sensitized cattle (Okafor *et al.* 2011a); however, the percentage of cattle retaining a positive result in the IFN-γ assay at exsanguination was higher with sensitized cattle. The findings of this study suggest that collection of blood at exsanguination for use in the IFN-γ assay is practical; however using currently recommended assay methods and interpretations, the assay has moderate agreement compared to result from sample obtained ante mortem.

In herd A of Group 1, 3 cattle were found bTB IFN-γ assay positive on blood collected at exsanguination. One of these three cattle did not present any gross lesion during slaughter examination. Subsequently, *M. bovis* was cultured from the tissues of the three cattle. This observation further supports that bTB IFN-γ assay is able to identify bTB infected cattle before gross lesions are observable (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006; Neill *et al.* 1994; Schiller *et al.* 2010; Wood *et al.* 1991). Because all cattle that were reactors to the tuberculin tests had been removed from this herd 2 months before this study commenced, it is important to note that cattle that were found positive by the IFN-γ assay were thought to be negative for bTB. Additionally, unlike in most cases of the IFN-γ assay in cattle, the use of the assay in this study was not secondary to the CFT reactors. Clearly, the IFN-γ production from the sampled cattle could not have been amplified by any previous tuberculin exposure. Hence, the IFN-γ assay would be a complement to the current visual slaughter surveillance of bTB. This

further supports the integration of bTB IFN-γ assay for POC screening of cattle at slaughter as part of a regional or national bTB eradication program.

No bTB IFN-γ assay positive animals were found in Group 3 and in one of the herds in Group 1. The absence of an animal with a positive bTB IFN-γ assay result from Group 3, the low risk exposure group, could be explained in two ways. One possibility is the reassurance of the IFN-γ assay's performance because those cattle originated from areas without any recent history of bTB exposure. The other reason could be that bTB IFN-γ assay has specificity greater that 96.6% in the population of cattle used in Group 3. The lack of bTB IFN-γ assay positive cattle in herd C, a herd which had previously been found to be infected with bTB, could be explained by the bTB epidemiology in this herd. This herd was depopulated one year after it was found infected with bTB. At the initial diagnoses of bTB, a single yearling heifer was found infected. The epidemiologic investigation of the herd and the small size of the identified gross lesion suggested that the heifer was recently infected and that the bTB exposure most likely came via direct or indirect contact with bTB infected free-ranging deer. Based on this information, it was unlikely that other cattle had become bTB infected by contact with the heifer. Furthermore, the herd owner had instituted a wildlife risk mitigation plan to reduce the risk of bTB transmission from wildlife to his cattle, further reducing the possibility of widespread disease within his herd. This scenario could explain why no evidence of bTB infection was found using either bTB IFN-γ assay or visual observation at slaughter.

For most cattle, bTB IFN-γ assay result pre slaughter was maintained when blood was subsequently collected at exsanguination. Similar to experimental studies (Okafor *et al.* 2011a; Rothel *et al.* 1992), there are occasional animals that are bTB IFN-γ assay positive on blood collected before stunning but bTB IFN-γ assay negative on blood collected at exsanguination.

The loss in categorization as bTB positive by the IFN-γ assay occurs primarily in cattle whose IFN-γ assay response before stunning was marginally above the cutoff-line for classification of an animal as bTB positive or negative (Okafor *et al.* 2011a). In addition, co-infection with other organisms that depress the function of lymphocytes such as Bovine Leukosis Virus and Bovine Viral Disease Virus (BVDV) could be responsible for the differences in the number of animals detected at the different screening points in this study. It has been reported that acute infections of cattle with non-cytopathic BVDV may temporarily result in a failure of bTB IFN-γ assay to identify bTB infected cattle (Charleston *et al.* 2001). Hence, significant challenges with the immune system of an animal could cause a false negative bTB IFN-γ assay result in an animal.

Because of the close relationship between *Mycobacterium avium subsp paratuberculosis* (MAP*)* and *Mycobacterium bovis*, there is some belief that the risk for a false positive bTB IFN-γ assay result is higher in cattle infected with MAP (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006). In this study, MAP was not cultured from any animal that was bTB IFN-γ assay positive on blood collected at exsanguination. This observation suggests that the bTB IFN-γ assay positive cattle in this study were less likely influenced by MAP infection.

In this study, bTB IFN-γ assay positive cattle originated from herds where bTB was a concern. The IFN-γ assay performed on blood collected during exsanguination identified bTB positive animals from varying exposure levels of bTB. Assuming the bTB classification of the herds used in this study was unknown and the animals were sent to slaughter where bTB IFN-γ assay was performed alongside visual slaughter surveillance, the assay could have helped in the identification of herds A and B in Group 1 as well as Group 2 for further screening, whereas visual slaughter surveillance would have identified herd A only. Visual slaughter surveillance alone would have missed herd B (Group 1) and Group 2 because no gross lesion was found in

any of the cattle. The ability of bTB IFN-γ assay to identify animals in herds B of Group 1 and cattle in Group 2 is not surprising because the assay has been shown to detect animals at the preclinical stages of bTB infection (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006; Neill *et al.* 1994; Schiller *et al.* 2010; Wood *et al.* 1991). If used in parallel with routine visual slaughter surveillance, the at-slaughter bTB IFN-γ assay could enhance detection of bTB infected/exposed cattle herds.

To improve the agreement of bTB IFN-γ assay results between sampled collected before slaughter and during exsanguination, areas of potential improvement include adjusting the cut-off criterion for assay interpretation in samples obtained at exsanguination. This measure is important to account for the decreased production of IFN-γ by blood collected at exsanguination when compared to blood collected before stunning (Okafor *et al.* 2011a). Due to known decrease in IFN-γ responses at exsanguination, timing of blood collection following stunning is crucial to getting reliable assay results. Therefore, it is important that exsanguination process is performed as close to the time of stunning as possible.

Due to the small number of animals used in Group 2, the extent to which the result observed in this group would apply to the larger population of animals from bTB exposed herds in the area of high bTB prevalence should be looked at cautiously. However, since several reports indicate that bTB IFN-γ assay is equivalent or better than intradermal skin tests for detection of early stage infection with bTB (Buddle *et al.* 2009; de la Rua-Domenech *et al.* 2006; Neill *et al.* 1994; Schiller *et al.* 2010; Wood *et al.* 1991), it is expected that the at-slaughter bTB IFN-γ assay would correctly identify bTB exposed herds before clinical lesions are observed. The subsequent use of larger numbers of cattle from areas of high bTB prevalence would improve our understanding on the usefulness and adaptation of bTB IFN-γ assay at slaughter.

CONCLUSION

The IFN-γ assay on blood collected at exsanguination of cattle at slaughter is capable of identifying cattle as bTB positive. Performing bTB IFN-γ assay at slaughter would aid in the identification of bTB suspect herds for subsequent targeted whole herd testing. Future application of bTB IFN-γ assay at slaughter, especially in parallel with the already in place visual slaughter surveillance, would improve upon current bTB surveillance strategies and help support continued eradications efforts of bTB.

Table 5.1: The IFN-γ assay results for bTB on blood obtained before and during exsanguination

Group	Total cattle	bTB IFN-γ assay positive		bTB-compatible gross lesion	*M. bovis* culture[†]
		Before stunning	During exsanguination[*]		
1[A]	55	4	3(3/2)	7	12
1[B]	16	0	1(0/0)	0	0
1[C]	78	0	0	0	NT
2	24	8	1(0/0)	0	0
3	60	0	0	0	NT

Group 1 (bTB infected herds: [A, B, C]), Group 2 (Potentially bTB exposed cattle), Group 3 (cattle from bTB non-exposed herds), [*]number of bTB IFN-γ assay positive animals (*M. bovis* culture positive animals/animals with bTB compatible gross lesions), [†]NT (not tested)

Table 5.2: bTB IFN-γ assay and gross lesion results of the *M. bovis* culture positive animals in a bTB infected herd (herd A)

ID	bTB IFN-γ assay result (pre-slaughter)	bTB IFN-γ assay result (at exsanguination)	bTB-like lesion at necropsy	*M. bovis* culture
1	+	+	+	+
2	+	+	+	+
3	+	+	-	+
4	+	-	+	+
5	-	NV	+	+
6	-	NV	-	+
7	-	-	+	+
8	-	-	+	+
9	-	-	+	+
10	-	-	-	+
11	-	-	-	+
12	-	-	-	+

+ (Positive), - (Negative), NV (Non viable)

REFERENCES

REFERENCES

Buddle, B. M., Livingstone, P. G., and de Lisle, G. W. (2009). Advances in ante-mortem diagnosis of tuberculosis in cattle. *New Zeal Vet J* **57**(4), 173-180.

Charleston, B., Hope, J. C., Carr, B. V., and Howard, C. J. (2001). Masking of two in vitro immunological assays for *Mycobacterium bovis* (BCG) in calves acutely infected with non-cytopathic bovine viral diarrhoea virus. *Vet Rec* **149**(16), 481-484.

de la Rua-Domenech, R., Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G., Christiansen, K. H., and Clifton-Hadley, R. S. (2006). Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, [gamma]-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci* **81**(2), 190-210.

Monaghan, M., Quinn, P. J., Kelly, A. P., McGill, K., McMurray, C., OCrowley, K., Bassett, H. F., Costello, E., Quigley, F., Rothel, J. S., Wood, P. R., and Collins, J. D. (1997). A pilot trial to evaluate the gamma-interferon assay for the detection of *Mycobacterium bovis* infected cattle under Irish conditions. *Irish Vet J* **50**(4), 229-232.

Neill, S. D., Cassidy, J., Hanna, J., Mackie, D. P., Pollock, J. M., Clements, A., Walton, E., and Bryson, D. G. (1994). Detection of *Mycobacterium bovis* infection in skin test-negative cattle with an assay for bovine interferon-gamma. *Vet Rec* **135**(6), 134-135.

Okafor, C. C., Grooms, D. L., Bolin, S. R., and Kaneene, J. B. (2011a). Detection of bovine interferon-γ response in blood collected during exsanguination of cattle sensitized with *Mycobacterium bovis*. *Am J Vet Res*. *Accepted June, 2011*.

Okafor, C. C., Grooms, D. L., Bruning-Fann, C. S., Averill, J. J., and Kaneene, J. B. (2011b). Descriptive Epidemiology of Bovine Tuberculosis in Michigan (1975-2010); Lessons Learned. *Veterinary Medicine International* **2011:874924**.

Pollock, J. M., Welsh, M. D., and McNair, J. (2005). Immune responses in bovine tuberculosis: Towards new strategies for the diagnosis and control of disease. *Veterinary Immunol Immunop* **108**(1-2), 37-43.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1990). A Sandwich Enzyme-Immunoassay for Bovine Interferon-Gamma and Its Use for the Detection of Tuberculosis in Cattle. *Aust Vet J* **67**(4), 134-137.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1992). The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle - Conditions affecting the production of gamma-interferon in whole-blood culture. *Aust Vet J* **69**(1), 1-4.

SAS Institute Inc. SAS/STAT user's guide: statistics, version 9.2 edition. Cary, North Carolina. 2009.

Schiller, I., Oesch, B., Vordermeier, H. M., Palmer, M. V., Harris, B. N., Orloski, K. A., Buddle, B. M., Thacker, T. C., Lyashchenko, K. P., and Waters, W. R. (2010). Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transbound Emerg Dis* **57**(4), 205-220.

USDA APHIS VS. Tuberculosis sample submission manual for meat inspection personnel. http://www.aphis.usda.gov/animal_health/animal_diseases/tuberculosis/downloads/tb_guidebook.pdf . 2005.

Wood, P. R., Corner, L. A., Rothel, J. S., Baldock, C., Jones, S. L., Cousins, D. B., Mccormick, B. S., Francis, B. R., Creeper, J., and Tweddle, N. E. (1991). Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. *Aust Vet J* **68**(9), 286-290.

Wood, P. R., Corner, L. A., Rothel, J. S., Ripper, J. L., Fifis, T., Mccormick, B. S., Francis, B., Melville, L., Small, K., De Witte, K., Tolson, J., Ryan, T. J., de Lisle, G. W., Cox, J. C., and Jones, S. L. (1992). A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. *Vet Microbiol* **31**(1), 71-79.

Wood, P. R., and Jones, S. L. (2001). BOVIGAM (TM): an in vitro cellular diagnostic test for bovine tuberculosis. *Tuberculosis* **81**(1-2), 147-155.

Wood, P. R., and Rothel, J. S. (1994). In vitro immunodiagnostic assays for bovine tuberculosis. *Vet Microbiol* **40**(1-2), 125-135.

GENERAL DISCUSSION

In this dissertation, many lessons were learned from the descriptive epidemiology of bTB in Michigan cattle, privately owned cervids, and wild deer. The counties in Michigan where bTB has been isolated from both livestock and wildlife were described, providing areas that need greatest attention in the future eradication efforts. Cattle movement was found not to be a significant source of transmission of bTB in cattle which further supports that wildlife reservoirs play a significant role in the epidemiology of bTB in Michigan. The downward trend of bTB in both livestock and wildlife suggest that the Michigan bTB Eradication Project partners have been successful in the control of bTB. However, if complete eradication of the observed on-going bTB challenge in Michigan is to be accomplished, it may be necessary to explore new and more aggressive control strategies in both cattle and wildlife that transcends political as well as social barriers. Besides surveillance, other areas that would need improvement include increased cooperation between regulatory agencies, other stakeholders (for example hunters or local business owners), and livestock industry partners; sustaining practices that reduce wild white-tailed deer-cattle space interactions such as storage of feed in enclosures that protect it from deer access, limiting cattle access to stagnant water sources and areas of cover that are also attractive to wild white-tailed deer, and removing feedstuffs from cattle areas that are attractive to deer (e.g. wild apple trees); reduction of deer concentration and deer density; and research in bTB.

It was observed from the review of literature on surveillance on bTB that the development of sophisticated targeted screening strategies at POC of cattle would most likely produce a significant reduction in resource expenditure while at the same time maintaining the necessary rapid identification of bTB infected herds to eradicate the disease. The use of blood-based bTB assay in parallel with current slaughter surveillance for bTB could be very valuable.

Such prospective strategy could improve the detection of cattle herds exposed to or at early stages of bTB infection and subsequently lead to targeted surveillance for bTB to the originating herds.

To our knowledge, one of the studies presented in this dissertation was the first to demonstrate positive bTB IFN-γ assays interpretation using blood collected during exsanguination at slaughter from naturally infected cattle that were confirmed as positive for bTB on post mortem examination. Nine (75%) of the bTB sensitized cattle retained their positive classification at exsanguination. Although blood collected at exsanguination was found capable of producing bovine IFN-γ in response to antigen stimulation, there was a statistically significant decrease in the optical density reading for each stimulant (avian PPD, bovine PPD, and pokeweed mitogen) from before to after stunning. The observed decline in the IFN-γ response at exsanguination is in agreement with results of a previous study in which samples of blood collected from experimentally infected cattle at 5 minutes following exsanguination (Rothel *et al.* 1992). Test interpretation for animals that had comparatively higher IFN-γ assay optical density readings before slaughter was not affected by the decline in optical density readings observed after stunning. In animals that were borderline positive for bTB before slaughter (E, J, and K), the decrease in their IFN-γ assay resulted in their bTB classification changing to negative.

Useful data was obtained in a follow-up study, evaluating factors that can affect measurable interferon-γ production in cattle at time of slaughter. First, it was shown that under experimental conditions the interpretation of the IFN-γ likely would not change from before slaughter for most cattle if blood used for the IFN-γ assay is obtained immediately after commencement of exsanguination. Second, the number of total lymphocytes in flowing blood

drops quickly after the exsanguination process commences, which likely contributes to the drop in IFN-γ production observed with blood obtained at various time points during exsanguination. Finally, a relatively short period of transportation was sufficient to cause a reduction in IFN-γ production when blood obtained after transport was compared with blood obtained immediately before transport. This last finding is important should the IFN-γ assay be used to test cattle for bTB at POC. Based on these findings, specific protocols of the assay could be reconsidered and tested to improve the reliability of bTB IFN-γ assay for adoption as a surveillance tool at slaughter. For example, to compensate for the decline in lymphocyte count, an adjusted ELISA OD values cut-off limit for categorizing animals as either bTB positive or negative could be developed for blood collected at exsanguination. To understand the most appropriate cut-off limit, more number of animals would be useful and the use of cattle under natural conditions of *M. bovis* exposure would be valuable.

The final study was yet the first to demonstrate that under field conditions, the IFN-γ assay can detect bTB positive cattle when blood used in the assay is collected at exsanguination. Those results agree with results from a similar study that used experimentally sensitized cattle (Okafor *et al.* 2011a); however, the percentage of cattle retaining a positive result in the IFN-γ assay at exsanguination was higher with sensitized cattle. The IFN-γ assay positive cattle originated from herds with where bTB was a concern at varying levels of risks. Some of the IFN-γ assay positive cattle did not present a gross lesion compatible with bTB; from this group *M. bovis* was cultured from the tissues of one animal. This study suggest that collection of blood at exsanguination for use in the IFN-γ assay is practical; however using currently recommended assay methods and interpretations, the assay has moderate agreement compared to result from sample obtained ante mortem. To improve the agreement of bTB IFN-γ

assay results between sampled collected before slaughter and during exsanguination, areas of potential improvement include adjusting the cut-off criterion for assay interpretation in samples obtained at exsanguination. This measure is important to account for the decreased production of IFN-γ by blood collected at exsanguination when compared to blood collected before stunning (Okafor *et al.* 2011a). Due to known decrease in IFN-γ responses at exsanguination, timing of blood collection following stunning is crucial to getting reliable assay results. Therefore, it is important that exsanguination process is performed as close to the time of stunning as possible.

CLOSING THOUGHTS AND FUTURE APPLICATIONS

Counties in Michigan with the burden of bTB in both livestock and wildlife have been described. The counties should be the major focus of future eradication strategies in Michigan. Eradication strategies involving intensive wildlife risk mitigation practices and targeted surveillance in livestock would improve timely eradication of bTB in Michigan. A novel strategy of screening cattle at points-of concentration as a tool for targeted surveillance has been evaluated. The IFN-γ assay on blood collected at exsanguination of cattle at slaughter is capable of identifying cattle as bTB positive. Performing bTB IFN-γ assay at slaughter would aid in the identification of bTB suspect herds for subsequent targeted whole herd testing. Future application of bTB IFN-γ assay at slaughter, especially in parallel with the already in place visual slaughter surveillance, would improve upon current bTB surveillance strategies and help support continued eradications efforts of bTB. Besides bTB IFN-γ assay, other potential new assays such as MAPIA, ELISA, Enferplex TB, and Chembio rapid immunomigration assay should borrow from the novel strategy described in this dissertation and be evaluated for blood samples collected at various points of concentration cattle. The adaptation of these potential new assays for used in slaughter facilities or auction/sale barns could provide additional tools for targeted surveillance, a measure that would advance timely eradication of bTB.

REFERENCES

REFERENCES

Audige, L., and Beckett, S. (1999). A quantitative assessment of the validity of animal-health surveys using stochastic modelling. *Prev Vet Med* **38**, 259-276.

Bridget Patrick. (personal communications 2010). Animal Industry Division, Michigan Department of Agriculture, P O Box 30017 Lansing MI 48909 . 2010.

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., Cooley, T. M., Miller, J. M., and Muzo, D. P. (2001). Bovine tuberculosis in free-ranging carnivores from Michigan. *J Wildlife Dis* **37**(1), 58-64.

Bruning-Fann, C. S., Schmitt, S. M., Fitzgerald, S. D., Payeur, J. B., Whipple, D. L., Cooley, T. M., Carlson, T., and Friedrich, P. (1998). *Mycobacterium bovis* in coyotes from Michigan. *J Wildlife Dis* **34**(3), 632-636.

Buddle, B. M., Delisle, G. W., Pfeffer, A., and Aldwell, F. E. (1995). Immunological Responses and Protection Against *Mycobaterium bovis*in Calves Vaccinated with A Low-Dose of Bcg. *Vaccine* **13**(12), 1123-1130.

Buddle, B. M., Livingstone, P. G., and de Lisle, G. W. (2009). Advances in ante-mortem diagnosis of tuberculosis in cattle. *New Zeal Vet J* **57**(4), 173-180.

Cannon, R. M. (2002). Demonstrating disease freedom - combining confidence levels. *Prev Vet Med* **52**(3-4), 227-249.

Charleston, B., Hope, J. C., Carr, B. V., and Howard, C. J. (2001). Masking of two in vitro immunological assays for *Mycobacterium bovis* (BCG) in calves acutely infected with non-cytopathic bovine viral diarrhoea virus. *Vet Rec* **149**(16), 481-484.

Christensen, J., and Gardner, I. A. (2000). Herd-level interpretation of test results for epidemiologic studies of animal diseases. *Prev Vet Med* **45**(1-2), 83-106.

Conner, M. M., Ebinger, M. R., Blanchong, J. A., and Cross, P. C. (2008). Infectious disease in cervids of north America - Data, models, and management challenges. *Ann NY Acad Sci* **1134**, 146-172.

Corner, L. A. L. (2007). Bovine tuberculosis control in Australia, New Zealand and Ireland: Wild animals and the epidemiology of tuberculosis in domestic animals. *Cattle Practice* **15**, 3-12.

Cousins, D. V., and Roberts, J. L. (2001). Australia's campaign to eradicate bovine tuberculosis: the battle for freedom and beyond. *Tuberculosis* **81**(1-2), 5-15.

de Kantor, I. N., Lobue, P. A., and Thoen, C. O. (2010). Human tuberculosis caused by *Mycobacterium bovis* in the United States, Latin America and the Caribbean. *Int J Tuberc Lung D* **14**(11), 1369-1373.

de la Rua-Domenech, R., Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G., Christiansen, K. H., and Clifton-Hadley, R. S. (2006). Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, [gamma]-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci* **81**(2), 190-210.

de Lisle, G. W., Bengis, R. G., Schmitt, S. M., and O'Brien, D. J. (2002). Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Revue Scientifique et Technique de l Office International des Epizooties* **21**(2), 317-334.

Dhabhar, F. S. (1998). Stress-induced enhancement of cell-mediated immunity. *Neuroimmunomodulat* **840**, 359-372.

Dhabhar, F. S., and Mcewen, B. S. (1996). Stress-induced enhancement of antigen-specific cell-mediated immunity. *J Immunol* **156**(7), 2608-2615.

Dhabhar, F. S., and Mcewen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. *Brain Behav Immun* **11**(4), 286-306.

Dhabhar, F. S., Miller, A. H., Mcewen, B. S., and Spencer, R. L. (1995). Effects of Stress on Immune Cell Distribution - Dynamics and Hormonal Mechanisms. *J Immunol* **154**(10), 5511-5527.

Dhabhar, F. S., Miller, A. H., Stein, M., Mcewen, B. S., and Spencer, R. L. (1994). Diurnal and Acute Stress-Induced Changes in Distribution of Peripheral-Blood Leukocyte Subpopulations. *Brain Behav Immun* **8**(1), 66-79.

Doherr, M. G., and Audige, L. (2001). Monitoring and surveillance for rare health-related events: a review from the veterinary perspective. *Philos Trans R Soc Lond B Biol Sci* **356**, 1097-1106.

Doherr, M. G., Heim, D., Fatzer, R., Cohen, C. H., Vandevelde, M., and Zurbriggen, A. (2001). Targeted screening of high-risk cattle populations for BSE to augment mandatory reporting of clinical suspects. *Prev Vet Med* **51**, 3-16.

Doherr, M. G., Hett, A. R., Cohen, C. H., Fatzer, R., Rufenacht, J., Zurbriggen, A., and Heim, D. (2002). Trends in prevalence of BSE in Switzerland based on fallen stock and slaughter surveillance. *Vet Rec* **150**, 347-348.

Dohoo, I., Martin, W., and Stryhn, H. (2003). Veterinary Epidemiologic Research, pp. 47-48. AVC Inc. Charlottetown, Prince Edward Island, Canada.

Dorn, M. L., and Mertig, A. G. (2005). Bovine tuberculosis in Michigan: Stakeholder attitudes and implications for eradication efforts. *Wildlife Society Bulletin* **33**(2), 539-552.

Ebel, E. D., Williams, M. S., and Tomlinson, S. M. (2008). Estimating herd prevalence of bovine brucellosis in 46 U.S.A. states using slaughter surveillance. *Prev Vet Med* **85**(3-4), 295-316.

Fenton, K. A., Fitzgerald, S. D., Kaneene, J. B., Kruger, J. M., Greenwald, R., and Lyashchenko, K. P. (2010). Comparison of three immunodiagnostic assays for antemortem detection of *Mycobacterium bovis* stimulation in domestic cats. *J Vet Diagn Invest* **22**(5), 724-729.

Frawley, B. J. Michigan deer harvest survey report 2009 seasons. http://www.michigan.gov/documents/dnr/report3513_327318_7.pdf (MDNR Wildlife Report No. 3513). 2010.

Goff, B. S. L. (1996). Effect of dexamethasone treatment of tuberculous cattle on results of the gamma-interferon test for *Mycobacterium bovis*. *Veterinary Immunol Immunop* **53**(1-2), 39-47.

Grange, J. M. (2001). *Mycobacterium bovis* infection in human beings. *Tuberculosis* **81**(1-2), 71-77.

Green, L. R., Jones, C. C., Sherwood, A. L., Garkavi, I. V., Cangelosi, G. A., Thacker, T. C., Palmer, M. V., Waters, W. R., and Rathe, C. V. (2009). Single-Antigen Serological Testing for Bovine Tuberculosis. *Clin Vaccine Immunol* **16**(9), 1309-1313.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J. B., Harris, B., Sofranko, D., Waters, W. R., and Lyashchenko, K. P. (2009). Highly Accurate Antibody Assays for Early and Rapid Detection of Tuberculosis in African and Asian Elephants. *Clin Vaccine Immunol* **16**(5), 605-612.

Griffore, R. J., and Phenice, L. A. Farm families and bovine tuberculosis: a critical perspective. Critical Social Work 9(1). 2008. 8-24-2011.

Hickling, G. J. Dynamics of Bovine Tuberculosis in Wild White-Tailed Deer in Michigan. http://ww2.dnr.state.mi.us/publications/pdfs/HuntingWildlifeHabitat/Reports/WLD-library/3301-3399/3363.pdf MDNR Wildlife Division Report(3363). 2002.

IDEXX. IDEXX *M. bovis* Ab Test. http://www.idexx.com/view/xhtml/en_us/livestock-poultry/ruminant/bovine-tuberculosis.jsf?SSOTOKEN=0 . 9-1-2011. 9-1-2011.

Jolley, M. E., Nasir, M. S., Surujballi, O. P., Romanowska, A., Renteria, T. B., De la Mora, A., Lim, A., Bolin, S. R., Michel, A. L., Kostovic, M., and Corrigan, E. C. (2007). Fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in bovine sera. *Vet Microbiol* **120**(1-2), 113-121.

Kaneene, J. B., Bruning-Fann, C. S., Dunn, J., Mullaney, T. P., Berry, D., Massey, J. P., Thoen, C. O., Halstead, S., and Schwartz, K. (2002a). Epidemiologic investigation of *Mycobacterium bovis* in a population of cats. *Am J Vet Res* **63**(11), 1507-1511.

Kaneene, J. B., Bruning-Fann, C. S., Granger, L. M., Miller, R. A., and Porter-Spalding, B. A. (2002b). Environmental and farm management factors associated with tuberculosis on cattle farms in northeastern Michigan. *JAVMA* **221**(6), 837-842.

Kaneene, J. B., VanderKlok, M., Bruning-Fann, C. S., Palmer, M. V., Whipple, D. L., Schmitt, S. M., and Miller, R. (2002c). Prevalence of *Mycobacterium bovis* infection in cervids on privately owned ranches. *JAVMA* **220**(5), 656-659.

Kaneene, J. B., Miller, R., and Meyer, R. M. (2006). Abattoir surveillance: The U.S. experience. *Vet Microbiol* **112**(2-4), 273-282.

Lobue, P. A., Enarson, D. A., and Thoen, C. O. (2010). Tuberculosis in humans and animals: an overview. *Int J Tuberc Lung D* **14**(9), 1075-1078.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Chambers, M. A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B. M., Jackson, R., O'Brien, D. J., Schmitt, S., Palmer, M. V., Delahay, R. J., and Waters, W. R. (2008). Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. *Vet Microbiol* **132**(3-4), 283-292.

MDNR. History of Legislation and Regulations for Bovine Tuberculosis in Michigan Wildlife. http://www.michigan.gov/documents/Bait_Feed_History_138339_7.pdf . *Accessed Novemeber 14, 2010.*

MDNR. Bovine Tuberculosis. http://www.michigan.gov/emergingdiseases . *Accessed Novemeber 14, 2010.*

Michel, A. L., Mnller, B., and van Helden, P. D. (2010). *Mycobacterium bovis* at the animal-human interface: A problem, or not? *Vet Microbiol* **140**(3-4), 371-381.

Miller, R., Kaneene, J. B., Fitzgerald, S. D., and 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. *J Wildlife Dis* **39**(1), 84-95.

Monaghan, M., Quinn, P. J., Kelly, A. P., McGill, K., McMurray, C., OCrowley, K., Bassett, H. F., Costello, E., Quigley, F., Rothel, J. S., Wood, P. R., and Collins, J. D. (1997). A pilot trial to evaluate the gamma-interferon assay for the detection of *Mycobacterium bovis* infected cattle under Irish conditions. *Irish Vet J* **50**(4), 229-232.

Morignat, E., Ducrot, C., Roy, R., Baron, T., Vinard, J. L., Biacabe, A. G., Madec, J. Y., Bencsik, A., Debeer, S., Eliazsewicz, M., and Calavas, D. (2002). Targeted surveillance to assess the prevalence of BSE in high-risk populations in western France and the associated risk factors. *Vet Rec* **151**, 73-77.

Munyeme, M., Muma, J. B., Samui, K. L., Skjerve, E., Nambota, A. M., Phiri, I. G. K., Rigouts, L., and Tryland, M. (2009). Prevalence of bovine tuberculosis and animal level risk factors for indigenous cattle under different grazing strategies in the livestock/wildlife interface areas of Zambia. *Tropical Animal Health and Production* **41**(3), 345-352.

Neill, S. D., Cassidy, J., Hanna, J., Mackie, D. P., Pollock, J. M., Clements, A., Walton, E., and Bryson, D. G. (1994). Detection of *Mycobaterium bovis*Infection in Skin Test-Negative Cattle with An Assay for Bovine Interferon-Gamma. *Vet Rec* **135**(6), 134-135.

Nol, P., Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Waters, W. R., Palmer, M. V., Nonnecke, B. J., Keefe, T. J., Thacker, T. C., Rhyan, J. C., Aldwell, F. E., and Salman, M. D. (2009). Humoral Immune Responses of White-Tailed Deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG Vaccination and Experimental Challenge with *M. bovis*. *Clin Vaccine Immunol* **16**(3), 323-329.

Nol, P., Palmer, M. V., Waters, W. R., Aldwell, F. E., Buddle, B. M., Triantis, J. M., Linke, L. M., Phillips, G. E., Thacker, T. C., Rhyan, J. C., Dunbar, M. R., and Salman, M. D. (2008). Efficacy of oral and parenteral routes of *Mycobacterium bovis* bacille Calmette-Guerin vaccination against experimental bovine tuberculosis in white-tailed deer (*Odocoileus virginianus*): A feasibility study. *J Wildlife Dis* **44**(2), 247-259.

O'Brien, D. J., Bernardi, P., Dubay, S., Mayhew, S., Moritz, W. E., and Purol, D. (2005). A Risk-based audit of the captive/privately owned cervid industry in Michigan. http://www.michigan.gov/documents/CPOCAuditReport_Final_118651_7.pdf . *Accessed August 10, 2010.*

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., and Berry, D. E. (2001). Tuberculous lesions in free-ranging white-tailed deer in Michigan. *J Wildlife Dis* **37**(3), 608-613.

O'Brien, D. J., Schmitt, S. M., Berry, D. E., Fitzgerald, S. D., Lyon, T. J., Vanneste, J. R., Cooley, T. M., Hogle, S. A., and Fierke, J. S. (2008). Estimating the True Prevalence of Mycobacterium Bovis in Free-Ranging Elk in Michigan. *J Wildlife Dis* **44**(4), 802-810.

O'Brien, D. J., Schmitt, S. M., Berry, D. E., Fitzgerald, S. D., Vanneste, J. R., Lyon, T. J., Magsig, D., Fierke, J. S., Cooley, T. M., Zwick, L. S., and Thomsen, B. V. (2004). Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan. *J Wildlife Dis* **40**(1), 42-52.

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., and Kaneene, J. B. (2002a). Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995-2000. *Prev Vet Med* **54**(1), 47-63.

O'Brien, D. J., Schmitt, S. M., Fitzgerald, S. D., Berry, D. E., and Hickling, G. J. (2006). Managing the wildlife reservoir of *Mycobacterium bovis*: The Michigan, USA, experience. *Vet Microbiol* **112**(2-4), 313-323.

O'Brien, D. J., Schmitt, S. M., and Hickling, G. J. (2002b). Comments offered on Michigan bovine TB study. *JAVMA* **221**(10), 1380-1381.

O'Brien, D. J., Schmitt, S. M., Lyashchenko, K. P., Waters, W. R., Berry, D. E., Palmer, M. V., McNair, J., Greenwald, R., Esfandiari, J., and Cosgrove, M. K. (2009). Evaluation of Blood Assays for Detection of Mycobacterium Bovis in White-Tailed Deer (*Odocoileus virginianus*) in Michigan. *J Wildlife Dis* **45**(1), 153-164.

Odore, R., Badino, P., Re, G., Barbero, R., Cuniberti, B., D'Angelo, A., Girardi, C., Fraccaro, E., and Tarantola, M. (2011). Effects of housing and short-term transportation on hormone and lymphocyte receptor concentrations in beef cattle. *Res Vet Sci* **90**(2), 341-345.

OIE. FAO/OIE/WHO call for targeted strategy including poultry vaccination to help curb avian flu - Experts issue recommendations to manage crisis. http://www.oie.int/en/for-the-media/press-releases/detail/article/faooiewho-call-for-targeted-strategy-including-poultry-vaccination-to-help-curb-avian-flu-expert/ . 2-5-2004. 8-23-2011.

OIE. Bovine Spongiform Encephalopathy. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.11.5.htm#surveillance . 8-23-2011. 8-23-2011.

Okafor, C. C., Grooms, D. L., Bolin, S. R., and Kaneene, J. B. (2011a). Detection of bovine interferon-γ response in blood collected during exsanguination of cattle sensitized with *Mycobacterium bovis*. *Am J Vet Res*. *Accepted June, 2011*.

Okafor, C. C., Grooms, D. L., Bruning-Fann, C. S., Averill, J. J., and Kaneene, J. B. (2011b). Descriptive Epidemiology of Bovine Tuberculosis in Michigan (1975-2010); Lessons Learned. *Veterinary Medicine International* **2011:874924**.

Olea-Popelka, F. J., Costello, E., White, P., McGrath, G., Collins, J. D., O'Keeffe, J., Kelton, D. F., Berke, O., More, S., and Martin, S. W. (2008). Risk factors for disclosure of additional tuberculous cattle in attested-clear herds that had one animal with a confirmed lesion of tuberculosis at slaughter during 2003 in Ireland. *Prev Vet Med* **85**(1-2), 81-91.

Olmstead, A. L., and Rhode, P. W. (2004). An impossible undertaking: The eradication of bovine tuberculosis in the United States. *Journal of Economic History* **64**(3), 734-772.

Oreilly, L. M., and Daborn, C. J. (1995). The Epidemiology of *Mycobaterium bovis*Infections in Animals and Man - A Review. *Tubercle and Lung Disease* **76**, 1-46.

Palmer, M. V. (2007). Tuberculosis: A reemerging disease at the interface of domestic animals and wildlife. *Wildlife and Emerging Zoonotic Diseases: the Biology, Circumstances and Consequences of Cross-Species Transmission* **315**, 195-215.

Palmer, M. V., Thacker, T. C., and Waters, W. R. (2007). Vaccination of white-tailed deer (*Odocoileus virginianus*) with *Mycobacterium bovis* bacillus Calmette Guerin. *Vaccine* **25**(36), 6589-6597.

Palmer, M. V., Thacker, T. C., and Waters, W. R. (2009). Vaccination with *Mycobacterium bovis* BCG Strains Danish and Pasteur in White-tailed Deer (*Odocoileus virginianus*) Experimentally Challenged with *Mycobacterium bovis*. *Zoonoses Public Hlth* **56**(5), 243-251.

Palmer, M. V., Thacker, T. C., Waters, W. R., Robbe-Austerman, S., Lebepe-Mazur, S. M., and Harris, N. B. (2010). Persistence of *Mycobacterium bovis* Bacillus Calmette-Guerin in White-Tailed Deer (*Odocoileus virginianus*) after Oral or Parenteral Vaccination. *Zoonoses Public Hlth* **57**(7-8), E206-E212.

Palmer, M. V., Waters, W. R., and Whipple, D. L. (2004). Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact. *Am J Vet Res* **65**(11), 1483-1489.

Palmer, M. V., Waters, W. R., and Whipple, D. L. (2002). Milk containing *Mycobacterium bovis* as a source of infection for white-tailed deer fawns (*Odocoileus virginianus*). *Tuberculosis* **82**(4-5), 161-165.

Palmer, M. V., and Whipple, D. L. (2006). Survival of *Mycobacterium bovis* on feedstuffs commonly used as supplemental feed for white-tailed deer (*Odocoileus virginianus*). *J Wildlife Dis* **42**(4), 853-858.

Palmer, M. V., Whipple, D. L., Payeur, J. B., Alt, D. P., Esch, K. J., Bruning-Fann, C. S., and Kaneene, J. B. (2000). Naturally occurring tuberculosis in white-tailed deer. *JAVMA* **216**(12), 1921-1924.

Palmer, M. V., Whipple, D. L., and Waters, W. R. (2001). Experimental deer-to-deer transmission of *Mycobacterium bovis*. *Am J Vet Res* **62**(5), 692-696.

Pollock, J. M., Welsh, M. D., and McNair, J. (2005). Immune responses in bovine tuberculosis: Towards new strategies for the diagnosis and control of disease. *Veterinary Immunol Immunop* **108**(1-2), 37-43.

Prattley, D. J., Cannon, R. M., Wilesmith, J. W., Morris, R. S., and Stevenson, M. A. (2007a). A model (BSurvE) for estimating the prevalence of bovine spongiform encephalopathy in a national herd. *Prev Vet Med* **80**(4), 330-343.

Prattley, D. J., Morris, R. S., Cannon, R. M., Wilesmith, J. W., and Stevenson, M. A. (2007b). A model (BSurvE) for evaluating national surveillance programs for bovine spongiform encephalopathy. *Prev Vet Med* **81**(4), 225-235.

Radunz, B. (2006). Surveillance and risk management during the latter stages of eradication: Experiences from Australia. *Vet Microbiol* **112**(2-4), 283-290.

Riley, S. J., Muter, B. A., and Gore, M. L. (2011). Expert perspectives on bovine tuberculosis management policies in Michigan and Minnesota. *Accessed May 5, 2011*.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1990). A Sandwich Enzyme-Immunoassay for Bovine Interferon-Gamma and Its Use for the Detection of Tuberculosis in Cattle. *Aust Vet J* **67**(4), 134-137.

Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C., and Wood, P. R. (1992). The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle - Conditions affecting the production of gamma-interferon in whole-blood culture. *Aust Vet J* **69**(1), 1-4.

Salman, M. D. (2008). Surveillance and Monitoring Systems for Animal Health Programs and Disease Surveys, pp. 3-13. Iowa State Press.

Salman, M. D., Stark, K. D. C., and Zepeda, C. (2003). Quality assurance applied to animal disease surveillance systems. *Rev Sci Tech OIE* **22**(2), 689-696.

SAS Institute Inc. SAS/STAT user's guide: statistics, version 9.2 edition. Cary, North Carolina. 2009.

Schiller, I., Oesch, B., Vordermeier, H. M., Palmer, M. V., Harris, B. N., Orloski, K. A., Buddle, B. M., Thacker, T. C., Lyashchenko, K. P., and Waters, W. R. (2010). Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of their Relevance for Disease Control and Eradication. *Transbound Emerg Dis* **57**(4), 205-220.

Schiller, I., RayWaters, W., Vordermeier, H. M., Jemmi, T., Welsh, M., Keck, N., Whelan, A., Gormley, E., Boschiroli, M. L., Moyen, J. L., Vela, C., Cagiola, M., Buddle, B. M., Palmer, M., Thacker, T., and Oesch, B. (2011). Bovine tuberculosis in Europe from the perspective of an

officially tuberculosis free country: Trade, surveillance and diagnostics. *Vet Microbiol* **151**(1-2), 153-159.

Schmitt, S. M. (2010). Bovine *Tuberculosis in Wildlife*. http://www.michigan.gov/documents/emergingdiseases/DNRESchmittPublicMeeting062210_325662_7.pdf (MDA-DNRE TB Informational Meetings June 22-23,2010). *Accessed May 2, 2011*

Schmitt, S. M., Fitzgerald, S. D., Cooley, T. M., BruningFann, C. S., Sullivan, L., Berry, D., Carlson, T., Minnis, R. B., Payeur, J. B., and Sikarskie, J. (1997). Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *J Wildlife Dis* **33**(4), 749-758.

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

Sears, P. M., Paape, M. J., Pearson, R. E., and Gwazdauskas, F. C. (1978). Comparison Between Tail Vein and Jugular Vein Cannulation in Cattle. *Journal of Dairy Science* **61**(7), 974-979.

Stanger, K. J., Ketheesan, N., Parker, A. J., Coleman, C. J., Lazzaroni, S. M., and Fitzpatrick, L. A. (2005). The effect of transportation on the immune status of Bos indicus steers. *J Anim Sci* **83**(11), 2632-2636.

Stark, K., Regula, G., Hernandez, J., Knopf, L., Fuchs, K., Morris, R., and Davies, P. (2006). Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: Review of current approaches. *BMC Health Serv Res* **6**(1), 20.

StatTools Web site. Programs for sample size estimation, McNemar test. http://www.stattools.net/SSizMcNemar_Pgm.php . *Accessed January10, 2011*

Tavornpanich, S., Gardner, I. A., Carpenter, T. E., Johnson, W. O., and Anderson, R. J. (2006). Evaluation of cost-effectiveness of targeted sampling methods for detection of *Mycobacterium avium* subsp paratuberculosis infection in dairy herds. *Am J Vet Res* **67**(5), 821-828.

Tavornpanich, S., Munoz-Zanzi, C. A., Wells, S. J., Raizman, E. A., Carpenter, T. E., Johnson, W. O., and Gardner, I. A. (2008). Simulation model for evaluation of testing strategies for detection of paratuberculosis in Midwestern US dairy herds. *Prev Vet Med* **83**(1), 65-82.

Thacker, T. C., Palmer, M. V., and Waters, W. R. (2009). T-Cell mRNA Expression in Response to *Mycobacterium bovis* BCG Vaccination and *Mycobacterium bovis* Infection of White-Tailed Deer. *Clin Vaccine Immunol* **16**(8), 1139-1145.

The Center for Food Security & Public Health. Bovine Tuberculosis. Iowa State University . 7-20-2009. 8-28-2011.

Thoen, C. O., Lobue, P. A., and de Kantor, I. (2010). Why has zoonotic tuberculosis not received much attention? *Int J Tuberc Lung D* **14**(9), 1073-1074.

Thoen, C. O., Lobue, P. A., Enarson, D. A., Kaneene, J. B., and de Kantor, I. N. (2009). Tuberculosis: a re-emerging disease in animals and humans. *Veter Ital Ser* **45**(1), 135-181.

Thurmond, M. C. (2003). Conceptual foundations for infectious disease surveillance. *J Vet Diagn Invest* **15**, 501-514.

Tweddle, N. E., and Livingstone, P. (1994). Bovine Tuberculosis-Control and Eradication Programs in Australia and New-Zealand. *Vet Microbiol* **40**(1-2), 23-39.

USDA APHIS. Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective January 1, 2005.

USDA APHIS. Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective January 22, 1999.

USDA APHIS VS. Tuberculosis Sample Submission Manual for Meat Inspection Personnel. http://www.aphis.usda.gov/animal_health/animal_diseases/tuberculosis/downloads/tb_guidebook.pdf . 2005.

USDA APHIS VS. Guidelines for the Importation of Cattle (ruminants) into the United States (except from Canada and Mexico). http://www.aphis.usda.gov/import_export/animals/live_animals.shtml . 9-8-2011. 9-8-2011.

USDA Census of Agriculture. (2007). http://www.agcensus.usda.gov/Publications/2007/Full_Report/Volume_1,_Chapter_2_US_State_Level/st99_2_011_011.pdf . *Accessed May 15, 2009.*

van Asseldonk, M. A. P. M., van Roermund, H. J. W., Fischer, E. A. J., de Jong, M. C. M., and Huirne, R. B. M. (2005). Stochastic efficiency analysis of bovine tuberculosis-surveillance programs in the Netherlands. *Prev Vet Med* **69**(1-2), 39-52.

Waters, W. R., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Vordermeier, H. M., Hewinson, R. G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J. M., Andersen, P., and Lyashchenko, K. P. (2006). Early Antibody Responses to Experimental *Mycobacterium bovis* Infection of Cattle. *Clin Vaccine Immunol* **13**(6), 648-654.

Waters, W. R., Palmer, M. V., Whipple, D. L., Slaughter, R. E., and Jones, S. L. (2004). Immune responses of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG vaccination. *J Wildlife Dis* **40**(1), 66-78.

Webb, C. R., Wilesmith, J. W., Simmons, M. M., and Hoinville, L. J. (2001). A stochastic model to estimate the prevalence of scrapie in Great Britain using the results of an abattoir-based survey. *Prev Vet Med* **51**, 269-287.

Whipple, D. L., Bolin, C. A., and Miller, J. M. (1996). Distribution of lesions in cattle infected with *Mycobacterium bovis*. *J Vet Diagn Invest* **8**(3), 351-354.

Wilkins, M. J., Meyerson, J., Bartlett, P. C., Spieldenner, S. L., Berry, D. E., Mosher, L. B., Kaneene, J. B., Robinson-Dunn, B., Stobierski, M. G., and Boulton, M. L. (2008). Human *Mycobacterium bovis* infection and bovine tuberculosis. outbreak, Michigan, 1994-2007. *Emerg Infect Dis* **14**(4), 657-660.

Wilkins, M. J., Bartlett, P. C., Judge, L. J., Erskine, R. J., Boulton, M. L., and Kaneene, J. B. (2009). Veterinarian injuries associated with bovine TB testing livestock in Michigan, 2001. *Prev Vet Med* **89**(3-4), 185-190.

Williams, M. S., Ebel, E. D., and Wells, S. J. (2009a). Poisson sampling: A sampling strategy for concurrently establishing freedom from disease and estimating population characteristics. *Prev Vet Med* **89**(1-2), 34-42.

Williams, M. S., Ebel, E. D., and Wells, S. J. (2009b). Population inferences from targeted sampling with uncertain epidemiologic information. *Prev Vet Med* **89**(1-2), 25-33.

Wood, P. R., Corner, L. A., Rothel, J. S., Baldock, C., Jones, S. L., Cousins, D. B., Mccormick, B. S., Francis, B. R., Creeper, J., and Tweddle, N. E. (1991). Field Comparison of the Interferon-Gamma Assay and the Intradermal Tuberculin Test for the Diagnosis of Bovine Tuberculosis. *Aust Vet J* **68**(9), 286-290.

Wood, P. R., Corner, L. A., Rothel, J. S., Ripper, J. L., Fifis, T., Mccormick, B. S., Francis, B., Melville, L., Small, K., De Witte, K., Tolson, J., Ryan, T. J., de Lisle, G. W., Cox, J. C., and

Jones, S. L. (1992). A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. *Vet Microbiol* **31**(1), 71-79.

Wood, P. R., and Jones, S. L. (2001). BOVIGAM (TM): an in vitro cellular diagnostic test for bovine tuberculosis. *Tuberculosis* **81**(1-2), 147-155.

Wood, P. R., and Rothel, J. S. (1994). In vitro immunodiagnostic assays for bovine tuberculosis. *Vet Microbiol* **40**(1-2), 125-135.

Zhu, J. G., and Lin, Y. A. (2011). Surveillance of infection by *Mycobacterium bovis*. *Rev Med Microbiol* **22**(2), 17-21.