APPLICATION OF DIAGNOSTIC ASSAYS FOR THE DETECTION OF MYCOBACTERIUM BOVIS INFECTION IN SUSPECT MICHIGAN RESERVOIR SPECIES

By

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A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Pathology

2011

ABSTRACT

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Mycobacterium bovis, the etiologic agent of bovine tuberculosis, is present on multiple continents and has one of the broadest host ranges of any known zoonotic pathogen. The broad host range of this microbe has created a complex ecosystem, in which diagnostic assay development, disease discovery, epidemiological characterization, surveillance, and control have proven to be a global challenge. In the state of Michigan, an endemic focus of bovine tuberculosis is maintained within the sole wildlife reservoir, the white-tailed deer; however, additional surveillance and assay application for the discovery of other potential wild and domestic animal reservoirs are currently being researched. This dissertation discusses four projects in which traditional diagnostic assays and three new assays utilizing a macrophage cell line and two antibody based assays; the multiantigen print immunoassay (MAPIA) and rapid test (RT), were implemented in the surveillance and disease characterization of *Mycobacterium bovis* in potential Michigan reservoir species. The first study was an evaluation of the immune response in specific pathogen-free (SPF) cats stimulated with Sensitinogen, a heat killed *Mycobacterium bovis* product, using the RT, MAPIA, and bovine purified protein derivative (bPPD) single skin test. The bPPD test at 72 hours had a mean skin thickness of 0.3 mm for stimulated cats and 0.1 mm for controls. Rapid test identified 4 of 6 stimulated cats after bPPD injection. The MAPIA detected antibody against MPB83, 16/83, 16kDa, and *M. bovis* culture filtrate (MBCF) antigens. All assays differentiated between stimulated and control cats; however 7 of 49 non-SPF control cats had a reaction for either antigen MBCF or 16/83. These preliminary

studies show potential for antemortem detection of *M. bovis* among domestic cats. The second study was an evaluation of disease spread by experimentally assessing intra-species lateral transmission of virulent *M. bovis* among four replicate inoculated/exposed, co-habitating wild caught *Didelphis virginiana* (Virginia opossum). Gross and histologic examinations were consistent with caseogranulomatous pneumonia in all four inoculated opossums. Additionally, these four inoculated opossums had a positive test band on the RT and were *M. bovis* culture positive. The exposed and control groups were unremarkable on gross, histology, rapid test, and culture. These findings support that there was no appreciable lateral transmission of *M. bovis* after aerosol inoculation and 45 days of cohabitation between opossum. The third study was a surveillance of 124 hunter harvested feral in the state of Michigan from November of 2006 until September 2010. Three swine were noted having gross lesions of tuberculosis; however, these were not confirmed to be tuberculous lesions histologically. All other animals in the study had no evidence grossly or histologically of tuberculosis. The fourth study utilized the monocytic cell line TIB-202 to propagate *M. bovis* in order to investigate a more rapid, sensitive, flexible, and cost effective diagnostic culture method. The phagocytic cells were incubated with virulent *M. bovis*, lysed, and evaluated for *M. bovis* propagation by PCR amplification of the IS6110. The preliminary PCR results were able to detect a marked increase in amplifiable DNA within lysated cells after six days of incubation, supporting idea that this cell line could potentially be used as a rapid detection diagnostic assay; however, continued research is needed. In conclusion, domestic cats, Virginia opossum, and feral swine appear to have a minimal impact on bovine tuberculosis spread and persistence in the state of Michigan. However minimal the current impact maybe, continued research and surveillance is empirical for disease characterization and the ultimate global eradication of tuberculosis.

ACKNOWLEDGEMENTS

I would like to extend thanks to everyone who made it possible for me to achieve my doctorate of philosophy while in my residency training. It has been a long challenging journey but very rewarding. The guidance, expertise, and patience of my major professor, Dr. Scott Fitzgerald, and committee members, Dr. Steve Bolin, Dr. John Kaneene, and Dr. John Kruger, is appreciated, thank you. Additionally, I would also like to extend my thanks to the technical staff and faculty at Michigan State University and collaborative institutes for their assistance in animal care, gathering of data, and data interpretation. Thank you: Dr. Konstantin Lyashchenko, Dr. Rena Greenwald, Dr. James Sikarskie, David Marks, Janet Hengesbach, Shannon Griffin, Cindy Garrison, Dr. Ailam Lim, Dr. Jean Gaymer, Dr. Danielle Ferguson, RoseAnn Miller, and Joseph Hattey. A special thank you to my fellow graduate students, resident mates, and close friends, here's to wishing you success in your own careers. Last but not least, I would like to thank my parents, David and Janie Fenton, who have been a positive influence in my life by supporting my personal goals no matter how challenging.

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CHAPTER 1

General Microbiology and Pathology of *Mycobacterium* Species

The genera of *Mycobacterium* contain numerous diverse species that encompasses multiple important diseases within animals and humans including leprosy, Johne's disease, and tuberculosis. These bacterial species are organized into categories as leprosy, mycobacterium other than tuberculosis (MOTT), and the mycobacterium tuberculosis complex (MTC), respectively. Each category contains multiple *Mycobacterium* species that produce similar disease within each of the respective classification. This dissertation will largely focus on the mycobacterium tuberculosis complex. This complex includes the following species, *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium microti*, *Mycobacterium africanum*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis*[2].

Mycobacteria in general are classified as a gram positive bacterium; however, due to the high levels of mycolic acids and lipids in the cell membrane it is better classified as an acid-fast bacillus, which resists decolorization[7]. Mycobacteria are related to other mycolic acid containing organisms such as *Nocardia* and *Rhodococcus.* These bacteria measure approximately 0.2-0.6um by 1.0-10 um, are slightly curved rods, largely aerobic, non-spore forming, and non-motile[4]. Pigmentation varies across the different *Mycobacterium* species. The strains that produce no pigment are classified as nonphotochromogen and those strains that produce pigment are considered scotochromogen. Additionally, those strains that require light to form pigment are termed, photochromogen. The generation time or duplication time ranges from 2-24 hours, which is considerably long when compared to other bacteria; such as, *Escherichia coli*, having a duplication time near 20 minutes[7]. This long generation time hinders isolation of a pure culture of mycobacteria, as contaminants often over grow the media.

The appropriate pretreatments and processing of samples by homogenization, decontamination, and concentration, will minimize the effects of potential contaminants. Additionally, the appropriate selection of culture media and incubation conditions must be optimized to facilitate recovery of *Mycobacterium*. The selected agent for decontamination, which aids in the reduction of non-specific bacterial over growth, is commonly sodium hydroxide. This compound minimizes bactericidal effects on *Mycobacterium*, optimizes the mucolytic effects, and is largely compatible with most commercially available broth culture systems, including the radiometric BACTEC 460TB system[4]. However, some systems such as the Mycobacterium Growth Indicator Tube (MGIT) method are not suitable with this form of decontamination; therefore, adjustments are required during processing[4]. In general, after homogenization and decontamination, concentration is achieved by centrifugation at greater than or equal to 3,000 times gravity for 15 minutes. The remaining sediment is smeared on to a glass slide then inoculated onto the appropriate liquid and solid media. Smear microscopy is one of the quickest, simplest, and easiest ways to detect Mycobacterium presence[4]. Traditional gram stains do not yield satisfactory results, largely due to the high mycolic acids on the surface of the bacteria which resist the proper uptake of dyes. Additional staining procedures are applied to *Mycobacterium* yielding in satisfactory results, such as Kinyoun's cold carbol fuchsin method, acid fast staining procedures of Ziehl-Neelsen, and fluorochrome methods[4]. The second and third staining methods respectively were recently identified as superior to the Kinyoun's cold carbol fuchsin method[4]. Culture methods have been more successful at detection of 10^1 or 10^2 viable organisms per milliliter than smear procedures; therefore, multiple identification procedures are often applied to one sample. Typically, parallel inoculation of liquid media for a

rapid initial isolation of mycobacteria and solid media to aid in the phenotypic characterization of the colonies are performed. The two main categories of solid media include egg-based and agar based, of the egg based media the most commonly used is Lowenstein-Jensen (LJ)[4]. This media is largely selective for *Mycobacterium tuberculosis*, with minimal growth for other mycobacteria. There are multiple elaborate liquid culture isolation systems available to isolate mycobacteria ranging from simple broths and tubes (MGIT and MB Redox), to semi-automated systems (BACTEC 460B), and finally to fully automated systems (BACTEC 9000 MB and BACTEC MGIT 960, ESP Culture systems II, and MB/BacT ALERT 3D System)[4]. A brief description of the above described *Mycobacterium* isolations systems are as follows: the MIGT systems is composed of a modified Middlebrook 7H9 broth incorporated with a fluorescence quenching-based oxygen sensor which is able to detect the growth of mycobacteria, as the bacteria grow, the oxygen depletes leading to an increase in intensity of florescence. The MB Redox is a nonradiometric medium system composed of a modified Kirchner medium enriched with growth-promoting additives, antibiotics, and a colorless tetrazolium salt as a redox indicator; as the bacteria grow, reduction of the tetrazolium salt to the colored formazan is visualized. The semi-automated system, BACTEC 460TB media is enriched with a carbon 14 labeled palmititc acid source, as the bacteria grow, release of carbon 14 labeled carbon dioxide is released and monitored, which is directly proportional to the growth rate. The remaining fully automated systems are similar to the semi-automated; however, these systems no longer rely on radioisotopes[4]. Additives of antibiotics into culture media will selectively enhance the growth of *Mycobacterium* and retard the growth of potential contaminates. Common antibiotic for *Mycobacterium* selective media include, penicillin, nalidixic acid, cycloheximide, lincomycin,

nalidixic acid, carbecicillin, polymyxin B, trimethoprim lactate, and amphotericin B[4]. Regardless of system or selective media the optimal incubation environment is important, generally the optimal incubation parameters are, 35 to 37 degrees Celsius, 5-10% carbon dioxide, and 6-8 weeks duration of incubation[4]. Speciation of *Mycobacterium* is approached by multiple laboratory techniques including phenotypic tests, mycolic acid analysis, and genotypic analysis. Specific phenotypic tests include the following: growth rate, growth temps, pigmentation, photoreactivity, colony morphology, arylsulfatase reactivity, catalase reactivity, iron uptake, niacin accumulation, nitrate reducation, pyrazinamidase reactivity, sodium chloride tolerance, inhibition by thiophene-2-Carboxylic Acid Hydrazide, Tellurite reduction, Tween 80 hydrolysis, and urease reactivity[4]. Additionally, mycolic acids maybe analyzed via high pressure liquid chromatography. Multiple approaches for genomic analysis are available; however, the most widely used to identify the mycobacterium tuberculosis complex is the restriction fragment length polymorphism (RFLP) pattern of the insertion sequence 6110 (IS6110)[4].

The mycobacterium tuberculosis complex is largely classified as obligate pathogens; however, other *Mycobacterium* species can principally be classified as obligate, opportunistic, or saprophytic pathogens[7]. Mycobacteria possess the ability to infect a wide range of hosts these include, domestic animals (e.g. cattle, swine, dogs, cats, and chicken), wildlife (e.g. deer, elk, coyotes, raccoons, opossums, and black bear), aquatic animals (e.g. fish and amphibians), and humans[8]. The prominent route of infection often differs with each animal's susceptibility to infection and the infectious dose[2]. The disease of tuberculosis is largely considered an aerosol-inoculation; however, oral-inoculation and direct contact of wounds are well documented[8].

The immunopathogenesis of tuberculosis is intensely studied; however, the full cascade of events has yet to be fully understood. Recent identification of two specialized secretion systems associated with virulence, ESX-1 and ESX-5, now termed the type VII secretion system are instrumental for the pathogenic nature of tuberculosis[1]. The virulence factors encoded within the RD1 locus, such as, the early secreted antigenic target of 6kDa (ESAT-6) and culture filtrate protein of 10 kDa(CFP-10) are thought to be secreted by means of these systems and have proven to be important as T-cell antigenic targets[1,3]. *Mycobacterium* is an intracellular pathogen, which elicits predominately a cell mediated immune response. The bacterium is phagocytosized by resident macrophages and subsequently disrupts the fatal fusion of the *Mycobacterium* containing phagosome and the pre-formed lysosome. The orchestrated interaction of the *Mycobacterium* within the phagosome is the initial stimulus for the host immune response. This response is critical for the formation of the characteristic gross pathologic features of tuberculosis, the tubercle[5].

Tuberculosis is well suited as a respiratory pathogen, as the infectious particle “the droplet nucleus” is ideal for alveolar infection[2]. Inhaled particles measuring less than 1um in diameter are considered to be too small to contain infectious organisms. Inhaled particles that are less than or equal to 5um may harbor up to 5 infectious organism per droplet nucleus and are still considered small enough for passage into the alveolar spaces[2]. Particles measuring 6-10um travel to the secondary bronchi and terminal bronchioles where as larger particles measuring 20-60um reach the mid-region of the trachea and primary bronchi. The largest particle, measuring greater than 60um become lodged within the mucoid sediment of the upper respiratory tract[2].

Pulmonary alveoli lack the mucocillary apparatus present in the upper portions of the respiratory tract; therefore, making alveoli the most susceptible portion of the respiratory tract to disease[7].

The ability of mycobacteria to penetrate to this region is crucial for disease progression. In cattle and guinea pigs, the infectious droplet nucleus is 0.7-7um with a minimum infectious dose of 1 organism in the alveolus. In the alveolus, the alveolar macrophage is the first line of defense for the host; however, this system is largely circumvented by the bacterium. Phagocytosis by non-activated macrophages is critical for the survival of the bacterium, as activated macrophages have the ability to kill phagocytized *Mycobacterium* species. The fate of the internalized microbe was described in the late 1960s and early 1970s, with the identification of a "non fusogeneic" phenotype of *Mycobacterium* and it's positive correlation with active tuberculosis infection[6].

The identification of this phenotype spurred a marked increase of phagosome physiology research leading to the discovery in the 1990s that the phagosomes containing *Mycobacterium* were less acidic then the adjacent lysosomes. Specifically, the *Mycobacterium* containing vacuoles were restricted to an acidity of a pH 6.2-6.3, deviating from the expected pH 5.2[6].

Upon entry into the host cell, phagosomes quickly mature in a highly dynamic and multiphase event. Two stages that posses different biologic markers have been classically noted, the early phagosome and late phagsome[6]. GTPases of the Rab family are well known phagosome maturation marker proteins that are present on organelles. Other well studied markers of the endocytic pathway include but are not limited to, the early endosomal antigen1 (EEA1), lysosome-associated membrane glycoprotein 1 (LAMP1), transferrin, coronin I, and cathepsin D[6]. The acquisition and or loss of these markers aids in the identification of the maturation process. Rab5, an early endosome recruiter of pre-phagosomes, dissociates when Rab7 is

acquired, a late endosomal marker. The acquisition of lysosome-associated membrane glycoprotein 1 (LAMP1) is indicative of phagosome and lysosomal fusion and eventual maturation that leads to acidification of the vacuole. Interestingly, in *Mycobacterium* infected cells Rab5 is retained, indicating the arrest of phagosome maturation[6]. *Mycobacterium* containing phagosomes have the ability to acquire multiple markers such as, transferrin (a marker of the cell membrane recycling pathway), major histocompatibility complex II (HMCII), lysosome-associated membrane glycoprotein 1 (LAMP1), coronin I (an actin-binding protein associated with early phagosomes), and calmodulin D (calcium-binding protein associated with early phagosomes)[6].

Mycobacterium survives within the host by implementing two main tactics: 1. Evading or inhibiting an effective immune response and 2. Suppressing essential pathways to the production of an effective immune response[5]. Evasion of the humoral immune response is first implemented by the internalization or phagocytosis of the bacteria, which is initiated by Toll like receptors or by complement associated opsonization. This internalization elicits a release of multiple pro-inflammatory cytokines and chemokines such as, tumor necrosis factor alpha (TNF-alpha), CCL2, CXCL10, IL1-alpha, IL1-Beta, and IL18, this combination of signaling molecules initially recruit neutrophils which in turn release additional signaling molecules to recruit macrophage and lymphocytes via the pro-inflammatory cytokines and chemokines of TNF-alpha, CCL2, CXCL10, CXCL9, CCL5, CCL3, and CCL4. IFN-gamma is released from the recruited lymphocytes; the T-helper CD4+ contributes the bulk of the signal with a lesser contribution from the cytotoxic CD8+ T-cells. INF-gamma down regulates the pro-inflammatory response by inhibition of TNF-alpha, increased expression of MHC molecules, increased expression of antigen processing components, and activation of macrophages[5]. INF-gamma

activated macropahges are able to override the arrestment of the *Mycobacterium* containing phagosome and allow for the acidification of the vacuoles to a pH 5.2. The viability of the engulfed *Mycobacterium* is dependant on both the acidification of the vacuoles and the production of nitric oxide via the inducible nitric oxide synthase (NOS), both of which occur in activated macrophages[7]. The recruitment of additional activated macrophages leads to fusion and the formation of Langhans type multinucleated giant cells (MNGCs)[5]. The characteristic granuloma is constructed as inflammatory cells are recruited to the site of infection. The stable granuloma has multiple distinct regions noted histologically; the central portion is composed of one or more phagocytized *Mycobacterium* surrounded by numerous foamy macrophages (activated), multinucleated giant cells, macrophages (nonactivated), lymphocytes, and ultimately a rim a fibrosis[7]. The mature granuloma undergoes central regional necrosis and mineralization as the vascular supply becomes compromised; this necrosis is thought to precede the cassation, break down of the surrounding fibrosis, and ultimate release of the microbe[5]. Maintenance of the fibrotic encapsulation is indicative of latent tuberculosis[7]. During times of stress (nutritional, environmental, pregnancy, etc…) the immune system maybe compromised and the tubercle may actively start shedding organisms. Animals infected with *M. bovis* have varying clinical signs depending on the number of organisms and the host immune response. In general animals will be lethargic, thin, and exhibit mild respiratory distress[8]. If the immune system is compromised the risk of secondary infections is also a concern.

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CHAPTER 2

General Epidemiology, Classification, and Diagnostic Assays for *Mycobacterium* Species

Tuberculosis is estimated to be one of the oldest diseases to mankind. Prior to the identification of the etiologic agent, this disease was known as consumption, white plague, and a multitude of similar names that described the clinical and gross features of the disease. The earliest evidence of tuberculosis was noted in neolithic bone remains that show evidence of the stereotypical angulation often seen with spinal tuberculosis. The first documented mortality caused by tuberculosis was recorded in the 1850s and ever since humans have been in a battle for the eradication of tuberculosis. In 1882, Koch first identified *Mycobacterium* as the causative agent for tuberculosis. In the early 1920s the discovery and subsequent human trials for the Bacillus Calmette-Guérin (BCG) vaccine, an attenuated *Mycobacterium bovis* strain, was attempted. Beyond the discovery of the BCG vaccine, public health advances to decrease the transmission and infection of tuberculosis were implemented, these included, the pasteurization of milk in the 1930s and discovery of effective antibiotics in the 1940s and 1950s. In the 1960s, Crofton designed the first coordinated public health initiative for prevention and treatment of tuberculosis which included the use of a combination of antibiotics, pasteurization of milk, tuberculin testing of cattle, BCG vaccinations of whole populations, chest radiographs for early detection, and reduction of overcrowding. This intensive initiative was successful, as indicated by the lowest recorded case load of tuberculosis infections during this era; however, closely following this new initiative, the first antibiotic resistant tuberculosis was identified with a progressive resurgence of tuberculosis[6]. Currently the World Health Organization (WHO) estimates that approximately one third of the world's population (near 2 billion people) have latent tuberculosis with 7-8 million cases of active tuberculosis noted each year. The first recorded multidrug resistant tuberculosis (MDR-TB) was documented in 1995[6].

Running in parallel with the above described human health initiatives were US federal supported eradication program for bovine tuberculosis, started in 1917[24]. This program includes testing of livestock on farms and monitoring of animals sent to slaughter or transported across state lines. As a result of this program, bovine tuberculosis is nearly eradicated from cattle in the United States.

In 1979, Michigan was declared bovine tuberculosis free and maintained this status until 2000, when bovine tuberculosis was apparent in wild white-tailed deer and subsequently domestic livestock[8,16,24]. Today Michigan has been granted a split state status[8]. The five United States Department of Agriculture (USDA) designated bovine tuberculosis status levels are accredited free zone (AFZ), modified accredited advanced zone (MAAZ), modified accredited zone (MAZ), accredited preparatory zone (MPZ), and non accredited zone (NAZ)[8]. The highest status level is accredited free, with zero percent prevalence of bovine tuberculosis in a state or zone. The lowest designation is non accredited. Requirements for movement, surveillance, testing, record keeping, and animal identification are specific for each state or zone. As stated previously, in 1979 Michigan was declared bovine tuberculosis free and was revoked this status in 2000 due to the identification of a positive white-tailed deer. At this time an intense comprehensive surveillance plan was established to determine the extent of disease. In 2000, the upper and lower peninsulas of Michigan were considered modified accredited, which resulted in restrictions on animal movement. An extensive bovine tuberculosis surveillance program that tested every known cattle, goat, bison, and privately owned cervid herd in the state of Michigan was lead by the Michigan Department of Agriculture (MDA), USDA, and private veterinarians from January of 2000 to December of 2003[9]. Additionally, the Department of Natural Resources

(DNR), Michigan Department of Community Health (MDCH), and Michigan State University (MSU) tested thousands of hunter-harvested white-tailed deer for bovine tuberculosis. This surveillance identified that cattle were at risk for infection when bovine tuberculosis positive white-tailed deer were in close proximity. In April 2004, the MDA requested split state status and the USDA granted approval. Eleven counties and portions of two surrounding counties were considered modified accredited zone (MAZ), the remaining portion of the state was classified as modified accredited advanced zone (MAAZ). In 2005 the upper peninsula was considered free of bovine tuberculosis (accreditation free zone), leading to three zones of accreditation, accreditation free zone (AFZ), modified accredited advanced zone (MAAZ), and modified accredited zone (MAZ). In 2009, the MAAZ was expanded to include 6 more counties, with 3 subzones of decreasing prevalence of bovine tuberculosis within both cattle and white-tailed deer populations (Figure 2.1)[8]. Full eradication of bovine tuberculosis is the ultimate goal; therefore, identification, tracking, and testing of cattle and deer is essential. The requirements for identification of all privately owned livestock include premise identification. All Michigan cattle must be officially identified with a radio frequency identification tag (RFID) prior to movement from the farm of origin[9]. These tags are linked to the premise identification and allows for quick identification of herd origin, in the case of a disease investigation. Surveillance testing of cattle depends on the zoning and/or the subzoning, where the farm is located, class, purpose, and age of the animal. All cattle in the MAZ are required to have RFID prior to tuberculosis testing, for tuberculosis testing in the MAAZ and free zones all cattle must be officially identified. The bovine tuberculosis eradication effort requires continued testing of cattle outside the MAZ. A random surveillance system has been implemented since 2003, to determine if tuberculosis was present outside the MAZ. The computer generated system picks a herd at random based on the

number of herds in each region. To increase the ability to detect bovine tuberculosis in cattle, a risk based surveillance program was established. This random surveillance was able to test those herds with the greatest risk of exposure; such as, animals from the MAZ and counties surround the MAZ, animals originating from an infected herd, and those in proximity to white-tailed deer infected with bovine tuberculosis.

There are three phases implemented for individual animal testing[9]. Phase I of animal testing is the caudal fold tuberculin test (CFT), this test casts a wide net in determining those cattle that are potentially infected with bovine tuberculosis. The intradermal injection of bovine purified protein derivative (bPPD) is given and manually read in 72 hours. The CFT will generate nearly a 5-7% positive response rate in Michigan, and these cattle will be identified as responders. Individual responder animals deem the entire herd to quarantine. Phase II is implemented on the individual responder animals. One of two tests maybe implemented the comparative cervical skin test (CCT) or the IFN-gamma test. There are specific requirements for running these tests in sequence; the CCT can only be done within 10 days of the CFT test, if the test was not implemented within the 10 days the animal can only be tested after 60 days. The procedures for the CCT are as follows: the cervical neck is bilaterally shaved, one site is injected with avian tuberculin (aPPD), one site with bovine tuberculin (bPPD), and the injections are manually read at 72 hours post injection. The results of the skin test principally measure change in thickness; this measurement is plotted on the official USDA CCT scattergram. The results of the scattergram are read as negative, suspect, or a reactor. The herd quarantine is released when all animals on the premise are negative for the CCT. Animals that are classified as suspects have two options: 1. Removal of animal with euthanasia, necropsy, and testing at MSU, or 2. Retesting of suspect animals on the premise after 60 days. Animals that are classified as reactors

are required to be confined and all products that are intended for consumption shall be disposed. These reactor animals are sent for a full necropsy, which includes the associated histopathology and bacteriology tests. There is a potential for a whole herd bovine tuberculosis testing 60 days after removal of the phase II reactors. The second laboratory test option for phase II testing is the IFN-gamma test, which measures the individual animal IFN-gamma in response to both avian and bovine tuberculin. This test requires a sample of blood to be drawn, usually at the same time as the CFT test is read or within 30days of the tuberculin injection. The results for this test take 5-7 days and results are classified as either negative or positive. The herd quarantine is released when all animals on the premise are negative for the IFN-gamma test. Animals that are classified as positive have two options: 1. Removal of animal with euthanasia, necropsy, and testing at MSU, or 2. Retesting of suspect animals on the premise within 30 days. If an animal is deemed positive on the retest, they are considered positive and removed for full necropsy and laboratory testing. The final phase, phase III is necropsy and lab testing. This phase includes histopathology and bacterial culture. Positive animals at this phase may result in depopulation of the herd.

Despite this highly successful eradication effort, bovine tuberculosis remains a serious health concern in several countries. In the United States, recent bovine tuberculosis cases in Michigan, California, Minnesota, New Mexico, South Dakota, and Indiana, continue to emerge, indicating that tuberculosis is far from eliminated[25]. The current classification scheme for the state of Michigan is given to those regions with positive bovine tuberculosis in domestic livestock and/or certain species of wildlife species; including cattle, bison, and white-tailed deer; however, other potential reservoir hosts remain unaccounted. Surveillance in the state of Michigan during 2003 identified numerous potential untraditional reservoir and/or spill over host

species. The list included a wide variety of wildlife species such as, Virginia opossum, black bear, bobcat, coyote, raccoon, and red fox[1,10,13,14,15,19,20,22,23,26]. In addition, domestic animals (e.g. dogs and cats) residing in close proximity with infected cattle may also represent potential reservoirs. The current diagnostic tests used in the surveillance program (the skin tests, IFN-gamma assay, gross and histologic pathology, and culturing) are not validated in all species; therefore, continued research, and application of current diagnostic assays for potential reservoir host species is important.

Impedance of the eradication of tuberculosis in domestic livestock has been attributed to the presence of the disease in wildlife species. Historically, it is thought that the majority of infection originates in livestock and crosses over to the wildlife. Tuberculosis in some regions has become an important disease in wildlife, either through the direct effect of infection and disease spread, or through regulation of control measures. Internationally, control of bovine tuberculosis has proved difficult in situations where the disease persists in wildlife reservoirs. Identification of potential wildlife maintenance hosts will greatly aid in the eradication of tuberculosis worldwide. Tuberculosis has been reported in many animals; yet, only a few known wildlife species have the ability to maintain the disease via interspecies transmission in the absence of an external source of infection. These maintenance hosts include the Eurasian badger *(Meles meles)* in Ireland and the United Kingdom, the brush-tailed possum *(Trichosurus vulpecula)* in New Zealand, African buffalo (*Syncerus caffer*), lechwe (*Kobus lechwe*), warthog (*Phacocoerus africanus*) and kudu (*Tragelaphus strepsiceros*) in Africa, white-tailed deer (*Odocoileus virginianus*) in the United States (Michigan), and in Spain the red deer (*Cervus elaphus*) and the European wild boar (*Sus scrofa*)[2,11,23]. Other animal species are thought to be

spillover hosts in which tuberculosis does not persist indefinitely without reinfection from other species.

In Canada, *M. bovis* infection in multiple wildlife species did not cause great alarm until the disease was almost eliminated from domestic livestock. The plains bison, elk, moose, and mule deer in Buffalo National Park (BNP) harbored tuberculosis during the 1920s and 1930s. Infected bison were transported from BNP to Wood Buffalo National Park (WBNP), where tuberculosis remains endemic in the regional bison[23].

New Zealand has struggled with the control of bovine tuberculosis since the first detection of tuberculosis in the brushtail possum (*Trichosurus vulpecula*) during the 1950s. This wildlife species is currently considered a key reservoir impeding eradiation of tuberculosis in this country. Similarly since 1971 the United Kingdom (UK) has had numerous Eurasian badgers *(Meles meles)* culled after the identification of a tuberculosis positive badger near an infected livestock farm. Additionally, the first known case of tuberculosis in wild ungulates in Africa occurred in 1967 in the Kruger National Park and was subsequently identified in African buffalo (*Syncerus caffer*). African buffalo, much like the Eurasian badger and the brushtail possum, are ideal for the maintenance of tuberculosis due to their high susceptibility to the disease, survival and reproduction for years with the disease, and their communal behaviors which enhances spread of the bacterium[23].

Control and surveillance of tuberculosis in animals was largely based on a test and slaughter approach; however, improvements in antemortem and postmortem diagnostic assays has attributed to the reduction of excessive culling. The skin test is one of the most widely used antemortem screening assays for identification of bovine tuberculosis[21,24]. Cattle are injected

intradermaly at the base of the tail (caudal fold) with 0.1mL bPPD (bovine purified protein derivative). The injection of bPPD consists of inactivated proteins of *Mycobacterium bovis*. The overall purpose of the bPPD was to stimulate the immune system, resulting in recruitment of cells to the site of injection allowing for visual inspection of the immune reaction. However, differences in strains have resulted in slight inconsistencies among each bPPD product from different manufacturers[21]. Injection of bPPD mimics natural infection and stimulates a delayed type cell mediated immune reaction which can be best visualized (increased thickness and reddening) between 48 to 72 hours. This skin test is referred to as the caudal fold single skin test or the comparative cervical skin test when used in combination with avian purified protein derivative (aPPD). Weaknesses of this test include cross reactivity with other *Mycobacterium spp.* and false positives. Additionally, those animals in late stages of the disease may not respond (anergy) creating false negatives[18]. In cattle, to help eliminate other *Mycobacterium* reactivity the comparative cervical skin test is performed. The procedure and theory are similar to the caudal fold single skin test but injection of purified proteins from *Mycobacterium avium subspecies avium* are also administered on the opposite side of the cervical region. Alleviation of false negatives is achieved by implementation of additional diagnostic assays.

The LBA (Lymphocyte Blastogenic Assay) was one of the earlier cell mediated in vitro assays created to increase sensitivity, specificity and shorten identification time. The assay was performed by titrating thymidine after exposure to *Mycobacterium spp.* antigens as a way to measure cell response. Experimental application of this assay was promising but performed poorly detecting natural infection. Most laboratories implement the IFN-gamma assay over the LBA due to ease of protocol and increased sensitivity and/or specificity[23].

The INF-gamma assay (Bovigam) measures the INF-gamma release in response to *M. bovis* antigens[23]. T-cells are stimulated to release the IFN-gamma in order to active macrophages. IFN-gamma levels are measured using an ELISA, which eliminates the radioactive labeling used in the LBA. Overall this assay is well received and is used often as an ancillary assay with skin test and culturing. The major limitations of this assay are sample handling, as blood samples need to be processed with in hours of collection and inconsistency in test results.

Histopathology at time of necropsy or from a biopsy can aid in identification of Mycobacterium infections[3,4,5,17]. Identification of suspect cases can be determined within 2-3 days. Submitted tissues are fixed in 10% neutral buffered formalin and embedded within wax parafilm. The classical tubercle granuloma is visualized on standard staining with hematoxylin and eosin. The granulomatous lesion consists of a central area of caseous necrosis and mineralization rimmed by epithelioid macrophages and variable numbers of Langhans-type multinucleated giant cells (MNGCs), and an outer perimeter containing a rim of lymphocytes and a pseudocapsule of fibrosis. Typically, very few intra-phagocytic organisms are noted (pacibacillary) which can be highlighted using acid fast stains, such as Ziehl-Neelsen[18,23].

Additional assays are available but have failed to be more sensitive or specific than those listed above and ultimately culture is considered the gold standard for identification of tuberculosis positive animals. The most frequent scenario for tissue culture is at the time of necropsy although biopsy and other antemortem samples maybe processed. Samples are thoroughly decontaminated with a mixture of antibiotics to reduce the amount of background overgrowth and are then subsequently grown on soild and/or liquid media. The sample is incubated at 37 degrees. Phenotypic, biochemical, and finally genetic traits are identified

allowing for a specific classification. Culturing methods have been altered to speed up the traditional 6-8 week incubation[12].

Multiple antemortem diagnostic assays are emerging though continued research, these include multiple antigen print immunoassay (MAPIA), rapid test (RT), fluorescence polarization assay (FPA), rapid immunochromatographic test, 96-well plate multiplex system, dual path platform assay, chemiluminescent platform, and an improved ELISA. Currently, the two most promising antemortem diagnostic assays gaining validation in multiple host species are the MAPIA and RT[7]. These antibody-based assays are currently being optimized to identify the immunodominant antigens for each potential host species. The ultimate goal is to increase the speed along with the sensitivity and specificity of antemortem diagnostic assays. The MAPIA has identified multiple immunodomiant antigens such as, MBP70 (cattle), MPB83 (cattle, deer and badgers), and ESAT-6 (elephants, non-human primates) that will serve in optimizing the sensitivity and specificity of these upcoming antibody based assays for each host species[7]. Preliminary data has shown that the RT and MAPIA, using a multiple mycobacterial antigen cocktail have been reported to have a higher sensitivity and specificity in badgers (*Meles meles*) and were able to maintain serological reactivity longer than existing ELISA tests[7].

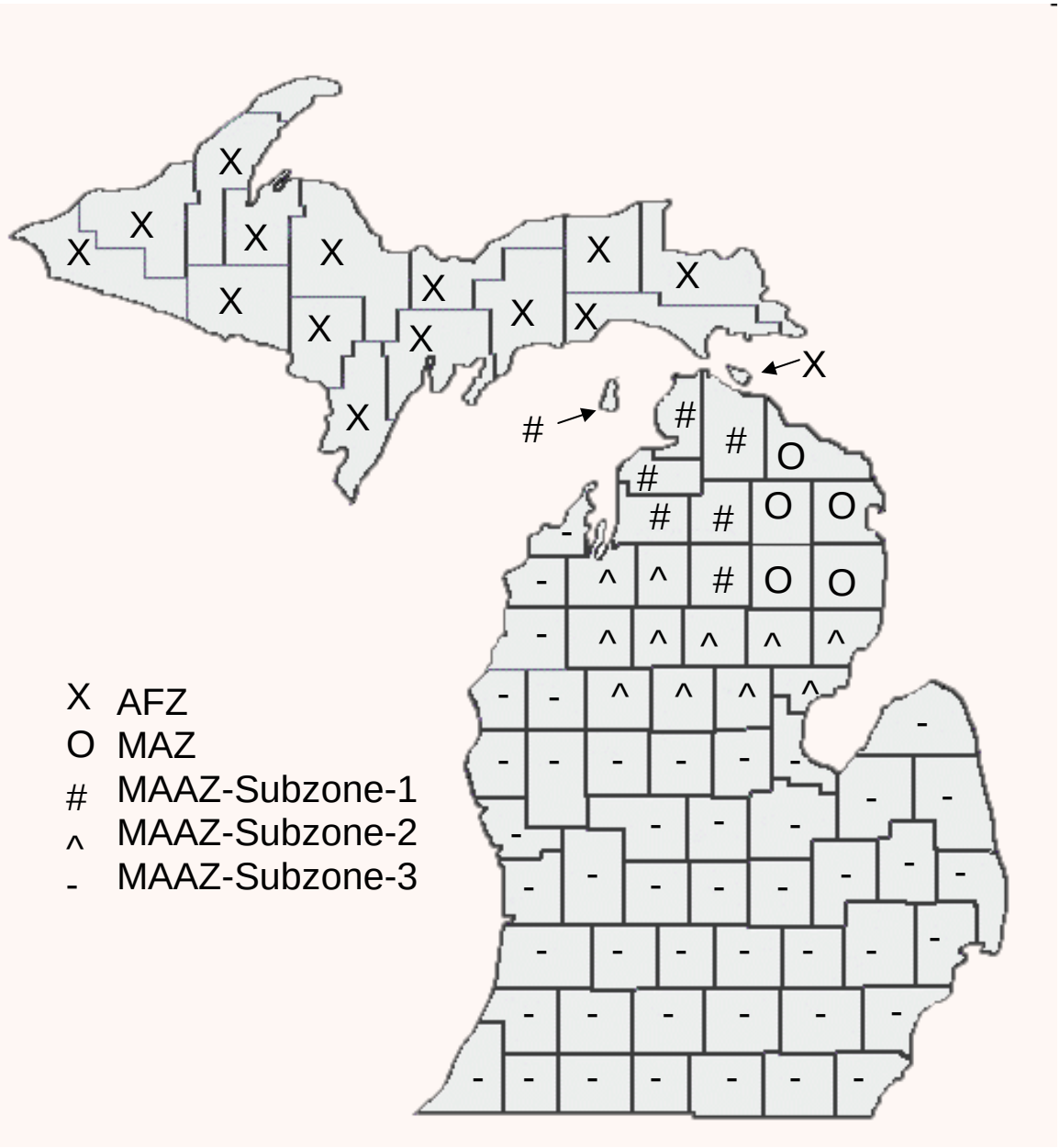

Figure 2.1 Michigan 2010 bovine tuberculosis zoning designation[4]
For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. This map indicates the zoning of United States Department of Agriculture (USDA) designated bovine tuberculosis status levels for the state of Michigan. The zones labeled as X for accredited free zone (AFZ), O for the modified accredited zone, # for modified accredited advanced zone (MAAZ)- Subzone-1, ^ for modified accredited advanced zone (MAAZ)- Subzone-2 , - for modified accredited advanced zone (MAAZ)- Subzone-3.

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CHAPTER 3

Comparison of Three Immunodiagnostic Assays for Antemortem Detection of *Mycobacterium bovis* Stimulation in Domestic Cats

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Reformatted version as published in J Vet Diagn Invest. 2010 Sep;22(5):724-9.

ABSTRACT

Mycobacterium bovis causes disease in numerous mammalian species including humans, thus making research, surveillance, and control important in the eradication of tuberculosis. Domestic cats are susceptible to multiple mycobacterial species including *Mycobacterium bovis*; however their role in the epidemiology of bovine tuberculosis is not fully documented. This study was an evaluation of the immune response in specific pathogen-free (SPF) cats stimulated with Sensitinogen, a heat killed *Mycobacterium bovis* product, using the rapid test, multiple antigen print immunoassay (MAPIA), and bovine purified protein derivative (bPPD) single skin test. Six cats were inoculated with Sensitinogen subcutaneously on days 0 and 24; two noninoculated cats and 49 non-SPF cats were controls. Serial serum samples were collected during 135 days and assayed for *M. bovis* antibodies by rapid test and MAPIA. On day 123, bPPD skin test was performed and read at 48 and 72 hours. The bPPD test at 72 hours had a mean skin thickness of 0.3 mm for stimulated cats and 0.1 mm for controls. Rapid test identified 4 of 6 stimulated cats after bPPD injection. The MAPIA detected antibody against MPB83, 16/83, 16kDa, and *M. bovis* culture filtrate (MBCF) antigens. All assays differentiated between stimulated and control cats; however 7 of 49 non-SPF control cats had a reaction for either antigen MBCF or 16/83. These preliminary studies show potential for antemortem detection of *M. bovis* among domestic cats. Additional studies to better characterize virulent *M. bovis* infection in cats would be of value.

Key Words: Antemortem; assay; cats; *Mycobacterium bovis*; sensitinogen.

INTRODUCTION

Domestic cats are susceptible to many different mycobacterial species, and disease is classified into 3 categories: classical tuberculosis, leprosy, and opportunistic.[11,19,22] Multiple species of *Mycobacterium* are able to cause tuberculosis; however, the classic form of tuberculosis in cats is thought to be due to *Mycobacterium bovis* which can produce clinical disease in a wide range of mammalian species including humans, thus making research, surveillance, and control of *M. bovis* important in the eradication of tuberculosis.[1-3,6,11,21,22,24]

Antemortem diagnosis of *M. bovis* infection in potential reservoir species, especially cats, has been difficult.[8,19] Antemortem diagnosis of *M. bovis* infection within cattle uses the tuberculin skin test, the single intradermal comparative tuberculin test, and the gamma interferon assay; however, skin testing is reportedly inconsistent when used in domestic cats.[4,5,11,13,14,19,24] In an effort to increase sensitivity and specificity in antemortem assays, laboratories have expanded their research to include both cell mediated and humoral immune responses with diagnostic tests such as enzyme linked immunosorbent assay (ELISA), multiple antigen print immunoassay (MAPIA), and rapid test. The most recent immunodiagnostic tests developed for cats are the gamma interferon (IFN-γ) ELISA and enzyme-linked immunospot (ELISPOT) assay.[23] These blood assays incorporate the following antigens: avian-purified protein derivative (aPPD), bovine PPD (bPPD), positive mitogen (PMA/CA) and a cocktail of ESAT-6 and CFP10 which aid in predictability of *M. bovis* and *M. microti* infection, but were unable to reliably predict *Mycobacterium avium* infections. In other host species, MAPIA has identified MBP70 (cattle), MPB83 (cattle, deer, and badgers), and ESAT-6 (elephants, nonhuman primates) as immunodominant antigens.[6,9,10,12,16,17,26] The rapid test and MAPIA,

when used with the mycobacterial antigen cocktails, have been reported to have a higher sensitivity and specificity in Eurasian badgers (*Meles meles*)[10] and were able to maintain serologic activity longer than existing ELISA tests.[17] To date, the gold standard for diagnosis of feline tuberculosis still remains bacterial culture from biopsy or at time of necropsy. The present study was aimed at identifying antemortem methods to detect *M. bovis* infection by evaluating serologic assays, rapid test and MAPIA, and the single skin test using bPPD in cats experimentally stimulated with sensitinogen, a heat-killed *M. bovis* strain AN-5 suspended in mineral oil.[7]

OBJECTIVE

Mycobacterium bovis is endemic in the wild deer population of northern Michigan and previous reports have identified the domestic cat as a possible frequent contact species. This project will investigate possible antemortem tests in the domestic cat therefore decreasing indiscriminate culling or propagation of *M. bovis*.

HYPOTHESIS

If experimental stimulation of cats with Sensitinogen produces a detectable immune response then the antemortem tests of MAPIA, RT, and bPPD single skin test can be compared.

MATERIALS AND METHODS

Eight, 6-month-old, specific pathogen free (SPF), purpose breed, domestic short hair cats were purchased from a commercial vendor[a]. All cats were vaccinated with a killed virus preparation at 5 months of age for protection against panleukopenia, rhinotracheitis, and calicavirus (FELOVAX PCT[b]) before purchasing. Institutional Animal Care and Use Committee (IACUC)-approved guidelines were implemented. Cats were group housed, monitored daily, housed at a temperature range of 22°C +/- 2°C humidity range of 40-50%, and were maintained on a commercially available cat food with water ad libitum within the animal containment facility at Michigan State University (MSU; Lansing, Michigan). In addition, serum was also collected from 9 deceased domestic cats received at the Diagnostic Center for Population and Animal Health (MSU) and from 40 SPF control cats that participated in a previous research project.

Lot number 0401 sensitinogen[c] was administered at a dose of 0.4ml subcutaneously over the lateral body wall to 6 of the 8 cats on days 0 and 24 to induce a cell-mediated hypersensitivity to *M. bovis*. The 2 remaining cats that were not injected served at controls. Approximately 4 mls of blood was collected by jugular venipuncture approximately every 30 days. Immediately following venipuncture, blood samples were clotted at 4°C for 1 hr, centrifuged at 5,000 x g for 5 min, serum was then separated into sterile tubes and frozen at -20°C until all samples were collected for the entirety of the study.

On day 123, approximately 2 weeks before the last blood draw, the right pinna of each cat was measured with dermal calipers to the nearest 0.05 mm, and 0.1 ml of bPPD[d] at a concentration of 1.0 +/- 0.1 mg/ml was injected intradermally to the right inner pinna. Pinnae

were monitored for heat, reddening, and thickness. Pinna thickness was measured by dermal calipers at 48 and 72 hours post bPPD injection.

The rapid test, performed at a commercial laboratory[e], is a lateral-flow, blue latex bead signal-based, qualitative antibody detection assay that utilizes a nitrocellulose membrane impregnated with a cocktail of selected *M. bovis* antigens (ESAT-6, CFP10, Acr1, MPB83). Thirty microliters of test serum and 3 drops of diluent buffer were added to the test well and the result of the reaction was read by visual evaluation after 20 min.[15] An antibody-positive sample was indicated by a visible band at both the test and control lines, whereas an antibody-negative sample was indicated by a visible band at the control line but no band at the test line.[15]

The MAPIA, performed at a commercial laboratory[e], used purified *Mycobacterium tuberculosis*-complex antigens (ESAT6, CFP10, MPB64, MPB59, MPB70, rMPB83, 16kDa, and 38kDa), fusion proteins (E6/P10 and16/83), and native antigens (bPPD), and *M. bovis* culture filtrate (MBCF) at a concentration of 0.05 mg/ml[f]. The antigen imprinted nitrocellulose membrane was cut into strips and processed as described previously.[17] Test strips were then incubated with serum samples diluted 1:40 in blocking solution for 1 hr at room temperature. Antibody/antigen reaction bands were visualized by using protein A-horseradish peroxidase conjugate and substrate 3,30,5,50-tetramethyl benzidine.[g,h] The most prominent bands noted as positive results were also analyzed semiquantitatively by densitometry.[18]

Two representative sensitinogen-stimulated cats were sedated with a cocktail of ketamine hydrochloride (11 mg/kg) and acepromazine (0.01 mg/kg) before euthanasia by intracardiac injection of sodium pentobarbital (200 mg/kg). These two sensitinogen-stimulated cats were

euthanized, and full necropsies were performed. Two large ulcerated granulomas were noted grossly on each cat. These lesions correlated with the site of sensitinogen injection. Tissue samples collected included skin at sensitinogen injection site, submandibular and retropharyngeal lymph nodes, and tonsil. Harvested tissues were fixed in 10% neutral buffered formalin, routinely processed, sectioned, and stained with hematoxylin and eosin. Light microscopy revealed chronic granulomatous dermatitis with multifocal fibrosis, necrosis and mineralization in both cats. The tonsil of one cat was determined to have a mild multifocal tonsillitis. Regional lymph nodes of both cats had reactive lymphofollicular hyperplasia, but no inflammation.

Mean and standard deviation were calculated for the numerical data collected.[20] The 2-sample *t*-significance test was used to assess statistical difference between tuberculin skin test results for the stimulated and control cats. The t-statistic obtained from the data was compared to the *t*-distribution critical values table using the smallest degrees of freedom and P value of 0.05 for a 1-sided test and 0.025 for a 2-sided test.[20] Repeated measures analysis of variance (ANOVA) was performed on SAS 9.1 software for both tuberculin skin test and MAPIA densitometry data[i].

RESULTS

In all cats, thickness of the pinna before the bPPD injection was 0.1mm (Table 3.1). Change in pinna thickness for sensitinogen stimulated cats ranged from 0.15 to 0.3mm, whereas change in pinna thickness for control cats ranged from 0 to 0.05. Pinnae of senitinogen-stimulated cats after the skin test were grossly thickened and reddened, and warm to the touch; the controls had no notable change. Using the 2 sample *t*-test, the *t*-statistic was 7.08 with degrees of freedom equal to 1 resulting in a value of $P < 0.05$. Using the repeated measures ANOVA for determining significance of pinna skin thickness over time shows a F value of 9.67 and a P value of 0.0191, and a F value of 6.49 and P value of 0.0408 when looking at effects of sensitinogen stimulation over time. Quantifiable measurements from dermal calipers yielded greater than twice the increase in thickness of pinnae and a $P < 0.05$, suggesting a significant difference between stimulated and control groups at 72 hr after bPPD injection. Previous reports on the intradermal test in domestic cats suggested poor prediction of disease in naturally infected animals.[11,19] In contrast, the current study correctly identified all 6 sensitinogen stimulated cats by the bPPD single skin test. It should be recognized, however, that this experimental model using a killed *M. bovis* preparation may not fully reflect the dose and complexity of the delayed-type hypersensitivity responses induced during a virulent *M. bovis* infection. Therefore, the diagnostic performance of the skin test in cats with naturally acquired *M. bovis* infections may be different from that observed among the sensitinogen stimulated cats. Limitations of intradermal testing in cats echo concerns seen within bovine tuberculosis control and eradication programs for cattle, such as cross-reactivity from other *Mycobacterium spp.* and the potential effects on subsequent antemortem assays.[23]

The rapid test assay identified 4 of 6 stimulated cats after injection of bPPD; all other cats were uniformly negative. The rapid test antigen cocktail (ESAT-6, CFP10, Acr1, and MPB83) used in the current study was originally designed for species other than feline. The current cocktail of antigens may contribute to the low reactivity seen in the present experiment, as the only antigen identified by MAPIA as a potential reactive antigen in the sensitinogen-stimulated cats was MPB83. Reformulation of the rapid test to include 16/83, 16kDa, and MBCF may enhance the results of the rapid test. Recent studies have proven the rapid test to be useful in detecting *M. bovis* infections in llamas (*Llama glama*), elk (*Cervus elaphus*), white-tailed deer (*Odocoileus virginianus*), wild boar (*Sus scrofa*), Eurasian badgers, brushtail possums (*Trichosurus vulpecula*), and meerkats (*Suricata suricatta*); however validation of the rapid test for domestic cats has yet to be achieved .[6,9,10,12, 15,16,17,26]

Multiple antigen print immunoassay identified 16/83, 16kDa, MPB83 and MBCF as major antigens involved in the humoral immune response in sensitinogen-stimulated cats. Visually all sensitinogen-stimulated cats reacted consistently to antigen MBCF and less consistently to 16/83, 16kDa, and MPB83 antigens (Figure 3.1). The experimental controls had no reaction band early in the experiment, and by day 109 a faint band was noted and quantified by optical densitometry. Sensitinogen-stimulated and control cats were group housed, allowing for social interactions such as grooming between individuals. All Sensitinogen stimulated cats developed ulcerative granulomas at the site of injection and it is possible that control cats were exposed to sensitinogen during grooming and contributing to a mild immune response prior to administration of bPPD or this could be simply due to non-specific responses. The non-SPF controls were mostly unremarkable with 5 of 49 reacting to MBCF and 2 of 49 reacting to 16/83 antigens. Using densitometry, semiquantitative estimates of band signal intensities for

sensitinogen-stimulated cats were compared for antigens 16/83 and MBCF (Table 3.2). Using the repeated measures ANOVA, significance was determined for MBCF band intensity over time; days 0 - 109 (F value = 51.98, P = 0.0042), and when looking at effects of sensitinogen-stimulation over time (F value = 49.35, P = 0.0045). Using the repeated measures ANOVA, a lack of significance was determined for 16/83 band intensity over time; days 0 - 109 (F value = 0.59, P = 0.6930) and when looking at effects of sensitinogen-stimulation over time (F value = 0.55, P= 0.7142). A repeated measures ANOVA was not determined for days 0 - 135 due to overlap in values between senitinogen-stimulated and control cats indicating no statistical significance. The mean optical density for MBCF reactions obtained before the bPPD skin test within sensitinogen-stimulated cats was significantly greater than the mean for the control cats. In addition, antigens ESAT-6 and CFP10, commonly used for stimulation in the INF-γ assays are also cited to cross-react with nontuberculosis mycobacteria such as, *M. kansasii*, *M. marinum* and *M. gordonae*.[25] These findings suggest that antigens 16/83, 16kDa, and MPB83 play a major role in the humoral immune response of domestic cats stimulated with sensitinogen.

DISSCUSSION

The identification of immunodominant antigens 16/83, 16kDa, MPB83 and MBCF in domestic cats had not been elucidated prior to this experiment and identifies the need for an experiment using virulent *M. bovis* strains as there are inherent limitations of using a heat killed product, such as senitinogen. Documentation on the humoral and cell-mediated immune responses for sensitinogen versus a live *M. bovis* strain are lacking; however, one can speculate, antigens that are actively secreted especially during replication or those easily destroyed during heat processing will likely be represented at reduced or absent responses. In the present study, the only available option due to low numbers of known feline cases of natural *M. bovis* infection and standard animal care guidelines was to evaluate the antigenic profile of senitinogen-stimulated cats. In addition, this experiment did not include cats infected with nontuberculous mycobacteria. Future research comparing the antigenic profiles of cats infected with virulent *M. bovis* and nontuberculous mycobacteria are needed to optimize these potential diagnostic assays.

The epidemiology of feline tuberculosis, its relationship to bovine tuberculosis, and formulation of practical management strategies has been hindered by lack of reliable methods of antemortem detection of mycobacterium infections in cats. The 3 immunodiagnostic assays for bovine tuberculosis evaluated in the present study show the potential of detecting specific immune responses in experimentally senitinogen-stimulated SPF cats. The preliminary results show promise for the development of a serologic antemortem assay for domestic feline *M. bovis* infection. All tests (bPPD single skin test, MAPIA, and rapid test) would greatly benefit from a controlled study to determine the sensitivity and specificity of these tests within domestic cats infected with virulent *M. bovis*.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Janet Hengesbach, Jean Gaymer, Danielle Ferguson, and the MSU animal containment facility staff for help in animal care and restraint, and RoseAnn Miller for help in data analysis.

SOURCES AND MANUFACTURES

a. Harlan Laboratories Inc., Indianapolis, IN.

b. FELOVAX PCT, Fort Dodge Laboratories Inc., Fort Dodge, IA.

c. National Veterinary Service Laboratory, United States Department of Agriculture, Ames, IA.

d. Synbiotics Corp., San Diego, CA

e. Chembio Diagnostic Systems Inc., Medford, NY

f. Linomat IV, Camag Scientific Inc., Wilmington, DE.

g. Sigma-Aldrich, St. Louis, MO.

h. Kirkegaard & Perry Laboratories, Gaithersburg, MD.

i. SAS Institute Inc., Cary, NC.

Cats	0 hours	48 hours	72 hours
C 145	0.10	0.30	0.30
D 109	0.10	0.30	0.30
C 153	0.10	0.40	0.40
C 145	0.10	0.25	0.20
C 155	0.10	0.25	0.30
D 099	0.10	0.25	0.25
Mean (Standard Deviation)	-	0.30 (0.06)	0.30 (0.07)
D 115-Control	0.10	0.15	0.10
D 117-Control	0.10	0.10	0.10
Mean (Standard Deviation)	-	0.10 (0.10)	0.10 (0.00)

Table 3.1 Feline bPPD single skin test results in millimeters

Cats D 115 and D117 represent control cats and the remaining 6 cats are Sensitinogen stimulated. Measurements by dermal calipers were measured in millimeters prior to bPPD at 0 hours and post bPPD injection at 48 and 72 hours.

16/83	Day 0	Day 24	Day 51	Day 80	Day 109	Day 135
C145	0.00	0.00	0.00	0.00	0.00	67.08
C147	0.00	0.00	20.43	59.40	62.01	83.18
C153	0.00	0.00	0.00	0.00	67.00	85.31
C155	0.00	6.96	30.11	13.49	11.81	74.92
D099	0.00	0.00	35.31	37.46	33.57	83.42
D109	0.00	1.32	3.86	9.54	12.50	23.23
Mean (Standard Deviation)	-	-	-	-	31.15 (28.06)	69.52 (23.69)
D115	0.00	0.00	0.00	0.00	0.00	23.50
D117	0.00	0.00	0.00	0.00	1.75	2.54
Mean (Standard Deviation)	-	-	-	-	0.88 (1.24)	13.02 (14.82)
MBCF	**Day 0**	**Day 24**	**Day 51**	**Day 80**	**Day 109**	**Day 135**
C145	0.00	71.15	83.33	84.29	67.22	67.52
C147	0.00	82.67	98.11	99.88	85.93	106.88
C153	0.00	69.39	75.44	93.43	104.30	95.43
C155	0.00	58.95	75.55	79.90	82.30	78.60
D099	0.00	39.44	77.91	85.05	66.78	79.00
D109	0.00	36.95	74.64	88.91	70.32	61.14
Mean (Standard Deviation)	-	-	-	-	79.48 (14.57)	81.43 (17.10)
D115	0.00	0.00	0.00	0.00	1.65	69.45
D117	0.00	0.00	0.00	0.00	3.38	39.57
Mean (Standard Deviation)	-	-	-	-	2.52 (1.22)	54.51 (21.13)

Table 3.2 Feline MAPIA densitometry readings for antigens 16/83 and MBCF from day 0 to day 135

Cats labeled D115 and D117 represent control cats and the remaining 6 cats are Sensitinogen stimulated. Day 135 readings were collected 12 days after bPPD injection for skin testing. Data represents the actual densitometry readings for antigens 16/83 and MBCF. The mean and standard deviations at days 109 and 135 were calculated for Sensitinogen stimulated and control cats.

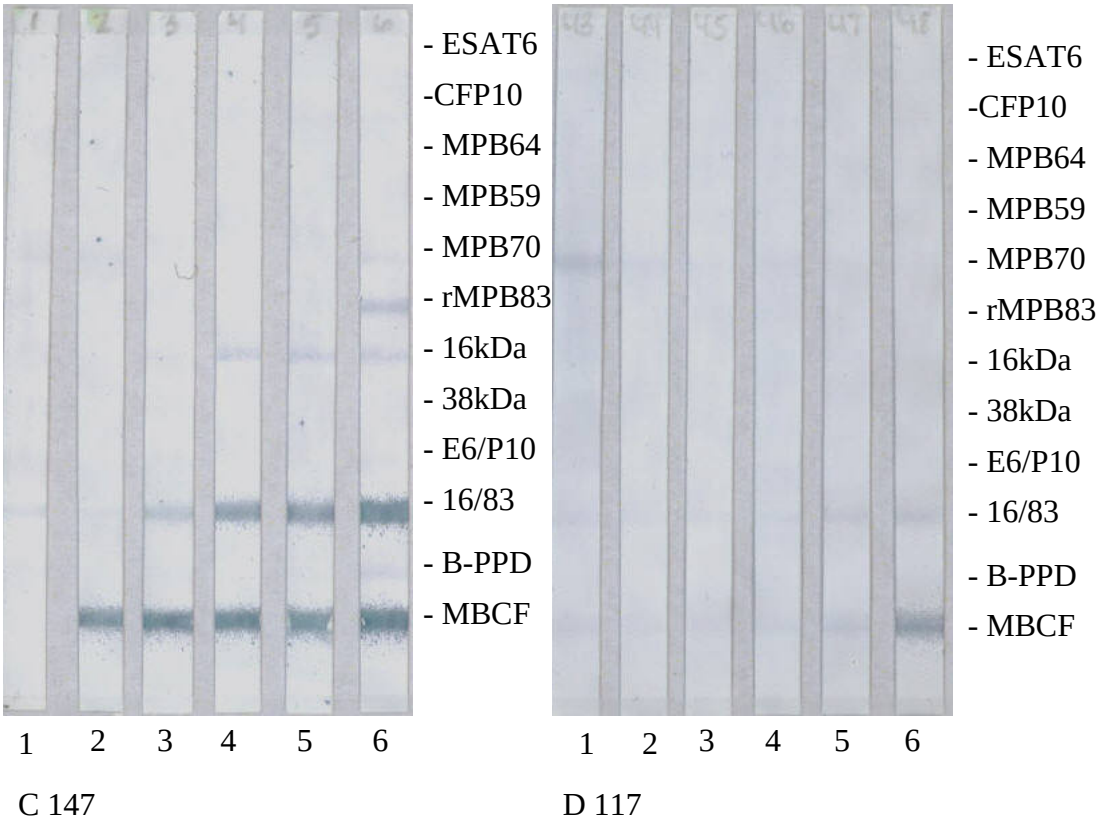

Figure 3.1 Multiantigen print immunoassay (MAPIA) results for a representative sensitinogen stimulated and control cat, C147 and D117 respectively.

Mycobacterium tuberculosis-complex antigens are indicated to the right of the test strips, a visible band indicates antibody presence and a positive reaction. The MAPIA strips are organized into groups of 6 strips per cat with each strip representing a sampling time point 1 = day 0, 2 = day 24, 3 = day 51, 4 = day 80, 5 = day 109 and 6 = day 135. Day 135 readings were collected 12 days after bPPD injection for skin testing. Among the stimulated cats MBCF, 16/83, 16kDa and rMPB83 were the most reactive.

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CHAPTER 4

Aerosol Inoculation and Experimental Lateral Transmission of *Mycobacterium bovis* In Virginia Opossum (*Didelphis virginiana*)

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This chapter will be submitted to the Journal of Wildlife Diseases.

ABSTRACT

An endemic focus of *Mycobacterium bovis (M. bovis)* infection in the state of Michigan has contributed to a regional persistence in the animal population. The objective of this study is to determine if *Didelphis virginiana* (Virginia opossum) are contributing to disease persistence by experimentally assessing intra-species lateral transmission. One wild caught pregnant female Virginia opossum bearing 11 joeys and one age matched joey were obtained for the study. At approximately 10 weeks of age four joeys received aerosolized *M. bovis (*inoculated), four joeys were non-inoculated (exposed), and four joeys plus the dam were controls. Four replicate groups of one inoculated and one exposed joey were housed together for 45 days. At day 84, Virginia opossums were sacrificed and a full necropsy was performed. Serum was collected for antibody testing via the rapid test and organs were weighed, cultured, and examined histologically. Gross and histologic examinations were consistent with caseogranulomatous pneumonia in all four inoculated Virginia opossums. Additionally, these four inoculated Virginia opossums had a positive test band on the rapid test and were *M. bovis* culture positive. The exposed and control groups were unremarkable on gross, histology, rapid test, and culture. In conclusion, *M. bovis* infection within the inoculated Virginia opossums was confirmed by gross pathology, histopathology, bacterial culture, and antibody tests. However, *M. bovis* was not detected in the control and exposed Virginia opossums. There was no appreciable lateral transmission of *M. bovis* after aerosol inoculation and 45 days of cohabitation between Virginia opossum.

Key Words: *Mycobacterium bovis*, Virginia opossum, *Didelphis virginiana*, lateral transmission

INTRODUCTION

Numerous wildlife species have proven to be a significant reservoir of *Mycobacterium bovis (M. bovis):* the European Badger (*Meles meles*) in Great Britain, the African Buffalo (*Syncerus caffer*) in South Africa, the Brushtail possum (*Trichosurus vulpecula*) in New Zealand, and the White-Tailed Deer (*Odocoileus virginianus*) in the United States[1,3,8,11-15]. Since the first reported case of tuberculosis within these reservoir species, multiple countries have been struggling to eradicate this disease. *Mycobacterium bovis* has the ability to produce disease within a wide range of species including humans, thus making collaborative efforts to discuss research, surveillance, and control essential to understanding the epidemiology of this disease. Virginia opossum (family Didelphidae) and the Brushtail possum (family Phalangeridae) both belong to the same order, Marsupialia; however distant, these relatives share similar behavioral traits that may contribute to the spread of bovine tuberculosis[6]. It has been established that Brushtail possums are an ideal host for bovine tuberculosis due to the fact that they are highly susceptible to *M. bovis*, shed the organism through multiple routes, and have shared dens[4,7,12]. Virginia opossum (*Didelphis virginiana*) is a known host of bovine tuberculosis in the state of Michigan and previous studies have shown them to be susceptible to *M. bovis* by aerosol inoculation[6,15]. Additionally, Virginia opossum utilizes shared dens, and in the state of Michigan, has a high potential for significant interaction with other animals harboring bovine tuberculosis[15]. This project aims to answer whether or not the Virginia opossum is contributing to disease spread by characterizing the intra-species lateral transmission after aerosol inoculation and 45 days of cohabitation.

OBJECTIVE

Determine if wild mammals other then deer, such as the Virginia opossum (Didelphis virginiana), are shedding M. bovis and contributing to spread of disease

HYPOTHESIS

If wild Virginia opossum are aerosol inoculated with virulent *M. bovis* and are cohabitating with non-inoculated Virginia opossum then significant lateral transmission will occur by direct contact.

MATERIAL AND METHODS

Virginia opossum

One wild caught, pregnant female Virginia opossum bearing 11 joeys plus one age matched joey from outside of the litter were obtained. Animals were assessed and clinically judged to be in good health at Michigan State University, College of Veterinary Medicine, Zoo and Wildlife Services. The dam was prophylactically treated with oral fenbendazole (50 mg/kg). Animals were monitored daily and offered a commercially available dry cat food and water ad lib with weekly supplements of granny smith apples or moist canned cat food. Institutional Animal Care and Use Committee (IACUC) approved guidelines were implemented

The stock *M. bovis* isolate was obtained from the Michigan Department of Community Health (MDCH), Lansing, Michigan, USA animal 08 TB 883 AF 327 DEER 269398. This pure culture was quantified by plating 100ul of culture onto Middlebrooks 7H10 agar and incubated at 37^{o} C. The undiluted stock culture was estimated to have 10,000cfu/ml, aliquotes were diluted to the desired concentration of 1×10^{6} colony forming units (cfu) per unit of volume. Sedation of the approximately 10 week old joeys was achieved by intramuscular injection of Telazol (Fort Dodge Animal Health) 100mg/kg. Four sedated joeys received aerosol inoculation of *M. bovis* (inoculated group), four served as non-inoculated in-contact joeys (exposed group), and three joeys, the dam, and the additional age matched joey from outside of the litter served as the control group. *Mycobacterium bovis* was administered to the joeys in the designated inoculated group at a concentration of 1×10^{6} cfu via nebulization for a total of 10 minutes. Inoculated joeys were ear notched for identification purposes. Inoculated and non-inoculated (exposed) joeys were housed individually for one week prior to the forty-five days of co-habitation in a BL-

3 Horsfall isolator. One non-inoculated (exposed) joey was housed with one inoculated joey making four replicate co-habitation groups. The control animals were individually housed in a separate containment room.

Gross and Histopathology

Individual weight measurements were taken every two weeks until the animals were sacrificed. At day eighty-four post inoculation or post exposure, joeys were sacrificed by initial sedation with an intramuscular injection of Telazol (100mg/kg) and subsequent intracardiac exsanguination. Immediately after exsanguination the whole blood samples were clotted at $4^{o}C$ for 1 hour, centrifuged at 5,000 time gravity for 5 minutes, and serum was then separated into sterile tubes and frozen at $-20^{o}C$ until all samples were collected for the entirety of the study.

A complete post-mortem examination was performed. Brain, eye, nasal turbinates, trachea, lungs, heart, liver, kidney, spleen, stomach, pancreas, gonad, adrenal gland, small intestine, large intestine, tonsil, lymph nodes (cranial, thoracic, and abdominal), urinary bladder, skeletal muscle, and pinea were harvested, fixed in 10% neutral-buffered formalin, and trimmed for histopathology. All major organs (lungs, liver, kidney, and spleen) were individually weighed and collected for *M. bovis* culture. Slides were stained with hematoxylin and eosin and Ziehl-Neelsen's acid-fast stain followed by light microscopy examination.

Bacteriology

Tissues were processed for *M. bovis* isolation at Michigan Department of Community Health (MDCH). Four tissue groups were pooled for culture: Group A: Cranial lymph nodes and tonsil, Group B: Thoracic lymph nodes and lungs, Group C: Liver, kidney, spleen, abdominal lymph nodes, Group D: Small intestine and large intestine.

Serology Assay- Rapid Test

Serum was sent to a commercial laboratory for rapid test analysis (Chembio Diagnostics Systems Inc., Medford, NY). The rapid test is a lateral-flow, blue latex bead signal-based, qualitative antibody detection assay that utilized a cocktail of selected *M. bovis* antigens (ESAT-6, CFP10, Acr1, MPB83). The assay uses a ready-to-use plastic cassette containing a nitrocellulose membrane impregnated with the cocktail of test antigens. Thirty microliters of test serum and 3 drops of diluent buffer were added to the test well and the result of the reaction was read by visual evaluation after 20 minutes.[9] An antibody positive sample was indicated by a visible band at both the test and control lines, while an antibody negative sample was indicated by a visible band at the control line but no band at the test line.[9]

Statistical Analysis

The two-sample *t*-significance test was calculated on all data sets to determine difference between the inoculated, exposed and control groups. The student's t-test was chosen based on the minimal sample size and distribution of values.[10] The t-statistic obtained from the data was compared to the *t* distribution critical values table using the smallest degrees of freedom and p value of 0.05 for a one-sided test and 0.025 for a two sided test.[10]

RESULTS

Gross and Histopathology

All of the animals gained weight during the extent of the study. The average bi-weekly weight gain between the three groups were not remarkably different, inoculated (425g), exposed (385g) and controls (502g) and no significant difference was noted for total body weight gain. Additionally, there was no significant difference noted for any of the major organs across any of the groups. There was no significance noted when comparing total body weight gain of control versus the inoculated opossums $p<0.15$, controls versus exposed opossums $p<0.10$, and inoculated versus exposed opossums $p<0.25$. There was no significance noted when comparing major organ weight of controls versus inoculated opossums for lung $p<0.10$, liver $p<0.20$, kidney $p>0.25$, and spleen $p>0.25$. There was no significance noted when comparing major organ weight of controls versus exposed opossums for lung $p>0.25$, liver $p>0.25$, kidney $p>0.25$, and spleen $p<0.25$. And finally, there was no significance noted when comparing major organ weight of exposed versus inoculated opossums for lung $p<0.15$, liver $p<0.20$, kidney $p>0.25$, and spleen $p<0.20$.

On gross examination, the lungs of all four inoculated opossums were characterized by marked multifocal to coalescing, raised, white, firm caseogranulomatous nodules. These nodules were distributed throughout all lung lobes. The exposed and control groups were grossly unremarkable. Histological examination of all the inoculated opossums had marked, multifocal, caseogranulomatous pneumonia (Figure 4.2 and Figure 4.3).

Bacteriology

Identification of *M. bovis* from pulmonary tissue was successful in all the inoculated opossums and was additionally identified in the pooled samples of liver, kidney, and spleen in half of the

inoculated group (Table 4.1). Bacterial cultures for *M. bovis* were negative for all control and non-inoculated (exposed) opossums.

Serology- Rapid Test

The rapid test identified positive results in 4 of 4 inoculated opossums. The exposed and control opossums were rapid test uniformly negative (Figure 4.1).

DISCUSSION

In summary, the ability to identify *M. bovis* infection in potential wildlife reservoirs in the state of Michigan is essential for the forward progression of tuberculosis eradication. This study investigated the potential for intra-species lateral transmission of *M. bovis* in Virginia opossum. Partial basis for this investigation was attributed to the well-known role of the Brushtail possum in bovine tuberculosis spread in New Zealand[12]. The Brushtail possum is a distant relative of the Virginia opossum; however, little information is known for the potential of *M. bovis* disease spread within Virginia opossums[6].

This study demonstrated that there was no appreciable lateral transmission after aerosol inoculation of *M. bovis* between four replicate groups of Virginia opossums. All of the inoculated opossums had gross, histologic, bacterial culture, and serologic positive tests for bovine tuberculosis; where as, the exposed and control opossums had negative tests. Classic gross and histologic lesions of multifocal ceseogranulmatous pneumonia were noted within all of the inoculated opossums. The control and exposed opossums had no appreciable gross or histologic lesions of tuberculosis. All inoculated opossums were culture positive for *M.bovis* from the representative respiratory samples (pooled thoracic lymph nodes and lung) and half of these were also positive for *M. bovis* from the representative systemic samples (pooled liver, kidney, spleen, and abdominal lymph nodes). The control and exposed opossums were uniformly negative. Bacterial culture is considered the gold standard for positive infection; therefore, these results were conclusive that the aerosol inoculation was successful and at day 84 post inoculation disease was present and widely disseminated in half of the inoculated opossums. Clinically, these opossums did not show signs of illness, emaciation, or draining tracts throughout the duration of the study. In comparison to Brushtail possums with natural *M. bovis* infection, disease has

proven to be a highly progressive and fatal disease in which the mean survival time of clinically tuberculous Brushtail possums has been recorded as 4.7-14 months and experimentally by intratracheal inoculation fulminant pneumonia and death was often seen by 8 weeks post inoculation[4,12]. The immune response to *M. bovis* in the Brusthtail possum and the Virginia opossum is speculated to be different[2]. Additionally, the specific immune response and disease progression of *M. bovis* in Virginia opossums has not been fully elucidated; however, based on previous studies identifying infection by 90 days, the financial limitations of this study, and the known progressive disease by 8 weeks (56 days) in experimentally inoculated Brushtail possums, incubation and evaluation of inoculated Virginia opossum at of 84 days post inoculation was considered sufficient for disease assessment[6,12]. This study did not address the clinical manifestation of chronic disease progression or bacterial shedding in Virginia opossum, future studies may benefit from these observations.

Recent advancements in serologic assays to determine antemortem disease status of *M. bovis* include the multiple antigen print immunassay (MAPIA) and rapid test[9]. In this experiment the rapid test was able to identify all inoculated opossums as positive and the exposed and control opossums as negative. Interestingly, as previously mentioned two of the four opossums had positive *M. bovis* cultures from the representative respiratory samples and the systemic samples, these two positive opossums also had very prominent test bands on the rapid test. It is uncertain if disease burden could be correlated to the intensity of the rapid test. The MAPIA for this experiment was unrewarding and yielded non-interpretable reactions, further investigation is warranted (data not shown). Other ante mortem assays often performed in animals to determine tuberculosis disease status include the intradermal injection of bovine purified protein derivative

(bPPD)[8]. Administration and evaluation of pinnal bPPD injections were difficult and unrewarding in this experiment and subsequently were terminated (data not shown).

In conclusion, the inoculated group was infected with *M. bovis* and confirmed by classic gross lesions, histologic lesions, bacterial culture, and antibody tests; however, *M. bovis* was undetectable in the control and exposed groups. Future studies assessing shedding pattern and chronic disease progression maybe warranted in Virginia opossum. The conclusion of this study states that there was no appreciable lateral transmission after aerosol inoculation of *M. bovis* after 45 days of cohabitation between Virginia opossums.

ACKNOWLEDGEMENTS

Authors would like to acknowledge Janet Hengesbach, Jean Gaymer, Danielle Ferguson, and the MSU animal containment facility staff for help in animal care and restraint, and RoseAnn Miller for help in data analysis.

Figure 4.1 Representative results for the rapid test

The cassette to the left displays a positive band at the control (C) and test (T) window, representing a positive *M. bovis* result. The cassette to the right only displays a positive band at the control (C) window and no band at the test (T) window, representing a negative *M. bovis* result.

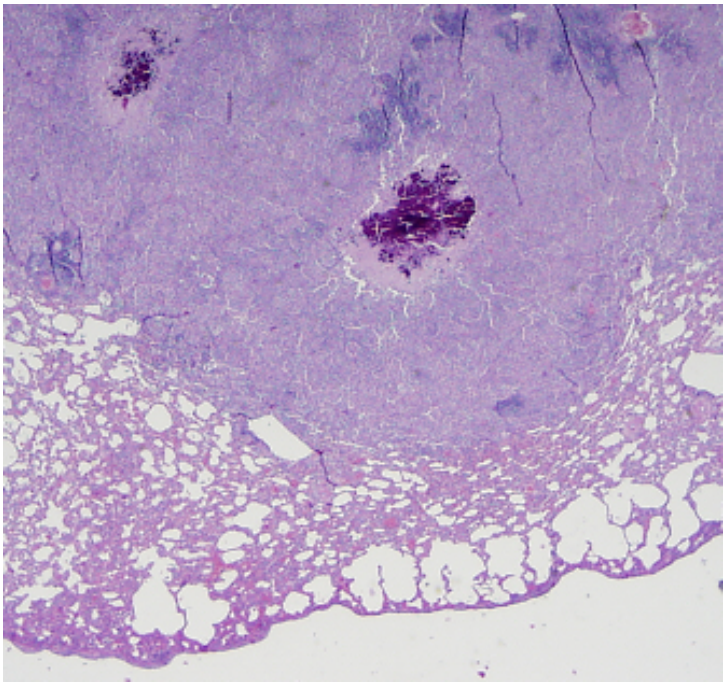

Figure 4.2 Photomicrograph of a pulmonary tubercle obtained from a *M. bovis* inoculated opoosum (2 X magnification)

Light microscopic features included marked, multifocal, caseogranulomatous pneumonia with variable amounts of central mineralization.

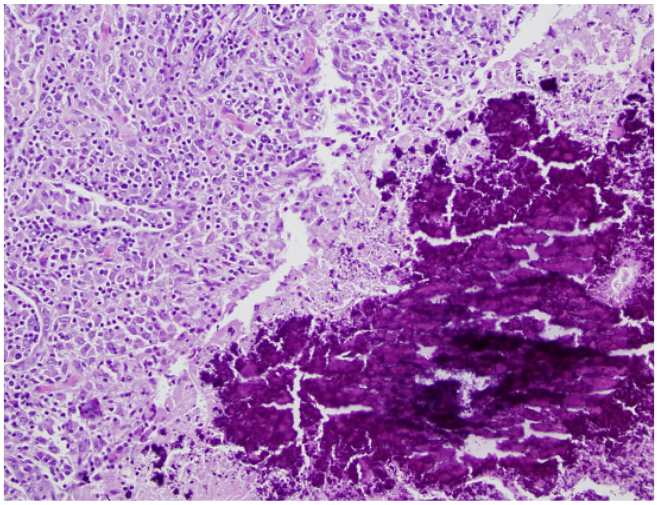

Figure 4.3 Photomicrograph of a pulmonary tubercle obtained from the opossum described in Figure 4.2 (40 X magnification)

Higher magnification of a representative *M. bovis* inoculated opossums characterized by marked, multifocal, caseogranulomatous pneumonia with variable amounts of central mineralization.

Inoculation Group	Culture Group A Upper Respiratory		Culture Group B Lower Respiratory		Culture Group C Systemic		Culture Group D Alimentary	
	Pos	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Inoculated	0	4	4	4	2	4	0	4
Exposed	0	4	0	4	0	4	0	4
Controls	0	4	0	4	0	4	0	4

Table 4.1: *Mycobacterium bovis* culture group results

The column to the right indicates the opossum group as *M. bovis* inoculated, exposed, or control. The top row indicates the culture group as either A, B, C, or D. A correlates with the upper respiratory tissues, B correlates with the lower respiratory tissues, C correlates the systemic tissues, and finally D correlates with the alimentary. The other boxes are split, numbers on the right indicate the positive results and the number on the right indicates total number of opossums in the group.

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CHAPTER 5

Surveillance for *Mycobacterium bovis* in Hunter Harvested Feral Swine from 2006 to 2010, in the State of Michigan

INTRODUCTION

Tuberculosis is a costly disease in which numerous domestic and wildlife species are susceptible, thus making surveillance, control, and research important in regional and global eradication efforts. This chapter provides a brief discussion on the historical data, historical epidemiology, and current disease surveillance of bovine tuberculosis within the wild and feral swine population.

The current swine population is classified within one of the three following categories, wild and feral swine, transitional swine, or commercial production swine. Wild and feral swine are defined as those swine that have lived all (wild) or any part (feral) of their lives as free-roaming animals. Transitional swine are those feral swine that are captive or those that have reasonable opportunities to be exposed to feral swine. Commercial production swine are those swine that are continuously managed and have adequate facilities with practices to prevent exposure to either transitional or feral swine. During the US fiscal year of 1995 nearly 94,500,000 commercial production swine were slaughtered in which approximately 200,000 contained gross lesions attributed to tuberculosis[10]. Tuberculous lesions in commercial production swine are attributed to multiple species of *Mycobacterium*; however, classical swine mycobacteriosis is historically associated with *Mycobacterium tuberculosis* and *Mycobacterium avium* complex[10]. The clinical symptoms of disease are often nonspecific; therefore, the antemortem diagnosis of tuberculosis was difficult in swine. The tuberculin skin test was established for swine by intradermal injection of the pinnea or vulva with both bPPD and aPPD followed by observation at 48hrs[1]. Antemortem testing for swine mycobacteriosis was not routinely performed; therefore, detection of mycobacteriosis was historically identified at

slaughter[1]. Tuberculous lesions caused by *M. bovis* in swine are often limited to the lymph nodes of the cervical and mesenteric regions. The lesions are characterized grossly as multiple, small, coalescing, yellow to white, caseous foci that range from a few millimeters to diffuse enlargement of the entire lymph node[10]. Lesions attributed to *M. bovis* and *M. avium* are often confused as only minor differences maybe noted. Additionally, other higher bacteria, including partially acid fast organisms such as *Rhodococcus equi*, have often confounded the identification of tuberculosis grossly and histologically. Lesions noted at necropsy and subsequent conformation via histopathologic examination are merely suggestive of tuberculosis; where as, culture is still considered the gold standard[10].

The zoonotic potential and concern for public health is widely recognized for tuberculous swine in the area of food safety. These concerns are largely attributed to the isolation of *M. avium* complex in immunocompromised human individuals; however, sporadic cases of *M. bovis* and *M. tuberculosis* have been documented[9]. Isolation of multiple zoonotic *Mycobacterium* species within the global population of swine remains a concern for public health; even beyond, those who maybe immunocompromised. Recent literature has noted that the prevalence of *Mycobacterium bovis* is increasing within the wild and feral swine population[10]. Surveillance and control programs for tuberculosis were implemented in 1917 to decrease this potential threat to public health. This 1917 campaign focused on the eradiation of bovine tuberculosis within the North American cattle population; however, despite this large campaign the percentage of tuberculous swine remained fairly high, peaking at 16.38% in 1922 and then dipping down to 1.35% in 1968. In 1972 to 1995 a marked downward tread was noted, starting at 0.85% in 1972 and ending at 0.21% in 1995. In the early 1970s the incidence of tuberculosis in poultry

dramatically dropped leading to a correlative drop in prevalence of tuberculous swine, indicating that infection with *M. avium* was the prominent cause of swine tuberculosis [10].

Despite the campaign efforts to eradiate tuberculosis, sporadic cases of *M. bovis* were reported in feral swine and other domestic and wildlife hosts. Germany had the first documented case of *M. bovis* in the wild boar. Since this case in the early 1930s, bovine tuberculosis has been isolated from feral swine in many regions of the world, including Australia, New Zealand, Hawaiian Islands, Spain, and Italy[3,5,7,9]. However the role of feral swine in the epidemiology of bovine tuberculosis differs greatly by region and population. In Australia and New Zealand, ingestion of infected carcasses had earned a classification of spill over hosts, in contrast wild boars in the Mediterranean (Spain) appear to be maintenance hosts[8]. In Italy, feral swine are also considered dead end hosts; however, prevalence varied with exposure to infected cattle carcasses[8]. Bovine tuberculosis has not been identified in the North American feral swine population, but similar risks for disease emergence are present, especially with the rapid northern expansion of feral swine and potential for interactions with tuberculous reservoirs in these regions.

Texas has the largest concentration of feral swine within the United States, estimated at 5 million head, followed by Florida, California, and Hawaii. Historically feral swine were limited to the southern United States; however the active spread towards the northern United States and Canada (currently occupying 38 states and 3 Canadian provinces) have nearly double the number of feral swine since 1988[4]. This spread north is particularly worrisome for those northern states harboring small endemic foci of tuberculosis, such as Minnesota and Michigan. In the state of Michigan, the potential exist for the development of a new bovine tuberculosis maintenance host

by direct interactions of feral swine with tuberculous white-tail deer and cattle. Historically, reports of infected swine were linked to positive bovine tuberculosis cattle after ingestion of unpasteruized milk, unpasteruized dairy by products, or by the practice of feeding swine offal from abattoirs or uncooked garbage. Other potential routes of infection included sharing of pasture and exposure to feces laden with viable organisms. New pathways of infection may emerge based on the rapid northern expansion of feral swine game ranches intended for recreational hunting within the United States.

Once tuberculosis has been established, eradication is often difficult due to the minimal, nonspecific clinical signs, the lack of reliable multi-species antemortem diagnostic tests, and the lack of an effective vaccination. Australia has had the only successful eradiation program where prevalence of *M. bovis* within feral swine dropped from 31% in the 1960s to 0.25% in 1990s which lead to complete eradication[9]. The decrease in prevalence was largely due to the depopulation of infected water buffaloes, which were considered a maintainence host. A similar decrease in swine tuberculosis prevalence was noted during the 1960s in California, as a focused depopulation of bovine tuberculosis positive cattle was managed. In an effort to prevent feral swine from becoming established within Michigan, a coordinated, comprehensive feral swine control program was implemented. The objective of this surveillance program was to identify and characterize the impact of feral swine diseases, specifically bovine tuberculosis on domestic livestock, wildlife, humans, and pets by conducting a continuous surveillance from hunter harvested feral swine.

OBJECTIVE

Identify and characterize bovine tuberculosis in hunter harvested feral swine in the state of Michigan.

HYPOTHESIS

If feral swine are a significant reservoir host of *M. bovis* in the state of Michigan then hunter harvested feral swine will display gross lesions, histologic lesions, and positive culture results.

MATERIALS AND METHODS

Feral Swine

Hunter killed feral swine were submitted to the Michigan Department of Natural Resources (MDNR). The whole animal or more commonly the head was submitted to the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University (MSU).

Gross and Histopathology

A complete post-mortem examination was performed. Tissues samples were collected for histopathology, when available, samples included: cranial lymph nodes (submandibular, retropharyngeal, and parotid), thoracic and abdominal lymph nodes, tonsil, skeletal muscle (tongue and masseter), brain, trigeminal ganglion, and any other organ with gross lesions. Samples were fixed in 10% neutral-buffered formalin and trimmed for histopathology. Slides were stained with hematoxylin and eosin and Ziehl-Neelsen's acid-fast stain followed by light microscopy examination.

Microbiology and Serology

During the necropsy, representative fresh tissue samples were collected for microbiology, when available, samples included: tonsil, spleen, trigeminal ganglion, nasal swabs, cranial lymph nodes (submandibular, retropharangeal, and paroid), thoracic lymph nodes, and abdominal lymph nodes. Collected lymph nodes were frozen in the event that bacterial culture was warranted.

RESULTS and DISCUSSIONS

One hundred and twenty four feral swine are on record for the hunter killed feral swine surveillance in the state of Michigan from November of 2006 until September 2010. The submitted feral swine were approximately 54% adults, 11% juveniles, 9% subadults, and 25% unknown. The collected animals were nearly evenly split between males (30%), females (32%), and unknown sex (38%). In the first year of the program 30 animals were submitted, followed by 41 the next year, 45 the following and only 8 in the final year (Figure 5.1-5.5) Three swine were noted having gross lesions of tuberculosis; however, these were not confirmed to be tuberculous lesions histologically. All other animals in the study had no evidence grossly or histologically of tuberculosis. As of 2010 the tested hunter harvested feral swine have not been identified as having tuberculosis

This surveillance to date has yet to identify any potential devastating infectious agents, including tuberculosis within the hunter harvested feral swine in the state of Michigan. These negative results are promising; however, continued surveillance in the state of Michigan is desirable, especially as the feral swine population spreads to the northern portions of the lower peninsula. Active sampling of feral swine within the MAZ or even the MAAZ would be of the interest of the state of Michigan, as feral swine are highly susceptible to bovine tuberculosis.

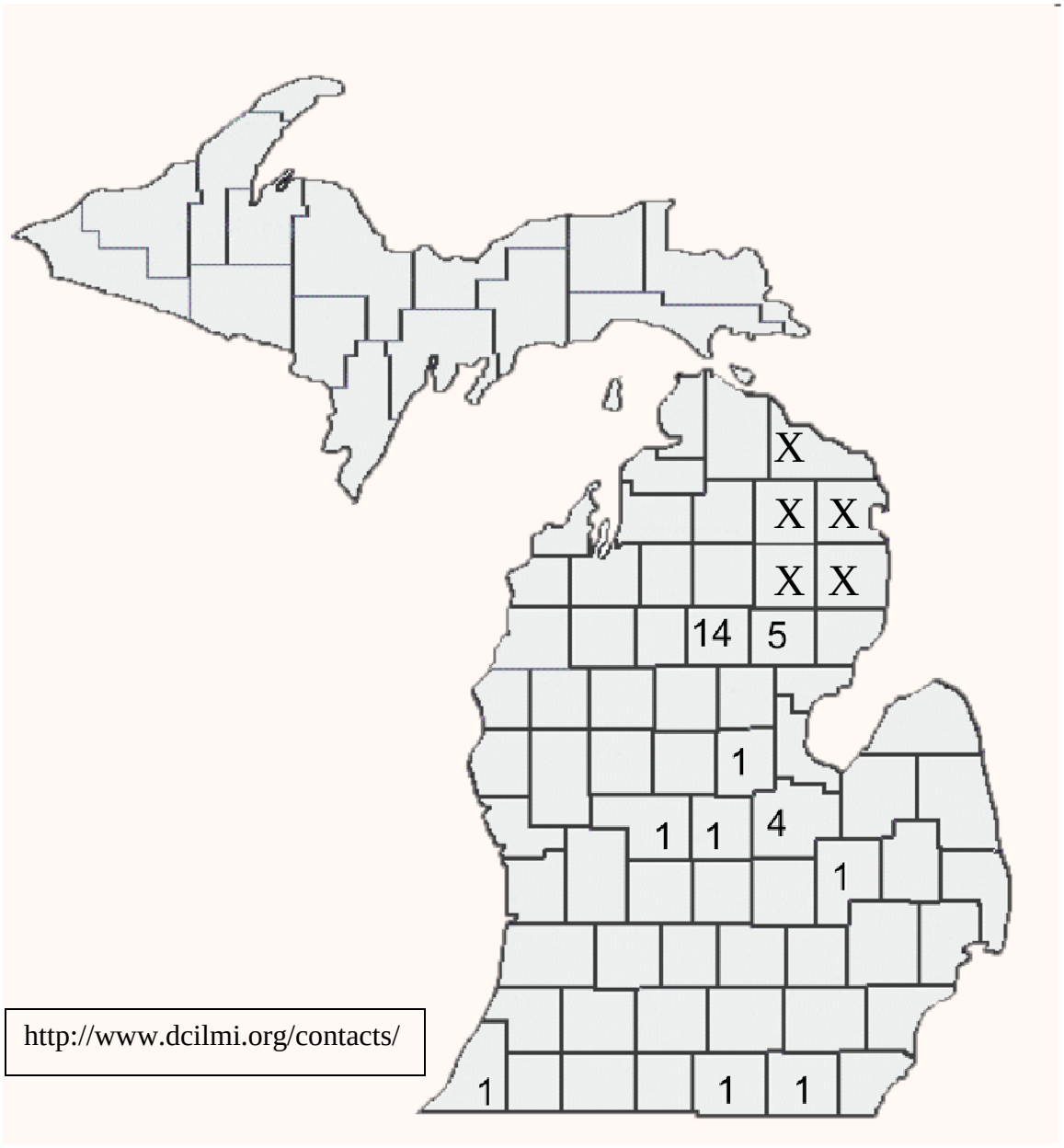

Figure 5.1: Feral swine submitted from November 2006-September 2007

Feral swine were submitted for evaluation from Roscommon (14), Ogemaw (5), Saginaw (4), Gratiot (1), Midland (1), Hillsdale (1), Genesee (1), Lenawee (1), Moncalm (1), and Barrien (1) during the 2007 fiscal year. An “X” represents one of the five modified accredited zones (MAZ) as indicated by the United States Department of Agriculture.

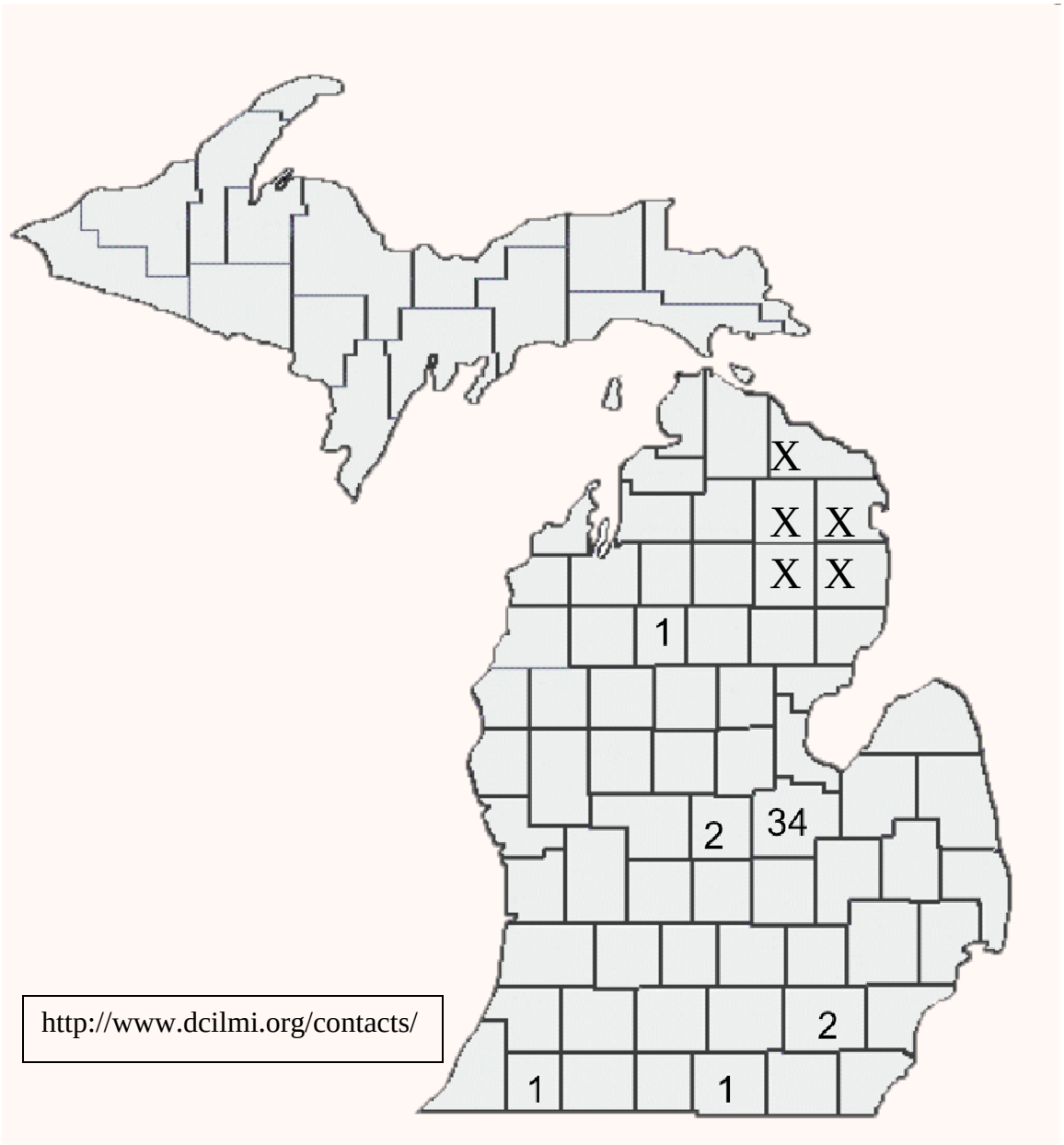

Figure 5.2: Feral swine submitted November 2007-September 2008

Feral swine were submitted for evaluation from Saginaw (34), Washtenaw (2), Gratiot (2), Cass (1), Hillsdale (1), and Missaukee (1) during the 2008 fiscal year. An “X” represents one of the five modified accredited zones (MAZ) as indicated by the United States Department of Agriculture.

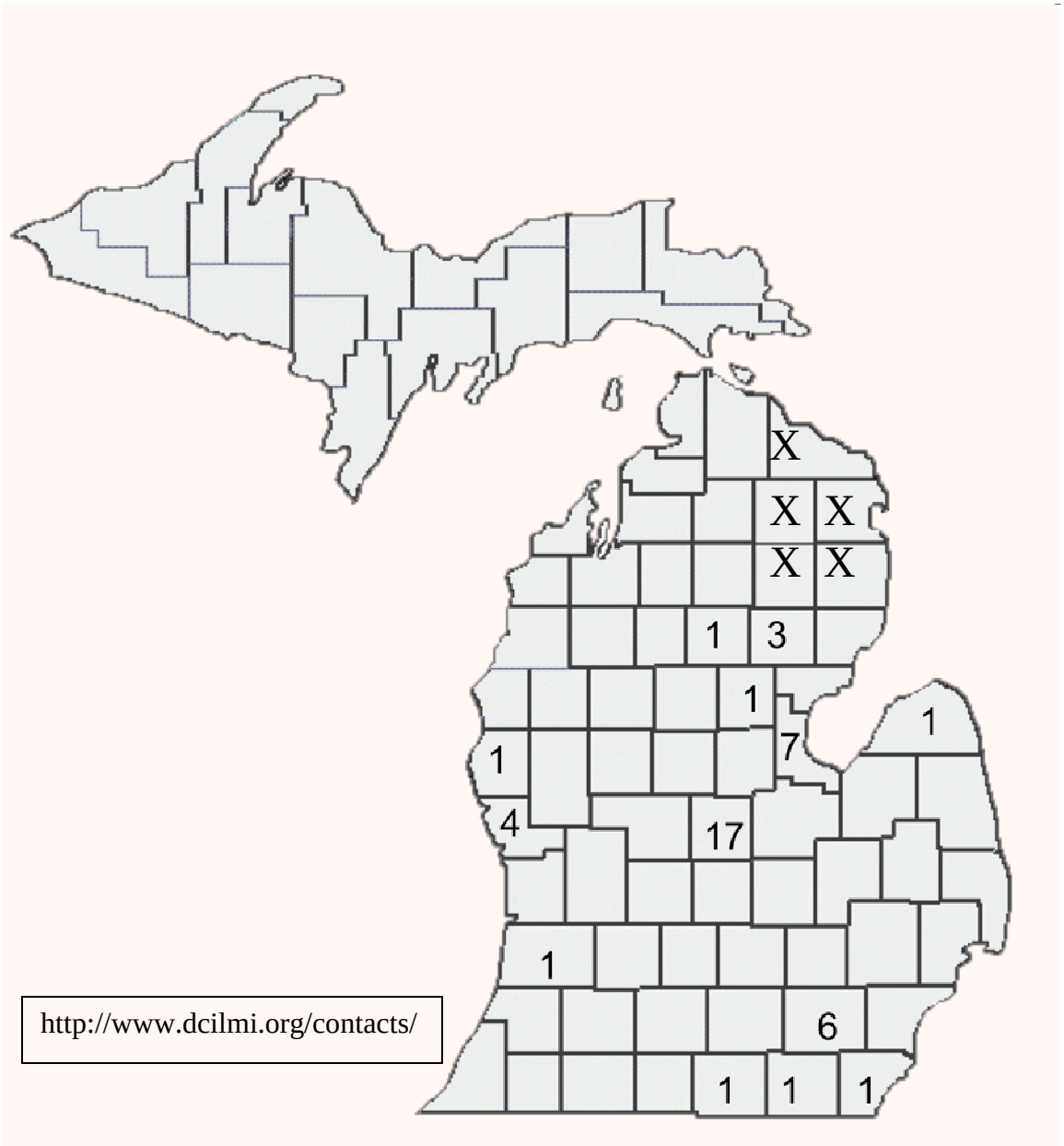

Figure 5.3: Feral swine submitted November 2008-September 2009

Feral swine were submitted for evaluation from Gratiot (17), Bay (7), Washtenaw (6), Muskegon (4), Monroe (1), Ogemaw (3), Huron (1), Hillsdale (1), Gladwin (1), Allegan (1), Lenawee (1), Roscommon (1), and Oceana (1) during the 2009 fiscal year. An "X" represents one of the five modified accredited zones (MAZ) as indicated by the United States Department of Agriculture.

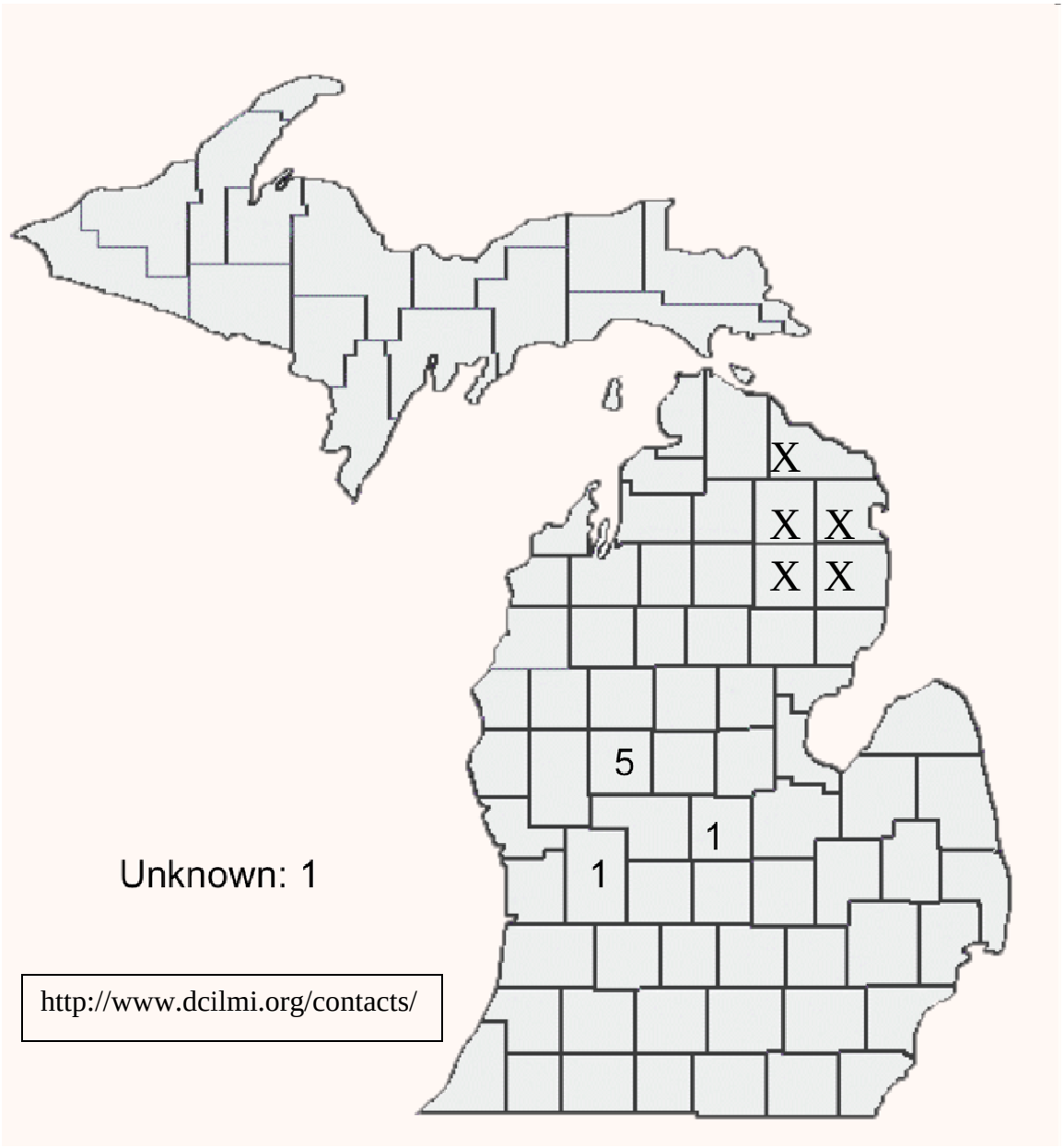

Figure 5.4: Feral swine submitted November 2009-September 2010

Feral swine were submitted for evaluation from Mecosta (5), Gratiot (1), Kent (1), and Unknown (1) during the 2010 fiscal year. An "X" represents one of the five modified accredited zones (MAZ) as indicated by the United States Department of Agriculture.

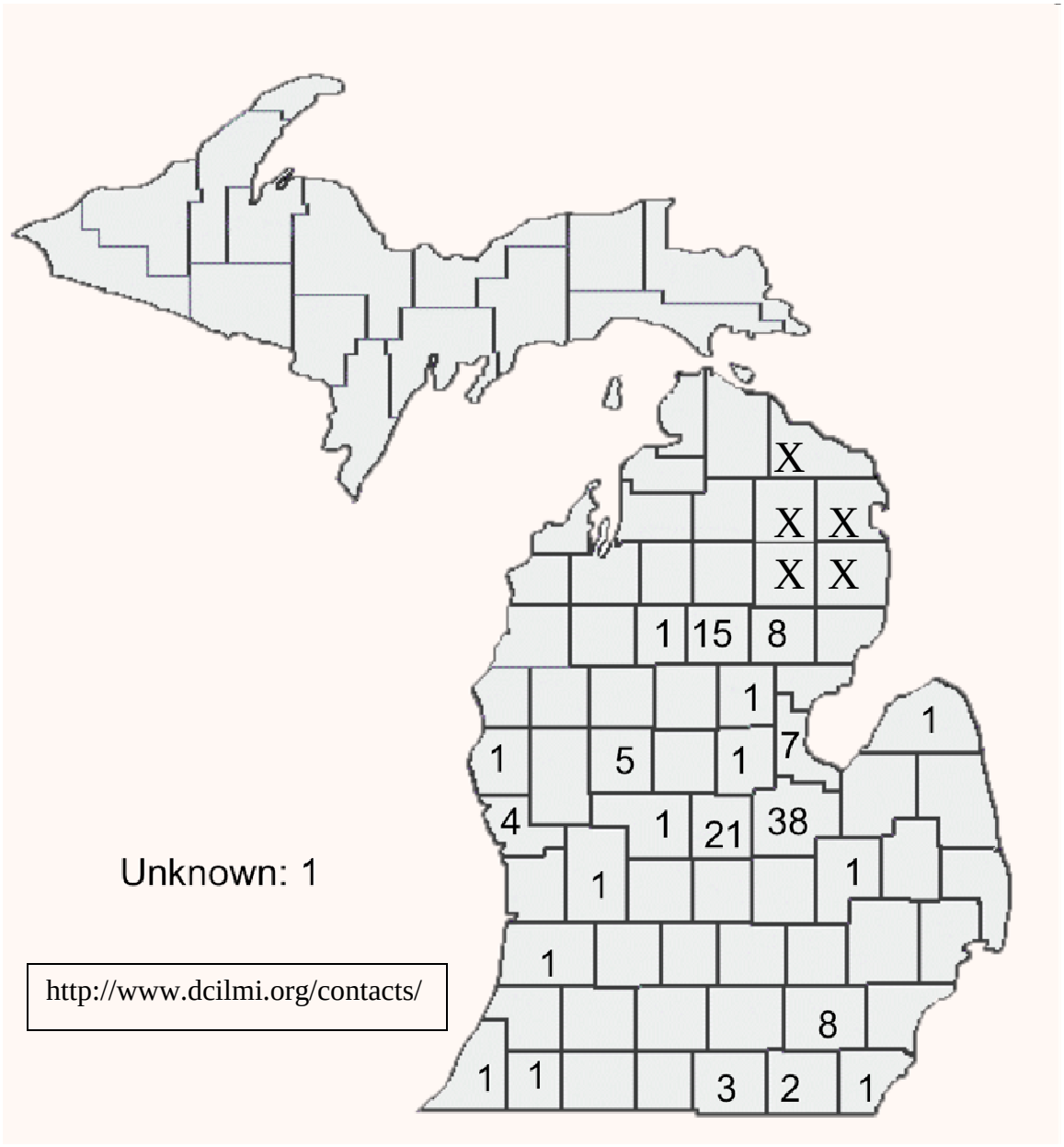

Figure 5.5: The total number of feral swine submitted November 2006-September 2010

Feral swine were submitted for evaluation from Roscommon (15), Ogemaw (8), Saginaw (38), Gratiot (21), Midland (1), Hillsdale (3), Genesee (1), Lenawee (2), Moncalm (1), Barrien (1), Washtenaw (8), Cass (1), Missaukee (1), Bay (7), Muskegon (4), Monroe (1), Huron (1), Gladwin (1), Allegan (1), Oceana (1), Mecosta (5), Kent (1), and Unknown (1) during the 2007-2010 fiscal years. An "X" represents one of the five modified accredited zones (MAZ) as indicated by the United States Department of Agriculture.

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CHAPTER 6

Rapid Detection and Identification Techniques of *Mycobacterium bovis*: Macrophage cell Line TIB-202 Versus Traditional Culturing of Cervid and Bovine Tissue Samples

INTRODUCTION

Mycobacterium species are considered facultative or obligate intracellular bacteria that are able to replicate in non-activated macrophages. The specific characteristic and pathogenesis of this organism leads to evasion of the humoral and cell mediate immune response leading to a chronic insidious disease. This has hindered the discovery of a quick, reliable, and cost effective antemortem diagnostic assay for tuberculosis. The two main challenges for a diagnostic assay hinge on the ability of a assay to correctly detect diseased animals (sensitivity), and the ability to correctly answer if the animal is not diseased (specificity). Historically, validated and experimental diagnostic assays have remained inferior to traditional culture methods, as they lack sensitivity and specificity. The lack of sensitivity and specificity leads to errors of false positives and false negatives. Although the sensitivity and specificity are compromised the speed in which these assays can be performed largely out weight the time consuming 6-8 weeks required for traditional bacterial culture, which is considered the current gold standard. Even those culture techniques that utilize radiometric analyses are not considered replacements for traditional culturing. Combinations of these less specific and sensitive diagnostic assays with traditional culture techniques have proven to be effective; however, final confirmation of disease status often results in delayed results or excessive culling of farm animals. Improved culture methods are expected to expedite results while retaining sensitivity and specificity. Utilizing the natural behavior of the microbe through intraphagocytic propagation has experimentally proven to reduce the length of time required to identify *Mycobacterium* species[1,4,5]. The objective of this project is to utilize the macrophage cell line TIB-202 to propagate *Mycobacterium bovis* in order investigate a more rapid, sensitive, flexible, and cost effective diagnostic culture assay.

OBJECTIVE

Identify macrophage cell line TIB-202 as a rapid, sensitive, flexible, cost effective diagnostic test for the identification of *M. bovis* in cervid and bovine lymph node tissue samples.

HYPOTHESIS

If the macrophage cell line TIB-202 can propagate *M. bovis* from infected tissue samples then it will be a more rapid, sensitive, flexible and cost effective diagnostic tool when compared to traditional culturing.

MATERIAL, METHODS, AND RESULTS

Cell Line

Human, acute monocytic leukemia cell line TIB-202 (THP-1) was utilized for this project. The cells were maintained as a suspension in culture media M199 and supplemented with fetal bovine serum. Cell suspensions were incubated at 37^{O}C, 0% CO2, and refed every 3 days. Cells were grown to a cell density of approximately 5,000 cells per milliliter. Cells were harvested via centrifugation at 1000 RPM for 10 minutes. Ten microliters of the cell suspension were added to a hemocytomer glass slide. The concentration of the cell suspension was quantified by manual cell count per grid covering an area of 1 mm^2 or a volume of 0.1mm^3. At least 100 cells were observed over multiple grids, the average number per chamber was multiplied by the dilution factor. Two vessels were used to house the activated cells, chamber slides (containing 8 chambers of 1cm^2) and 25cm^2 flasks. Approximately 5,000 cells were applied to each chamber of the chamber slides and nearly 1,000,000 cells per flask. The supernatant liquid was removed and the monocytes were activated to phagocytic macrophages by activation with M199 plus 2 mercaptoethanol (1.3 x 10 ^-5M) and Phorbol 12-Myristate 13-Acetate (PMA) 20nM was added (Table 6.1).

Phagocytosis Assay

The activated suspension of TIB-202 cells was incubated for 24 and 48 hours. As indicated in Table 6.1, the activated TIB-202 cells yielded a higher absorbance at 24 hours with a standardized supplementation of 20nM PMA. The activated and attached monolayer of macrophages was visualized via microscopy. The CytoSelect TM 96-Well Phagocytosis Assay (Red Blood Cell, Colormetric Format) from Cell Biolabs, INC. was applied to ensure the

phagocytic nature of the selected cell line. Briefly, IgG opsonized sheep erythrocytes were incubated for 30 minutes at 37^{o} C with the activated cell line, this solution was removed, followed by multiple washes with the wash solution and PBS. The cells are then lysed, transferred to a 96-well microtiter plate, and the substrate solution is added. Absorbance is measured at 650nm in a 96-well microtiter plate reader. The right portion of the table 6.2 compares the concentration of 2-METOH from the stock solution ($5x10^{5}$ M) and subsequent dilutions of 1 to 16, 1 to 8, 1 to 4 and finally 1 to 2 along the top row. The third column from the left indicates the decreasing concentration of PMA added to each of the concentrations of 2-METOH. The optimal absorbance was with 20nM PMA and 1:4 dilution of 2-METOH (5x 10 -5) at 24 hours of incubation.

Bacterial Strain

The stock *M. bovis* was obtained from the Michigan Department of Community Health (MDCH) animal 08 TB 883 AF 327 DEER 269398. This pure culture was quantified by plating 100ul of culture onto Middlebrooks 7H10 agar and incubated at 37^{o} C. The undiluted stock culture was estimated to have 10,000cfu/ml, diluted culture of 10^{1} was estimated at 1000cfu/ml, and the diluted culture of 10^{2} was estimated at 100cfu/ml.

DNA Extraction

Mycobacterium bovis DNA was extracted from the stock culture, supernant, and lysed cells. Cells were lyzed using a digestion buffer composed of 10nM Tris-HCL pH 9.83 and 50mmM KCL with 0.5% PSM 100mls H2O and supplementation with 20ul (5mg/ml) proteinase k per 500ul of buffer. Briefly, glass beads were added to the samples, placed in a sonicator bath for 5 minutes, heated to 100^{o} C or greater for 10 minutes, snap frozen in an ethanol and dry ice

bath, heated to 100^{o} C or greater for 5 minutes, and finally centrifuged at 10,000 RCF time gravity for 10 minutes.

Nested PCR

Primers used for isolating the portion of the *Mycobacterium tuberculosis* insertion sequence 6110 are listed in Table 6.3. The outer nested PCR amplifications of DNA of 252 base pairs were performed using 25ul reactions, containing 5ul DNA, 0.2ul each of the outer forward and reverse primers (SB 474 and SB 269), 12.5ul GoTaq Green Master Mix, and 7.1ul water. The inner nested PCR amplifications of DNA of 116 base pairs were performed using 25ul reactions, containing 1ul of the previously amplified DNA, 0.2ul each of the inner forward and reverse primers (SB 268 and SB 475), 12.5ul GoTaq Green Master Mix, and 11.1ul water.

The PCR protocol for the outer primer set included 5 minutes at 95^{o} C for the initialization step, 20 cycles of 30 seconds at 95^{o} C, 30 seconds at 67^{o} C, 30 seconds at 72^{o} C for denaturation, annealing, and elongation, and 5 minutes at 72^{o} C for the final elongation . The PCR protocol for the inner primer set included 5 minutes at 95^{o} C for the initialization step, 40 cycles of 30 seconds at 95^{o} C, 30 seconds at 65^{o} C, 30 seconds at 72^{o} C for denaturation, annealing, and elongation, and 5 minutes at 72^{o} C for the final elongation. The PCR products were subjected to 300V (approximately 190mA) for 10 minutes in 1% agarose gel. Figure 6.1 displays the PCR implication of IS6110 and 10 fold dilutions for the original inoculum at day 0 (1cfu/100ul), the supernants from day 2 and 6, and lysates from day 2 and 6. For each sample collection day, the numbers 0-7 indicates a 10 fold dilution out to 10^{-7}. Amplicons were noted one dilution out for the original inoculum at day 0, and for the supernatant at day 2; where as,

amplicons were noted at two dilutions out for the lysate, indicating a ten fold increase in the detectable *Mycobacterium bovis* DNA. Amplicons were noted three dilutions out from the original inoculum at day 6.

DISCUSSION

The objective of this project was to utilize the monocytic cell line TIB-202 to propagate *Mycobacterium bovis* in order to investigate a more rapid, sensitive, flexible, and cost effective diagnostic culture method. The preliminary results are promising as the manipulations of this cell line suspension for maintenance, proliferation, quantification, and activation were relatively easy. Additionally, the total amount of cells for this experiment ranged from 5,000 to near 1,000,000 per individual reaction which allowed for a flexible and customizable assay. The preliminary PCR results were able to detect a marked increase in amplifiable DNA within the lysated cells after six days of incubation, supporting the hypothesis that this cell line could potentially be used as a rapid detection diagnostic assay.

The biggest challenge thus far has been decreasing laboratory contaminates and determining the lowest detectable limits of this assay. As noted in figure 6.1, on day 2 the undiluated lysate was unable to produce a band, however clear bands were noted in the two subsequent dilutions (10^{-1} and 10^{-2}). Additionally, a bright band was aberrantly noted at the 10^{-7} dilution of the day 6 lysate. Mycobacterium in suspension often clumps due to the characteristic of the organisms cells wall; the aberrant band at the 10^{-7} dilution was attributed to the transfer of these clumped organisms. Even with these unexpected results, the overall picture indicates that this assay has potential. Figure 6.1 is representative of inoculation with 1 colony forming unit (CFU); however, additional parallel inoculations with 1CFU, 10CFU, and 100CFU maybe a good comparison to examine trends. In addition, PCR for specific genes that are upregulated following phagocytosis may aid in identification of virulent *Mycobacterium*[2,3].

The ultimate goal of this project was to inoculate the optimized TIB-202 cell line with cervical lymph node samples collected from cattle or deer that were submitted to the diagnostic center for population and animal health (DCPAH) at Michigan State University. The tissues would be homogenized and subjected to decontamination in order to reduce the amount of background or contaminate bacteria. The homogenized tissue suspension would be fed to the activated human monocyctic leukemia cell line TIB-202, maintained, and eventually lysed to yield the proprogated *Mycobacterium*. Traditional culturing ran in parallel with the cell line propagation will allow for verification of sensitivity, specificity, and for a detailed cost analysis.

48 Hour 20nM PMA		24 Hour 20nM PMA	
Sample 1	0.191	Sample 1	0.275
Sample 2	0.189	Sample 2	0.344
Sample 3	0.295	Sample 3	0.316
Average	0.225	Average	0.312

Table 6.1 Absorbance values for comparison of the phagocytic ability of cell line TIB-202 at 24 and 48 hours activation time

Samples were ran in triplicate; the optimal activation incubation time was nearly 1.4 times greater at 24 hours versus 48 hours when activated with 20nM of PMA.

	Stock 2-METOH ($5X10^5$ M)	**1:16 dilution 2-METOH**	**1:8 dilution 2-METOH**	**1:4 dilution 2-METOH**	**1:2 dilution 2-METOH**
200nM PMA	0.225	0.256	0.267	0.199	0.241
100nM PMA	0.344	0.279	0.325	0.374	0.228
20nM PMA	0.316	0.339	0.378	**0.411**	0.309

Table 6.2 Absorbance values for the optimized activation and phagocytic activity of the cell line TIB-202

This table compares the concentration of 2 mercaptoethanol (2-METOH) from the stock solution ($5x10^5$ M) and subsequent dilutions of 1 to 16, 1 to 8, 1 to 4, and finally 1 to 2 along the top row. The first column on the left indicates the decreasing concentration of Phorbol 12-Myristate 13-Acetate (PMA) added to each of the concentrations of 2-METOH. The optimal absorbance was with 20nM PMA and 1:4 dilution of 2-METOH (5x 10 -5) at 24 hours of incubation.

Primer	Nucleotide sequence
SB 268 (Out Forward)	5’ CTCGTCCAGCGCCGCTTCGG 3’
SB 268 (Out Reverse)	5’ CCTGCGAGCGTAGGCGTCGG 3’
SB 474 (In Forward)	5’ CGTGAGGGCATCGAGGTGGC 3’
SB 475 (In Reverse)	5’ GCGTAGGCGTCGGTGACAAA 3’

Table 6.3 Nucleotide sequence for primers of the *Mycobacterium tuberculosis* insertion sequence 6110

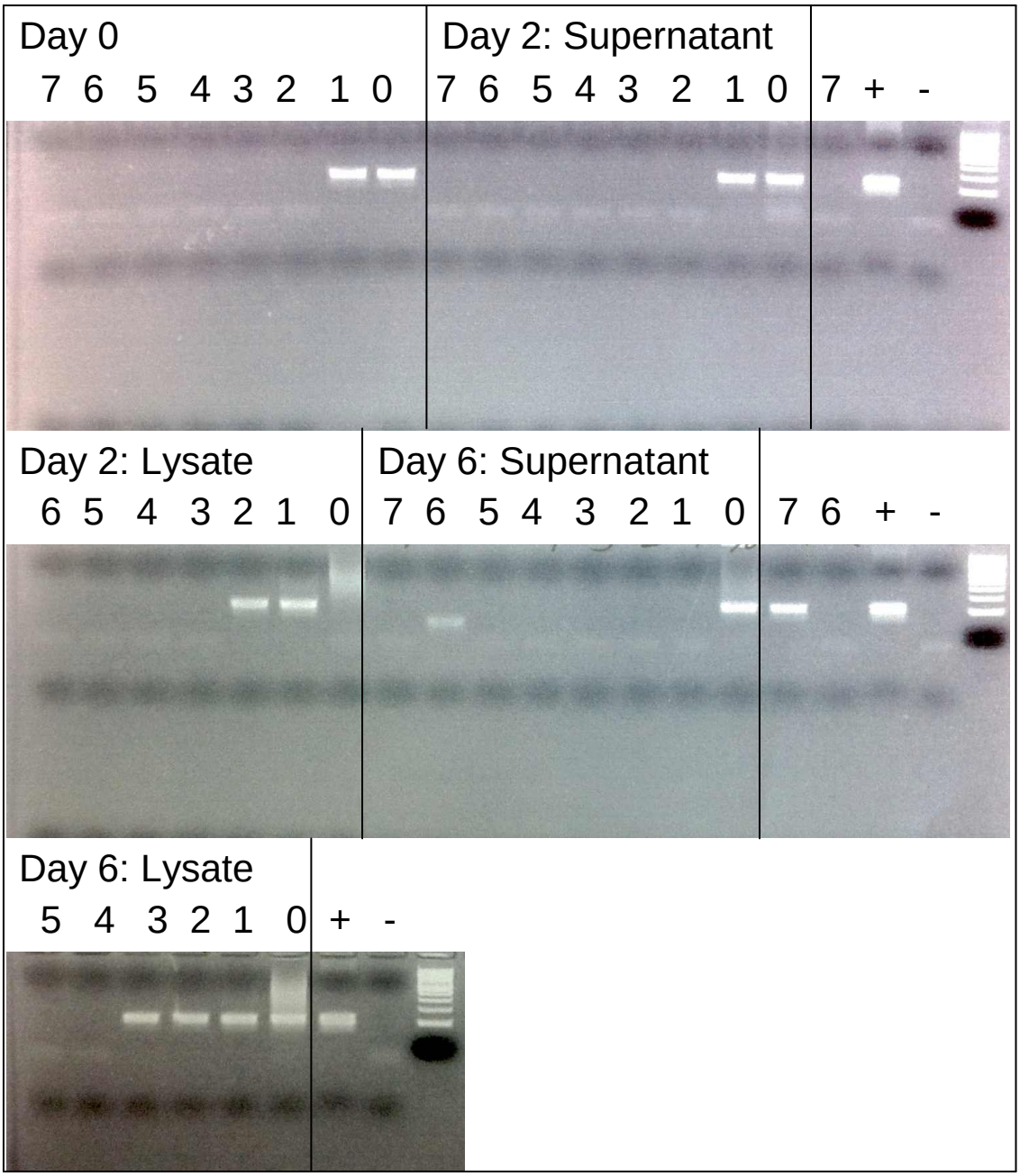

Figure 6.1 Argrose gel of PCR IS6110 amplication and 10 fold dilutions for days 0, 2, and 6

This figure displays the PCR amplication of IS6110 and 10 fold dilutions for the original inoculum at day 0(1cfu/100ul), the supernants from day 2 and 6, and lysates from day 2 and 6. The + symbol indicates the positive control and the – symbol indicates the negative control. For each day, the numbers 0-7 indicate the dilution. The 0 indicates no dilution, 1 indicates 10^{-1}, 2 indicates 10^{-2} and so on until 10^{-7}. The unmarked lane is the 100 base pair DNA ladder (N3231S Biolabs).

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Chapter 7

Conclusions, significance, and future direction for the application of diagnostic assays in the detection of *Mycobacterium bovis* infection in suspect Michigan reservoir species.

Tuberculosis has plagued mankind for many centuries with the earliest evidence of spinal tuberculosis diagnosed in Egyptian mummies dating back to many thousand years BC[1].

Advances in microbiology has allowed for the characterization and classification of this microbial family. *Mycobacterium bovis*, the etiologic agent of bovine tuberculosis, is categorized among the tuberculosis Mycobacterium. This group includes six other closely related species: *M. tuberculosis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae*, and *M. canetti*[1].

Mycobacterium bovis is present on multiple continents and has one of the broadest host ranges of any known zoonotic pathogen[8]. This pathogen has been isolated from domestic livestock, domestic pets, wildlife, and even humans. The broad host range of this microbe has created a complex ecosystem, in which disease discovery, epidemiological characterization, surveillance, and control have proven to be a challenge[7]. A focused global collaborative effort to discuss and implement bovine tuberculosis control measures is essential for the forward progression of tuberculosis eradication.

Six steps to manage bovine tuberculosis in free-ranging wildlife have been conceptualized, these include: discovery, epidemiological characterization, initial control, simulations/forecasting, focused control, and verification of control/eradication[6]. The first step, discovery, is defined by the presence of one or more species infected with *M. bovis*. These cases are often discovered by gross examination and laboratory tests. The second step, epidemiological characterization, focuses on the identification of maintenance hosts, reservoirs, and routes of transmission. If the first two steps are fulfilled then the third step of initial control is implemented using general procedures to reduce infected populations or to reduce the means by which disease is transmitted. As the management model progresses additional steps in disease modeling and

planning are initiated by complex simulation and forecasting. The knowledge acquired by simulation models will result in a focused control of tuberculosis by targeting specific populations for culling and vaccination. The final step of this management model is control/eradication, in which lack of disease is expected upon surveillance. Eradication of such an insidious disease requires constant research, diligence, and collaboration. These steps eloquently outline how multiple areas of research can contribute to the eradication process. The four investigative projects included in this dissertation are an example of how research within the areas of discovery and epidemiologic characterization can aid in the over all goal of control and eradication of tuberculosis.

Discovery is the key initiator of disease awareness. In the state of Michigan an endemic focus of bovine tuberculosis is maintained within the white-tailed deer population. White-tailed deer are identified as the sole wildlife reservoir for bovine tuberculosis in the state of Michigan; however, additional surveillance of other potential wild and domestic animals are underway to reduce the myopic view of reservoirs hosts[11]. In the state of Michigan, it was identified that raccoons, opossums and the grey fox could potentially serve as wildlife reservoirs in northern Michigan[11]. Other studies have suggested that black bears, bobcats, coyotes, feral cats, and the red fox are potential reservoirs hosts of bovine tuberculosis. Whatever the potential reservoir host species maybe, proper identification of diseased animals is paramount.

The first study described in this dissertation was aimed at evaluating the immune response in specific pathogen-free (SPF) cats stimulated with Sensitinogen, a heat killed *Mycobacterium bovis* product, using the rapid test, multiple antigen print immunoassay (MAPIA), and bovine purified protein derivative (bPPD) single skin test. The bPPD test at 72 hours had a mean skin thickness of 0.3 mm for stimulated cats and 0.1 mm for controls. Rapid test identified 4 of 6

stimulated cats after bPPD injection. The MAPIA detected antibody against MPB83, 16/83, 16kDa, and *M. bovis* culture filtrate (MBCF) antigens. All assays differentiated between stimulated and control cats; however 7 of 49 non-SPF control cats had a reaction for either antigen MBCF or 16/83. These preliminary studies show potential for antemortem detection of *M. bovis* among domestic cats; and therefore disease discovery; however, additional studies to better characterize virulent *M. bovis* infection in cats would be of value. Important areas to address in future research of tuberculous domestic felids include routes of infection, distribution of lesions, and shedding of the organism. Of the current data compiled it appears that domestic cats have minimal impact on disease spread in Michigan.

The second study described in this dissertation was focused on the epidemiological characterization of Virginia opossum in respect to disease spread. This was achieved by characterizing the intra-species lateral transmission after aerosol inoculation and 45 days of cohabitation. The Virginia opossum (*Didelphis virginiana*) is a known host of bovine tuberculosis in the state of Michigan and previous studies have shown them to be susceptible to *M. bovis* by aerosol inoculation[4,11]. Additionally, Virginia opossum utilize shared dens, and in the state of Michigan, has a high potential for significant interaction with other animals harboring bovine tuberculosis[11]. Recent advancements in serologic assays to determine antemortem disease status of *M. bovis* include the multiple antigen print immunassay (MAPIA) and Rapid test[6]. In this experiment the RT was able to identify all inoculated opossums as positive and the exposed and control opossums as negative. The MAPIA for this experiment was unrewarding and yielded non-interpretable reactions (data not shown), further investigation is warranted. Other ante mortem assays often performed in animals to determine tuberculosis

disease status include the intradermal injection of bovine purified protein derivative (bPPD)[5]. Administration and evaluation of pinnal bPPD injections were difficult and unrewarding in this experiment and subsequently were terminated. At the end of the experiment it was noted that the entire inoculated group was infected with *M. bovis*; this was confirmed by classic gross lesions, histologic lesions, bacterial culture, and antibody tests; however, *M. bovis* was undetectable in the control and exposed groups. Future studies assessing shedding pattern and chronic disease progression maybe warranted in Virginia opossum. The conclusion of this study states that there was no appreciable lateral transmission after aerosol inoculation of *M. bovis* after 45 days of cohabitation between Virginia opossums. Of the current data compiled it appears that the Virginia opossum have minimal impact on disease spread in Michigan.

The third study described in this dissertation was focused on the disease discovery and surveillance of feral swine in Michigan by conducting a continuous surveillance for bovine tuberculosis. One hundred and twenty four feral swine are on record for the hunter killed feral swine surveillance in the state of Michigan from November of 2006 until September 2010. Three swine were noted having gross lesions of tuberculosis; however, these were not confirmed to be tuberculous lesions histologically. All other animals in the study had no evidence grossly or histologically of tuberculosis. As of 2010 the tested hunter harvested feral swine have not been identified as having tuberculosis. This surveillance to date has yet to identify any potential devastating infectious agents, including tuberculosis within the hunter harvested feral swine in the state of Michigan. These negative results are promising; however, continued surveillance in the state of Michigan is desirable, especially as the feral swine population spreads to the northern portions of the lower peninsula. In Europe, the Eurasian wild boar (*Sus scrofa scrofa*) has been identified as a true wildlife reservoir of bovine tuberculosis[2]. Sampling of feral swine within the

MAZ or even the MAAZ should be of the interest to the state of Michigan, as feral swine are highly susceptible to bovine tuberculosis.

The first three studies discussed in this dissertation cover two of the six conceptualized steps in management of bovine tuberculosis in free-ranging wildlife, discovery and epidemiological characterization. Research devoted to diagnostic assay development and testing is also considered an important aspect of tuberculosis eradication prior to these six management steps. (Figure 7.1) Current approved testing for bovine tuberculosis first includes variations of the purified protein derivative (PPD) skin test (caudal fold test [CFT] or the neck [CIT]), followed by the comparative cervical skin testing (CCT) concurrently with the INF-gamma assays and finally, post-mortem evaluation. Although these assays have been historically used in combination with a fairly good success rate, bacterial culture is still considered the gold standard for *Mycobacterium bovis* identification. In order to circumvent the long incubation time required for traditional culture, new antibody-based assays have erupted within tuberculosis research. This dissertation implements two current, highly researched, multi-antigen, antibody based assays, the MAPIA and rapid test. These new assays have proven to be a promising, quick, reliable, diagnostic test; however, these assays must be considered preliminary only as continued research is needed to characterize the immunodominant antigens for each host species. The fourth study described in this dissertation was focused on other potential assays to circumvent traditional culture by utilization of the monocytic cell line TIB-202 to propagate *Mycobacterium bovis.* The preliminary results are promising as the manipulations of this cell line suspension for maintenance, proliferation, quantification, and activation were relatively easy. The preliminary PCR results were able to detect a three fold increase of amplifiable DNA within the lysated cells after six days of incubation, supporting the hypothesis that this cell line could potentially be used

as a rapid detection diagnostic assay. The ultimate goal of this project, which will need to be continued by the future personal, was to inoculate the optimized TIB-202 cell line with cervical lymph node samples collected from cattle or deer that were submitted to the diagnostic center for population and animal health (DCPAH) at Michigan State University. The tissues would be homogenized and subjected to decontamination in order to reduce the amount of background or contaminate bacteria. The homogenized tissue suspension would be fed to the activated human monocyctic leukemia cell line TIB-202, maintained, and eventually lysed to yield the propagated *Mycobacterium bovis*. Traditional culturing ran in parallel with the cell line propagation will allow for verification of sensitivity, specificity, and for a detailed cost analysis.

In conclusion, domestic cats, Virginia opossum, and feral swine appear to have a minimal impact on bovine tuberculosis spread and persistence in the state of Michigan. However minimal the current impact maybe, continued research and surveillance is empirical for disease characterization and the ultimate global eradication of tuberculosis. “I need hardly add that the fight against cattle tuberculosis only marks a stage on the road which leads finally to the effective protection of human beings against the disease” Emil von Behring Nobel Prize acceptance speech[8].

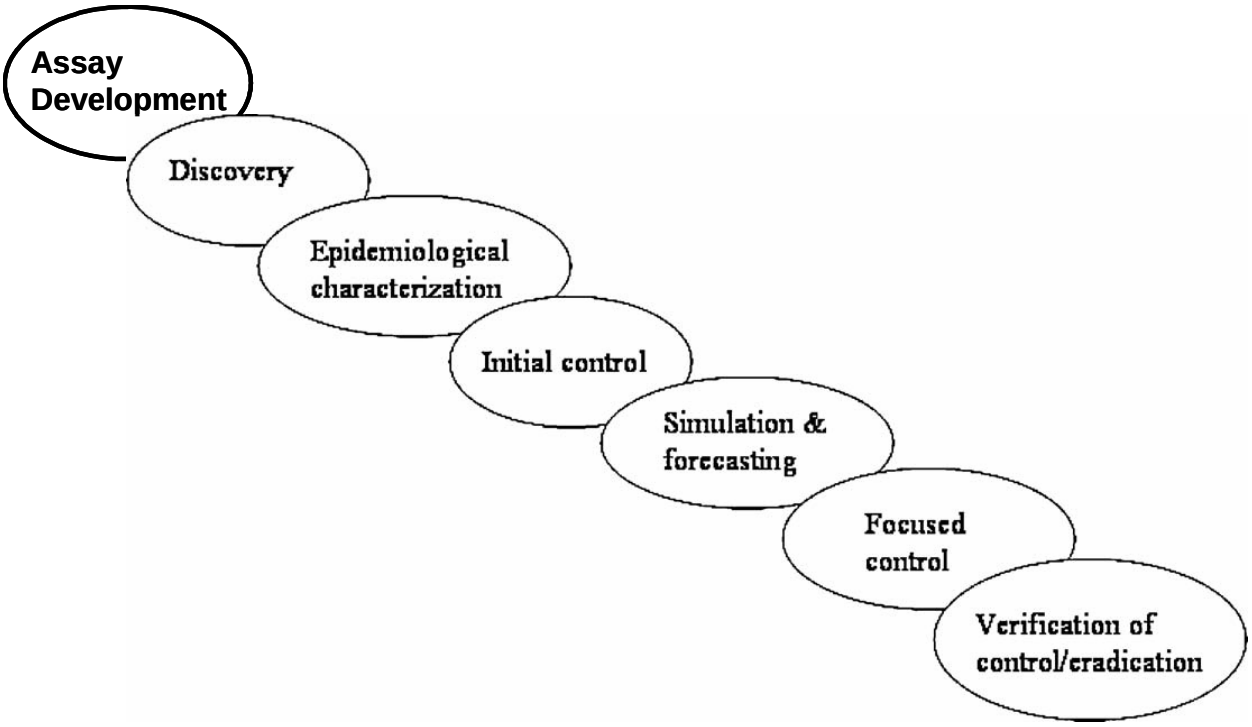

Figure 7.1 Seven conceptualized steps to manage bovine tuberculosis in free-ranging wildlife

This figure was adapted with permission from O'Brien et al. and displays the seven steps suggested for the management and ultimate complete eradication of bovine tuberculosis in free-ranging wildlife.

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APPENDICES

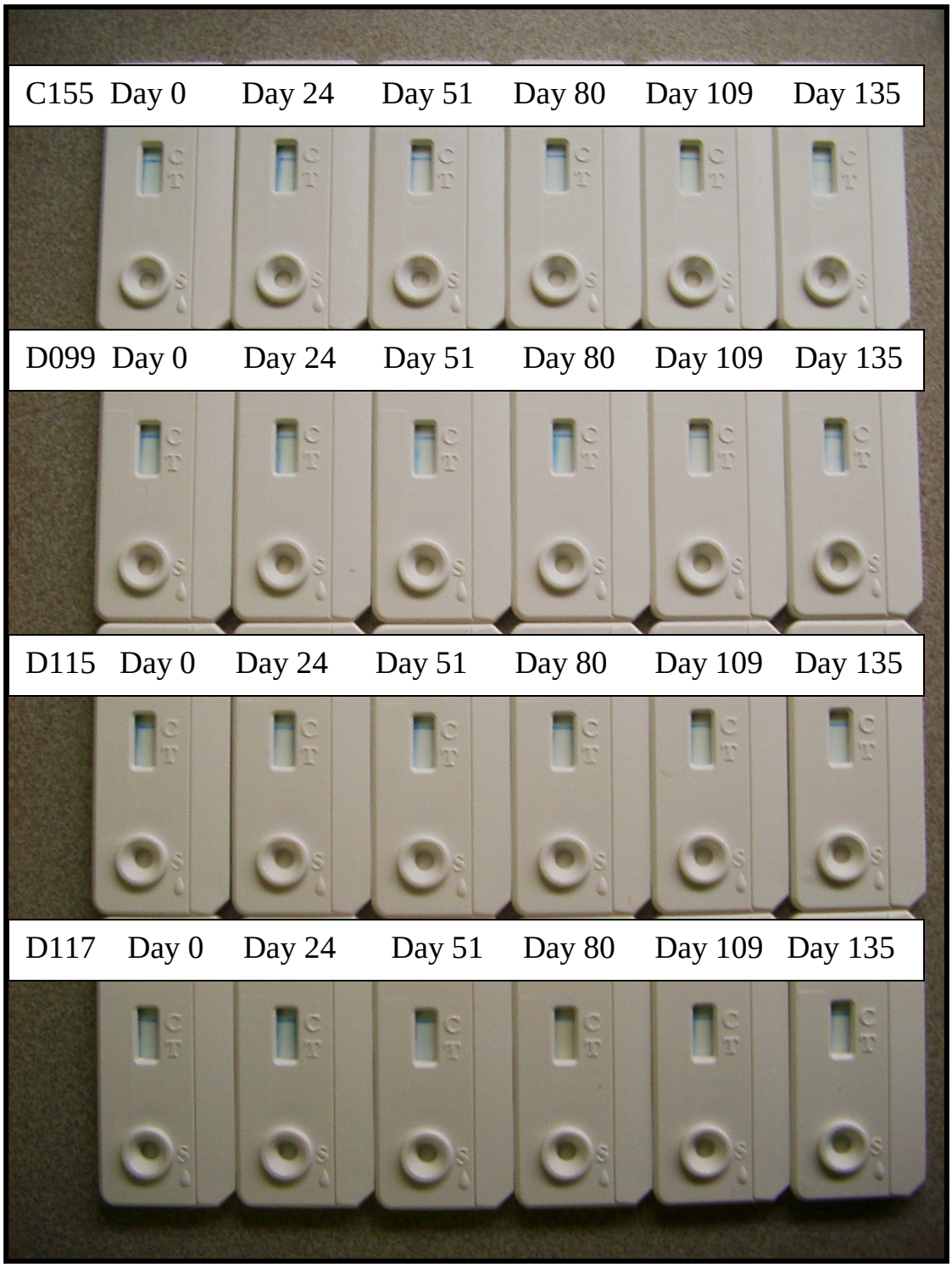

Figure A.1 Rapid test raw data for Sensitinogen stimulated domestic cats.

Figure A.2 Rapid test raw data for Sensitinogen stimulated domestic cats.

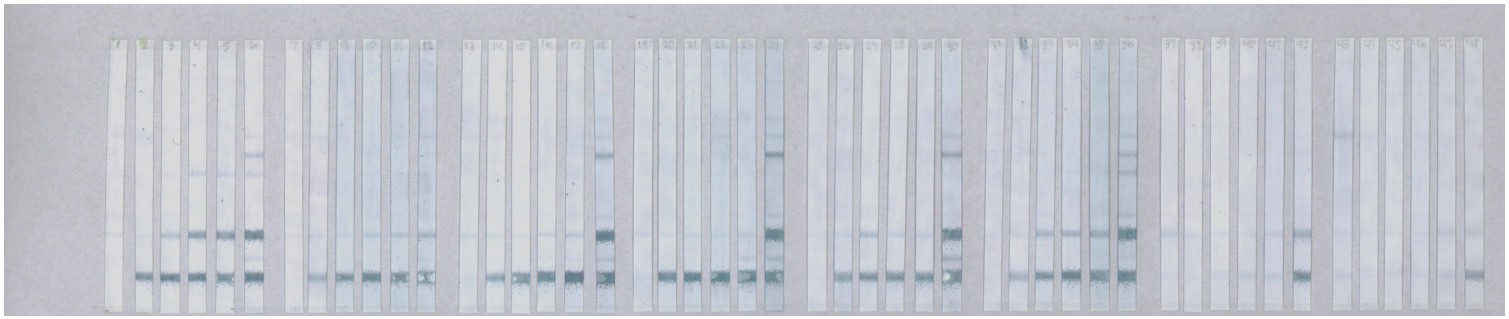

Figure A.3 MAPIA raw data for Sensitinogen stimulated domestic cats.
There are 6 test strips per animal, in which from left to right indicate day 0, 24, 51, 80, 109, and 135.
Animals D115 and D117 were controls.

Figure A.4 MAPIA raw data for non-SPF domestic cats. One test strip indicates one non-SPF cat.

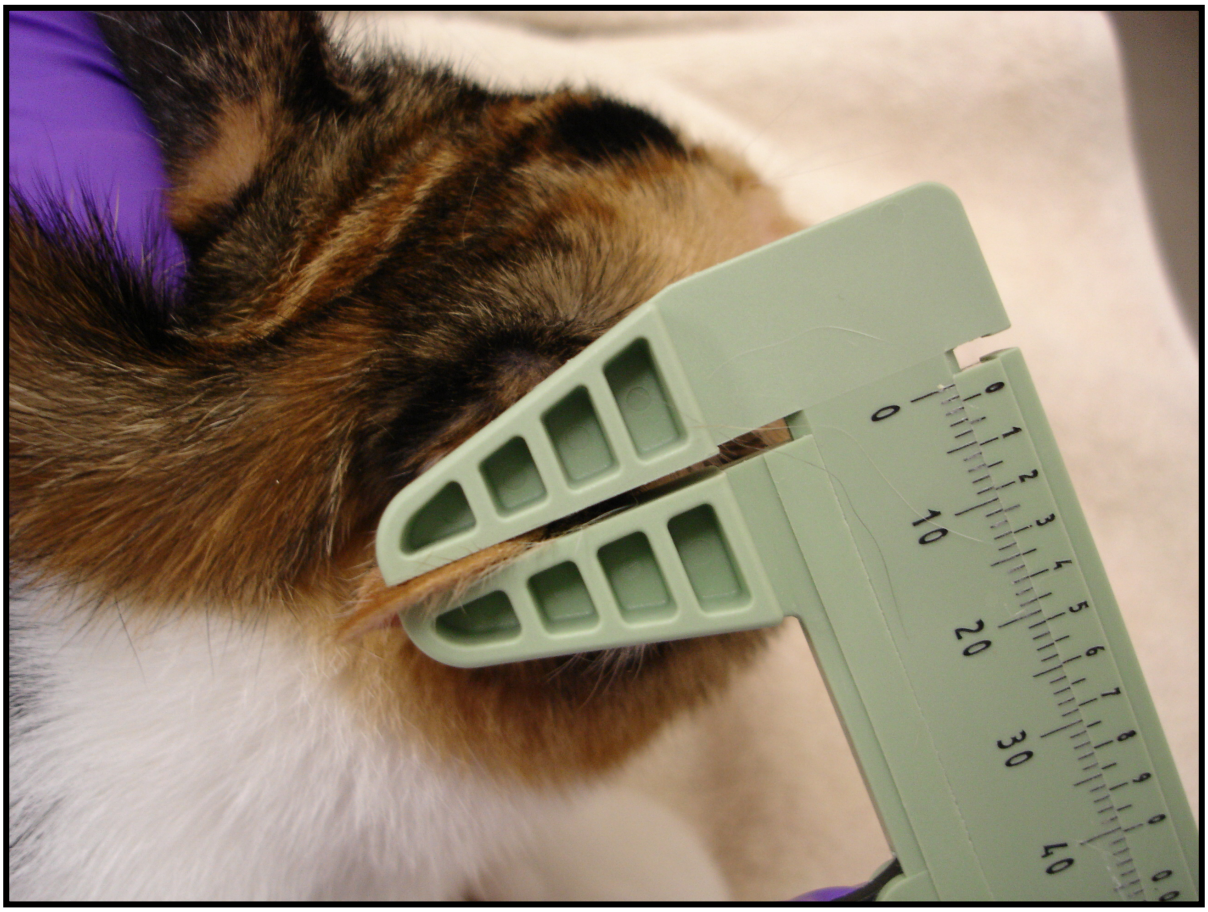

Figure A.5 Bovine tuberculosis PPD skin test, control domestic cat.

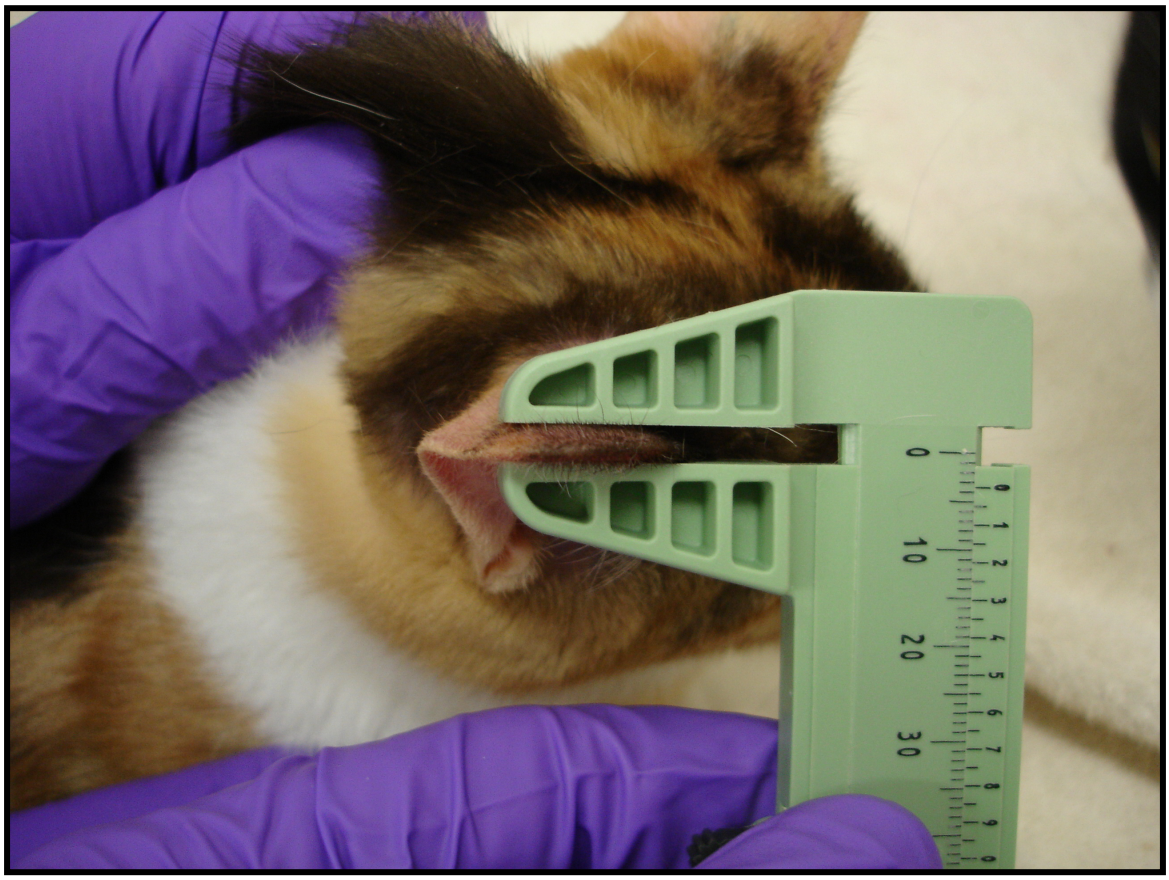

Figure A.6 Bovine tuberculosis PPD skin test, Sensitinogen stimulated domestic cat.

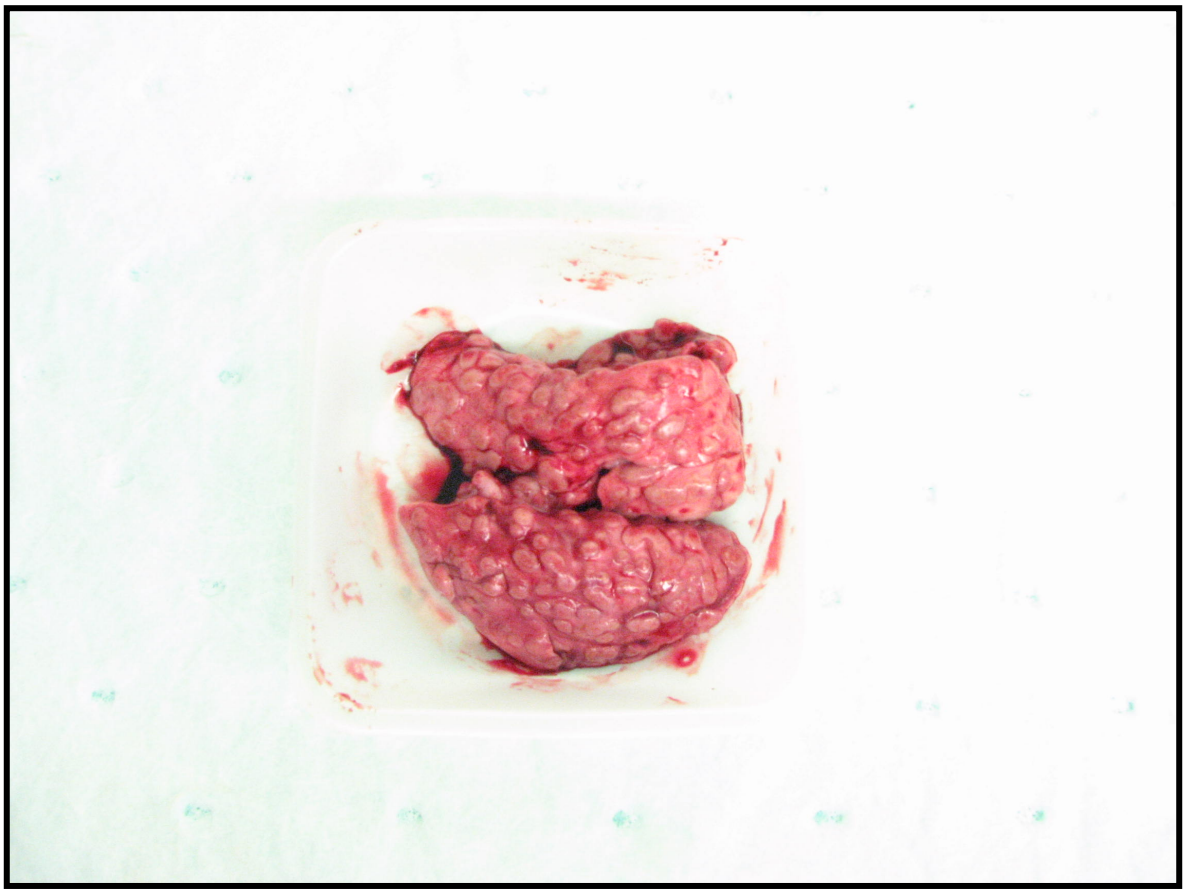

Figure A.7 Pulmonary gross pathology of *M. bovis* inoculated Virginia opossum.

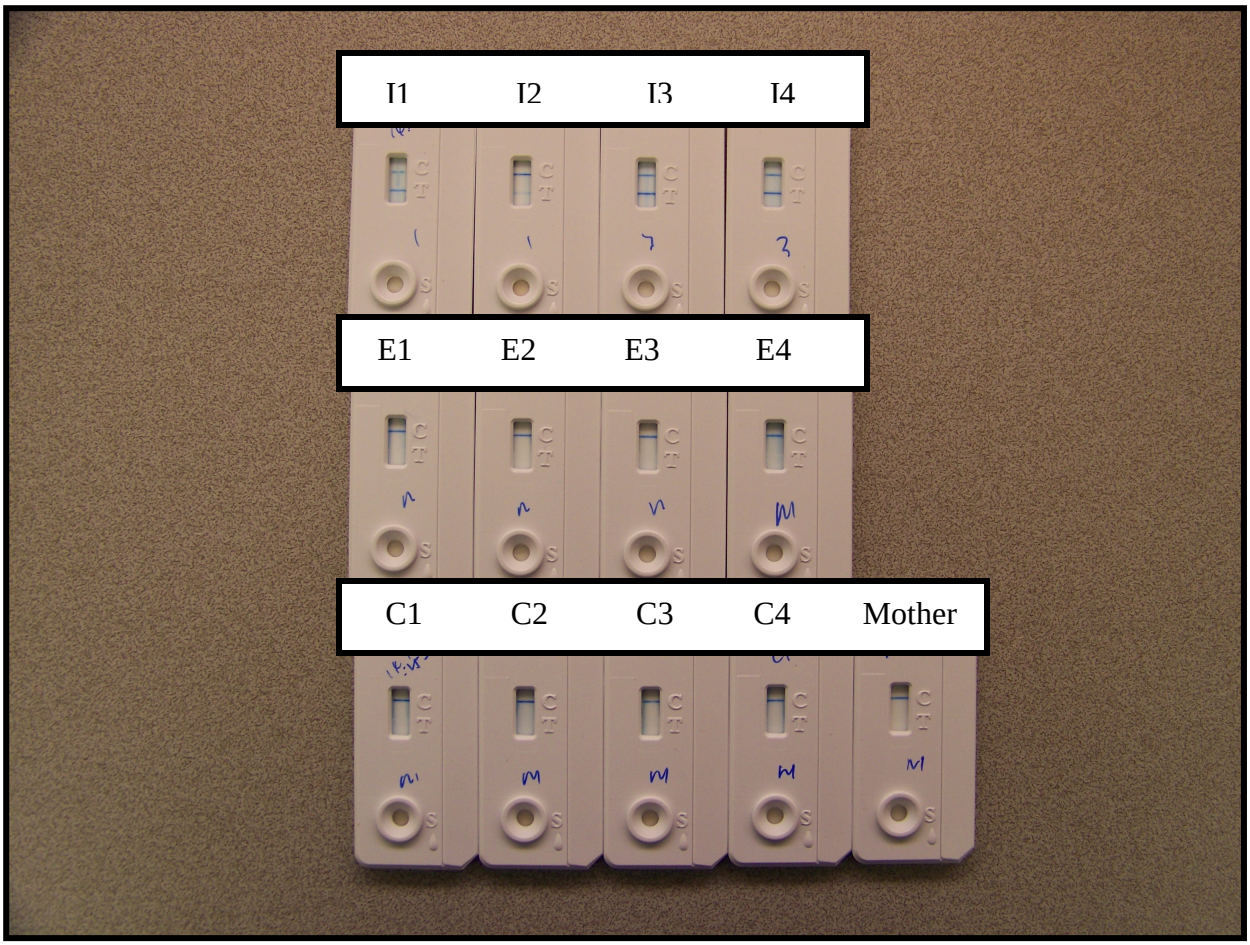

Figure A.8 Rapid test raw data for *M. bovis*, inoculated, exposed and control Virginia opossum.