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THE PATHOLOGY AND TRANSMISSION OF *MYCOBACTERIUM BOVIS* IN MICHIGAN WILDLIFE

By

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ABSTRACT

THE PATHOLOGY AND TRANSMISSION OF MYCOBACTERIUM BOVIS IN MICHIGAN WILDLIFE

By

Kelly Lynn Butler

Michigan currently has an endemic strain of *Mycobacterium bovis* tuberculosis in its free-ranging white-tailed deer (*Odocoileus virginianus*) herd. The disease has been identified in deer and a large number of small mammalian species throughout the Lower Peninsula of the state, has spread to domestic cattle herds, and has been identified in a captive cervid herd. Cases identified through surveillance programs in wildlife are primarily concentrated in the northeastern portion of the Lower Peninsula thus far, but new counties with positive cases are identified each year as the span of surveillance widens and the numbers of animals included in the program grow.

The objectives of this body of work all sought to better define this endemic strain of tuberculosis in Michigan wildlife. A primary objective was to evaluate several common species for their potential in the transmission of the disease, two native passerine bird species, the European starling (*Sturnus vulgaris*) and the American crow (*Corvus brachyrhynchos*), and the North American opossum (*Didelphis virginianus*) were studied. Although they would certainly figure prominently in transmission if they were efficient hosts of the disease, none of these species had ever been challenged with the *M. bovis* organism experimentally, and no reports describing natural infection exist, so a large part of this thesis was to describe any pathology resultant from experimental inoculation of these animals. Birds were challenged intraperitoneally and orally with *M. bovis*, but

infections were only established by the intraperitoneal exposure route. Opossums were challenged intramuscularly and orally with *M. bovis*. Infection was established via both exposure routes in opossums.

Major concerns with tuberculosis in all species include the zoonotic potential of the disease and the emerging threat of these microorganisms becoming increasingly resistant to drugs used in treatment. A fundamental question that had not been examined until the time of this work was whether or not the strain of *M. bovis* causing endemic tuberculosis in Michigan's wildlife had any evidence of drug resistance. The objective to determine the susceptibility of the strain to common antimycobacterials was achieved successfully, it was found that there is no evidence of resistance to date in this strain. Moreover, comparison of the strain to that of several human cases of *M. bovis* identified in Michigan showed differences in both susceptibility profiles and in genetic fingerprints when compared to the animal case strain.

The final objective of this dissertation was to evaluate a novel cytologic technique for its potential application in the detection of mycobacteria in clinical samples. Cells were obtained from the tracheal, nasal, and oral mucosae of white-tailed deer experimentally inoculated with *M. bovis*. These samples were examined for the presence of intracellular mycobacteria after being prepared using the new technique, the ThinPrep 2000 automated cytology device. Results were compared to mycobacterial cultures taken from the same sites in these deer. The technique showed some promise in the antemortem diagnosis of mycobacterial disease, but was less sensitive and specific than existing veterinary diagnostic tests.

DEDICATION

For Mom, Dad, and Rob...

I suppose this would be where I say "I couldn't have done it without you"-
I consider myself very lucky that I'll never have to find out whether
or not I could have done so.

For being my strength, my humor, and my reality check
in times when all of these things are so hard to find on my own-
Thank you. I love you.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AHDL	Animal Health Diagnostic Laboratory
αβ-T	alpha-beta T cell
BALT	bronchial-associated Lymphoid tissue
BCG	bacillus Calmette-Guerin
BTB	blood tuberculosis test
CD	cluster of differentiation
CI	confidence interval
CIP	ciproflozacin
CFU	colony forming unit
CM	capreomycon
CMI	cell mediated immunity
CS	cycloserine
DMU	deer management unit
DTH	delayed type hypersensitivity
EA	ethionamide
ELISA	enzyme-linked immunosorbent assay
EMB	ethambutol
GALT	gut-associated lymphoid tissue
γδ-T	gamma-delta T cell
GI	growth index
GSF	granulocyte stimulating factor
H&E	hematoxylin and eosin
HEPA	high efficiency particulate air
HPF	high power field/ 40X
IFN-γ	gamma interferon
IL	interleukin
IM	intramuscular
INH	isoniazid
IP	intraperitoneal
K	kanamycin
LAM	lipoarabinomanan
MAC	*M. avium* complex
MCP I	monocyte chemoattractant protein I
MDR	multidrug-resistant

MHC	major histocompatibility complex
MOTT	mycobacteria other than tuberculosis
MDCH	Michigan Department of Community Health
MDNR	Michigan Department of Natural Resources
MSU	Michigan State University
NADC	National Animal Disease Center
NK	natural killer T cell
PBS	phosphate buffered saline
PI	post inoculation
PPD	purifed protein derivative
PZA	pyrazinamide
RA	rifampin
RFLP	restriction fragment length polymorphism
SM	streptomycin
TAD/ TDM	trehalose-6,6' dimycolate
TCH	thiophen-2-carboxylic acid Hydrazide
Th1/ 2	helper T cell subset 1/ 2
TGFβ	transforming growth factor β
TNF-α	tumor necrosis factor alpha

Chapter One

Literature Review and Surveillance Background

Literature Review

Mycobacteria: Structure and Virulence

Organisms classified as *Mycobacterium spp.* are numerous, and include those ranging from nonpathogenic or opportunistic saprophytes, to highly pathogenic, zoonotic disease agents. A large part of the virulence of these organisms is determined by certain structural components and biochemical or enzymatic functions inherent to each species. In general, mycobacteria are non-spore forming, rod-shaped (bacillar), slow-growing, variably obligate aerobes. Unique to mycobacteria (and to a lesser extent, corynebacteria and nocardiae) is their characteristic acid-fastness. This term describes the fact that once stained, mycobacteria will retain stain after treatment with acid or alcohol (Brooks et al 1991). Both the difficulty in initial staining and the resistance to decolorization that mycobacteria exhibit are due to the composition of their cell walls. The largest part of the cell wall of mycobacteria is an amalgamation of lipids and lipid-containing compounds, including waxes, which contribute to most of their acid-fastness. Proteins and polysaccharides make up the remaining constituents. Together, these components elicit both delayed type hypersensitivity and cell mediated immunity to varying degrees. Humoral immunity is not protective in mycobacterial disease, and does not play a significant role in lesion development (Thoen and Bloom 1995; Dungworth 1993). The components of the specific chemical makeup of cell walls differ proportionately between species of mycobacteria and are intimately linked to the virulence of the strain in question. Generally speaking, those

bacteria that have the "biochemical ingredients" which allow them to most efficiently multiply and survive within host cells while eliciting the least significant inflammatory response in the host will prove most virulent (Lagrange et al 1999).

Among the defenses of the mycobacteria leading to disease are the variably effective, species-specific abilities to survive and grow in number both intracellularly, within unactivated host macrophages, and extracellularly, within caseous centers of lesions resulting from infection (Dannenberg 1999; Thoen and Bloom 1995). The ability of mycobacteria to prevent fusions of phagosomes and lysosomes within macrophages is one of its most effective defense mechanisms. Acidic lipids known as sulfatides were thought to be primarily responsible for this property (Thoen and Bloom 1995), but data does exist that raises questions toward the validity of this finding (Goren 1988). It is known that these sulfolipids do contribute to the inflammatory response, however, possibly by inducing prostaglandin synthesis and altering superoxide production in inflammatory cells (Moulding 1999).

Regardless of the yet to be determined mechanism, it is known that virulence does typically coincide with sulfolipid content of cell walls in mycobacteria (Goren et al 1974). Another primary virulence factor is cord factor, trehalose-6,6' dimycolate (TAD;TDM) (Glickman 2001; Dungworth 1993; Brooks et al 1991). The specific effects of this cell wall glycolipid include it's leukotoxic abilities (partially due to its stimulation of cachectin) and inhibition of chemotaxis of leukocytes (Silva and Faccioli 1988; Noll et al 1956). It also has been noted as toxic to mitochondria, is linked to ribosome detachment from the rough endoplasmic reticulum, and contributes

to the inhibition of phagolysosome membrane fusion (Glickman 2001; Spargo et al 1991; Thoen and Himes 1986). Interestingly, bacilli will morphologically form ropelike cords due to the presence of this factor, and their ability to do so can be positively correlated with their species-specific virulence (Dungworth 1993).

Also important to mycobacterial virulence is lipoarabinomanan (LAM), a glycolipid thought to be responsible for not only deterring adherence of the bacteria by macrophages, but also having unique free radical scavenging capabilites (Moulding 1999; Strohmeir and Fenton 1999; Thoen and Bloom 1995). Adherence and uptake of bacilli into macrophages is due to the binding of LAM to the CD14 and macrophage mannose receptors. This nonspecific binding then allows mycobacteria to enter the cytoplasm of the macrophage to begin replication. Interesting effects of LAM also include its abilities to not only attract lymphocytes to the site of infection by stimulating the production of chemokines, but also its ability to suppress the function of these same lymphocytes once attracted to the site through promotion of enhanced transforming growth factor β (TGFβ) production (see immunology review below) and reduced gamma interferon (IFN-γ) production (Glickman 2001). Such duality in effects may lead to enhanced mycobacterial proliferation within cells while at the same time, the overall immune response to infection is thought to diminish. Poorly understood mechanisms leading to suppressed lymphocyte activation have also been attributed to LAM (Strohmeir and Fenton 1999).

Activation of monocytes may also be impaired by the mycobacterial protein antigen 85A. In fact, of the specific mycobacterial proteins studied in detail, antigen

85A is among the most important of those that directly enhance virulence by binding fibronectin, preventing it from adhering to these bacilli (Thoen and Bloom 1995). Another protein from this complex, antigen 85B, seems to have a role in mycolic acid production (Cooper and Flynn 1995). Proteins in the antigen 85 complex, along with ESAT-6, another mycobacterial protein, have the ability to potently stimulate the proinflammatory cytokine IFN-γ (Cooper et al 2001; Weinrich et al 2001; Cooper 1995). Most of the remaining mycobacterial structural proteins, and indeed many of the lipids as well, have immunomodulation of the host as their primary function (Brooks et al 1991). These antigens are among the important contributors to those soluble fractions harvested as purified protein derivatives (PPD) from *M. bovis* or *M. avium* cultures for use in the tuberculin skin test (Adams 2001).

Pathogenesis and the Immune Response in Mycobacterial Disease:

Introduction

As mentioned in the previously section discussing virulence, the main host defenses against mycobacterial infection in all species in which they are of importance are primarily the cell mediated immune responses (CMI). CMI and delayed type hypersensitivity (DTH) are both major (separate) factors in tuberculosis, with humoral immunity playing a much less significant role that does not contribute to memory immunity (Dungworth 1993). Humoral responses are, when detected, most often present in chronic and/ or disseminated infections (Reddy and Andersen 1998). This is not to say that antibody opsonization of organisms and resultant enhanced phagocytosis does not occur in all stages of this disease, but it is primarily the T cell

generated response to the bacteria that is responsible for resolution of infection and generation of memory immunity in mycobacterial infections (Thoen and Himes, 1986). Interestingly, the host cellular response to mycobacterial invasion is of great importance, not only because it determines the course of the disease, but also because it is largely involved in the development of the unique pathologic features of tuberculosis that ensue as clinical disease develops. In fact, it is the host's own immune response, and the balance between protective acquired immunity and delayed type hypersensitivity that determine much of the course of the disease (Dannenberg 1999).

Basic Pathophysiology and Immunology

Mycobacteria enter, most typically, as inhaled droplets (less than 20 μm in diameter and containing one to three bacilli) into pulmonary alveoli or, less commonly, are ingested and invade the gastrointestinal system mucosa (Dannenberg 1999; Thoen and Himes 1986). As organisms are engulfed by local tissue polymorphonuclear cells and non-activated monocytes, their intracellular existence begins. It is at this stage that infection may be initially limited as the host's immune system clears itself of infectious bacilli (Dannenberg 1999). But unique properties that allow mycobacteria to escape degradation by this, the body's first defense against them, immediately become important to the survival of the bacteria within host cells. These defenses include an overall impaired difficulty by the host in the destruction of the relatively rigid cell walls containing mycolic acids, waxes, cord factor, and other lipids or protein complexes which enhance virulence and prevent phagolysosome

formation; survival of mycobacteria within phagolysosomes by microorganism-generated pH elevations, or by structural defenses; and phenolic glycolipids which directly deter superoxide radical degradation of bacteria, even allowing intracytoplasmic replication of the organisms (Rhoades and Ullrich 2000; Brooks et al 1991). These specific virulence factors were discussed in the first section of this chapter and will not be reviewed in detail.

Products resulting from the degradation of bacilli and inflammatory cells cause an initial release of tumor necrosis factor alpha (TNF-α), various interleukins (interleukins one, six, and ten most importantly; IL-1, IL-6, and IL-10) and other chemokines, particularly monocyte chemoattractant protein I (MCP I) and IL-8, which modulate the activation of further inflammatory cells and the chemotaxis of these cells to the site of initial infection (Skinner et al 2001). An important subset of cells involved in the production of MCP I in infection are the gamma-delta receptor-bearing subset (γδ) of T cells, which have some evidence of participation in driving the formation of tuberculosis lesions towards more purely monocyte-type reactions than suppurative, neutrophilic ones (Orme 1999). Dendritic cells travel from the forming "primary complex" to regional lymph nodes in response to antigen presentation from initially insulted macrophages. Antigen presentation to CD (cluster of differentiation) 4+ T cells allows for expansion of subsets of these cells specific to mycobacterial antigens. The involved subsets of CD 4+ cells are primarily class one helper T cells, Th1 cells, which are recruited in tuberculosis infections, until lesion resolution and chronic immune responses begin to be developed by type 2, Th2, subsets and Th1

involvement subsides (Ortana and Cauda 1998; Griffin et al 1995). Interleukin two is important in initial signals to activate macrophages and recruit new circulating monocytes towards the now-forming granuloma in this cascade. Activated macrophages in turn produce IL-12, furthering recruitment of CD 4+ helper cells. Natural killer (NK) T cells are activated by IL-12 and produce IFN-γ, along with $\gamma\delta$ T cell subsets (Orme and Cooper 1999).

CD 8+ cytotoxic T cells are also important in the elimination of mycobacteria from the site of initial infection, and as they spread throughout the body. Cytotoxic T cells are primarily involved in destruction of those phagocytes with major histocompatibility complex (MHC) class I expression, which induce immediate cytolysis by CD 8+ cells, whereas CD 4+ cells produce lymphokines in response to recognition of MHC II associated presentation (Reddy and Andersen 1998). Both CD 4+ and CD 8+ T cells secrete IFN-γ, which enhances overall inflammatory cell activity in the body by recruiting both specific and non-specific phagosomes. Although both cell types are implicated in this defense, CD 4+ cells apparently are the more important contributors (Dannenberg 1999). Both CD 8+ and CD 4+ Th1 cell types are specific to the mycobacterial antigens produced by the infection and presented to them on associated MHC receptors (Reddy and Andersen 1998). Certain non-peptide components of the cell wall are presented by macrophages to lymphocytes on non-MHC receptors. The lymphocytes that recognize these antigens are sometimes referred to as CD1 lymphocytes or CD 4-8- lymphocytes, based on the molecular structure of the receptor. Such cells are important in the cytotoxic killing of microbe-

laden cells and in producing IFN-γ, not only in tuberculosis infections, but in other bacterial diseases as well (Dannenberg 1999; Cooper and Flynn 1995).

Tumor necrosis factor alpha (TNF-α) is secreted by both CD 4+ and CD 8+ T cells as well. This cytokine is quite paroxysmal in its mechanisms, in that it not only helps to enhance bactericidal activity of immune cells, but also leads to host tissue destruction that results in the formation of caseous granulomas typical of tuberculosis (Cooper and Flynn 1995). A balance is thought to exist between TNF-α and transforming growth factor beta (TGF-β) in tuberculous lesions, with cells located centrally in granulomas producing more of the former, and cells peripherally (within the fibro-epilthelioid "capsule", see basic pathology section below), producing more of the latter (Aung et al 2000; Vanham et al, 1997). Interestingly, TGF-β may even play a role in the regression of tubercles (Maeda 1993).

TGF-β expression typically increases in the typical tuberculosis immunologic cascade of events as infection subsides, while TNF-α expression is most extreme during the initial stages of infection and during primary tissue destructive phases (Hernandez-Pando 1997). Interleukin ten and IL-4 are also associated with lesion resolution phases in tuberculosis, and are secreted by type 2 CD 4+ lymphocytes, which dominate the immune response at these late stages of infection (Ortona and Cauda 1998). Along with IL-13, these cytokines downregulate type 1 CD 4+ lymphocyte activity, and promote the limited humoral immune response typical of mycobacterial disease (Dannenberg 1999).

The majority of the T cell response to mycobacterial infection involves alpha-beta ($\alpha\beta$) receptor type, CD 4+ and CD 8+ lymphocytes. Recently, interest has formed regarding the potentially intriguing role of $\gamma\delta$ receptor type T cell subsets as modulating mycobacterial infections in very specific ways (Delves 2000a). Current research indicates that these cells are important in establishing secretory IgA in response to repeat intracellular bacterial infections (Delves 2000b). They may play a role in chronic, granulomatous stages of disease or in infectious recrudescence (Delves 2000b; Orme and Cooper 1999), but do have a significant part in nonspecific killing of macrophages laden with organisms along with NK cells very early in infection (Dannenberg 1999). Their diagnostic value (the ability to identify them immunohistochemically in tissue sections) for the investigation of responses to PPD and other antigen-derived injection responses plays a potentially beneficial role in vaccine technologies. They may form part of the link between cell mediated immunity and delayed-type hypersensitivity in tuberculosis, a line which is currently relatively still blurred pathophysiologically (Cooper and Flynn 1995).

The inflammatory events described lead to the formation of the primary and secondary, or post-primary, complexes of infection. These lesions may bring resolution to infection and a level of memory immunity in the host to the mycobacterial agent involved. Conversely, they may lead to devastating and widespread tissue destruction and death; or they may lead to temporary cessation of inflammation, as viable, yet dormant, mycobacteria become essentially walled-off to further body inflammatory defenses by the host's own defenses. Immunologic

compromise of any sort in the host with such surviving mycobacteria-laden granulomas can lead to reinstatement of the entire disease process.

Cell Mediated Immunity Versus Delayed-Type Hypersensitivity

Perhaps the most unique feature of the pathogenesis of mycobacterial disease are the intimately related cellular events and antigenic responses that lead to both protective immunity and lesion formation, often simultaneously. A fine line exists between the immunologic mechanisms by which a beneficial host response is created and those mechanisms which lead to tissue destruction.

In a beneficial host response (CMI), memory T cell immunity develops and activated macrophages are signaled to sites of bacterial invasion in order to kill multiplying bacilli. In delayed-type hypersensitivity (DTH), tubercle bacilli are contained by non-activated macrophages which eventually rupture, inciting caseous inflammation, but at the same time limiting the more favorable environment for mycobacterial growth, that within inflammatory cells. Each response can be regarded as protective in its own right, yet it is that of DTH which leads to host tissue damage and the hallmark caseous granulomas of tuberculosis (Dannenberg 1999).

Essentially these responses are indivisible, but the course of disease depends on which predominates. A dose-dependent response of mononuclear phagocytes entering the site of the developing lesion and the amount of mycobacterial antigen available for destruction is seen (Reddy and Andersen 1998). As antigen load increases, the number of macrophages, activated and non-activated, will increase, and a DTH type response will predominate. As the growth of tubercle bacilli is held in check, CMI

will predominate and healing will begin as bacteria are eliminated (Dungworth 1993). Interestingly, studies indicate that most species will "obey the laws of immunity" to mycobacteria in that those with numerous bacilli present in tuberculosis lesions have poor *in vitro* lymphocyte transformation readings, indicating a poorly mounted host immune response (Thorns et al 1982).

The tuberculin skin test is the classic example of the cellular response elicited by memory T cells, and relies heavily on the concepts of CMI and DTH. These immunologic consequences are also important considerations in vaccine development, as antigen sets most like those present in natural infection clearly provide the best protection because they include both mycobacterial structural components and secreted proteins (Collins 1994; Fifis et al 1994). Because beneficial cellular responses in the host can be characterized and measured, studying specific antigen subsets leading to these responses may lead to more efficacious vaccine strategies in the future, for both humans and animals.

Pathologic Presentation of Tuberculosis in Animals

Species of Mycobacteria

The primary mycobacteria implicated as causing disease in animals in the United States are *Mycobacterium bovis*, and those within the *M. avium* complex (MAC; *Mycobacterium avium, intracellulare, lepraemurium and paratuberculosis*). The human tuberculosis agent, *Mycobacterium tuberculosis*, has been known to cause disease in both domestic and captive wild species, but is typically due to transmission from human caretakers (Timoney et al 1988). It follows logically then that most

human cases of *M. bovis* tuberculosis are due to contact with infected cattle. Many human *M. bovis* cases diagnosed in recent decades represent recrudescence of extrapulmonary infections established prior to milk pasteurization and tuberculosis eradication programs being established (Wilkins et al 1986). Numerous additional species of mycobacteria are reported in both animals and man, but are of much lesser epidemiologic significance, are less virulent save for cases involving immunocompromised hosts, and are much easier to manage clinically. Cases involving mycobacteria other than tuberculosis group (MOTT) organisms and MAC organisms in animals usually involve saprophytes (*M. scrofulaceum, kansasii, marinum, fortuitum*, etc.), and are typically associated with individual, localized infections.

The species *Mycobacterium africanum* and *Mycobacterium microti*, the remaining organisms in the *M. tuberculosis* group along with *M. tuberculosis* and *M. bovis*, are of medical concern in their regions of greatest import, Africa and Europe (the United Kingdom, the Netherlands, and Germany) respectively (Rastogi et al 2001; Niemann et al 2000; Kremer et al 1998). Disease caused by these species can affect both animals and man in these areas, but is not present in the United States at this time. Cases of tuberculosis are much rarer for these mycobacteria, and are of much less importance than those previously mentioned, even in their countries of origin (Horskotte et al 2001; Skinner et al 2001; Collins 2000; Alfredsen and Saxegaard 1992). All disease-causing mycobacteria can be considered zoonoses, but the transmission of some of the less virulent, more opportunistic pathogens in the group is

typically of much less concern either between animals and man or within groups (Rastogi et al 2001). For the purpose of this discussion, "tuberculosis" in animals will be that due to *M. bovis*, except where described in birds, where the described lesions are those caused by *M. avium* infection.

Mammalian Versus Avian Presentations of Tuberculosis

Immunology

The hallmark lesion of tuberculosis in any of the various susceptible species is the granuloma (Dungworth 1993). The characterization of this granuloma, and the events leading to its formation do differ in very specific ways in the response to tuberculosis infection in mammalian and avian (or reptilian) hosts (Montali 1988).

Perhaps most important and most basic are the distinct differences that exist between avian and mammalian hosts in the organs responsible for the generation of inflammatory and immune-mediated responses. In particular, their lymphoid systems share very few similarities. The generation and maturation of B lymphocytes in birds takes place primarily in the bursa of Fabricius, whereas in mammals, generation of these cells is in the bone marrow, Peyer's patches, spleen, and lymph nodes, and in the thymus to some extent (Sharma 1991). That is not to say that the bone marrow and spleen of birds do not contribute to B lymphocyte development as well, more so that the task does have a more dedicated organ than it does in mammals. Both mammals and birds have a thymus as their primary T lymphocyte source, both during development and throughout life (Pope 1991).

The lack of a well-developed lymphatic system is notable when comparing avian anatomy to that of the mammal. Although some species (waterfowl primarily) do have rudimentary lymph nodes, when present, these structures do not serve in filtration as they do in mammals. Consequently, no need for well-developed lymphatics is present in birds, and as such, they have comparatively few of these vessels, and they figure much less prominently in immunogenesis than do those of mammals (Pope 1996). Because birds lack organized lymph nodes, the occurrence of secondary lymphoid tissues associated intimately with various organ parenchyma is much more prominent in these species. Gut-associated and bronchial-associated lymphoid tissues (GALT and BALT), cecal tonsils, hepatic, and even renal lymphoid reserves serve to protect organs in birds where lymph nodes defenses may be absent, along with disseminated lymphoid aggregates present in nearly all parenchymatous tissues (King and McLelland 1984). Also noteworthy is the avian spleen being a more central feature in many inflammatory processes than it is in mammals. The avian spleen is best seen as a defensive spleen, with nearly equal red and white pulp distribution, particularly when compared to that of mammals, which have spleens featuring a larger proportion of red pulp (King and McLelland 1984; Hodges 1974). Germinal centers occurring in the lymphoid tissues of birds are not as highly developed as are those of mammals- with well-mapped maturation sequences present. But these areas do often feature a reticular cell envelope that is not necessarily prominent in mammalian germinal centers (Pope 1996).

Although relatively unimportant in the defense against mycobacteria, some mention towards humoral immunity is necessary. Immunoglobulins shared by birds and mammals are IgG (also called IgY in birds), IgM, and IgA (Sharma, 1997). IgD and IgE, present in mammals, are absent in birds. Also relatively unimportant in the discussion of tuberculosis, but nonetheless absent in birds are lymphocytes having MHC class III antigen presentation expression. Mammals and birds both have MHC class I and II expression, but birds lack a true counterpart to mammalian MHC III, and mammals lack the exclusively avian MHC class IV (Lamont 1998). In both species, most immune responses, including that directed towards tuberculosis infection, do primarily involve lymphocytes with expression of MHC classes I and II (Lamont 1998; Sharma 1997). Lymphocytes bearing these class markers are similar in birds and mammals. Cytokine and chemokine cascades and the involved cellular responses in inflammation are quite comparable as well, particularly the initial monokine activities of IL-1, IL-2, TNF-α, and GSF (granulocyte stimulating factor)(Klasing, 1991). It should be noted that research to specifically define avian lymphokines has not been performed nearly as extensively as it has been in mammals. (Sharma 1991; Montali 1988). Many of those that have been researched and compared to date do, however, seem to be functionally similar but with limited cross-reactivity (Klasing 1998).

Clinical Presentation of Disease

When discussing clinical cases of tuberculosis, it is important to note differences in exposure routes typical of avian and bovine TB that lead to organ infection and resultant clinical signs. In avian tuberculosis, ingestion of infected feed, water, or litter is the primary route of exposure with the aerogenous route being second most important (Gerlach 1994). As such, dissemination of infection throughout the abdominal viscera is typical in birds (Goodwin 1996). Vertical and vector transmissions occur, but are less common. In cattle the primary route of exposure is aerogenous, from infective droplets produced by other infected animals (Neill et al 1994; Thoen and Himes 1986). The caudal lung lobes and draining lymph nodes may therefore be the only affected organs. Ingestion of infected sputum secondary to respiratory infection in individuals may lead to pharyngeal, intestinal, hepatic, and mesenteric lesions, as may ingestion of contaminated feeds or water, the second most common transmission mode. Congenital, cutaneous, and genital transmission of tuberculosis occur less frequently (Neill et al 1994). Exposure routes are also related to the environmental stability of the involved organisms. All mycobacteria are known to exist for long periods environmentally in organic substrates. Yet *M. avium* may persist in contaminated soil for up to two years, whereas *M. bovis* may lose infectivity in a few days when exposed to sunlight (it can persist for much longer in the absence of heat and sun)(Timoney et al 1988). Both agents (particularly *M. avium*) survive for very long periods in water supplies, soil, and dust. Disinfection of the environment

via prolonged contact with phenols is the method of choice for either agent (Falkinham 1998; Greene and Gunn-Moore 1998).

Clinically, tuberculous birds may or may not show evidence of disease. Inapparent infections may exist, particularly in young birds. As infections advance, weight loss in the presence of a hearty appetite and unthriftiness ensue. Weight loss may or may not be associated with gastrointestinal distress such as diarrhea and bleeding. As the disease disseminates, lameness due to bone and joint involvement and eventual death are typical (Gerlach 1994). Cattle infected with tuberculosis may harbor infections for very long periods without clinical signs (Smith 1990; Timoney et al 1988). Both mammals and birds may form primary complexes of mycobacterial infection that do not progress beyond the initial infection until much later in the animal's life. Typically such recrudescence is associated with concurrent illness or immunosuppressive states, and is more commonly noted in mammals than in birds. Fever and weight loss are typical in early active disease in both mammals and birds. If infection is not "walled off" as a primary focus in cattle, dissemination into lymph nodes draining areas of lesion formation is common. Progression therefore leads to cachexia, respiratory distress, and enlarged or draining lymph node lesions.

Comparative Pathology

The pathology and pathogenesis of tuberculosis in each species is inherently linked to the typical response to intracellular organism infections in each. Yet in order to understand this response, some discussion of comparison of the basic cellular inflammatory response is necessary. Although granulocytes (heterophils in birds, and

neutrophils in mammals) are called upon in the acute inflammatory response in both species, in birds this phase progresses much more rapidly and is quite dissimilar to that of mammals. A rapid influx of phagocytes in response to pathogens within the avian respiratory and gastrointestinal tracts is common, largely due to the low numbers of resident tissue macrophages available for defense (Qureshi et al 2000). Within hours, the avian heterophilic response is followed and enhanced by a macrophage response that may not be seen for days to weeks, if at all, in mammals (Barnes 1996). The formation of the heterophilic granuloma in birds is partially due to differences in granule composition of the heterophil. Avian heterophil granules lacks myeloperoxidase and alkaline phosphatase that are largely responsible for phagocytic pathogen elimination in mammals (Harmon 1998; Montali 1988). This is not to suggest that avian heterophils lack microbicidal activity, but this activity is limited to lysosomal cationic killing. Because heterophils lack these proteinases, the heterophilic granuloma tends to caseate centrally, whereas proteinases in neutrophils create lesions which are centrally liquefactive (Montali 1988). Mononuclear cells are recruited to the site of the developing lesion as events progress in both species, although, again, this response is seen typically quite early on in birds (Barnes 1996). CD4+ and CD8+ lymphocytes and natural killer cell involvement in delayed typed hypersensitivity are present in both species (Gobel et al 1996; Arstila et al 1994). As cellular immunity progresses, activated macrophages become part of the response. In birds, macrophages participating in heterophilic granulomas are often Langhans giant cell types, due to an elicited foreign body type response during tissue caseation. In

mammals, resolution of suppurative inflammation may only involve lymphocytes and plasma cells, with few macrophages participating. In birds, macrophage participation is imminent as a primary defense against most immunostimulants (Qureshi et al 2000). Aggregates of lymphocytes and plasma cells may or may not figure prominently in avian granulomas, but these cells will usually be present in at least small numbers (Barnes 1996; Montali 1988). Should the infection in a mammal be chronic enough to elicit a granulomatous response, multinucleate giant cell formation is not necessarily typical as it is in birds. Moreover, central caseation in mammals is more likely to mineralize, an event not at all common in birds. When present in birds, mineralization of caseogranulomas is most likely due to previous parasitic infection. In the latest of chronic states in both species, whether granuloma formation had resulted in mammalian tissue or not, complete resolution will usually involve eventual granulation and/ or fibroplasia at the site of the initial lesion in both species, as phagocytes continue to clear remnants of destroyed tissue parenchyma. Viable tubercle bacilli may or may not remain within the caseous centers of such regressing lesions (Barnes 1996; Thoen and Bloom 1995; Slauson and Cooper 1990).

Both similarities and differences between these species' responses to intracellular organisms exist as well. Initially, in each, mycobacteria are engulfed by resident tissue phagocytes after inhalation of infective droplets or ingestion of contaminated sources. As infection ensues, macrophages in each species produce cytokines which signal development of memory T cells, which in turn activate macrophages further through the production of further cytokines (see immune response discussion above).

These cytokines signal further phagocyte localization and blood monocyte adhesion and colonization. By this point in both species, the lesion forming is the granuloma. The histiocytic granuloma in birds consists of large, foamy to multinucleate giant cells, sometimes with small numbers of heterophils. As these macrophages coalesce, central caseation develops much as in heterophilic granulomas. Indeed, with chronicity, it may be difficult to tell the difference between the two lesion types, the primary difference being necrotic core cell composition, which can be amorphous and almost amyloid-like, but rarely mineralized as is common in bovine tuberculosis (Montali 1988). As mammalian granulomas develop, neutrophils are often featured secondarily, but are featured primarily only in acute stages of infection. In bovine tuberculosis, acute exudative lesions may be present, and these consist nearly entirely of neutrophils with lesser numbers of necrotic macrophages. Such exudative lesions are relatively typical in cervid *M. bovis* infections (Rhyan and Saari 1995). In both birds and mammals, epithelioid macrophages and aggregates of lymphocytes and plasma cells are featured in granulomas, particularly along the most peripheral edges of lesions. Epithelioid macrophages and fibroblasts congregate around caseogranulomas, forming a capsule that helps to prevent further spread of pathogens into surrounding tissues (Slauson and Cooper 1990). Large numbers of acid-fast bacilli are typically visible histologically in *M. avium* tuberculous granulomas. The number of organisms associated with lesions due to the bovine variant is variable, but often is minimal (Dungworth 1993; Mirsky et al 1992). Lesion resolution with replacement by fibrous tissue after infection is cleared, or pathogen dormancy in the

microaerophilic to anaerobic environments of encapsulated granulomas will then take place unless these lesions expand to include vascular supplies allowing for hematogenous spread (Thoen and Himes 1986). Such hematogenous spread without the benefit of lymph node filtration leads to the commonly disseminated distribution of lesions found in birds (Gerlach 1994). Since reticuloendothelial cell contributions to clearing of infections are common, organs rich in both blood supply and in these cell populations are often colonized by infected cells. These organs include, but are not limited to, the spleen, liver, bone marrow, lungs, and kidney. Since mammalian hosts do have the benefit of lymph nodes as defense, nodes draining the organ originally infected will often develop lesions. In mammals, containment of infection may take place in lymph nodes, or may progress further along lymphatics and into other viscera, particularly in immunocompromised hosts (Greene and Gunn-Moore 1998; Thoen and Himes 1986).

Tuberculosis in Cattle and Deer

The primary agent causing tuberculous lesions in both cattle and deer is *Mycobacterium bovis*. And although the basic modes of transmission and the molecular events which follow infection in both cervid and bovine tuberculosis are relatively similar, the cervid cellular response can be considered more dramatic and acute in some respects. Both species are known to develop primarily pharyngeal area and thoracic cavity lesions, particularly when aerogenously exposed (Griffin and Buchan 1994). Yet the suppurative component to lesions in deer is usually more pronounced, particularly when compared to the granulomatous to caseous lesions

present in most cases of tuberculosis in the bovine (Palmer et al 2000a; Clifton-Hadley and Wilesmith 1991). Cattle are typically found to have at least partially encapsulated granulomas with thick, caseous exudate, which is often gritty due to partial mineralization, in either the lung or lymph nodes draining the site of infection. Grossly, lesions in deer may often be large abscesses containing thick purulent exudate (see Figures 1.1-1.4). It should be pointed out that within the various cervid species known to be affected by bovine tubercle bacillus thus far, a wide range of lesion types have been reported, both between and within any given cervid species (Rhyan and Saari 1995; Griffin and Buchan, 1994). Lesions ranging from nearly entirely suppurative with fistulous tracts draining externally from affected lymph nodes, to more classically granulomatous and mineralized, can be identified in tuberculosis cases in cervidae (Palmer et al 1999; Rhyan and Saari 1995). Yet generally speaking, and even more importantly perhaps, speaking from personal observation of white-tailed deer tuberculosis cases identified in Michigan's ongoing surveillance program, deer often develop severely necrotizing, pyogranulomatous, liquefied to partially caseogranulomatous, occasionally cavitating or partially mineralized abscesses in response to infection with *M. bovis*. Such lesion development suggests poorer CMI in cervidae, as more liquefactive lesions are the result of the more tissue-damaging, cytotoxic DTH being more involved in the defense against mycobacteria than that of CMI (Dannenberg 1999). Also indicating poor CMI is the larger number of bacilli typically found present in cervine tuberculosis lesions compared to the often rare bacilli noted in bovine cases (Mirsky et al 1992). When

CMI is the primary mechanism by which mycobacteria are eliminated from the developing lesion, the result is usually a more "pure" granuloma. Such lesions are composed of large numbers of activated macrophages signaled to the area, often forming Langhans giant cells. These lesions will often have central caseation and mineralization also resulting from the cell death caused by DTH, but with containment of bacillary growth and fibrous capsule formation, as is seen in most cases of tuberculosis in cattle (Dannenberg 1999; Rhyan and Saari 1995; Dungworth 1993). It would logically follow to assume that deer are then somewhat more "susceptible" to the bovine tuberculosis agent when exposed than are the more "resistant" cattle when the general presentation of lesions are compared. Interestingly, studies have shown that deer, particularly those with disseminated disease and truly liquefactive lesions, tend to mount a larger humoral response when antibody production is measured (Palmer et al 2000b). Cattle rarely mount significant antibody responses until late in the course of the disease (Fifis et al 1994). Evolutionarily, it makes sense that the species most historically affected by the agent (the bovine) would have generated less dramatic and potentially less fatal inflammatory responses to the infectious agent, by having better genetically-established CMI towards *M. bovis* antigens. Perhaps most importantly however, is the noted potential for differences in lesion presentation both between and within any species infected with *M. bovis*. The differences suggest that there may not be any true "classic" presentation for this disease, and it should be considered a differential in all susceptible species wherein the described spectrum of lesions are found present at necropsy.

Surveillance Program Background-*M. bovis* in Michigan

Mycobacterium bovis

Tuberculosis in humans was suspected as being potentially linked to that in cattle several hundred years before the actual link was discovered by Robert Koch in 1882. (Grange 1995). Respiratory disease ("consumption") was linked to bovine wasting disease, cervical lymphadenitis ("scrofula") was noted as being present more commonly in those persons that were known to drink cow's milk. Even cutaneous mycobacterial lesions were suspected as being caused by animal carcass contact in those people who were in professions that would require potential contact with such sources, but no clear scientific proof existed for such connections prior to Koch's work in 1882 (Grange and Yates 1994). Initially, he isolated bacilli from both human and animal sources, but failed to distinguish any difference between the two. It wasn't until 1898 that Smith defined differences between the bacilli (Grange 1995). After Smith's reports, the differences in the strains were thought to be significant enough that each would be host-specific in its ability to cause disease. The primary researchers in mycobacterial work were all under the assumption that bovine strains were not pathogenic in humans. In fact, some of these scientists believed that animal strains may have been capable of providing protection from disease when used as a live vaccine in humans, and vice versa. It was actually Koch that first spearheaded such beliefs in the scientific community (Collins 2000). Fortunately, researchers (many of them veterinarians) that had suspected transmissibility of the organisms

between these hosts did not rely on Koch's ideas at that time and went on to demonstrate human cases of *M. bovis*. The work of these same researchers led to subsequent reports that the organism, in fact, was a potentially serious threat to humans. It was based on these findings that measures to eradicate the disease in cattle were eventually established by the United States Department of Agriculture, albeit several decades later in 1917 (Collins 2000; Grange 1995; Grange and Yates 1994).

Tuberculosis in Michigan Deer

Michigan was certified free of *Mycobacterium bovis* in its cattle herds in 1979, the conclusion of intense eradication efforts established in the early 1970's. Prior to the eradication of tuberculosis in cattle, *M. bovis* was only reported in a single deer park in Michigan in 1962 (Essey and Vantiem, 1995). During the time span of the cattle tuberculosis eradication program, there was a single reported case of *M. bovis* tuberculosis in a white-tailed deer (*Odocoileus virginianus*), identified in a hunter-killed deer in the northeastern part of the lower peninsula of the state in 1975. This case was not pursued further by the Michigan Department of Natural Resources (MDNR) at that time, as it was a rare disease in deer, and when found was typically associated with exposure to tuberculous cattle. The thought pattern at that time was that eradication from cattle would eliminate any further risk to deer in the state and as such, wildlife reservoirs of this disease were not a concern (Sikarskie et al 1999; Schmitt et al 1997).

Concern was raised when the second hunter-killed deer with lesions suggestive of tuberculosis was submitted to the Animal Health Diagnostic Laboratory, Michigan

State University (AHDL, MSU) in 1994. This deer was from the same area in the state as was the deer identified in 1975, and was also confirmed as positive for *M. bovis* (Schmitt et al 1997).

Surveillance for additional infected deer began in 1995 as an attempt to determine whether *M. bovis* infection was in fact a rarity in the Michigan free-ranging white-tailed deer population, or whether this species could actually have been maintaining the disease in the wild since the eradication program for cattle had been discontinued due to its postulated success decades earlier. The diagnostic procedures involved in surveillance primarily involve gross inspection of cranial lymph nodes followed by culture and microscopic evaluations of tissue determined as suspect of tuberculosis infection based on gross inspection at the AHDL. Parotid, submandibular, and medial retropharyngeal lymph nodes are those inspected in all cases. Each year, a variable number of carcasses and/ or additional organs (largely liver and lungs) are submitted along with the heads of the deer to the AHDL from hunters or MDNR staff. Typically, carcasses or tissues other than heads are submitted only if questionable lesions or discoloration are found present during field inspection.

The presentation of tuberculosis has varied in Michigan surveillance deer cases, as is typical of most cervid species (Rhyan and Saari 1995). Yet by far, the medial retropharyngeal lymph node has been the most common site in which lesions are found (O'Brien et al 2001; Fitzgerald et al 2000). Typically, the gross lesion in these deer is that of a unilaterally or bilaterally enlarged and abscessed retropharyngeal lymph node, featuring purulent exudate which varies in color and consistency from

thick and yellow-green to more fluctuant and tan. True caseation and mineralization are present less commonly (Figures 1.1-1.4). Histologically, these lesions often feature multinucleate giant cells and epithelioid macrophages surrounding variably-sized zones of caseous necrosis, with or without mineralization. A suppurative component to the lesions is common, with degenerate neutrophils accenting the more typical tuberculosis granuloma features mentioned. Acid-fast bacilli are present on histologic examination of tissues in nearly 90 percent of the positive cases (Fitzgerald et al 2000), although usually these are only found in low numbers.

The original year of surveillance indicated that deer were very much a potential reservoir for bovine tuberculosis, at least in the limited area of the state in which it had been performed (see Figure 1.5). Geographic expansion of surveillance has continued each year since 1995 from the original "DMU 452" zone in the lower peninsula to include surrounding counties, and eventually began to include representative samples of deer from every county in the state annually (Figures 1.6-1.12).

Prevalence of the disease in deer in Michigan has apparently decreased from its peak in 1997 over the past several years, and appears to be somewhat stable at the present time (Table 1.1). The dramatic decrease in prevalence indicated by the 1998 surveillance results is likely the goal result of management procedures instituted and enforced largely by the MDNR, including an increase in antlerless deer permit distribution and the placing of restrictions on supplemental feeding and baiting of deer. Both management tools appear to have contributed to decreased disease prevalence by helping to decrease overall deer numbers in the wild, with subsequent

decreases in contacts between infected and noninfected animals. The elimination of feeding and baiting in areas of highest prevalence has served to decrease incidences of deer concentrated deer around feed piles, an activity which allows for an increase in potential transmission of disease and which may lead to disease spread. Feeding restrictions also serve to decrease the overall population of deer in the state, as cases of starvation increase as supplemental feeding decreases, particularly during severe winters (Sikarskie et al 1999).

A trend towards decreased prevalence of tuberculosis in deer in Michigan should continue as population management programs continue to be enforced. Allowing deer numbers to match rather than exceed, or more ideally to be lower than, the biologic carrying capacity of this species in the state is the primary goal towards which these efforts are directed. Complete eradication may never be achieved realistically, but if the disease can be reduced to a prevalence in deer such that domestic species (particularly cattle) are no longer at significant risk, successful management may eventually be realized. Continued surveillance to document the disease prevalence in deer will be necessary at least until the time such a goal is met.

Tuberculosis in Other Michigan Wildlife Species

Non-cervid reservoir hosts for *M. bovis* tuberculosis are very important in maintaining this disease in the wild in other countries. Because species such as the badger, ferret, and brushtail possum are implicated as reservoir hosts in other countries (O' Reilly and Daborn 1995), examining the potential for similar species that are native to Michigan which may serve as reservoirs became an important objective of

surveillance very soon after the disease was proven to be well-established in the white-tailed deer population.

The search for additional potential reservoir hosts in Michigan began in 1996, when various species of wild omnivores and carnivores were added to the surveillance program, albeit on a much smaller scale than that of deer. Because these hosts may often be infected with mycobacteria without having any gross evidence of disease, surveillance in these species is relatively more involved than is that of deer. All animals submitted as part of the carnivore and omnivore project have a complete necropsy performed. Not only are those lymph nodes from cranial sites collected for histologic and culture evaluations, but also those nodes from the abdominal and thoracic cavities, as well as portions of any other organ with suspect lesions noted on gross examination of the carcass (Buddle et al 1999).

Each year, new species are added to the small mammal surveillance program. To date, fifteen potential hosts have been investigated (Table 1.2). Since submissions are primarily those found dead or trapped, the total surveyed vary significantly between species. All hosts investigated thus far appear more likely to be "spillover hosts" rather than true reservoirs. Lesions, when present, are primarily found in mesenteric lymph nodes in these animals. Such lesion distribution suggests ingestion of materials infected with *M. bovis* as the primary mode of transmission, and does not lead to further spread of disease by animals infected as such, as mesenteric node lesions would tend to contain bacilli within granulomas unless hematogenous spread to other viscera occurred. Very few cases of disseminated disease have been noted in any of

the species examined in this surveillance program. In contrast, disseminated disease is common in non-cervid reservoir hosts in other areas of the world (O'Reilly and Daborn 1995).

Free-ranging elk have been included in surveillance since 1996. Elk surveillance is essentially identical to that performed on white-tailed deer in that gross inspection of cranial lymph nodes is performed, followed by additional testing of suspect tissues only. The first positive elk case was identified in surveillance for the year 2000 (Table 1.3). Interestingly, this animal had a bilateral suppurative tonsilitis detected grossly. Tonsilar lesions have not been widely featured in positive white-tailed deer cases identified in Michigan to date. In elk, as in deer, the primary site for lesions of tuberculosis are reportedly the retropharyngeal, mesenteric, and mediastinal lymph nodes (Rohonsky et al 1996). This elk also did not have acid-fast organisms detected histologically. Apparently, this is often the case in elk, as the positive predictive value of necropsy or histologic results identifying a true culture positive case can range from 44 to 80 percent, depending which results are used, and depending on if these are used in combination either serially or in parallel (Rohonsky et al 1996). In deer, the positive predictive value for gross lesion identification alone has not been reported, yet that of histology performed on tissues found suspect on gross examination is much higher than that reported for elk (Fitzgerald et al 2000).

Objectives for Research

The primary goal of the research topics presented in this dissertation is to, in combination, provide new insight to some very basic questions that have to date remained unanswered regarding *M. bovis* tuberculosis in wildlife species, particularly those in Michigan.

Specifically, these objectives can be divided into three categories. The first of these is to address whether or not certain animals may be potential vectors of disease, serving as either capable transmission hosts or as reservoirs similar to white-tailed deer. Two hypotheses were generated from this objective. The first was that American crows and European starlings could become infected with *M. bovis* by oral and intraperitoneal inoculation with the organism. The second was that North American opossums could become infected with *M. bovis* by oral and intramuscular inoculation routes.

The second objective was to explore potential antibiotic resistance patterns in the endemic strain of *M. bovis* in Michigan wildlife. The hypothesis to be tested based on this objective was that wild animal strains of *M. bovis* from tuberculosis cases in Michigan would differ in their resistance patterns from human cases of *M. bovis* tuberculosis diagnosed in the state, and that these animal strains would not have acquired resistance to any of the drugs commonly used to treat *M. bovis* tuberculosis.

The third objective was to assess the reliability and accuracy of a novel, cytology-based diagnostic test for mycobacterial infection that may have applications in human

medicine. The hypothesis for this objective was that *M. bovis* organisms would be detectable in cells gathered from several sites (oral, nasal, and tonsilar mucosae) of infected white-tailed deer, when cytology preparations made using ThinPrep 2000 technology were examined using light microscopy

Although the presented objectives may appear to be separately quite broad in their scope, together they most definitely aim to expand the existing knowledge base of that which exists concerning the transmission and pathology of *M. bovis* tuberculosis in wildlife.

Figure 1.1: Tuberculosis in a white-tailed deer (*Odocoileus virginianus*). Gross photograph of lung, cut section. Numerous tan to yellow-green, purulent to fluctuant abscesses, ranging in size from approximately 3 mm to 3 cm diameter, distributed throughout the pulmonary parenchyma.

Figure 1.1. Tuberculosis in the lung of a Michigan white-tailed deer (*Odocoileus virginianus*).

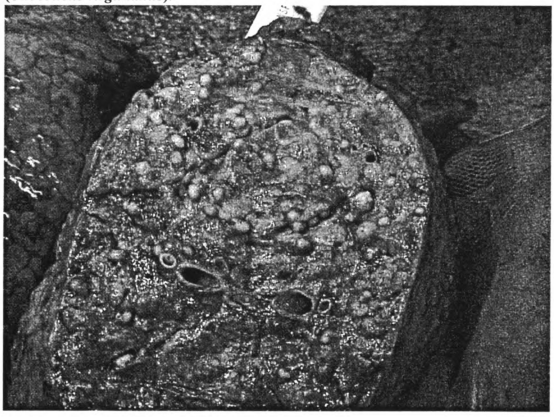


Figure 1.2: Tuberculous granuloma on the pleural surface of the lungs of a Michigan white-tailed deer. The nodule is singular, smooth, raised, and tan to white in color in this case. Similar nodules are often multiple and variably-sized, and distributed over both the parietal and visceral pleurae in more advanced cases of *M. bovis* tuberculosis involving the thoracic cavity in deer.

Figure 1.2. Tuberculous granuloma on the pleural surface of the lungs of a Michigan white-tailed deer.

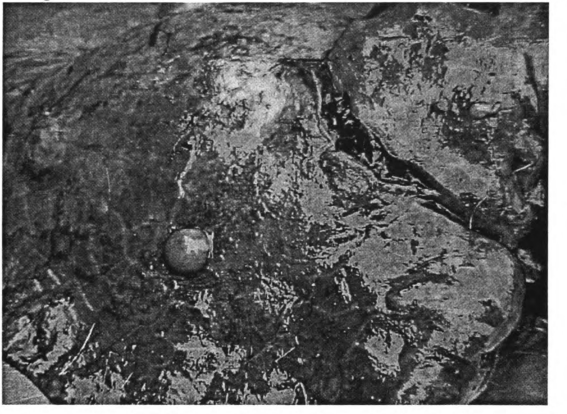


Figure 1.3: Purulent tuberculous abscesses in the lymph nodes of a Michigan white-tailed deer. Presentation is typical for M. bovis tuberculosis in cranial lymph nodes of white-tailed deer. Centrally, lesions contain vast amounts of thick, green-tan to yellow, purulent to viscous fluid, which may be contained by a fibrous capsule. Variable amounts of caseation or mineralization are often featured, either within or subtending such nodal parenchymal lesions.

Figure 1.4: Caseous tuberculous abscess in the lymph node of a Michigan white-tailed deer, cut section. Although such true caseogranulomas featuring thick, gritty, mineralized inflammatory debris encased within a well-defined fibrous connective tissue capsule are less commonly encountered as cranial lymph node lesions in white-tailed deer, they do still occur frequently, particularly in smaller lesions or in lesions affecting other tissues.

Figure 1.3. Purulent tuberculous abscesses in the lymph nodes of a Michigan white-tailed deer.

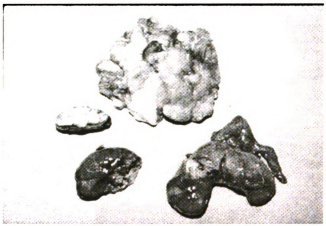
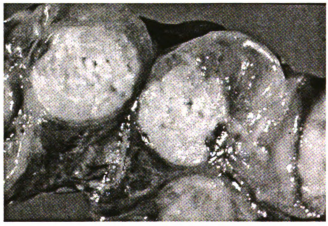


Figure 1.4. Caseous tuberculous abscess in a lymph node of a Michigan white-tailed deer, cut section.



Figures 1.5-1.12: Suveillance maps for *M. bovis* in Michigan's white-tailed deer population. Figure 1.5 shows the location of the initial tuberculous white-tailed deer (a 9 year old female) identified in 1975. Not until 1994 was another hunter-killed deer with tuberculosis found (Figure 1.6). Surveillance began in Deer Management Unit (DMU) 452 in 1995 (Figure 1.7). Each year, surveillance expanded. In 1996, intensive surveillance was performed in the 4 counties comprising DMU 452 and lesser numbers of deer were examined from other counties in the state (Figure 1.8). In 1997, surveillance was primarily in the 5-county area composed of the 4 counties surveyed in 1996 and that county outside of the original 4 in which a positive case had been found the year prior (Figure 1.9). Surveillance in 1998 included those 5 counties surveyed intensively in 1997 and a buffer zone extending to all sides of this block of counties in the Northeast portion of the Lower Peninsula (Figure 1.10). Statewide surveillance began in 1999 and continues to date, with larger numbers of deer taken each year from all counties, particularly in those from which positive cases are found for the first time (Figures 1.11 and 1.12). Images are presented in color.

Figure 1.5. Geographic location of initial *M. bovis* case in Michigan, 1975.

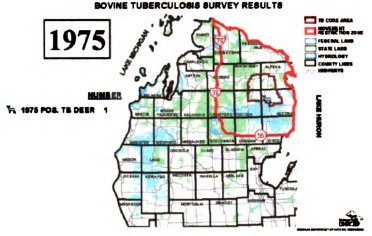


Figure 1.6. Geographic location of current propositus of endemic *M. bovis* in Michigan wildlife, identified in 1994.

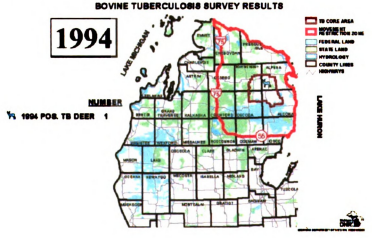


Figure 1.7. White-tailed deer *M. bovis* cases identified in first year of surveillance, 1995.

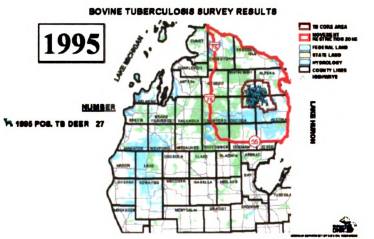


Figure 1.8. White-tailed deer *M. bovis* cases identified in 1996.

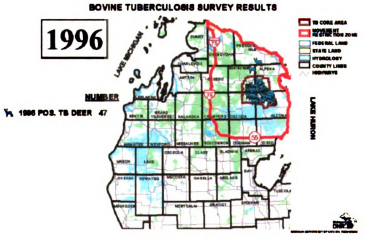


Figure 1.9. White-tailed deer *M. bovis* cases identified in 1997.

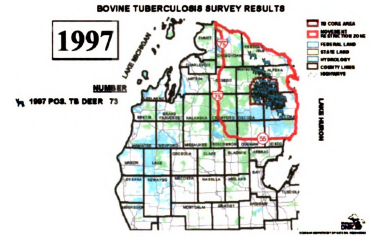


Figure 1.10. White-tailed deer *M. bovis* cases identified in 1998.

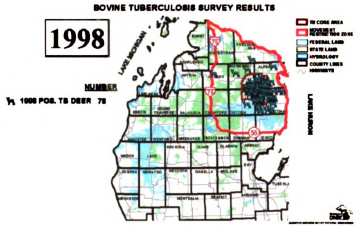


Figure 1.11. White-tailed deer *M. bovis* cases identified in 1999.

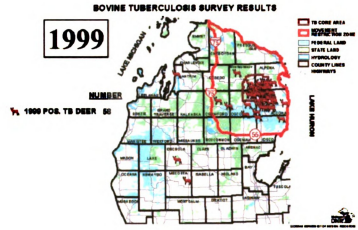
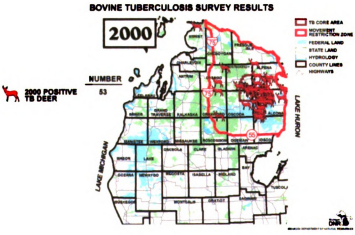


Figure 1.12. White-tailed deer *M. bovis* cases identified in 2000.



Figures 1.13-1.14: Surveillance data from first (Figure 1.13) and most recent (Figure 1.14) years of carnivore/ omnivore project, 1996 and 2000 respectively. The first *M. bovis*-positive animal identified in this project was a coyote found in DMU 452. An interesting trend to note is the close geographic proximity of these positive small mammal cases to the positive white-tailed deer cases. For the most part, positive carnivore/ omnivore cases have been found in areas in which the highest concentrations of positive deer cases exist. The presence of positive animals in "outlying" areas in which even small numbers of positive deer have also been found is intriguing as well. These geographic data seem to support the idea that transmission to these species is the result of contact with infected deer or deer carcasses (spillover from the deer reservoir of disease) rather than these species being true reservoir hosts themselves. Images are presented in color.

Figure 1.13. Geographic location of index case of *M. bovis* identified in carnivore surveillance, a coyote from DMU 452, 1996.

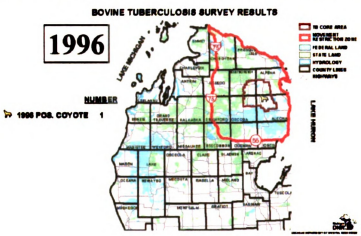


Figure 1.14. Carnivore/ omnivore surveillance data (positive *M. bovis* cases) from the most current year of surveillance, 2000.

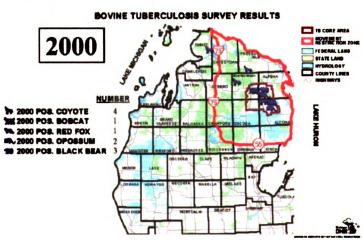


Table 1.1: Yearly prevalence rates for *M. bovis* tuberculosis in white-tailed deer in Michigan, 1995-2000.

[A] Number of deer cases included in surveillance for the year listed.

[B] Total number of deer found positive for *M. bovis* for the year listed.

[C] Number of positive cases divided by total number surveyed, multiplied by 100.

[D] Prevalence rate for *M. bovis* in originally surveyed DMU 452 area. Note this was the only area surveyed in 1995.

[E] Prevalence rate for *M. bovis* in the 5-county intensive surveillance zone.

Table 1.1. Yearly prevalence rates for *M. bovis* tuberculosis in deer in Michigan, 1995-2000.

YEAR	TOTAL NO.[A]	TOTAL POS.[B]	PREV. RATE[C]	CORE PREV.[D]	5-CO PREV.[E]
1995	814	27	3.32	3.32	NA
1996	3718	47	1.26	2.32	1.26
1997	3681	73	1.98	4.42	1.98
1998	9067	78	0.86	2.43	0.94
1999	19503	58	0.29	2.16	0.69
2000	25706	53	0.21	2.32	0.83

Table 1.2: Positive cases of *M. bovis* tuberculosis in Michigan carnivore/ omnivore surveillance through the year 2000.

[A] Number of this species surveyed through the end of the year 2000.

[B] Total number of culture-positives for *M. bovis* in this group.

[C] Number of positive cases in this species divided by total number surveyed, multiplied by 100.

Table 1.2. Positive cases of *M. bovis* tuberculosis in Michigan carnivore/ omnivore surveillance through the year 2000.

SPECIES	SCIENTIFIC NAME	TOTAL NUMBER[A]	TOTAL POSITIVE [B]	PREVALENCE RATE [C]
BADGER	*Taxidea taxus*	25	0	0.0
BEAR, BLACK	*Ursus americanus*	163	4	2.5
BOBCAT	*Felis rufus*	40	2	5.0
COYOTE	*Canis latrans*	249	11	4.4
FOX, GRAY	*Urocyon cinereoargenteus*	4	0	0.0
FOX, RED	*Vulpes vulpes*	16	2	12.5
MINK	*Mustela vison*	2	0	0.0
OPOSSUM	*Didelphis virginiana*	228	2	0.9
PORCUPINE	*Erethizon dorsatum*	1	0	0.0
RACCOON	*Procyon lotor*	194	2	1.0
SKUNK	*Mephistis mephistis*	12	0	0.0
WEASEL	*Mustela sp.*	1	0	0.0
TOTALS		**935**	**23**	**2.5**

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Chapter 2

Experimental Inoculation of European Starlings (*Sturnus vulgaris*) and American Crows (*Corvus brachyrhynchos)* with *Mycobacterium bovis*

Abstract

The purpose of this series of pilot studies was to determine whether the passerine species studied are susceptible to infection with *M. bovis*. Separate experiments were conducted on wild-caught starlings (*Sturnus vulgaris*) and American crows (*Corvus brachyrhynchos*). In each experiment, four birds were challenged intraperitoneally and four were challenged orally with microorganisms. Challenge dose was 1 x 10^5 colony forming units of *M. bovis* cultured from a white-tailed deer (*Odocoileus virginianus*) case in Michigan. Birds were sacrificed at one and two months post-inoculation. Histologic lesions suggestive of mycobacteriosis, without the presence of acid-fast bacilli, were noted in all experimental groups. Mycobacterial cultures performed on pooled tissue samples were positive for *M. bovis* only in some of the intraperitoneal inoculates of each species.

Introduction

Mycobacterium bovis is endemic in Michigan's free-ranging white-tailed deer (*Odocoileus virginianus*) population in the northeastern section of the Lower Peninsula of the state (Schmitt et al 1997). Surveillance to assess the spread of this disease within the deer population and into additional species has been ongoing since 1995. Through this surveillance, *M. bovis* has been detected in several carnivore and omnivore spillover host species. Avian species have not been included in the surveillance program and as such, have not been assessed as potential reservoirs of *M.*

bovis. The foundation for the described research involves the ideas that the passerine species used in the project are known to gather in agricultural settings in Michigan and also commonly feed on organic materials (feed, fecal matter, viscera) that may harbor *M. bovis*. Exposure of starlings or crows in such a manner would allow for considerable widespread transmission, if the birds can, in fact, become infected with or carry *M. bovis* agent and shed microorganisms in distant sites. No published reports of *M. bovis* infections in passerines exist to our knowledge, although some citations do report occurrence(s) in psittacine species (Gerlach 1994; Ackerman et al 1974). The closely related organism, *Mycobacterium tuberculosis*, is well-documented as being infectious in psittacines (Hoop et al 1996; Gerlach 1994; Ackerman et al 1974). Yet very little is known about the infectivity of *M. tuberculosis* complex microorganisms in other avian orders/ species. The objective of this study was to investigate the potential for native Michigan passerine species to become infected with *M. bovis* when experimentally exposed orally or intraperitoneally.

Materials and Methods

Birds

A total of eleven starlings were caught in mist nets placed strategically in doorways of dairy barns on the campus of Michigan State University. Three of the eleven birds were used as controls. These birds were caught and sacrificed prior to the capture of the eight birds to be inoculated with *M. bovis*. Twelve crows were caught in one capture with a rocket net after baiting birds to a specific site on the mink farm

of Michigan State University for several weeks prior (Federal Fish and Wildlife Permit number MB000836-0; State Scientific Collector's Permit for Michigan number SC1057). Control and inoculated birds were housed in separate wards. All inoculated birds were housed in biosafety cabinets in hepa-filtered rooms (biolevel 3 conditions) at the University Laboratory Animal Research Facility.

Inoculum

M. bovis culture isolates from positive white-tailed deer cases resulting from surveillance activities in Michigan are maintained by the tuberculosis laboratory at the Michigan Department of Community Health (MDCH). Inocula for this study were prepared using a typical strain common to all cases. Seven day growth in Middlebrook 7H9 broth was adjusted to a 0.5 McFarland turbidity standard, diluted 1:100 with sterile water and tested by plate counts to determine the CFU's (colony forming units) per unit of volume. Single dose, 1 ml, aliquots containing approximately 1 X 10^5 CFU/ml were used. The dose CFU/ml was confirmed again at the time that the birds were inoculated. Single dose oral inoculations were administered to birds via a tomcat catheter gavage. Intraperitoneally (IP) inoculated birds were injected using 1cc tuberculin syringes.

Experimental Design

Starling experiment

Prior to inoculation of experimental birds, three control birds were caught and euthanatized for necropsy. Euthanasia of all birds in these studies was performed via intracardiac injection of sodium pentobarbital solution (Fatal Plus®,Vortech

Pharmaceuticals, Dearborn, MI). Data recorded at necropsy included body weight of each bird, individual organ weights (spleen, liver and lung), and gross lesions where present. Sections of lung, liver, kidney, and spleen were collected and pooled for mycobacterial isolation and identification at necropsy. Sections of brain, lung, heart, kidney, liver, spleen, pancreas, small and large intestines, and ceca were collected at necropsy and fixed in 10% formalin solution for histologic examination. Tissues were stained with both routine hematoxylin and eosin (H & E) and either Ziehl-Neelsen or peanut oil acid-fast stains prior to histologic examination. Eight birds were randomly divided into equal groups of four orally inoculated and four IP inoculated animals. Fecal samples were collected from birds designated as those to be inoculated on the day prior to inoculation, these samples were submitted for mycobacterial isolation and identification. Inoculated birds were weighed on the day of inoculation and every two weeks thereafter throughout the length of the experiment. Fecal samples of all birds were collected twenty-four hours post inoculation (PI) and submitted for mycobacterial isolation and identification. Birds were fed a diet consisting of ad libitum access to pelleted zoo fowl maintenance diet 9037 (Master Mix Feeds, Fort Wayne, IN) which was supplemented with canned lamb and rice-based dog food (Iams Co., Dayton, OH) once weekly. Birds were monitored for inappetence and for inadequate fluid intake throughout the study. The clinical condition of each bird was recorded daily as well. Half of each inoculation group (two birds from each group) were euthanatized and necropsied one month after inoculation, with tissue processing

performed as described for control birds. The remaining birds were euthanatized and necropsied at two months PI.

Crow experiment

The study design for the crow experiment was similar to that described for the starlings, save for the inclusion of four control birds which were maintained as an experimental group throughout the length of the experiment. No control birds were sacrificed prior to inoculation of experimental birds. The four control crows were housed separately from the eight inoculated crows, which were kept in biosafety level 3 housing identical to that of the starlings. All birds received ad libitum access to the same pelleted diet as the starlings. Crows were supplemented twice weekly with canned rabbit and rice canine diet (Nature's Recipe Foods, Corona, CA). Fecal collections and recordings of body weights were carried out as described for the starlings. Necropsy dates were also at one and two months PI, with half of each group (two birds from each: control, oral, and IP) randomly chosen and euthanatized. Necropsy tissue collections for mycobacterial culture were identical to those of the starlings. Histopathologic analyses of those tissues collected from starlings as well as of proventriculus and ventriculus were performed on crow tissues. Both H & E and Ziehl-Neelsen acid-fast stained sections of each tissue were examined.

Mycobacterial Isolation and Identification

Mycobacterial cultures were performed at the MDCH, Tuberculosis Laboratory. Tissue specimens were ground, digested, and concentrated in a manner previously described (Kent and Kubica 1985). Fecal samples were cultured directly after

digestion and concentration. One each of a Lowenstein-Jensen medium slant (Becton-Dickinson, Cockeysville, MD), a Middlebrook 7H11S medium slant (Becton-Dickinson, Cockeysville, MD), and a Bactec12B broth vial (Becton-Dickinson, Sparks, MD), were inoculated with the resultant material. Media were examined for growth on a weekly basis for eight weeks. Bacterial growth, determined to be acid-fast by slide examination (Kent and Kubica 1985), were tested by genetic probe (Accuprobes, Gen-Probe, San Diego, CA) to determine whether the bacteria were members of the *M. tuberculosis* complex (Reisner et al 1994). Complete species identification was performed using biochemical testing and high performance liquid chromatography to differentiate *M. bovis* from the other members of the *M. tuberculosis* complex and to speciate other mycobacteria (Nolte and Metchock 1995; Butler et al 1991; Kent and Kubica 1985).

Statistical Analysis

SAS Version 8 statistical software was used for all calculations (Statistical Analysis Systems, SAS Institute, Inc., Cary, NC). The Wilcoxon Rank-Sum test was used to assess whether there were statistically significant ($p < 0.05$) changes in overall body weight and in individual organ weights within each treatment group and between groups. Associations between routes of exposure and the outcomes of interest (gross and histologic lesions and mycobacterial culture results) were assessed using the two-tailed Fisher's Exact test, and associations were expressed as odds ratios with 95% confidence intervals (CI).

Results

Clinical Condition of Birds

All birds remained clinically healthy throughout the trials. No depressions in food or water consumption were noted. No mortality prior to assigned sacrifice dates occurred.

Weight Change Data

No treatment group had significant changes in weight, though weight changes in orally inoculated starlings approached significance ($X^2 = 9.33$, $p = .0533$).

In crows, there were statistically significant differences in kidney weight, when comparing intraperitoneal exposure (mean = 3.2 g) to controls (mean = 4.05 g; Wilcoxon Rank-Sum $X^2 = 4.08$, $p = 0.0433$), and in spleen weights when comparing oral exposure (mean = 0.75 g) to controls (mean = 0.43 g; Wilcoxon Rank-Sum $X^2 = 5.4$, $p = 0.0202$). There was also a statistically significant difference in lung weights when comparing intraperitoneal exposure (mean = 7.85 g) with oral exposure (mean = 5.63 g; Wilcoxon Rank-Sum $X^2 = 4.08$, $p = 0.0433$). There were no statistically significant differences in organ weights for starlings.

The small numbers of subjects included in each experimental group may have influenced the power of statistical analyses, so general trends in body weight were also recorded. Changes in overall body weights are illustrated in Figure 2.1 (starlings) and Figure 2.2 (crows). In both experiments, both IP inoculated groups (one and two month sacrifice dates) showed reductions in mean group body weights over time. The

trend toward weight loss in IP inoculated birds was more steady in crows (Figure 2.2) than it was in starlings (Figure 2.1). Weight gain of all oral inoculated birds and control birds was the trend noted in the starlings (Figure 2.1). In crows, overall weight gain was also noted in all groups save for those orally inoculated birds sacrificed at one month (Figure 2.2). In these crows, mean body weight of the group increased during the first two weeks of the experiment, but dropped slightly from two to four weeks. Final mean weight of the group was still higher at the sacrifice date than at the inoculation date, however. A similar trend of gain until two weeks followed by slight loss until six weeks was noted in the two month sacrifice date, orally inoculated crows. These birds gained weight during the last two weeks of the experiment, however, and were greater in weight at the termination of the study than they were at inoculation.

Gross Necropsy Findings

Gross lesions suggestive of mycobacteriosis were not present in any starlings. One IP inoculated starling sacrificed at one month PI had a single, 0.5 cm diameter, green to brown, friable nodule present in the mesentery subtending the proventriculus. Dissemination of granulomas was not noted. The other IP inoculated starling sacrificed at one month had grossly detectable splenomegaly. Hepatomegaly and splenomegaly were found at necropsy in one IP inoculated bird sacrificed at two months. The other IP inoculated starling sacrificed at this time had only hepatomegaly.

No gross lesions suggesting disseminated mycobacteriosis were present in any crows. One IP inoculated crow sacrificed at one month PI had a prominent keel bone

with breast muscle atrophy and an enlarged spleen. The other IP inoculated crow sacrificed at this time had fibrinous adhesions attaching the gall bladder to the small intestine. Splenomegaly was noted in one orally inoculated bird sacrificed at two months. One IP inoculated crow sacrificed on this date had a prominent keel bone and breast muscle atrophy.

Microscopic Lesions

All tissues examined histologically from all birds were scored as either having lesions suggestive of mycobacteriosis or not having lesions consistent with a diagnosis of mycobacteriosis. Presence of bacilli on acid-fast stained tissue sections was also recorded as positive or negative. These results are compiled for the starlings in Table 2.1 and for the crows in Table 2.2. Lesions considered histologically consistent with mycobacterial infection included histocytic granulomas, with or without giant cells, featuring central caseation but not associated with central mineralization and either containing small numbers of heterophils or no heterophils. Often such granulomas featured mild fibroplasia, which is also typical of tuberculous lesions in birds. Primarily heterophilic granulomas were regarded as negative lesions, as were granulomas with or without giant cells that featured central mineralization (Figures 2.3 and 2.4). In starlings (Table 2.1), primarily heterophilic lesions were present in the liver or pancreas of three IP inoculated birds and in one control bird. No acid-fast bacilli were histologically noted in any sections of tissues from these birds, however. Additional non-specific lesions noted in starlings included reactive, hyperplastic bronchial- associated lymphoid tissues (BALT); hepatic granulomas with moderate to

large numbers of associated heterophils (in three orally inoculated birds; Figure 2.3); foci of primarily heterophilic hepatic necrosis with small numbers of associated mononuclear inflammatory cells; and multifocal granulomatous hepatitis with giant cells and central mineralization of necrotic hepatocytes, associated with histologically detectable portions of a necrotic cestode parasite larva (Figure 2.4). The liver of this same bird was marked by a mild, diffuse, vacuolar hepatopathy consistent with fatty degeneration. One IP inoculated starling also had a moderate, multifocal, nonsuppurative interstitial nephritis noted histologically.

In crows, three inoculated birds (one oral and two IP), and one control had lesions that could be considered histologically consistent with mycobacterial infection (Table 2.2). Small numbers (one to two bacteria) of acid-fast bacilli were noted within cytoplasms of cells forming one granuloma (Figure 2.5), in one specially-stained section of liver harvested from an IP inoculated crow. Lesions additionally noted that were not specific to the diagnosis included hyperplasia of gut associated lymphoid tissues (in orally and IP inoculated crows); splenic follicular reactivity with reticuloendothelial cell hyperplasia (in birds from all groups); multifocal heterophilic hepatic necrosis; and multifocal, primarily nonsuppurative hepatitis (also found in all groups). Granulomas consisting of giant cells with or without central mineralization, associated with fragments of degenerate cestode parasite larvae were present in sections of liver, kidney, and pancreas from several birds. One IP inoculated crow had adult cestodes noted within the lumen of sections of small intestine noted histologically. One IP inoculated crow had a mild, multifocal, heterophilic to

nonsuppurative interstitial nephritis. A mild, focal nonsuppurative myocarditis was present in one orally inoculated crow and a moderate, multifocal, necrotizing myocarditis and myocardial vasculitis was present in one IP inoculated crow.

Mycobacterial Isolation and Identification

All birds were scored as positive or negative for the presence of *M. bovis* in fecal and tissue cultures (Tables 2.1 and 2.2). *Mycobacterium bovis* was not recovered from the fecal samples of any birds, from either pre- or post inoculation sampling. *Mycobacterium gordonae* was cultured from the pooled tissue culture of a single control starling. Three IP inoculated starlings cultured positive for *M. bovis*, two after one month and one after two months. No further starlings cultured positive for any mycobacteria (Table 2.1). One IP inoculated crow cultured positive for *M. bovis* after one month, and one after two months. No further crows cultured positive for any mycobacteria.

Statistical Analysis

Compared to control birds, birds with intraperitoneal exposure were significantly more likely to have positive mycobacterial culture (Fisher's Exact p =0 .0256; odds ratio = 3.46, 95% CI = 1.25 - 9.55) and gross or histologic lesions (Fisher's Exact p = 0.0256, odds ratio = 3.49, 95% CI = 1.29 - 9.45). No significant effects were seen comparing controls to orally exposed birds. When comparing oral exposure to intraperitoneal exposure, birds with intraperitoneal exposure were significantly more likely to have a positive mycobacterial culture (Fisher's Exact p = 0.0256; odds ratio = 13.61, 95% CI = 1.19 - 155.9).

Discussion

In both bird species studied, *M. bovis* infection was apparently capable of being established via IP inoculation. In three of four starlings and in two of four crows inoculated by this route, infection was supported by microbiologic isolation of the organism. All birds that cultured positive for *M. bovis* had histologic lesions suggestive of mycobacterial infection. The experimental group that had the largest number of birds with histologic lesions and positive cultures in each species, the IP group, was also that which exhibited the most regular trend in weight loss. Yet in only one crow were all criteria typically considered as suggestive of positive infection (positive culture, gross lesions, histologic lesions, and acid-fast bacilli detection histologically) noted. No further inoculates had gross lesions suggesting mycobacterial infection. This bird had a mesenteric granuloma, which may or may not have been related to intraperitoneal infection. No acid-fast bacilli could be detected within this lesion histologically, yet histologic features were consistent with a mycobacterial, centrally caseous, histiocytic granuloma. If this lesion was exclusively a foreign-body type injection reaction, a more heterophilic granuloma would be expected (Barnes 1996; Montali 1988). Late in their development, both types of these granulomas may appear quite similar histologically, so the true nature of its development cannot be determined. Some birds did have nonspecific changes such as liver and spleen enlargement noted grossly, yet neither of these changes are restricted to being typical of mycobacterial infections alone. No birds other than this single

inoculated crow had histologically detectable acid-fast organisms in any sections of specially-stained tissues.

Organ weight analyses did have significant results in some groups. Yet differences in individual animal weights may have contributed to these data. No clear explanation exists as why IP inoculated crows had smaller kidney weights. No gross or histologic basis for this finding was present. Lymphoid infiltrates in the spleens of inoculated animals were present histologically in some cases, possibly explaining the relative heaviness of these organs in some groups in comparison to controls. Yet no clear reason exists that explains why orally inoculated birds had significant differences in weights of these organs compared to controls while IP inoculates did not. Lymphoid (BALT) hyperplasia noted more frequently in the lungs of IP inoculated crows could reasonably explain heavier lungs in these animals in comparison to orally inoculated crows.

Some birds that did not culture positive for *M. bovis* had histologic lesions suggestive of mycobacterial disease. In one control starling, this result appears to be the result of infection by *M. gordonae*, which was cultured from pooled tissues. In one control crow, these lesions are most likely in response to the same cestode parasite larvae which were identified in tissues of other crows, although actual parasite remnants no longer remained in the sections of tissues harvested for histology. Clear speciation of these parasites was not achieved, but literature reviews suggest that *Plagiorhynchus cylindraceus* is a likely agent in these birds, particularly in starlings (Moore and Bell 1969). The similarity between mycobacterial-type granulomas and

parasite-induced granulomas in birds can be striking. Central mineralization is much more suggestive of parasite involvement (in lieu of, or in addition to, finding actual parasites in section) than are histiocytic granulomas alone (Barnes 1996; Montali 1988). Another reasonable explanation for such lesions could be that other bacteria not microbiologically detected (only cultures for mycobacteria were requested, due to limited project funding) were involved in lesion formation. Additional bacteria that may produce granulomas similar to those noted in mycobacterial infections include salmonellae, enterococci, and *E. coli*, amongst others (Goodwin 1996). Unidentified bacterial infections were likely responsible for most of the primarily heterophilic lesions detected and reported in several birds of both groups as well. Histologic changes such as lymphoid tissue hyperplasia (in the spleen, gastrointestinal tract, and gut) were noted in many birds. Such changes suggest nonspecific immune stimulation, which could be attributed to any number of infectious agents, systemic or otherwise, but are certainly not specific to mycobacteriosis. Failure of the systems used to detect mycobacteria may have been involved in some instances in which inoculated birds with lesions could not be definitively diagnosed with *M. bovis* infection. Even the "gold standard" of mycobacterial isolation and identification will not detect all positive infections (Fitzgerald et al 2000).

One IP inoculated starling cultured positive, but did not have any histologic lesions suggestive of mycobacteriosis. Since this bird was sacrificed at one month PI, lesion formation may not have progressed to those histologically detectable in randomly harvested sections of tissue. Immune status plays an important role in the

progression of intracellular bacterial infections in birds, with lymphoid depletion in important organs such as the spleen being part of the pathophysiology (Gerlach 1996). This animal may have had a stronger overall immune status than others that had histologically detectable lesions in combination with being culture positive at the same sacrifice date. Also possible is that a small number of non-grossly detectable lesions had developed in one or more tissue sections not harvested for histologic examination, but instead submitted for mycobacterial isolation, in this bird's case.

In both crows and starlings, oral gavage with mycobacteria did not result in mycobacterial infection that could be discerned grossly, histologically or by pooled tissue cultures run on specimens collected from these animals. Body weight trends throughout the experiment in both orally inoculated crows and starlings suggest that overall weight loss was not featured as a result of such inoculation, unlike those results seen in IP inoculated birds. However, this observation could not be confirmed statistically. Fecal cultures run immediately PI suggest that even large doses of mycobacterial organisms are inactivated in the gastrointestinal tracts of the species studied, as no shedding could be detected by these cultures. Serial fecal culture results obtained throughout the experiment might have allowed this to be a more definitive conclusion, but the combination of negative fecal and tissue cultures obtained in all orally inoculated birds is supportive of resistance to *M. bovis* infection when organisms are introduced by this route in both of these species.

Orally inoculated birds appeared resistant to *M. bovis* infection, yet IP inoculated birds did develop lesions suggestive of mycobacterial infection in

statistically significant numbers. Perhaps even more importantly, many of these animals actually cultured positive for the organism to explain the true nature of these lesions, giving proof that this organism can indeed survive within the realms of avian physiology when inoculation is used to bypass natural protective mechanisms. Oral exposure in the wild is undoubtedly a more likely route by which these birds could be infected than is the IP route. But survival of *M. bovis* intraperitoneally in these species may warrant additional investigation into other potential exposure routes that might commonly occur in the wild, namely inhalation of infectious mycobacterial droplet nuclei.

Overall, these experiments demonstrate the remarkable resistance that several avian species have to infection with *M. bovis*. Both studied species are quite susceptible to avian tuberculosis and are implicated as potential sources of this disease in both wild and domestic avian and mammalian populations (Bickford et al 1966, Mitchell and Cuthrie 1950). Yet these passerines may be less susceptible to all of the genetically related *M. tuberculosis* complex organisms (*M. tuberculosis, M. bovis, M. microti*, and *M. africanum*) than are psittacines, as several case reports of *M. tuberculosis* in psittacines exist, yet none have been noted in passerines (Washko et al 1998; Hoop et al 1996; Gerlach 1994; Ackerman 1974). Alternatively, all bird species may be relatively resistant to infection by *M. bovis*, as is the commonly held belief. It is generally accepted that the higher core body temperatures of birds may prevent active infection with *M. tuberculosis* complex organisms in these animals. No convincing reports of infection with *M. bovis* in any bird species exist. Fecal shedding

appears to be quite uncommon in these birds after oral gavage with a large number of microorganisms, a dose of which they would be very unlikely to encounter in nature. It is therefore a reasonable conclusion that these animals do not play a large role, if they play any at all, in the dissemination of bovine tuberculosis within and between Michigan wildlife and domestic livestock populations. Fecal shedding did not occur in IP inoculated birds either, suggesting that even if a bird could somehow become intraperitoneally "inoculated" with infected materials in the wild, that spreading disease in such a manner is not of major concern. Further exposure routes such as inhalation that may lead to droplet spread after infection by these birds would be reasonable exceptions to this statement, as they have not yet been studied.

Also of consideration in this particular set of experiments was the timing between inoculation and sacrifice. Because the development of tuberculosis in most of the species it affects is often realized in its most chronic stages, longer time periods between inoculation and sacrifice of birds may still prove to be worthy of investigation. Funding was unfortunately available neither for a more chronic study nor for serial culture work in this experimental series. Multiple fecal cultures of living birds throughout the experiment may have given a better assessment of potential intermittent fecal shedding of organisms that may have gone unnoticed in the more restricted culture work included in the described protocol.

Table 2.1: Laboratory findings in starlings. [A] Number of birds tested/ number of birds positive for feature. Mycobacterial culture positives indicate *M. bovis* cultured from pooled tissue samples.

Table 2.2: Laboratory findings in crows. [A] Number of birds tested/ number of birds positive for feature. Mycobacterial culture positives indicate *M. bovis* cultured from pooled tissue samples.

Table 2.1. Laboratory data for starlings

Experimental Group	Group Size	Mycobacterial Culture Positive Result [A]	Histologic Lesions [A]	AFB Noted On Histology [A]
Control	3	0/ 3	1/ 3	0/ 3
Oral	4	0/ 4	0/ 4	0/ 4
IP	4	3/ 4	2/ 4	0/ 4

Table 2.2. Laboratory data for crows

Experimental Group	Group Size	Mycobacterial Culture Positive Result [A]	Histologic Lesions [A]	AFB Noted On Histology [A]
Control	4	0/ 4	1/ 4	0/ 4
Oral	4	0/ 4	1/ 4	0/ 4
IP	4	2/ 4	3/ 4	1/ 4

Figure 2.1: Weight data for starlings. X axis: weeks post inoculation that weight was recorded. Y axis: MEAN body weight for entire group in grams (indicated in legend) at given date.

Figure 2.2: Weight data for crows. X axis: weeks post inoculation that weight was recorded. Y axis: MEAN body weight for entire group in grams (indicated in legend) at given date.

Figure 2.1 Weight data for starlings

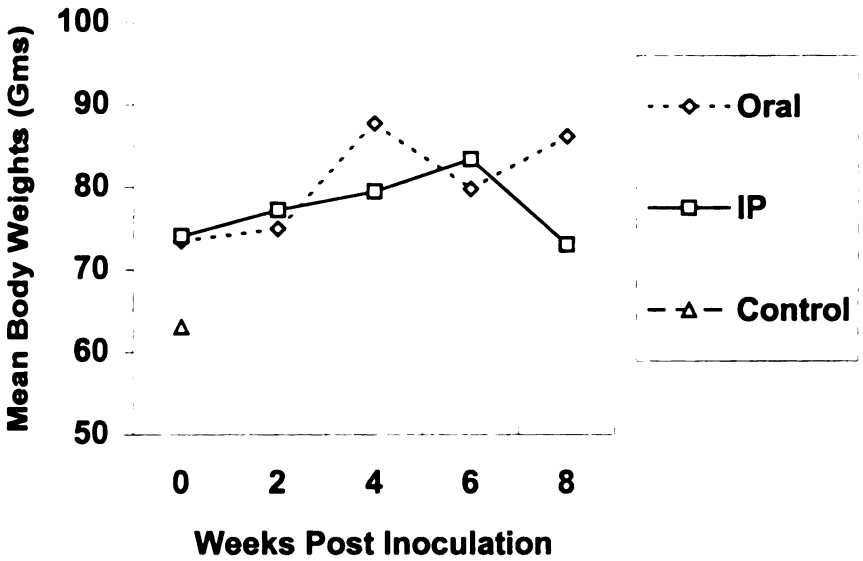


Figure 2.2. Weight data for crows

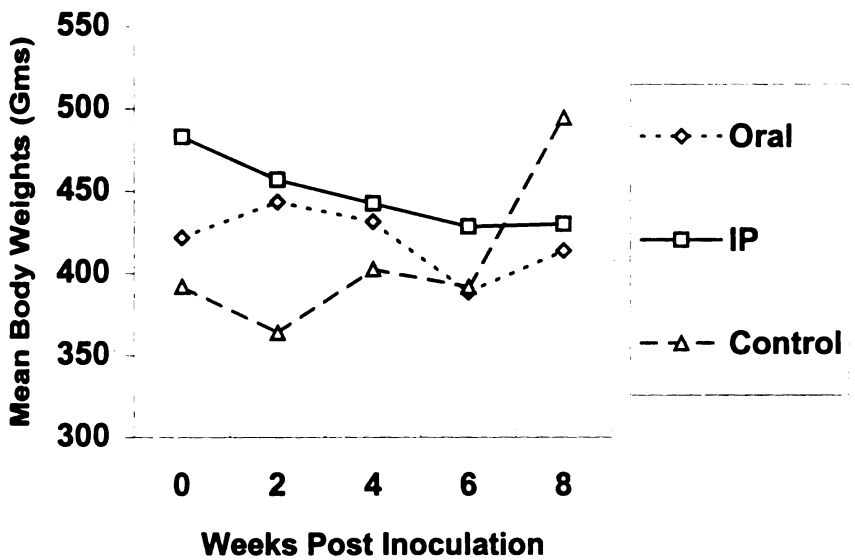


Figure 2.3: Photomicrograph of liver from a starling orally inoculated with *M. bovis*. Heterophilic hepatic granuloma not consistent with mycobacterial infection. Hematoxtlin & Eosin (H & E); 40X.

Figure 2.4: Photomicrograph of liver from a starling orally inoculated with *M. bovis*. Histiocytic hepatic granulomas with central mineralization (white arrows) and central necrosis associated with cestode larvae (black arrows); diffuse microvesicular hepatopathy consistent with hepatic lipidosis. H & E; 20X.

Figure 2.5: Photomicrograph of liver from a crow intraperitoneally inoculated with *M. bovis*. Histiocytic hepatic granuloma with giant cells associated with mild nonsuppurative inflammation and fibroplasia. H & E; 40X.

Figure 2.3

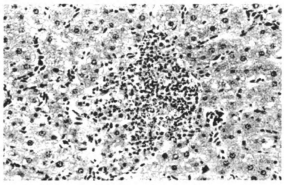


Figure 2.4

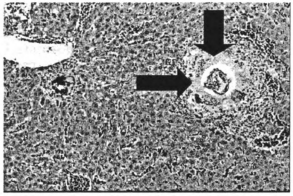
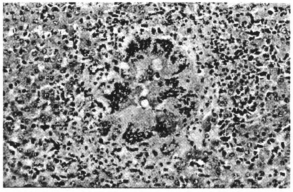


Figure 2.5



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Chapter 3

Experimental Inoculation of North American Opossums (*Didelphys virginiana*) With *Mycobacterium bovis*

Abstract

In order to investigate their potential as reservoir hosts for bovine tuberculosis in Michigan, eight North American opossums (*Didelphis virginiana*) were inoculated with 1 X 105 CFU (colony forming units) of *Mycobacterium bovis*. Half of the animals (four) received this dosage orally, and half were inoculated intramuscularly (IM). In each group, two animals were euthanatized at one month post inoculation (PI) and two at two months PI. Four control animals were housed separately and sacrificed in the same manner as those inoculated. One of the four orally inoculated opossums, and three of the four intramuscularly inoculated opossums were positive for *M. bovis* on tissue specimen cultures obtained at necropsy. The oral recipient had positive tissue cultures on both intestine and pooled lymphoid samples. All IM-inoculated animals that had positive tissue cultures had these positive results from pooled lymphoid samples. Two of the three tissue culture-positive IM recipients also had positive liver and lung samples. One animal with gross and histologic lesions compatible with tuberculosis did not have any positive tissue cultures.

Introduction

In Michigan, surveillance for *Mycobacterium bovis* tuberculosis in wild and captive cervids has been ongoing since 1995 (Schmitt et al 1997). As an attempt to identify spillover hosts in the wild, and also to search for potential reservoir hosts for this disease other than white-tailed deer (*Odocoileus virginianus*), additional carnivore and omnivore wildlife species have been added to the surveillance program over the

past six years (Bruning-Fann et al 2001). No additional reservoir hosts have been conclusively defined, yet positive cultures representing spillover infections have been identified in black bears (*Ursus americanus*), bobcats (*Felis rufus*), coyotes (*Canis latrans*), raccoons (*Procyon lotor*), red foxes (*Vulpes vulpes*), and North American opossums (*Didelphis virginiana*) via surveillance through the end of the year 2000. For the first several seasons of carnivore/ omnivore surveillance, no opossums were identified as positive, although they, along with coyotes, represented the largest in numbers of cases submitted (over one hundred each at that point). Although North American opossums are only distantly related to the New Zealand brushtail possum (*Trichosurus vulpecula*), the possibility of their resistance to *M. bovis* tuberculosis in comparison to such a susceptible reservoir host species was speculated upon. In order to determine whether North American opossums were uniquely resistant to *M. bovis*, a pilot study was designed to challenge these animals with the bovine tuberculosis agent by either the oral or intramuscular routes.

Materials and Methods

Opossums

The total number of animals to be used for the study was, based on available funding and biolevel-3 laboratory space restrictions, limited to twelve. Initially, five adult North American opossums were humanely live-trapped in Ingham County, Michigan. (State Scientific Collector's Permit for Michigan number SC1057). Upon collection, one of these adults was noted to be a late-term pregnant female. This

female was observed for several days when she was noted to have given birth to seven offspring. These young were captive-raised, weaned as juveniles, and included as subjects in this study, necessitating fewer live wild captures. Juveniles and adults (seven females and five males) were acclimated to the diet to be used throughout the experiment. During this diet acclimation period, each animal received 100 mg/kg fenbendazole (Panacur®, Hoechst-Roussel Agri-Vet Co., Somerville, NJ) orally, once daily, for three consecutive days. Control and inoculated opossums were moved to separate wards one week prior to inoculation to allow acclimation to the new laboratory. One adult male became suddenly and severely clinically depressed and anorectic one week after intramuscular inoculation and was found dead the next morning. This animal was replaced with a new, wild-caught juvenile male.

Inoculum

M. bovis culture isolates from positive white-tailed deer cases resulting from surveillance activities in Michigan are maintained by the Tuberculosis Laboratory at the Michigan Department of Community Health (MDCH, Lansing, MI). Inocula for this study were prepared using a typical strain (evaluated by the restriction fragment length polymorphism analyses, RFLP) common to all cases. Seven-day growth in Middlebrook 7H9 broth was adjusted to a 0.5 McFarland turbidity standard, diluted 1:100 with sterile water and tested by plate counts to determine the CFU's (colony forming units) per unit of volume. Single dose, 1 ml, aliquots containing approximately 1 X 10^5 CFU/ml were used for intramuscular (IM) recipients and 0.2 ml containing the same CFU were used for oral recipients. A more concentrated solution

was used for these oral recipients to assure complete delivery of the entire inoculum dosage. The dosage CFU/ml was confirmed again at the time that the opossums were inoculated. Single dose oral inoculations were administered to opossums mixed in a teaspoon of strawberry preserves, served on a one inch square of bread. Animals were monitored to assure the complete inoculation dosage was consumed. Intramuscular (IM) recipients were injected in the musculature of the left hind limb using 1ml tuberculin syringes.

Experimental Design

Twelve opossums were randomly divided into groups of four receiving oral inoculation, four receiving IM inoculation, and four controls. All inoculated opossums were housed in separate Horsfal units in hepa-filtered rooms (biolevel 3 conditions) at the Michigan State University Containment Facility until the date of sacrifice. Control opossums were housed in standard rabbit cages in a separate ward in the Facility. Animals received a diet of approximately 4 ounces of Hill's Science Diet Canine Maintenance Dry daily and approximately 2 ounces of Hill's Canine p/d Canned every other day (Hill's Pet Nutrition, Inc., Topeka, KS). This primary diet was supplemented with fresh fruit and strawberry preserves weekly prior to inoculation, and weekly with fruit alone post inoculation (PI). The clinical condition, food consumption, and water consumption of each opossum were recorded daily. Save for the single aforementioned adult male, no opossums showed signs of debilitation prior to sacrifice.

Animals were weighed on the day prior to inoculation and every two weeks thereafter throughout the experiment. Fecal samples for mycobacterial culture were collected from all animals on the day prior to inoculation and again at 24 hours post inoculation in order to assess shedding of microorganisms. Fecal samples were collected for mycobacterial culture from all animals again at 31 days PI. Half of the animals in each experimental group were euthanatized at thirty-one days PI and the remaining animals were sacrificed at 61 days PI. Euthanasia was performed via intracardiac injection of sodium pentobarbital solution at a dosage of 120 mg/kg (Fatal Plus®,Vortech Pharmaceuticals, Dearborn, MI) after initial sedation was achieved using 5 mg/kg xylazine (Rompun®, Miles, Inc., Shawnee, KS) injected intramuscularly. Fecal samples for mycobacterial culture were submitted at the same time as tissue samples collected from those animals sacrificed on that day. Fecal cultures were performed for all remaining opossums sacrificed at 61 days PI, also with pooled tissue cultures.

Data recorded at necropsy included total body weight of each animal, individual organ weights (spleen, liver, kidneys and lung), and gross lesions where present. Tissues harvested and submitted for mycobacterial isolation and identification included pooled liver and kidney, pooled small and large intestine, pooled spleen and lymph nodes, and lung. Sections of brain, heart, lung, tracheobronchial lymph node, stomach, small intestine, pancreas, large intestine, mesenteric lymph node, liver, kidney, adrenal gland, spleen, urinary bladder, teste or ovary, skin, hindlimb musculature, and an entire eyeball were collected from each

animal at necropsy and fixed in 10% neutral buffered formalin solution for histologic processing and examination. Tissues were stained with both routine hematoxylin and eosin and Ziehl-Neelsen acid-fast stains prior to histologic examination (Prophet et al 1992).

Mycobacterial Isolation and Identification

Mycobacterial cultures were performed at the MDCH Tuberculosis Laboratory. Tissue specimens were ground, digested, and concentrated in a manner previously described (Kent and Kubica 1985). One each of a Lowenstein-Jensen medium slant (Becton-Dickinson, Cockeysville, MD), a Middlebrook 7H11S medium slant (Becton-Dickinson, Cockeysville, MD), and a Bactec12B broth vial (Becton-Dickinson, Sparks, MD), were inoculated with the remaining material. Media were examined for growth on a weekly basis for eight weeks. Bacterial growth, determined to be acid-fast by slide examination (Kent and Kubica 1985), were tested by genetic probe (Accuprobes, Gen-Probe, San Diego, CA) to determine whether the bacteria were members of the *M. tuberculosis* complex (Reisner et al 1994). Complete species identification was performed using biochemical testing and high performance liquid chromatography to differentiate *M. bovis* from the other members of the *M. tuberculosis* complex (*M. bovis, M. tuberculosis, M. microti*, and *M. africanum*), and from other mycobacteria (Butler et al 1991; Kent and Kubica 1985; Nolte and Metchock 1995).

Surveillance Data

Carnivore and omnivore surveillance has continued in a method previously described (Bruning-Fann et al 2001), through the end of the year 2000. Cranial, thoracic, and abdominal lymph nodes from animals collected by the Michigan Department of Natural Resources (MDNR) were examined grossly and histologically as previously described. In cases where animals had lung lesions noted grossly, lung tissue was also harvested, fixed in 10 % neutral buffered formalin, paraffin embedded and sectioned for routing hematoxylin and eosin and Ziehl-Neelsen staining prior to histologic examination. Tissue mycobacterial cultures (pooled lymph nodes from all animals, and in those cases with lung lesions, pooled lymph nodes and lung) were performed as described for experimental subjects in this manuscript, at the MDCH Tuberculosis Laboratory.

Statistical Analysis

SAS Version 8 statistical software was used for all calculations (Statistical Analysis Systems, SAS Institute, Inc., Cary, NC). The Wilcoxon Rank-Sum test was used to assess whether there were statistically significant ($p < 0.05$) changes in weight within each treatment group over time. Associations between routes of exposure and the outcomes of interest (gross lesions, histologic lesions, mycobacterial cultures from fecal material, and mycobacterial cultures from organ samples) were assessed using the two-tailed Fisher's Exact test, and associations were expressed as odds ratios (OR) with 95% confidence intervals (CI).

Results

All animals gained weight during the experiment. The amount gained in controls was similar to that gained in inoculation groups. No statistically significant differences in organ weights were noted at necropsy. Gross lesions included hepatomegaly in one oral and one intramuscular recipient, splenomegaly in two of each inoculation group, and numerous gastric *Physaloptera turgida* in one IM recipient, one oral recipient, and one control animal. The same IM recipient had large numbers of cestodes throughout the entire gastrointestinal tract. At necropsy, the adult male replaced early in the experiment due to decline in clinical condition was found to have gastric ulceration and rupture with a resultant hemoperitoneum due to a severe *Physaloptera turgida* infection. One oral recipient had numerous, randomly scattered, firm, one to four millimeter diameter, pale tan to white nodules scattered throughout both the pulmonary and renal parenchyma. Two IM recipients had lesions in both the lungs and at the injection site. The lesion in the first of these animals was a 4.0 mm diameter, irregular, yellow to tan, firm nodule within the parenchyma of the caudal-dorsal right lung lobe. The lung lesion noted grossly in the second animal was a 0.5 mm diameter, irregular, gray to tan, firm nodule, also within the caudal right lung lobe. Both of these animals had large (3 cm X 2 cm and 3 cm X 3 cm, respectively) abscesses within the musculature of the left hind limb. The abscesses contained approximately three to five mls of exudate which ranged from thick and yellow-green

to watery and straw-colored. The abscesses extended into a deep cellulitis and fasciitis in one of the animals.

Histologic lesions attributable to *M. bovis* were not present in any of the routinely stained or acid-fast stained tissue sections examined in either control or oral inoculation groups (Table 3.1). In the IM inoculated group, two animals had acid-fast bacilli noted histologically in skeletal muscle tissue sections. Each case featured a severe, multifocal and coalescing to dissecting, chronic-active, pyogranulomatous to caseogranulomatous myositis, cellulitis and fasciitis, with foci of mineralization and featuring multinucleate giant cells (Figure 3.1). Acid-fast bacilli were present both within the cytoplasm of inflammatory cells in these lesions, and free within centers of caseous exudate. An additional IM recipient had similar skeletal muscle lesions present without histologically confirmed presence of bacilli. This same animal did, however, have acid-fast organisms within the cytoplasms of macrophages in sections of lung. Lesions in the lung of this animal were histologically characterized as a moderate to severe, multifocal, chronic, granulomatous interstitial pneumonia with foci of caseation and with multinucleate giant cells. Acid-fast bacilli within inflammatory cells in the lung, associated with a similar caseogranulomatous pneumonia were present in one additional IM recipient (Figures 3.2 and 3.3). This second case was one of the two recipients with a granulomatous myositis that featured acid-fast organisms histologically. No further significant histologic lesions were noted in any animals.

Compared to controls, opossums that were intramuscularly exposed to *M. bovis* were at increased risk for being histopathology positive (OR = 3.00, 95% CI = .97 - 9.30), having visible lesions (OR = 2.33, 95% CI = 0.99 - 5.49), and having positive culture results for organ tissues (OR = 9.0, 95% CI = 0.64 - 126.84), but none of these results were statistically significant ($p > 0.05$). Similarly, opossums that were orally exposed to *M. bovis* were at increased risk for having positive culture results from feces (OR = 3.0, 95% CI = 0.97 - 9.30) and organ tissues (OR = 2.33, 95% CI = 0.99 - 5.49) compared to controls, but none of these results were statistically significant ($p > 0.05$).

When comparing the two different routes of exposure, there were no statistically significant differences ($p > 0.05$) in body weight, organ weights, or any of the outcomes of interest. Compared to oral exposures, opossums exposed intramuscularly were at increased risk for having gross lesions (OR = 2.33, 95% CI = 0.99 - 5.49), positive fecal culture (OR = 3.0, 95% CI = 0.97 - 9.30) and positive tissue culture (OR = 6.0, 95% CI = 0.43 - 83.54), but were at decreased risk for positive histopathology (OR = .26, 95% CI = .02 - 3.46). None of these relationships were statistically significant ($p > 0.05$).

Several histologic lesions were noted in all groups, including control animals. Most common was a mild to moderate, diffuse, microvacuolar to granular hepatopathy which was occasionally associated with a mild, multifocal, nonsuppurative, portal hepatitis. Also common was a mild to moderate, diffuse, eosinophilic to nonsuppurative enterocolitis, occasionally associated with intraluminal nematode or

cestode parasites in cross-section. In one control animal, two oral recipients, and one IM recipient, a moderate to severe, multifocal and coalescing, chronic alveolar histiocytosis with a mild to moderate, multifocal, chronic nonsuppurative interstitial pneumonia was present within the pulmonary parenchyma when examined histologically. The macrophages comprising the lesion in these cases were large and lipid-laden and most consistent with a diagnosis of endogenous lipid pneumonia. Further nonspecific lesions common to all groups included splenic, pulmonary, and hepatic congestion, and nonspecific lymphoid reactivity in the spleen, lymph nodes, bronchial-associated lymphoid tissue (BALT), and gut associated lymphoid tissues (GALT). One control opossum had *Besnoitia darlingi* protozoal cysts distributed throughout the renal parenchyma. Similar cysts were also noted histologically within the skeletal muscle of one oral recipient, within the lung of one IM recipient, and within the adrenal gland, skeletal muscle, and the lung of another oral recipient. Tissue reaction to these cysts ranged from minimal, with little associated inflammation, to severe, with fibrous encapsulation of necrotic to mineralized cysts or with large numbers of eosinophils and mononuclear inflammatory cells associated with variable degrees of granulation in some instances. *Sarcocyst* spp. cysts were present in the skeletal muscle of one oral recipient with no noted tissue reaction.

Fecal cultures were negative for the presence of mycobacteria in all animals prior to inoculation. One oral recipient had *M. bovis* cultured from feces collected 24 hours after inoculation. A different oral recipient had positive *M. bovis* cultures in

feces collected at 31 days PI. No additional positive fecal cultures were obtained from any animal (Table 3.2).

Tissue cultures were positive in one oral recipient and in three IM recipients (Table 3.2). All animals with positive cultures were positive in the pooled spleen and lymph node submission. The oral recipient was also positive for *M. bovis* in the pooled small and large intestine submission. Two IM recipients were positive for *M. bovis* in the lung and in pooled liver and kidney in addition to being positive on the spleen and lymph node submission. Interestingly, the only animal with a combination of gross and histologic lesions compatible with tuberculosis did not have any tissue cultures from which mycobacteria could be isolated.

Updated carnivore/ omnivore surveillance activity results since the project's inception, and including the two opossums that have cultured positive for *M. bovis* are included in Table 3.3. Each of these animals had gross lesions noted in the lungs. A moderate to severe, multifocal to coalescing, caseogranulomatous pneumonia with intralesional acid fast bacilli noted on specially-stained tissue sections was present in each of these two cases.

Discussion

Most of the gross lesions found at necropsy in experimental subjects could not be attributed to inoculation. Gastrointestinal parasitism, hepatomegaly, and splenomegaly were present in all groups and therefore did not seem to be related to mycobacterial disease. One opossum with lung lesions that were suggestive of

granulomas grossly was determined to have pulmonary besnoitiosis based on histologic examination of the tissue. Also noted histologically in some animals was an endogenous lipid pneumonia. When accumulations of lipid-laden macrophages are subpleural, they may give a gross appearance of small granulomas of undeterminable pathogenesis based on gross inspection alone. This contributed to the array of lesion descriptions noted in the three animals with grossly visible lung lesions. Only one of these three animals had histologically confirmed evidence of gross lung lesions being attributable to tuberculosis. In both cases with myositis noted grossly nearly the injection site, the infection was directly caused by the injected organisms based on histologic examination of the tissues, rather than by a foreign-body-type injection reaction.

Nearly all animals in this study had some degree of hepatic glycogen or lipid storage detected histologically. Although animals were not fed ad libitum, access to food was relatively easier and more nutritious than that which they may encounter in the wild, and physical exercise was limited due to cage restrictions. Although no animal showed clinical evidence of hepatic disease, and no case was histologically severe, dietary restrictions for opossums in captivity may need to be considered based on these findings.

Interesting was the common finding of endogenous lipid pneumonia in these animals. This process is most typically reported in lab rodents and may be multifactorial in origin (Dungworth, 1993). There is no apparent explanation for its presence in these opossums, although a report describing it as being a rather common

finding in wild opossums exists (Brown, 1988). This report suggests lipid pneumonias in opossums may be related to pulmonary nematodiasis, but there were no instances in which pulmonary nematodes were found present on necropsy in this study.

Besnoitia darlingi protozoa were noted histologically in all groups. Typically, *Besnoitia darlingi* is not implicated as causing disease in opossums, yet the large numbers of cysts noted, particularly in some of the treatment group animals, is suggestive of some degree of pathogenicity in some animals in the study (Davidson and Nettles 1997). In fact, these protozoa, cestode, and nematode enteric parasites were present more frequently in inoculated animals than in control animals, suggesting possible immunosuppressive effects of *M. bovis*. Stress of capture and captivity alone may have been partially responsible for contributing to immunosuppression and thus, potentially, to parasitism in both control and treatment group animals. The *Sarcocyst* spp. present in one inoculated opossum was determined to be an incidental finding based on the limited tissue reaction and on the typical lack of pathogenicity of this organism in this species (Davidson and Nettles 1997).

At the time of completion of this study, no positive cases of *M. bovis* tuberculosis in North American opossums had yet been identified through wildlife surveillance activities in Michigan. Surveillance data through the end of the year 2000 now includes two positive cases of *M. bovis* tuberculosis out of 228 animals tested. This calculates to the one of the lowest prevalence rates (0.9 %) in any of the wild carnivore/ omnivore species with identified positive cases in surveillance activities to date (Table 3.3).

It was the original intent of the investigators to determine whether the North American opossum was at all susceptible to infection by *M. bovis*, as there had been no reports of the disease in this species. Also of interest was whether this species shared any similarity in infectivity with its distant marsupial relative, the brushtail possum (*Trichosurus vulpecula*), after *M. bovis* challenge. Along with the ferret (*Mustela furo*), the brushtail possum is known to be a potent, non-cervid wildlife reservoir host for *M. bovis* in New Zealand (O' Reilly and Daborn 1995). Both the laboratory and surveillance data presented here support the hypothesis that the North American opossum is indeed susceptible to infection, yet apparently to a less dramatic degree than either of these species.

In our experiment, opossums that were inoculated intramuscularly had more histologic evidence of lesions consistent with tuberculosis and had larger numbers of positive tissue cultures as a group than did those receiving oral inoculation. However, only those orally inoculated had positive fecal cultures at any time throughout the experiment, suggesting this transmission route as a plausible one in the wild. Interesting was the fact that two of the intramuscularly inoculated opossums developed pulmonary lesions consistent with tuberculosis. At least a partial affinity of the organism for the lung in this species is suggested by this finding. Additionally intriguing is the fact that the two opossums identified as positive for *M. bovis* tuberculosis in Michigan wildlife surveillance also had identifiable lesions limited to the thoracic cavity. This finding is especially interesting considering most of the additional carnivore and omnivore surveillance cases identified to date have had

abdominal cavity lesions (mesenteric lymph nodes in particular), if lesions were present at all (Bruning-Fann et al 2001).

Only one animal had met all three diagnostic criteria (gross lesions, histologic lesions, and positive mycobacterial isolation/ identification) for *M. bovis* tuberculosis. This stresses the importance of combining laboratory findings before making a final determination as to the presence or absence of this disease. Most obvious is the case of the "missed" histologic evidence of disease when only small sections of tissue may be available for examination. Yet equally important is the potential for problematic culture results, particularly when stringent tissue preparations/ digestions are necessary as is the case in some mycobacterial work performed on animal tissues, where microorganism recovery can prove quite difficult.

While this species is susceptible to *M. bovis*, the likelihood of it serving as a reservoir host under natural conditions may not be as great as is that of the brushtail possum. The dosage of microorganisms used in this pilot study was much higher than that used in studies with the brushtail possum, and at similar times post-inoculation, necropsy of North American opossums revealed far less extensive lesions than those that have been published for the brushtail possum (Corner and Presidente 1981). In fact, both experimentally and in the wild, brushtail possums are known to develop severe, generalized lesions, oftentimes including fistulous tracts that drain externally from infected lymph nodes, in response to exposure to *M. bovis* (Corner and Presidente 1981; Buddle et al 1994; Pfeffer et al 1995; Cooke et al 1995). When the lesions in either these laboratory inoculated, or in wild opossums that have cultured

positive through routine, ongoing carnivore/ omnivore surveillance are compared to those found naturally in brushtail possums (Cooke et al 1995), the difference in resistance to generalized infection after exposure to this organism becomes striking between species. In our experiment, even those animals inoculated with very high doses of *M. bovis* that could arguably be difficult for any animal to encounter in the wild had limited tissue distributions of lesions that could be definitively diagnosed as resulting from mycobacterial infection. In the presently identified two surveillance cases of bovine tuberculosis found in North American opossums in Michigan, similar limitations in both lesion severity and in distribution to the organs of the thoracic cavity have been noted.

In contrast to the brushtail possum is the badger (*Meles meles*), which is a known reservoir host for *M. bovis* tuberculosis in the United Kingdom and in Ireland (Little et al 1982). These animals, although very efficient reservoir hosts, apparently often shed *M. bovis* organisms in the wild without any evidence of clinical disease or debilitation (Gallagher et al 1998). Even in laboratory inoculation studies, the badger may live several years without evidence of clinical disease that would be typical of that in the brushtail possum given similar inoculation routes and organism dosages (Corner and Presidente 1980; Pritchard et al 1987; Buddle et al 1994). So to speculate that a spectrum of potential reservoir hosts for bovine tuberculosis may exist, some of which may become severely affected by the infection, and some of which may be able to carry and shed organisms without apparent clinical disease, seems reasonable. Whether or not the North American opossum has a place in this spectrum remains to

be seen. More likely, this species represents a "spillover host" with relatively high resistance to infection when compared to some spillover species, and indeed to any true reservoir host.

Additional studies that further explore the transmission and excretion routes of *M. bovis* in this species are, however, warranted. Based on lesion distribution in this study and in surveillance cases, the North American opossum seems more apt to potentially spread tuberculosis by the more efficient respiratory route when compared to other spillover species in Michigan. Opossums, both in surveillance and in these lab-based analyses, have shown a tendency towards respiratory infections, whereas all other spillover host species identified at this time in Michigan have had enteric disease. As such, intratracheal inoculation study results may prove useful in providing a better understanding of the disease process in this species.

Table 3.1: Laboratory results. Histology and culture results. Histologic examination based on the presence or absence of acid-fast bacilli on Ziehl-Neelsen acid-fast stained tissue sections. Culture results are those animals with positive tissue cultures for *M. bovis* .

Table 3.2: Summary of cases with evidence of *M. bovis* tuberculosis. Specific descriptions of those animals positive for *M. bovis* on necropsy.

[A] J = juvenille; A = adult; [B] F = female; M = male;

[C] Mu = muscle, Lu = lung, I = pooled small and large intestine, Ln = pooled spleen and lymph nodes, Liv = pooled liver and kidney.

Table 3.1. Laboratory results.

Inoculation Group	Number of Animals In Group	Acid-Fast Organisms Noted Histologically	Mycobacterial Culture Positives
Control	4	0/ 4	0/ 4
Oral	4	0/ 4	1/ 4
Intramuscular	4	3/ 4	3/ 4

Table 3.2. Summary of cases with evidence of *M. bovis* tuberculosis.

Age[A]	Sex[B]	Inoculation Route/ Days PI Sacrificed	Gross Lesions/ Organs[C]	Positive on Histology/ Organs[C]	Positive on Fecal Culture/ Days PI Cultured Positive	Positive on Culture/ Organs[C]
J	M	Oral/ 31 days	No	No	Yes/ 31 days	Yes/ I, Ln
J	F	IM/ 31 days	Yes/ Mu, Lu	Yes/ Mu	No	Yes/ Lu, Liv, Ln
J	M	IM/ 31 days	No	Yes/ Mu, Lu	No	Yes/ Lu, Liv, Ln
J	F	IM/ 61 days	Yes/ Mu, Lu	Yes/ Mu, Lu	No	No
A	F	IM/ 61 days	No	No	No	Yes/ Ln
J	M	Oral/ 61 days	No	No	Yes/ 1 day	No

Table 3.3: Carnivore and omnivore surveillance results for *M. bovis* in Michigan (1996-2000).

[a] Number of this species surveyed through the end of the year 2000;.

[b] Total number of culture-positives for *M. bovis* in this group;

[c] Number of positives in this species divided by total number surveyed multiplied by 100.

Table 3.3. Carnivore and omnivore surveillance results for *M. bovis* in Michigan (1996-2000).

Species	Scientific Name	Total[a]	Number[b]	Prevalence[c]
Badger	*Taxidea taxus*	25	0	0.0
Bear, Black	*Ursus americanus*	163	4	2.5
Bobcat	*Felis rufus*	40	2	5.0
Coyote	*Canis latrans*	249	11	4.4
Fox, Gray	*Urocyon*	4	0	0.0
Fox, Red	*Vulpes vulpes*	16	2	12.5
Mink	*Mustela vison*	2	0	0.0
Opossum	*Didelphis virginiana*	228	2	0.9
Porcupine	*Erethizon dorsatum*	1	0	0.0
Raccoon	*Procyon lotor*	194	2	1.0
Skunk	*Mephistis mephistis*	12	0	0.0
Weasel	*Mustela sp.*	1	0	0.0
TOTALS		**935**	**23**	**2.5**

Figure 3.1: Section of skeletal muscle from an opossum inoculated intramuscularly with *M. bovis*. Severe caseogranulomatous myositis with central mineralization is featured. Hematoxylin & Eosin (H & E); 4X.

Figure 3.2: Section of lung from an opossum intramuscularly inoculated *M. bovis*. Note large numbers of macrophages filling alveoli, coalescing as early granulomas. H & E; 40X.

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Figure 3.3: Section of lung from an opossum intramuscularly inoculated with *M. bovis*. Acid-fast bacillus within the cytoplasm of one of several large macrophages in an alveolus (arrow). Ziehl-Neelsen acid-fast; 100X.

Figure 3.1

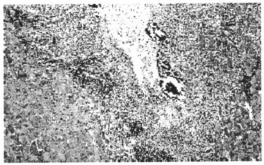


Figure 3.2

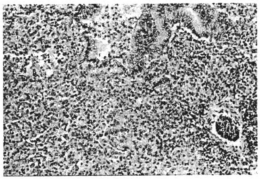
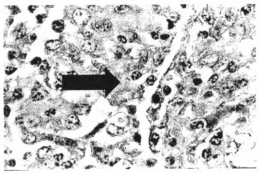


Figure 3.3



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Chapter 4

Antimicrobial susceptibility patterns of human and wildlife *Mycobacterium bovis* isolates from Michigan

Abstract

In Michigan, a continuing endemia of *Mycobacterium bovis* in wildlife has raised much concern for its potential impact on human health, particularly since the disease has been found present in domestic beef and dairy cattle and captive cervid herds in the state. No cases of human tuberculosis caused by the wildlife *M. bovis* strain have been identified to date, but preparedness for such an event has been warranted. As part of this preparedness, a better understanding of the strain in question, particularly that of its potential resistance to common antituberculous drugs is desirable. Cultures of this strain, obtained from positive cases found through both wild and domestic animal surveillance in Michigan, were tested by both agar plate and Bactec broth methods for resistance to the most commonly used antimycobacterial drugs. These results were compared to those from five human *M. bovis* tuberculosis cases identified in Michigan to date. No similarities between human and animal restriction fragment length polymorphism (RFLP) patterns were noted, and no cases were multidrug resistant. Some colony growth was present in the presence of isoniazid in several animal cases, and in the presence of cycloserine in one case. Although these cases are not considered as being resistant to these drugs, the growth patterns may be important in gaining a better understanding of this particular *M. bovis* strain in Michigan.

Introduction

Tuberculosis (both *Mycobacterium tuberculosis* and *Mycobacterium bovis*) infections in the world's population have increased dramatically in the last decade, necessitating declaration of the disease as a global emergency by the World Health Organization (Kamholz 1996; Lee et al 1995; Yew and Chau 1995). Risk factors including immigration, increased spread of the human immunodeficiency virus and other immunosuppressive diseases and treatments, poverty, and technological developments allowing increased international mobilization and travel have contributed to the spread of this disease worldwide, making tuberculosis the infectious process most commonly associated with death in the world (Bloom and Small 1998; Bradford and Daley 1998; Cohen 1997; Lee et al 1995; Yew and Chau 1995). Additionally problematic is the worldwide phenomenon of emergence of drug resistant strains of microorganisms, including mycobacteria responsible for tuberculosis infections (Castiglia and Smego 1997; Cohen 1997). The threat of continuing and, even increasing , tuberculosis case fatalities has become a great concern in the medical community largely because of the increasing number of multi-drug-resistant (MDR) strains (Cohen 1997; Yew and Chau 1995). MDR tuberculosis is defined as that which is resistant to both isoniazid (INH) and rifampin, with or without resistance to additional antimicrobials (Lee et al 1995). Although MDR *M. tuberculosis* is of primary concern as a human pathogen, human and wildlife *M. bovis* cases have been of increasing concern as well, both MDR strains and susceptible strains (Brett 1991).

Although relatively rare in humans, MDR *M. bovis* is of increasing concern due to this overall rise in serious mycobacterial illness. In Michigan specifically, awareness of *M. bovis* infections has been heightened dramatically in recent years due to recognition of an enzootic strain of the organism in Michigan wildlife (Bruning-Fann et al 1998; Schmitt et al 1997). Transmission of *M. bovis* to humans from wildlife is well documented in locales with endemically-infected feral animal populations (Brett 1991). In order to best eradicate *M. bovis* from endemically-infected Michigan wildlife, gaining understanding of potential drug resistances in the endemic strain of this microorganism is an important step in assuring safety for both humans and animal species in the future.

Specifically, the objective of this project was to define drug susceptibility patterns for isolates of *Mycobacterium bovis* obtained from Michigan human and wildlife cultures. The antituberculous antimicrobials isoniazid (INH), streptomycin (SM), rifampin (RA), ethambutol (EMB), ethionamide (EA), kanamycin (K), ciprofloxacin (CIP), cycloserine (CS), capreomycin (CM), and pyrazinamide (PZA) were tested by either Bactec^{R} methodology, proportion agar plate methodology, or both.

Antimicrobial susceptibility profiles were compiled using mycobacterial isolation and drug sensitivity testing procedures previously described (Kamholz 1996; Siddiqi 1996) and currently employed by the Tuberculosis Laboratory at the Michigan Department Of Community Health (MDCH).

Cultures from five human *M. bovis* cases, six carnivores (three coyotes, two raccoons, and one black bear), thirty cervid (white-tailed deer), and three bovine cases, all isolated in Michigan, were used for the study. Isolates were purified and were used as inocula for both Bactec broth media and Middlebrook 7H10 Agar plates impregnated with multiple antituberculous antibiotic discs for proportion plate susceptibility testing (Kent and Kubica 1985).

Materials and Methods

Growth from all isolates was adjusted to a 0.5 MacFarland suspension to prepare each for Bactec susceptibility testing (Nolte and Metchock 1995). The susceptible control for both Bactec (non-PZA) and proportion plate tests was TMC 102 *Mycobacterium tuberculosis*. The resistant control used for the Bactec PZA test was TMC 1011 *M. bovis*. The following antimicrobials: streptomycin, isoniazid, rifampin, and ethambutol were added to Bactec 12B vials (Middlebrook 7H12 medium, Bactec 460 TB system, Becton Dickinson and Co., Sparks, MD) resulting in final concentrations of 2.0, 0.1, 2.0, 2.5 μg/ml respectively. The 0.5 MacFarland suspensions were then inoculated into each of the drug containing vials (Siddiqi 1996). A 1:100 dilution of each 0.5 MacFarland suspension was also inoculated into a separate Bactec 12B vial to serve as a control vial for each test drug test panel. Bactec 12B vials were read daily for a growth index (GI) on the Bactec 460 TB system for a maximum of 14 days or until the control vial reached a GI of greater than or equal to 30. Susceptible isolates are those with a ΔGI (the difference between the most recent

GI value and the GI from the previous day) less than the ΔGI of the control vial. Resistant isolates are those with a ΔGI greater than the ΔGI of the control.

The Bactec method for PZA susceptibility testing was also implemented for each isolate. This required a Bactec 12B vial inoculated from the original 0.5 MacFarland with a GI of 300-499 to be inoculated into two separate, modified Middlebrook 7H12 medium (PZA test medium) vials with the pH adjusted to 6.0. PZA drug was added to one of these two vials to result in a final concentration of 100ug/ml. The other vial was maintained without PZA to serve as the test control vial. Results were interpreted when the GI of the control vial reached 200 or more.

Interpretations of Bactec PZA results were calculated as a % susceptibility using the following formula:

$$\frac{\textbf{GI Drug Vial}}{\textbf{GI Control Vial}} \times 100 = \textbf{\% Susceptibility}$$

Susceptible isolates were those resulting in percentages less than 9%, resistant isolates were those resulting in percentages greater than 11%, "borderline" isolates were those resulting in percentages between 9-11%.

Proportion plate susceptibility testing (1% method) was performed on each culture isolate as well. Middlebrook 7H10 agar plates (Becton Dickinson and Co., Sparks, MD) were prepared using drug-impregnated discs (Becton Dickinson and Co., Sparks, MD) resulting in the following final drug concentrations: INH (0.2 and 1.0 μg/ ml); SM (2.0 and 10.0 μg/ ml); RA (1.0 μg/ ml); EMB (5.0 μg/ ml); EA (5.0 μg/ ml); K (6 μg/ ml); and CIP (2.0 μg/ ml). Powdered CS, CM, and thiophen-2-carboxylic acid hydrazide (TCH) suspensions were used to prepare media plates at concentrations

of 30.0, 10.0, and 5.0 μg/ml, respectively. TCH testing was included as a part of the routine mycobacterial species determining protocol. Isolates were subcultured into Middlebrook 7H9 broth (Becton Dickinson and Co., Sparks, MD), and incubated for 2 to 5 days each. These broths were inoculated onto 7H10 Agar plates in two dilutions determined on a case-by-case basis for each culture, such that a colony count of 100 to 300 results from one of these dilutions. Dilutions were determined based on comparisons to MacFarland standards and were made with phosphate buffered saline (Nolte And Metchock 1995). Plates were examined weekly for three weeks, using a lighted magnification lens. Growth was recorded as 4+ (>500 colonies), 3+ (200-500 colonies), 2+ (100-200 colonies), or 1+ (50-100 colonies). Quadrants with less than 50 colonies present had the actual colony number counted and recorded. Inocula with no dilution showing 100-300 colonies in one of the quadrants were retested. The dilution that showed colony (desired) growth nearest to 200 was then used to determine resistance patterns. A percent resistance was calculated as follows:

$$\frac{\textbf{Number of colonies on drug quadrant of plate}}{\textbf{Number of colonies on control quadrant of plate}} \quad \textbf{X} \quad \textbf{100} = \textbf{\% Resistance}$$

A culture population with a percent of resistance greater than or equal to 1% was considered resistant to the tested drug. Cultures with less than 1% resistance were considered susceptible.

Results

All isolates were found to be PZA resistant, as is expected of *M. bovis*. Unlike other members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. microti*, and *M. africanum*), *M. bovis* lacks the enzyme pyrazinamidase which is necessary to activate

this drug, rendering all strains of the organism resistant to its activity. No isolates were found to be resistant to any of the other antimicrobials tested by Bactec methodology. The results of isolates showing growth on the susceptibility test agar plates in this study are provided in Tables 4.1 (animal isolates) and 4.2 (human isolates). No animal isolates were, by definition, resistant to any antimicrobials on agar plate protocols either. Several isolates did have slight growth noted, particularly in the presence of INH, but only when reading the inoculation dilution that generated 4+ control growth (Table 4.1). No growth was noted from these same isolates at the inoculation dilution that generated only 2+ control growth. Human isolates varied widely in growth patterns on agar plate testing (Table 4.2). Two human isolates were found to be single-drug resistant, one to EA, and one to CM. No human isolate tested was multidrug resistant.

Restriction fragment length polymorphism (RFLP) analysis was carried out on all isolates in this study using methods previously described (Chaves et al 1996; van Embden et al 1993). These "genetic fingerprints" were identical for all animal isolates, but varied widely both between those human isolates tested and between human and animal isolates.

Discussion

This is the first published report describing antimicrobial resistance patterns in Michigan's endemic strain of *M. bovis*. Except for PZA, no resistance was identified through conventional testing methods. The lack of resistance is possibly best attributed to the lack of "treatment pressure" that is often associated with

development of resistance in mycobacteria. Since positive animal cases are slaughtered rather than treated, the potential for selection of resistant mutant strains of the organism is eliminated. Although drug resistance does not appear to be a problem currently, some speculation can be given as to whether development of resistance in the future may be of concern based on the limited observed growth.

At-risk human populations in this endemic zoonosis include hunters, farmers, butchers, taxidermists, laboratory personnel, veterinarians, and veterinary professional students and their families. To date, no human cases of *M. bovis* have been caused by the endemic strain, as evidenced by both susceptibility and genetic typing (RFLP) assays. Only one additional case of *M. bovis* has been identified in Michigan since the time of conclusion of this study, and this case was found to be unrelated to all other human and animal cases as well. The human cases identified in the state thus far have been linked to exposure on farms in foreign countries, recrudescence of childhood infections established prior to eradication in Michigan, or from cancer therapy-induced infection by the bacillus Calmette-Guerin (BCG) strain of *M. bovis*.

Widespread education of hunters and farmers through outreach programs in Michigan has seemingly been of great benefit in controlling the spread of bovine tuberculosis in the state. These programs have included literature distribution, well-maintained web pages, lecture series, public radio broadcast updates, and well-advertised meetings, both local and statewide, with open invitations to concerned individuals. Yet certainly, careful monitoring of any new cases for potential relatedness to the endemic strain is of utmost necessity.

Colony growth in the presence of INH in the wildlife strain was minimal where observed. The clinical relevance of the growth, which was also typically noted at the more concentrated inoculum dilutions, is therefore insignificant from a therapeutic standpoint. Some below-standard growth is typical in tuberculosis susceptibility tests, so it is not the intent of this report to over-interpret the noted growth as definitive resistance. In fact, resistance in the endemic strain should not be problematic in Michigan at any time in the near future. Yet periodic susceptibility testing of the wildlife strain of *M. bovis* would nonetheless appear to be warranted. Such testing would be useful not only to assess potential development of resistance over time which exists in any strain of microorganism, but also that which may be already suggested by the findings in this report. And should any human case result from the described contact with infected animals or products, having a pre-existing understanding of which antimicrobials may be most efficacious for immediate administration of treatment rather than having to speculate and wait for final laboratory results would most certainly be beneficial.

Table 4.1: Wildlife-source *M. bovis* cases/ isolates from Michigan showing growth on proportion plate drug susceptibility testing.

[a] Case numbers assigned by Michigan Department of Community Health.

[b] INH = isoniazid; CS = cycloserine.

[c] Growth was recorded as 4+ (>500 colonies), 3+ (200-500 colonies), 2+ (100-200 colonies), or 1+ (50-100 colonies).

[d] All readings at –1 inoculum dilution log.

Table 4.2: Human-source *M. bovis* cases/ isolates from Michigan showing growth on proportion plate drug susceptibility testing. Bold italicized samples (cases 1201 and 252) are those determined resistant.

[a] Case numbers assigned by Michigan Department of Community Health.

[b] CM = capreomycin; EA = Ethionamide; INH = isoniazid; EMB = ethambutol.

[c] Growth was recorded as 4+ (>500 colonies), 3+ (200-500 colonies), 2+ (100-200 colonies), or 1+ (50-100 colonies).

Table 4.1: Wildlife-source *M. bovis* cases/ isolates from Michigan showing growth on proportion plate drug susceptibility testing.

Sample Species and Case Number [a]	Drug With Colony Growth Identified on 7H10 Agar [b]	Resistance Score [c, d] Or Number of Colonies Identified	Growth of Control At Same Dilution
Deer 5352	INH 0.2 µg/ ml	< 1+ (4 colonies)	4+
Deer 2673	INH 0.2 µg/ ml	< 1+ (3 colonies)	4+
Deer 7278	INH 0.2 µg/ ml	< 1+ (3 colonies)	4+
	INH 1.0 µg/ ml	< 1+ (4 colonies)	4+
Deer 5640	INH 0.2 µg/ ml	< 1+ (6 colonies)	4+
Coyote 1251	CS	1+	4+
Coyote 1865	INH 0.2 µg/ ml	< 1+ (5 colonies)	4+
	INH 1.0 µg/ ml	< 1+ (6 colonies)	4+
Raccoon 1966	INH 0.2 µg/ ml	< 1+ (3 colonies)	4+
Raccoon 1779	INH 0.2 µg/ ml	< 1+ (38 colonies)	4+
	INH 1.0 µg/ ml	< 1+ (31 colonies)	4+

Table 4.2: Human-source *M. bovis* cases/ isolates from Michigan showing growth on proportion plate drug susceptibility testing.

Sample Number [a]	Drug With Colony Growth Identified on 7H10 Agar [b]	Resistance Score [c] Or Number of Colonies Identified	Dilution Log of Inoculum	Growth of Control At Same Dilution
1201	***CM***	2+	-1	3+
252	***EA***	3+	-2	4+
	EA	1+	-4	2+
	INH 0.2 µg/ ml	< 1+ (25 colonies)	-2	4+
671	EMB	1+	-1	4+
	INH 0.2 µg/ ml	< 1+ (9 colonies)	-1	4+
	INH 1.0 µg/ ml	< 1+ (4 colonies)	-1	4+
1378	INH 0.2 µg/ ml	< 1+ (7 colonies)	-1	4+
	INH 1.0 µg/ ml	< 1+ (3 colonies)	-1	4+

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Chapter 5

A Novel Cytologic Evaluation Technique for the Detection of Mycobacteria

Abstract

Mycobacterial culture and identification is currently the "gold standard" technique for the diagnosis of mycobacteriosis in animals. Unfortunately, confirming infection using this technique can be time-consuming and cost-prohibitive in the veterinary setting, particularly when large numbers of samples require testing, as in a herd situation. The objective of this study was to compare results from a novel, cytology-based procedure for the diagnosis of tuberculosis in deer to culture results from these same animals. In order to test the new technique, cell-rich samples were collected from nineteen white-tailed deer (*Odocoileus virginianus*) inoculated by intratonsilar instillation of 2×10^8 colony forming units (CFU) *Mycobacterium bovis*. These cell samples were processed using both this new cytology method and by routine mycobacterial culture. The new test was found to be both less sensitive and less specific than is the culturing of samples for *M. bovis* tuberculosis, but was also found to be more time and cost-efficient. Moreover, this test may have substantial value in those species in which skin testing is an unreliable method for the antemortem diagnosis of tuberculosis.

Introduction

The diagnosis of mycobacterial disease has received much attention clinically in recent years, particularly due to the resurgence of tuberculosis. Tuberculosis is the only disease to be declared a global health emergency by the World Health Organization. Failures in both detection of the disease and in treatment of diagnosed

cases have been cited as contributing causes to this worldwide epidemic (Maher and Raviglione 1999).

Concerns over increased numbers of both *Mycobacterium tuberculosis* and *Mycobacterium bovis* tuberculosis cases in humans have been raised recently, although in industrialized countries, the prevalence of *M. bovis* tuberculosis is typically less than one percent of all diagnosed tuberculosis cases (Wilkins et al 1986). Many human *M. bovis* cases diagnosed in these countries in recent years have been found to be reactivated infections established prior to eradication programs in cattle and universally accepted milk pasteurization practices. Nonetheless, the disease remains a significant threat in third-world nations, along with that caused by *M. tuberculosis*. This increased prevalence in poorer countries is largely due to less adequate health care in both humans and animals, allowing the spread of this zoonotic disease to occur and oftentimes to go undetected.

M. bovis tuberculosis has recently regained much attention in the state of Michigan (Schmitt et al 1997). In Michigan, an endemic strain of *M. bovis* tuberculosis established in free-ranging white-tailed deer has become a major threat to livestock producers and has resulted in the loss of the tuberculosis-free status of the state. The need for efficient and reliable diagnostic testing for tuberculosis has been highlighted in the face of this endemic situation.

Currently, the gold standard diagnostic test for tuberculosis remains mycobacterial culture (isolation and identification), which, although very sensitive and specific, often takes weeks to complete. Histopathologic examination of acid-fast

stained tissue sections can be a very specific diagnostic tool, but depends on gross suspicion or actual detection of lesions and the number of bacilli associated with the section of tissue examined. Without histologic identification of actual bacilli, a diagnosis of tuberculosis cannot be confirmed on light microscopy alone and culture results are still necessary. Perhaps most importantly, in the veterinary world, both of these diagnostic procedures rely primarily on the collection of tissues or fluids during the post-mortem examination. The gathering of tissues by biopsy in order to diagnose or to treat the tuberculosis patient is a technique that is applied much less frequently in the veterinary clinical setting than in that of human medicine. As such, very few attempts at novel ante-mortem diagnostic techniques for tuberculosis in animals have been made in recent years, and those that do exist are predominantly serologic assays.

The widely accepted tuberculin (purified protein derivative, PPD) skin test varies in reported sensitivities and specificities, but still remains one of the best antemortem detection methods for the disease. Yet it is an accepted fact that the slaughter of healthy animals may be the result of false positives readings. And equally as important are the false negative readings that may overestimate sensitivity estimates of this test, and allow unhealthy cattle to remain sources of infection for other animals. Reported specificity and sensitivity values for tuberculin testing vary widely according to a review of the literature, from approximately 72% to over 99% in cattle, with an even wider range reported for deer (Palmer et al 2000; O'Reilly 1995). Results are often based on the type of skin test applied (single cervical, comparative cervical, or

caudal fold), whether this result is used in series with other diagnostic techniques, and on the geographic location of the cattle herd (O'Reilly 1995).

Lymphocyte transformation assays performed on blood collected from suspect cases, which measure the immune response to mycobacterial proteins have been of some use, but can be difficult to standardize, particularly when application across species lines is attempted. The blood tuberculosis test (BTB) test, a combination of the lymphocyte transformation test and an ELISA test run on blood of suspect cases, seems to offer higher sensitivities and specificities. However, this assay may produce variable results based on the species being tested, the antigens used in the assay, and the stage of disease or immune status of the patient (Palmer et al, 2000). Also being developed rapidly are gamma interferon (IFN-γ) measurement assays, which show promise in application to both cattle and cervid tuberculosis diagnosis. But at the present time, any immune response measurement test is best used in conjunction with skin tuberculin testing, based on the reasons listed above (Thoen et al 1995).

Gaining interest in the diagnosis of tuberculosis are those molecular techniques which incorporate genome amplification of the organism coupled with either chemiluminescent or colorimetric probes specific for the mycobacterial species being investigated. These tests have shown great promise in their application in human medicine, and are quite rapid, highly specific and reasonably sensitive when used as intended (Zheng and Roberts 1999). However, due to the fact that they are best applied on smear-positive human respiratory specimens (tracheal washes, etc.), their application in the veterinary field may be limited. Currently these tests are also much

more costly than is routine mycobacterial culture and identification, which would be somewhat prohibitive in the screening of large numbers of samples in a herd situation (Thoen et al, 1995).

One simple and relatively inexpensive technique which is rarely used in diagnosing tuberculosis in animals is cytology. Cytologic evaluations of proposed shedding sites have not been investigated prior to this report. Since mycobacteria are intracellular organisms, if present in adequate numbers in cells near shedding sites, they should be detectable within cells harvested from these sites. It was the purpose of this study to investigate whether such detection was possible. Moreover, since knowledge on transmission routes of tuberculosis in white-tailed deer is limited, this study sought to better define these potential shedding routes while investigating the potential applications of a novel cytologic mycobacterial preparation method previously used only in human medicine, the ThinPrep 2000 automated cytology equipment (Cytyc Corp., Boxborough, MA). This technology is currently applied primarily in the diagnosis of malignancy in gynecologic specimens (Pap-smears) in the human clinical setting. Reports of its use in clinical veterinary medicine, or for that matter for the diagnosis of infectious disease in either humans or animals, do not exist. This report describes the application of the device in preparing cytology samples used to detect *M. bovis* infection in white-tailed deer.

Materials and Methods

Deer subjects

Nineteen six month-old, white-tailed deer (*Odocoileus virginianus*) were experimentally inoculated with *Mycobacterium bovis* as part of a high-dose, short term inoculation study at the National Animal Disease Center (NADC) in Ames, Iowa. These eight castrated males and 11 females were experimentally inoculated by intratonsilar instillation of 2×10^8 colony forming units (CFU) of *M bovis* as previously described (Palmer et al 1999). Deer were housed inside a biosecurity level 3 building with directional airflow such that air from the animal pens was pulled towards a central corridor and passed through high efficiency particulate air (HEPA) filters before exiting the building. Airflow velocity was controlled to provide 10.4 air changes/minute in the animal pens. Deer in each pen had access to a circulating watering device and were fed a pelleted feed (deer and elk complete feed 55P3, Purina Mills, St. Louis, MO) and alfalfa hay. Pens were cleaned once daily, one at a time, by transferring deer to a holding pen with thorough washing of the floor and lower walls of the empty pen with a high-pressure hose. During cleaning, deer had contact with penmates only and not with deer from other pens.

Between days 21 and 63 after inoculation, 3 deer were euthanized due to injuries acquired during handling. Four deer were euthanized between 63 and 90 days after inoculation due to poor condition from advanced tuberculosis, and another 3 deer were euthanized due to injuries from handling. Between 90 and 113 days after inoculation, 1 deer was euthanized due to advanced tuberculosis and another due to trauma related

injuries. One hundred twenty days after inoculation all seven remaining experimentally inoculated animals were euthanized. Consequently, as the study progressed, the number of animals available for sampling decreased (Table 5.1).

For inoculation and sampling, deer were anesthetized with a combination of xylazine (Mobay Corporation, Shawnee, KS) (2 mg/kg of body weight) and ketamine (Fort Dodge Laboratories, Fort Dodge, IA) (6 mg/kg) injected intramuscularly. The effects of xylazine were reversed with tolazoline (Lloyd Laboratories, Shanandoah, IA) (4 mg/kg) injected IV.

Mycobacterial Strain

The strain of *M.bovis* used was strain 1315, originally isolated from a free-ranging tuberculous white-tailed deer killed by a hunter in Alpena county, Michigan in 1994. The isolate was incubated at 37° C for 6 weeks on Middlebrook 7H9 liquid media with 10% oleic albumin dextrose citrate enrichment (Bacto Middlebrook OADC Enrichment, DIFCO Laboratories, Detroit, MI). After incubation, the bacteria were harvested by centrifugation, and washed twice with 0.01M phosphate buffered saline (PBS) solution, pH 7.4. After resuspension in PBS solution, serial 10-fold dilutions were inoculated on Middlebrook 7H10 agar slants supplemented with OADC to determine the number of CFU. Inoculum was then frozen at -80 C for future use.

Specimen Handling

Nasal and oral swabs were collected for cytologic evaluation and bacteriologic culture on days 21, 63, 90 and 113 after inoculation. Swabs of the tonsilar crypts were collected 21 days after inoculation and at the time of necropsy. Swabs for cytologic

evaluation and bacteriologic culture were collected using a sterile 18 cm cytology brush (Puritan Medical Products, Guilford, ME.). For bacteriologic culture, swabs were rinsed thoroughly in 1.0 ml PBS. One half of the sample (0.5 ml) was added to 0.2% benzalkonium chloride solution (Zephiran chloride, concentrate 17%, Sterling Drug Inc., New York, NY) and let sit at room temperature for 15 minutes. After decontamination with benzalkonium chloride, samples were centrifuged for 20 min. at 2000 rpm and the supernatant decanted. To the sediment was added 0.5 ml of Bacto egg yolk enrichment 50% (DIFCO Laboratories, Detroit, MI). Samples of the sediment-egg yolk combination were inoculated onto separate agar slants containing Stonebrink's, Harrold's egg yolk, Middlebrook 7H10, or Middlebrook 7H11 media. Inoculated agar slants were incubated at 37° C for 8 weeks.

Cytology Preparation and Scoring

Tonsil swabs could not be obtained for either culture or ThinPrep 2000 sampling from any animals at 63 days PI (Tables 5.2 and 5.3). Only two of nine available animals could be sampled for cytologic evaluation at 90 days.

Samples were obtained from the NADC research deer described. Cytology brushes were placed in 20 mL PreservCyt® solution (Cytyc Corp., Boxborough, MA) contained in a 2 ounce plastic vial and were shipped to the Animal Health Diagnostic Laboratory (AHDL), Michigan State University (MSU) for preparation. Specimens were processed as recommended for mucoid samples according to the ThinPrep 2000 Operator's Manual (Cytyc Corp., Boxborough, MA), in a method previously described (Wang et al 1996). Briefly, this procedure involves vortexing the collected brush

sample, running the sample on the ThinPrep 2000 machine, fixing the slides in 95% ethanol for ten minutes or longer, and staining the slides with a modification of the Ziehl-Neelsen technique for the identification of acid-fast bacteria in cytology preparations (Prophet 1992). The modified staining procedure uses New Fuchsin blue to replace carbol fuchsin, and acetic alcohol is used for differentiation. While in the ThinPrep 2000, sample fluid in the plastic vial is first rotated to disperse cell materials from debris. Cells are then collected from the fluid across a filtration membrane using vacuum pressure. Filtered cells are then evenly pressed to a glass slide by the machine, in a circular area of approximately 20 mm diameter. Prepared slides with adherent cells are then placed in fixative for staining and evaluation.

In our study, slides were prepared and evaluated prior to any culture result being reported and were read without knowledge of animal history, with light microscopy. Subjective cellularity scores were assigned to each slide (poor, moderate or good cellularity). Slides were graded for the presence of mycobacteria at 40X magnification. Grades were assigned as follows: less than one acid-fast organism noted per high power field (HPF) = 1+; two to ten organisms noted per high power field = 2+; greater than ten organisms per HPF = 3+.

Statistical Analysis

Descriptive statistics (sensitivities, specificities, positive and negative predictive values) were calculated using Microsoft Excel software (Microsoft Corp., Redmond, WA). These values were generated by comparing the results of the ThinPrep technique to those of swabs obtained from the same anatomic sites, cultured for

mycobacteria. A swab from which *M. bovis* was cultured was considered a true positive result. Values were calculated separately for each anatomic site and also for the summed total of all results. Sensitivity defines the number of true positive samples (percentage positive by cytology that were positive by culture). Specificity defines the number of true negative samples (percent negative by cytology that were negative by culture). Positive predictive value is the probability that positive cytologies were truly positive when compared to the "gold standard", and negative predictive value is the probability that negative cytology samples were truly negative on culture.

Results

The number of deer determined to be positive for *M. bovis* by both mycobacterial culture (Table 5.2) and by the ThinPrep 2000 cytology technique (Table 5.3) are shown. Most samples that were positive had one or fewer organisms noted per HPF, a score of 1+ (Table 5.4). Eight of the 21 (38.1 %) positive samples examined from the tonsil had a score of 2+, and one of seven positive samples from the nasal cavity had a score of 2+ (14.3%). No samples received a 3+ grade for positivity. The new technique had an overall sensitivity of 73.17% and a specificity of 16.85 % when compared to the gold standard of mycobacterial culture (Table 5.5). Cytology preps obtained from the tonsil had the highest sensitivity and specificity of any of the three sites calculated individually (84.21% and 55.56% respectively). The overall positive predictive value for the test was 66.67%, and the overall negative predictive value 87.06%. The site generating the highest individual positive predictive value was the nasal cavity (85.71%), whereas the highest negative predictive value

resulted from those samples obtained from oral cavity swabs (91.18%). Subjective cellularity grades for the oral cavity were poorest. All samples from other sites had moderate to good cellularity reported for all slides, yet samples from the oral cavity obtained at all dates post inoculation had poor scores to a variable degree (one at 21 days, four at 63 days, four at 90 days, and three at 113 days).

Discussion

The ThinPrep 2000 (Cytyc Corp., Boxborough, MA) is currently Food and Drug Administration approved for use in diagnosis of multiple human samples, including but not limited to superficial scrapings, fluids, needle aspirates, mucoid samples (sputum, gastrointestinal, etc.), and gynecologic samples. To our knowledge however, despite its wide range of potential clinical applications, use of the process for the diagnosis of mycobacterial disease has never been evaluated. Use of this technology for processing veterinary clinical/ diagnostic samples has not been reported to date either, save for one reference mentioning its possible application in needle aspirate evaluation in research mice (Kobayashi et al 1997). In human literature, the technique has been evaluated to be as good as or better than cytocentrifuge or direct smear preparations (Wang et al 1996; Papillo and Lapen 1994).

In our study, cellularity was quite good in all samples, save for some of the oral cavity brush swabs. The likelihood that sampling technique was responsible for much of the noted decreased cellularity in these samples remains plausible. Subjectively, all slide preparations were easily read and did provide an adequate number of cells to determine whether acid-fast organisms were present or not. The stain procedure

worked very well in highlighting organisms where present, and did not stain non-mycobacterial organisms in any cases (Figures 5.1-5.3).

The more important shedding routes, both by culture and cytology, seem to be the nasal and tonsilar routes, as oral samples were rarely positive by either technique. Pathophysiologically, these results seem reasonable, as tuberculosis is primarily a respiratory and/ or lymphoid system-based disease in many large animal species. Since this is the first such study in deer, more data are necessary to confirm these findings, particularly because initial inoculation was intratonsilar in the study. Overall, of those sites assessed herein, the oral route appears to be the least diagnostically rewarding in cases of tuberculosis based on the low frequency of shedding and the relatively poor cellularity of oral cavity samples compared to tonsilar and nasal samples. These results are not surprising, as previous inoculation studies in white-tailed deer have suggested tonsilar and nasal shedding routes to be most important based on the distribution of those sites most commonly developing lesions after infection (Palmer et al, 1999).

Our evaluation of the ThinPrep technique for diagnosing mycobacterial infection resulted in an overall sensitivity comparable to that reported for detecting malignancy in human specimens (Wang et al 1996). Interestingly, the lack of specificity of the technique was the primary concern in that study as it appears to be in ours. The fact that the tonsil was the tissue with the highest sensitivity and specificity throughout the study was most reasonably due to the fact that inoculation of deer was intratonsilar, and shedding was most apparent in this tissue whenever samples were

taken. An intriguing finding is that tonsil swabs were positive by culture for all animals only at 21 days PI, with no further deer having positive tonsil culture results. Our technique detected several animals with intracellular acid fast organisms from tonsil samples at 90 and 113 days PI. Using culture as the gold standard technique would necessitate deeming these results as false positives. However, since cultures were performed on swabs rather than on macerated tissues, potentially decreasing the yield of organisms and overall specimen quality, it is proposed that perhaps all negative cultures may not have been truly negative if actual tissues had been sampled. One could similarly speculate that cultures performed on swabs from the oral cavity were falsely negative at 21 days PI. Conversely, the high negative predictive value does nevertheless suggest that shedding not detected by our test will not be able to be detected by the gold standard technique, mycobacterial isolation and identification, in most cases.

The number of animals used in this study was somewhat small to make conclusions using definitive descriptive statistics, but the resultant values do show that our technique may have some valid diagnostic application in the antemortem diagnosis of tuberculosis in animals. The difficulty in restraining captive cervidae for testing became apparent in the repeated collection of samples necessary for this study. Limiting the number of times animals require restraint in order to obtain an accurate diagnosis is of course warranted and is quite possible when samples or readings for multiple tests can be taken at one time. It certainly then follows that this test could be beneficial if used in conjunction with skin testing of deer, or even cattle. Results from

this technique, when used in conjunction with skin test results, would validate those cases in which active shedding may be occurring regardless of the certainty of a comparative cervical test result. And certainly in any animal species, this test could help distinguish active shedders from those animals that may have established but latent infections. Furthermore, in the case of many small, or even some exotic animals suspect of having tuberculosis, this test may be able to help diagnose active disease as well, and may even play a bigger role clinically in that no reliable skin testing protocol exists for many of these species.

In any of these potential clinical applications, the described procedure certainly surpasses culture for mycobacteria in cost-efficiency and timeliness of diagnosis. The estimated turn-around time for this test approximates 48 hours, from sample collection to reporting. The cost in our laboratory was eighteen dollars per sample including technician labor costs. When compared to the expected six to twelve weeks necessary for mycobacterial culture and the cost of culturing a clinical sample, approximately 110 dollars based on the charges we incurred through contractual laboratory work, clearly the ThinPrep method is the more rapid and cost-efficient technique. Of course, there are sacrifices of specificity and sensitivity of results to some degree with the technique, and no mycobacterial speciation can be performed without following cytology examination with the more expensive and time-consuming culture procedures. But when used as a screening process in cases with suspected active infections, particularly when large numbers of samples may need to be processed (e.g., in a herd situation), the benefits of the test are readily apparent.

No test will match the sensitivity and specificity of the available post-mortem methods of confirmation of mycobacterial infection. However, when used in conjunction with other available antemortem tests, ThinPrep 2000 cytologic analysis of veterinary samples may prove to be a quick and inexpensive tool for validation of established mycobacterial infections in some instances.

Table 5.1: Number of deer available for sampling at various times after inoculation.

Table 5.2: Deer which cultured positive for *M. bovis*. Number of deer culturing positive for listed tissue out of total number of deer sampled at that date, post inoculation (PI), are given.

Table 5.3: Deer with positive cytology readings (acid-fast bacilli noted with light microscopy). Number of deer with positive slide reading from listed tissue out of total number of deer sampled at that date, post inoculation (PI), are given.

Table 5.1. Deer numbers at post-inoculation (PI) sampling dates.

Days PI	Number of Deer
21	19
63	16
90	9
113	7

Table 5.2. Deer which cultured positive for *M. bovis*.

	Culture Positive Tissues		
Days PI	Tonsil	Oral	Nasal
21	19/19	0/19	2/19
63	0/0	6/16	6/16
90	0/9	3/9	2/9
113	0/7	2/7	1/7

Table 5.3. Deer with positive cytology readings.

	Cytology Positive Readings		
Days PI	Tonsil	Oral	Nasal
21	16/19	7/19	0/19
63	0/0	5/16	3/16
90	1/2	1/9	2/9
113	4/7	4/7	2/7

Table 5.4: Cytology scores for those samples with positive readings. Results are given as number of samples with listed score over total number of positive samples for listed tissue. [A] Scores assigned to average reading for entire slide as follows: 1+ = one or fewer organisms per high power field (HPF; 40X); 2+ = 2 to 10 bacilli per HPF; 3+ = greater than 10 bacilli per HPF.

Table 5.5: Results of descriptive statistical analysis of Thinprep 2000 cytology technique. Sensitivity defines the number of true positive samples (percentage positive by cytology that were positive by culture). Specificity defines the number of true negative samples (percent negative by cytology that were negative by culture). Positive predictive value is the probability that positive cytologies were truly positive when compared to the "gold standard", and negative predictive value is the probability that negative cytology samples were truly negative on culture.

Table 5.4. Cytology scores for those samples with positive readings.

	Scores Assigned to Cytology [A]		
Score[A]	Tonsil	Oral	Nasal
1+	13/21	17/17	6/7
2+	8/21	0/17	1/7
3+	0/21	0/17	0/7

Table 5.5. Results of descriptive statistical analysis of ThinPrep cytoprep technique.

Test	Tonsil	Oral	Nasal	Overall (All sites)
Sensitivity	84.21	72.73	54.55	73.17
Specificity	55.56	22.50	2.50	16.85
Positive Predictive Value	76.19	47.06	85.71	66.67
Negative PredictiveValue	57.14	91.18	88.64	87.06

Figure 5.1. Positive (2+) cytology obtained using ThinPrep technique, tonsilar sample. Intracellular acid-fast bacilli (arrows). New Fuchsin stain; 100X.

Figure 5.2. Positive cytology (2+) obtained using ThinPrep Technique, nasal sample. Large group of intracellular acid-fast bacilli (arrow). New Fuchsin stain, 100X.

Figure 5.3. Cytology example with large numbers of intracellular cocci and no acid-fast bacilli noted, ThinPrep technique. Photomicrograph displays clarity of cellular preparation for bacteria other than mycobacteria. New Fuchsin stain, 100X.

Figure 5.1. Positive (2+) cytology obtained using ThinPrep technique, tonsilar sample.

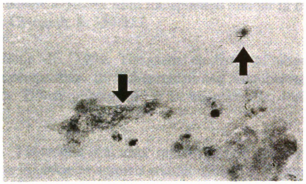


Figure 5.2. Positive cytology (2+) obtained using ThinPrep technique, nasal sample.

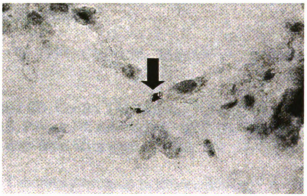
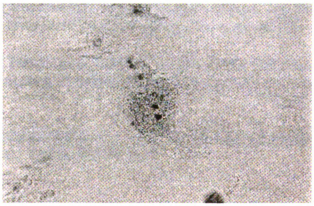


Figure 5.3. Cytology example with large intracellular cocci and no acid-fast bacilli noted, ThinPrep techinique.



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Chapter 6

Conclusions and Future Directions

The importance that the interaction of animals and man can have on the medical community is well exemplified in the case of a zoonotic disease. Tuberculosis is one of the most important zoonotic diseases both historically and currently. With the reemergence of the human disease globally as a world health emergency, a need to better understand the concepts of transmission and control of the disease in either an animal or human population is essential.

Although advanced technologies are the current focus of much of the research surrounding the diagnosis and treatment of tuberculosis, there are many essential, more basic questions about the transmission and pathology of the disease, both in animals and in man, that remain to be answered. Pathology is defined as the study of the nature of disease. The knowledge base that composes the understood pathology of any specific disease is best viewed as a continuum. This continuum constantly grows as new technologies are developed and new ideas are formulated involving the diagnosis and treatment of disease, the more clinical applications of medicine to which pathology is always inherently linked. And likewise, as better technologies are developed and implemented, the understanding of the nature of disease becomes more complete.

Answers to basic questions about tuberculosis are particularly lacking in the veterinary field, wherein understanding the true nature of the disease in a given animal population can become easily obscured by test and slaughter control methods. Although these methods are effective and probably even necessary to eradicate the

disease, they have to some extent eliminated much of the need for further study of the pathology of the disease in animals, as treatment is not typically a viable option.

The research involving *M. bovis* tuberculosis in the veterinary setting can in most instances be interpreted as primarily driven by need. When large outbreaks of the disease occur, particularly in wild animal populations, threats to the health of domestic livestock and humans become immediately important, and a need to answer basic questions to better understand the outbreak becomes imminent. The preceding chapters, in their simplest unified form, sought to answer some such basic questions that became imminently important in the face of Michigan's tuberculosis outbreak that has now become endemic in white-tailed deer.

Although it has been established in other countries that small mammals including badgers, ferrets, and brushtail possums can act very efficiently as reservoir hosts for *M. bovis* in the wild in addition to or in combination with cervids, little was known about transmission between deer and other wildlife hosts in Michigan. The first experimental chapter in this body of work sought to determine whether two bird species might reasonably be implicated in the transmission of *M. bovis* in Michigan. The second experimental chapter examined the same question in a mammalian species, the North American opossum.

The avian transmission study was perhaps most important due to the fact that no reports, experimental or clinical, of *M. bovis* in any bird species existed prior to its inception. European starlings and American crows were chosen because both occur commonly in wildlife and agricultural settings, and could reasonably be exposed to

materials infected with *M bovis*. Oral inoculation of the animals sought to simulate the natural ingestion of such materials (feed, carrion, feces, etc.), and intraperitoneal inoculation was used as an alternative route and contrast the type or extent of infection established, if any. It was found that these avian species are not likely to become infected by ingestion of *M. bovis*, but that they could reasonably maintain organisms intraperitoneally. The implication either of these species as viable wildlife reservoirs is considered unlikely based on our study results.

The transmission experiment using opossums was in some ways an extension of research carried out in its distantly related marsupial species, the brushtail possum, in New Zealand. Since the brushtail possum is known as a primary wildlife reservoir for *M. bovis* in New Zealand, it was reasonable to hypothesize that our own opossum species might play some role in the maintenance of the disease in Michigan, or at least be susceptible to experimental inoculation with the organism. Inoculation routes used in this study were intramuscular and oral. Both routes resulted in established systemic infections in some animals. Although it is unreasonable to assume that because they are susceptible to large doses of mycobacteria experimentally, these animals are reservoir hosts, there is a unique potential for infection in this species. Surveillance data suggest their potential role as reservoirs. Although very few cases of *M. bovis* in wild opossums have been identified to date through surveillance, it remains that they may play some part in the maintenance of the disease on a very small scale, or may become infected more readily than other small mammals when exposed.

The third project presented herein answered an essential question about Michigan's endemic strain of tuberculosis in wildlife: whether or not this strain specifically had any inherent resistance to antimicrobials. Although it would not be expected that the strain would have any secondary resistance due to treatment pressure or therapeutic noncompliance, the possibility of a primary resistance certainly does exist. A number of isolates from deer, cattle, and small mammal cases of *M. bovis* tuberculosis gathered through surveillance over the years in Michigan were tested for susceptibility to the standard panel of primary antimycobacterial drugs. As a comparison, all human isolates of *M. bovis* diagnosed in Michigan until the time of the realization of the study were tested for susceptibility to the same drugs. No animal isolates were resistant to any antimycobacterial tested based on the standards used by the Tuberculosis Laboratory at the Michigan Department of Community Health. Human *M. bovis* isolates were variably resistant, but none were found to be multidrug-resistant. All isolates, animal and human, were however resistant to pyrazinamide as is expected of *M. bovis*. Genetic analysis of the isolates did confirm that no human isolates were genetically identical, that these isolates differed substantially from animal isolates, and that all tested animal isolates were genetically identical. None of these resultant data were unexpected. Yet the study sets an important precedent for understanding the strain of microorganism involved in an outbreak on all levels including its resistance potential and genetic makeup, even one where treatment of infected animals may not be important. Even if bovine tuberculosis is best controlled by test and slaughter in animals, preparedness for the potential spread to humans,

because such an event would require treatment of human patients, remains an important consideration.

The final research objective presented in this dissertation was also partially inspired by an eventual application to human medicine. In this chapter, a new diagnostic technique for the detection of mycobacteria in cell-rich clinical samples was evaluated. Cellular swabs obtained from deer experimentally inoculated with *M. bovis* were processed on the ThinPrep 2000 automated cytology device, one which prepares very high quality cytologic slides, and were stained for acid-fast organisms. Results were compared to culture results of swabs taken from the same sites in the inoculated deer. The technique was developed as a rapid, inexpensive, antemortem test that would require relatively little technical training in its application. Although this cytology examination-based test was found to be less sensitive and specific than culturing clinical specimens, it has promise in its application in both human and veterinary medicine. As an inexpensive adjunct test that can be prepared and run at nearly any facility, without specialized knowledge of mycobacterial culture and identification techniques, it holds promise for being a useful procedure in the veterinary field.

While the work presented in this dissertation has contributed significantly to our understanding of the nature of *M. bovis* in Michigan wildlife, questions that have emerged from this work may prove equally as significant and will require additional, specifically-focused studies. The promise of a new antemortem veterinary diagnostic test for the detection of mycobacteria has been realized, but the extent to which it can

enhance existing and developing technologies remains to be seen. Larger numbers of samples, and perhaps even those from different animals sources should be tested before a final determination is made on the usefulness of ThinPrep processing of cytology samples for the diagnosis of tuberculosis. Moreover, the application of this technology for its use in the diagnosis of infectious diseases such as mycobacteriosis in the human clinical setting should be examined.

More final in the application of its results was the study of the susceptibility pattern of Michigan's endemic strain of *M. bovis*. Although it was determined that at present, no threat of drug resistance exists in the strain, tracking the isolate for genetic drift, particularly for that of acquired resistance over time, is now possible. Because there will always be a threat of transmission to humans as long as this disease remains prevalent in wild and domestic animals species in Michigan, assurance that this strain is not developing either primary or secondary, treatment pressure-based mutational resistance over time is important. The results of our study serve as a very good initial baseline in order to perform such monitoring.

The largest number of remaining questions was generated by our transmission studies. A limited number of potential reservoirs for *M. bovis* in Michigan were capable of being examined in the time period over which these studies were completed, and in these animals, a limited number of inoculation routes were assessed. Little remains known about the susceptibility of wild, non-passerine, avian species to *M. bovis*. An interesting follow-up study would be to compare the infection potential of certain herbivorous or non-passerine, native Michigan bird species. The reasoning

behind why certain carnivores and omnivores seem more easily infected with *M. bovis* in the wild remains uncertain. Continued inoculation studies that not only explore new inoculation routes in opossums, but also those in different species and the variable immune responses of these species when challenged with bovine tuberculosis will help to complete the understanding of the cycle of its transmission in and between wild and domestic animal hosts. Specifically, inoculation studies in wild-caught badgers, rodents, and other carnivores included in surveillance in Michigan are warranted. Moreover, in those species appearing to have the potential of becoming infected with *M. bovis*, inoculation studies determining specific doses of organisms necessary to establish infection, and time required for these species to shed bacteria from various sites after inoculation would certainly better our overall understanding of the precise pathogenesis of tuberculosis in these animals.

In summary, this body of work demonstrates that several very important objectives for researching various aspects of Michigan's endemic strain of *M. bovis* have been met. We have shown that European starlings, American crows, and North American opossums do not appear to be active reservoir hosts for this disease in the state at the current time. Yet it has been established that opossums have the potential to become a reservoir for the disease in the wild. The endemic strain of *M. bovis* in Michigan wildlife has been shown to be susceptible to most of the common antituberculosis therapies. A new technique for quickly and inexpensively documenting the shedding of mycobacteria from animals with active infection has been developed. All of these findings illuminate areas of bovine tuberculosis research

that had been ignored until the present time. The base of knowledge they provide for the continuing study of *M. bovis* in wildlife is therefore invaluable, and in all hopes will be built upon as future research continues to more completely define the pathology and transmission of this disease.