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EFFECTS OF *MYCOBACTERIUM BOVIS* INOCULATION IN SELECT POTENTIAL RESERVOIR OR SPILLOVER WILDLIFE HOST SPECIES

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Major Professor's Signature

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# EFFECTS OF MYCOBACTERIUM BOVIS INNOCULATION IN SELECT POTENTIAL RESERVOIR OR SPILLOVER WILDLIFE HOST SPECIES

By

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ABSTRACT

EFFECTS OF MYCOBACTERIUM BOVIS INOCULATION IN SELECT POTENTIAL RESERVOIR OR SPILLOVER WILDLIFE HOST SPECIES

By

Kathy-Anne Rochelle Clarke

White-tailed deer (*Odocoileus virginianus*) in the state of Michigan are recognized to be maintenance hosts for *Mycobacterium bovis,* the causative agent of bovine tuberculosis. It is believed that this organism was spread to the deer from tuberculous cattle prior to 1979 when the state was declared free of bovine tuberculosis. The first case of the disease in Michigan deer was identified in 1994. Since that time there have been just over five hundred deer diagnosed with this disease in the state. In addition to continued annual surveillance of hunter harvested deer, testing of domestic livestock for bovine tuberculosis and continued research efforts are necessary components of the plan to manage and eventually eradicate this endemic disease.

Thus, the primary objective of this dissertation was to experimentally introduce *M. bovis* into select wildlife species in order to provide necessary data on the transmission and pathogenesis in such species and ultimately to assess the attendant risk for each species in their ability to serve as either a reservoir or spillover host. Three rodent species (meadow voles [*Microtus pennsylvanicus*], house mice [*Mus musculus*] and rats [*Rattus norvegicus*]) and one avian species (the wild turkey [*Meleagris gallopavo*]) were experimentally inoculated. Meadow voles were found to be susceptible to both intranasal and oral inoculations. House

mice were also susceptible to oral exposure. The Norway rat was resistant to oral inoculation and wild turkeys were resistant to both intratracheal and oral inoculation.

As an extension of the inoculation study performed in house mice, expression of the *Bcg/Nramp-1/ Scl11a1* gene was also determined in this species. House mice were of the dominant/resistant phenotype, and thus it was concluded that the *Bcg* gene alone cannot determine the response to infection with *M. bovis* in this species.

The secondary objective of this dissertation was to develop a new technique for the diagnosis of *M. bovis* infection which utilized laser capture microdissection (LCM) for the procurement of granulomas from formalin-fixed paraffin-embedded tissues with subsequent PCR analysis of captured material. This study showed that the sensitivity of the LCM/PCR technique to be 69.2% when compared to the gold standard diagnostic technique, that of mycobacterial culture.

## DEDICATION

This work is dedicated to mummy and daddy, Montelle and James Clarke.
I am very thankful and proud to have both of you as my parents.
Your years of support and love are my 'fuel'.

This body of work would not have been possible without the grace of God Almighty, the cleansing shed blood of Jesus Christ, the Lamb, and the power of the Holy Spirit - the Trinity, one in three and three in one.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFB | acid-fast bacteria |
| AIDS | Acquired Immune Deficiency Syndrome |
| AHDL | Animal Health Diagnostic Laboratory |
| AM | alveolar macrophage |
| APC | antigen presenting cell |
| BCG | bacillie Calmette-Guerin |
| BL3 | biolevel-3 |
| bp | base pair |
| CCT | comparative cervical tuberculin test |
| CFU | colony forming unit |
| CD | cluster of differentiation |
| CI | confidence interval |
| CMI | cell mediated immunity |
| CON | control animals |
| DC | dendritic cell |
| DCPAH | Diagnostic Center for Population & Animal Health |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside triphosphates |
| DMU | deer management unit |
| DTH | delayed type hypersensitivity |
| EB | ethidium bromide |
| γδ-T | gamma-delta T cell |
| GM-CSF | granulocyte macrophage-colony stimulating factor |
| HD | high dose |
| H&E | hematoxylin and eosin |
| HEPA | high efficiency particulate air |
| HIV | Human Immunodeficiency Virus |
| HLA | human leukocyte antigen |
| HPF | high power field/ 40X |
| IFN-γ | interferon gamma |
| IL | interleukin |
| IN | intranasal |
| IP | intraperitoneal |
| IS | insertion sequence |
| IT | intratracheal |
| IV | intravenous |
| LAM | lipoarabinomannan |
| LD | low dose |
| LCM | laser capture microdissection |
| LN | lymph node |
| LT | leukotriene |

| | |
|---|---|
| MAIC | *Mycobacterium avium-intracellulare* complex |
| MDCH | Michigan Department of Community Health |
| MDNR | Michigan Department of Natural Resources |
| MHC | major histocompatibility complex |
| MOTT | mycobacteria other than tuberculosis |
| MSU | Michigan State University |
| M Tb | *Mycobacterium tuberculosis* |
| NADC | National Animal Disease Center |
| NK | natural killer T cell |
| NO | nitric oxide |
| Nramp | natural resistance associated macrophage protein |
| OMIM | Online Mendelian Inheritance in Man |
| PCR | polymerase chain reaction |
| PGL | phenoglycolipid mycosides |
| PI | post-inoculation |
| PPD | purified protein derivative |
| PZA | pyrazinamide |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| RNI | reactive nitrogen intermediates |
| ROI | reactive oxygen intermediates |
| SB | sodium borate |
| Scl11a1 | solute carrier family 11 member 1 |
| SQ | subcutaneous |
| *sst* | susceptibility to tuberculosis |
| TACO | tryptophane aspartate-containing coat protein |
| TB | tuberculosis |
| TBE | tris-borate/trisethylenediaminetetraacetic-acid (EDTA) |
| TCH | thiopen-2-carboxylic acid hydrazide |
| TE | tris-ethylenediaminetetraacetic-acid (EDTA) |
| TLR | toll-like receptor |
| TM | transmembrane |
| TNF-α | tumor necrosis factor α |
| VDR | vitamin D receptor |
| WTD | white-tailed deer |

# CHAPTER ONE

# LITERATURE REVIEW

## History of bovine tuberculosis

Robert Koch first described *Mycobacterium bovis*, the causative agent of bovine tuberculosis in 1882 in the seminal article of the tubercle bacillus isolated from human and animal sources. Theobald Smith in 1898 was further able to demonstrate predictable, small but definite differences between strains of tubercle bacilli of bovine and human origin. Although it was common to refer to tubercle bacilli of bovine origin as *M. bovis* this name was not legitimized until 1970 (Karlson and Lessel, 1970)

## Bovine tuberculosis in the United States (USA)

During the early 1800s and early 1900s, bovine tuberculosis was the most prevalent infectious disease of cattle and swine in the USA, causing more losses among farm animals than all other infectious diseases combined. The National Cooperative State-Federal Bovine Tuberculosis Eradication Program was initiated in 1917 by the United States Department of Agriculture (USDA) and has brought the USA close to eradicating bovine tuberculosis (Montali et al, 2001; Pritchard, 1988).

The regulatory agency in the USA with oversight for bovine tuberculosis eradication was known as the Bureau of Animal Industry up to 1955 when it was then changed to the Animal Disease Eradication Division until 1972. The agency is now referred to as the Animal and Plant Health Inspection Services (APHIS).

Area testing was the main method of testing during the first 50 years of the eradication program. It involved tuberculin testing of 15% of a State's cattle herds each

year, thus during a 6 year accreditation period all of the State's cattle herds should have been tested at least once. By 1940, all states had achieved Modified-Accredited State status, meaning that the prevalence of bovine tuberculosis did not exceed 0.5% in any State. Over the first 50 years, the prevalence of bovine tuberculosis in the United Sates had decreased from 55% to less than 0.3% (Essey and Koller, 1994). The national prevalence of bovine tuberculosis in infected herds in the United States was estimated to be 0.0011% in 2003 (USAHA, 2003).

In 1965, the program emphasis shifted to a slaughter surveillance policy whereby animals with lesions of tuberculosis detected at slaughter where traced back to the herd of origin. All animals in the herd of origin were then tested for tuberculosis. Extensive epidemiological investigations of infected herds were then carried out to locate all other herds that may have been exposed to animals in the source herd and to identify the origin of their infection (Frye, 1995).

**Agent**

*Mycobacterium bovis* is a member of the *Mycobacterium tuberculosis* (M Tb) complex that includes *M. tuberculosis*, *M. bovis*, attenuated *M. bovis* BCG (bacillie Calmette-Guerin) strains, *M. africanum*, *M. microti and M. canettii* (Brosch et al., 2002). It is widely accepted in both human and veterinary medicine that tuberculosis refers to infection with any one of the members of the M Tb complex. Mycobacteriosis is usually reserved for infection with mycobacteria that are not members of the M Tb complex [mycobacteria other than the M Tb complex (MOTT)], *M. leprae*, or *M.*

*lepraemurium* (Hines et al., 1995). Atypical mycobacteriosis refers to infection with species other that *M. bovis* and *M. tuberculosis* (Dungworth, 1993).

The genus *Mycobacterium* in which members of the M Tb complex are included encompasses about 85 species. Clinically mycobacteria can be subdivided in to one of three principal groups: 1) strict pathogens e.g. *M. tuberculosis*, *M. leprae*, *M. bovis* 2) opportunistic pathogens e.g. *M. simiae*, *M. avium*, *M. xenopi* and 3) rare pathogens e.g. *M. smegmatis*, *M. phlei* (Rastogi et al., 2001).

Speciation of mycobacteria is usually a challenging and time consuming exercise that often requires submission of samples to a diagnostic laboratory that specializes in such tests. Currently available methods for detection of mycobacterial species include but are not limited to: i) PCR, ii) nucleic acid probes, iii) gas liquid chromatography (GLC) and high-performance liquid chromatography (HPLC), iv) thin layer chromatograph, v) growth rate, vi) colony morphology, and vii) biochemical tests (Hines et al., 1995). The latter three tests will be further addressed in the following section.

**Morphology, staining characteristics and biochemical test reactions**

Mycobacteria are non-spore-forming nonmotile slender bacilli, 0.5 to 1.5μm in length, with a lipophilic waxy coat that resists regular staining with Gram stain but confers acid fastness (Rastogi et al, 2001; Samuelson, 1999; Hines et al, 1995; Timoney et al, 1988). Such bacteria were formerly thought to be strictly aerobic but *M. bovis* is now known to be microaerophilic. Acid fast indicates that mycobacteria are routinely stained with hot carbol dyes, usually carbol-fuchsin, and then resist decolorization by

acid alcohol. The mycolic acids in the cell wall enable mycobacteria to resist decolorization. Mycobacteria then appear red against a methylene blue counterstain (Dungworth, 1993). Additionally, mycobacteria can be stained with fluorescent dye and auramine-O is commonly used. Auramine-O, when combined with rhodanine is the procedure of choice for clinical specimens. Fluorochrome staining is more sensitive as mycobacteria are visualized at 25X objective whereas carbol-fuchsin techniques require oil-immersion 100X objective (Zheng and Roberts, 1999).

Growth characteristics, colony morphology and biochemical tests of the three species recognized to cause most disease in humans and animals, namely *M. tuberculosis*, *M. bovis* and *M. avium* are further defined. Each of these species is considered to be slow growing (mean division time of 12 h to 24 h, with a fully-grown culture requiring approximately 15 to 28 days). Colonies of the three species are not pigmented. However, *M. bovis* colonies on solid media are sparse (dysgenic) whereas *M. tuberculosis* is luxuriant (eugenic). Characteristics of these species demonstrated with select biochemical tests are listed in Table 1:1.

**Transmission**

*M. bovis* is reported to survive for variable periods on inanimate objects, soil, feces, urine and pasture if exposure to direct sunlight is limited. However, as the size of the minimum infective dose for oral infection is high, it is unlikely that the persistence of bacilli on fomites plays a significant part in transmission (Phillips et al,. 2003; Menzies and Neill, 2000; Morris et al., 1994).

In cattle, the aerosol route is currently accepted as the most frequent route of transmission between cattle. *M. bovis* is found to survive in water and can, therefore, enter the respiratory tract during drinking (Phillips et al., 2003). Spread to calves ingesting milk from tuberculous udders formerly factored significantly in transmission, but due to improved animal management practices and eradication programs in developed countries this mode is less common. However, in humans in developing countries spread of *M. bovis* to man via ingestion of raw or unpasteurized milk from infected animals remains a problem. Historically lesions on the penis of bulls and/or the external genitalia of cows also contributed to the spread of infection (Neill et al., 1994).

In pigs, oral transmission of tuberculosis remains the principal route of infection. Such animals are exposed via consumption of milk or milk products, kitchen and slaughterhouse scraps and excreta of tuberculous cattle (Morris et al, 1994)

Wildlife such as the brushtail possum (*Trichosus vulpecula*) in New Zealand and the badger (*Meles meles*) in Great Britain and the Republic of Ireland also serve as reservoirs of infection for domestic animals. Reservoir or maintenance hosts are those animals in which infection persists through horizontal transmission between individuals in the absence of any other source of *M. bovis* from either domestic or wild animals (de Lisle et al., 2001; O'Reilly and Daborn, 1995; Morris et al., 1994 ). In both the badger and possum, maintenance of infection within a local population is due to pseudo-vertical transmission (between birth and independence) from mother to young and horizontal transmission linked to breeding activity (Morris et al, 1994). The majority of spread within wildlife is via the airborne route. Similar to human tuberculosis it is thought that transmission by environmental contamination does not contribute to the

spread of infection (Menzies and Neill, 2000; Morris et al., 1994). An important exception is pasture contamination by excretion in badger urine.

Transmission among badgers is primarily via the respiratory route with a high component of pseudovertical transmission from mother to cub as well as via bite wounding in males. However, transmission from badger to cattle is due primarily to contamination of pasture by urine of tuberculous badgers with kidney lesions in addition to sputum, feces and discharges from bite wound fistulas (Gallagher and Clifton-Hadley, 2000).

Brushtail possums spread large numbers of *M. bovis* through draining open cutaneous lesions or the respiratory tract. Surface contamination of the pasture is not thought to be a factor in the spread from infected possums to cattle. Cattle that show much exploratory behavior are thought to be infected by contact with possum carcasses. Transmission from mothers to dependent joeys while in the pouch is another effective means of spreading infection (O'Reilly and Daborn, 1995; Morris et al., 1994).

Transmission to spillover wildlife hosts primarily occurs by scavenging of infected carcasses and predation. In spill-over hosts infection occurs sporadically when the challenge level is high and can only persist in such populations when a true maintenance host is present (de Lisle et al., 2001; O'Reilly and Daborn, 1995; Morris et al., 1994).

Reports to date clearly indicate that *M. bovis* is pathogenic for humans, but its pathogenicity may be less than that of *M. tuberculosis* (Grange, 1995). Zoonotic tuberculosis due to infection with *M. bovis* is currently more of a problem in developing countries where pasteurization is less often practiced (Grange, 2001). Such countries

also do not have the infrastructure in place to diagnose and monitor infection in domesticated animals (Cosivi et al, 1998)

Human to human transmission of *M. bovis* has been documented in HIV-positive individuals (Houde and Dery, 1988). Human to animal transmission, although rarely documented has been reported in the Netherlands, Germany, England, Sweden, New Zealand, Canada, Czechoslovakia and the USA (Grange, 1995; Pritchard, 1988). Transmission from humans to animal is usually via the respiratory route, although urinary tract infection (renal tuberculosis) in humans has also been implicated (Grange and Yates, 1994). This is a contentious issue as Dankner et al, 1993 state that most human tuberculin reactors do not develop progressive disease with *M. bovis* and are, therefore, incapable of transmitting disease.

The clinical presentation and severity of disease in humans infected with *M. bovis* is similar to that of *M. tuberculosis* (Collins, 2000; Moda et al., 1996). Scrofula or cervical lymphadenitis has been associated with drinking unpasteurized milk from cows with bovine tuberculosis (Ashford et al., 2001). Although 5% of human cases of tuberculosis were caused by *M. bovis* in children less than 5 years old this increased to 30% (Pritchard, 1988). However, it is not currently known what the frequency and severity of pulmonary disease is in humans who have reactivation of *M. bovis* later in life after an initial infection in childhood.

When contaminated cow's milk was eliminated as the primary vector for human infection, the distribution of anatomical sites of disease changed significantly. The lung became the predominant site of disease; genitourinary disease became relatively more common while lupus vulgaris became rare (Smith, 2003; Grange, 1995)

*Mycobacterium bovis* is resistant to isoniazid, thus it is important to determine the cause of tuberculosis in humans before treatment is initiated. In addition, detection of *M. bovis* in humans may act as a sentinel for infection in cattle.

**Pathogenesis**

The two most common portals of entry for *M. bovis* are respiratory and oral. However, the respiratory route is predominant in both humans and most animals (O'Reilly and Daborn, 1995; Neill et al., 1994).

The currently accepted pathogenesis of *M. bovis* in mammals is based on the rabbit model (Dannenberg, 2001). With inhalation of bacilli as the route of introduction, *M. bovis* organisms are first exposed to pulmonary alveolar macrophages (AM). Alveolar macrophages engulf the 0.5 x 2 μm bacilli. Multiplication of the bacilli occurs within the AM and the site of infection develops into a granuloma. It is at this stage that the infection can follow one of two divergent routes, delayed type hypersensitivity (DTH) or cell mediated immunity (CMI). Caseous necrosis occurs in DTH with liquefaction of the granuloma and dissemination of mycobacteria via blood or lymphatics. Direct implantation (extension) into adjacent tissues can also occur from the initial focus. In an immunocompetent host bacilli are destroyed. Alternatively in a susceptible host further multiplication occurs ultimately resulting in death. .With CMI, however, the caseous center is surrounded by activated macrophages which ingest and destroy bacilli at the periphery. Thus, the disease is arrested.

With infection via ingestion humans are usually exposed via contaminated milk. Animals may also be infected via milk (dam to offspring), contaminated foliage in herbivorous species, or via infected carcasses/viscera in carnivores. Lesions detected in orally acquired infection generally occur within the gastrointestinal tract (tonsils, mesenteric lymph nodes, liver, hepatic lymph nodes).

The ability of mycobacteria to survive and multiply intracellulary within macrophages is instrumental in their pathogenicity. The mycobacterial phagosome interacts with early endocytic compartments but does not mature into or fuse with lysosomes. This process is referred to as the *M. tuberculosis* phagosome maturation arrest or inhibition of phagosome-lysosome fusion (Vergne et al., 2004). Therefore mycobacteria not only avoid destruction within the lysosomes, but also avoid the generation of antigenic peptides in the endosomal compartments. By hiding within a mycobacterial phagosome, mycobacteria not only escape from the immediate antimicrobial macrophage host response but also interfere with the induction of adaptive immunity (Pieters and Gatfield, 2002; Russell, 2001).

Cholesterol is essential for the entry of mycobacteria into macrophages. In studies using macrophages which had their plasma membrane depleted of cholesterol such macrophages were unable to internalize mycobacteria (Gatfield and Pieters, 2000). The exact reason why cholesterol-depleted macrophages fail to phagocytose mycobacteria is currently unknown (Pieters and Gatfield, 2002)

Complement receptors are among the most widely used receptors for mycobacteria, although they may also use mannose receptors, Fc receptors and scavenger receptors. With the uptake of mycobacteria via complement receptors there is no upregulation of

bactericidal mechanisms. Thus, uptake via the complement system ensures that mycobacteria enter macrophages under relatively non-lethal conditions (Pieters and Gatfield, 2002)

The mycobacteriocidal ability of the macrophage depends on several factors including the virulence factors of the organism, immunologic factors and genetic factors of the host (Hines et al., 1995)

**Lesions**

The granuloma, the primary lesion seen with *M. bovis* infection and other *Mycobacterium* spp infection is formed by the host in an attempt to wall off or limit the spread of infection. Granulomas in tuberculosis also localize inflammatory and immune responses to the site of infection (Lagrange, 1984). Collins (1999) defines a granuloma as a focal area of granulomatous inflammation, which consists of a microscopic aggregation of macrophages that are transformed by cytokines such as Il-2 produced by T cells into epithelium-like cells which are surrounded by a collar of mononuclear leukocytes, principally lymphocytes and occasionally plasma cells. Granulomas in tuberculosis are referred to as tubercles when there is central caseous necrosis, resulting from the destructive effects of delayed type hypersensitivity (DTH). The word "tuberculosis" is derived from the Latin word "tuberculum" which means a lump or nodule. Such nodules are synonymous with tubercles (Hines et al., 1995). Caseous necrosis refers to the loss of cellular detail centrally with replacement by amorphous eosinophilic granular debris admixed with acid-fast bacilli.

Macrophages infected with mycobacteria produce TNF-α which in concert with IFN-γ produced by lymphocytes induces a chemokine gradient at the site of infection which causes migration of cells (monocytes/macrophages, T and B lymphocytes) to the infected macrophages, and a granuloma forms (Scott Algood, 2003) In addition, addressins, selectins and integrins are important in the recruitment, migration and retention of cell to and within the granuloma, that is granulomas are not static (Saunders and Cooper, 2000; Collins, 1999)

*Mycobacterium bovis* has a broad host range (O'Reilly and Daborn, 1995). Manifestation of disease following *M. bovis* infection shows subtle to significant variation according to the species infected. Lesions in cattle display typical caseated granulomas which may calcify. Cavitations may develop in cattle but are a more prominent feature of tuberculosis in humans. In swine, lesions are similar to those in cattle. Although infection is rare in cats and horses, when it does occur lesions are more indicative of a sarcoma and this may lead to misdiagnosis. Cavitation due to liquifactive necrosis often develops in these sarcoma-like lesions in dogs. In nonhuman primates, primarily Old World species, lesion development is similar to that of humans (Hines et al., 1995, Timoney et al., 1988).

**Virulence**

Although *M. tuberculosis* and *M. bovis* were identified by Koch as the respective causative agents of human and bovine tuberculosis over a century ago, to date, much remains unknown about virulence factors utilized by these microorganisms. Virulence factors in mycobacterial species incorporate those features which enable them to

survive, multiply and cause disease in the animal host (Collins, 2001). More specifically, mycobacterial species have demonstrated a remarkable ability to survive in diverse conditions encountered during the infection process. These include, but not limited to, surviving the bactericidal stresses within the macrophage, the anaerobic and nutritionally altered environment of the granuloma, and the metabolically inactive latent state (Mehrotra and Bishai, 2001)

The majority of known virulence factors occurs within the cell wall and includes:

i. **phenoglycolipid mycosides (PGL-1)** which suppress monocyte oxidative burst and are oxygen radical scavenger

ii. **cord factor** (trehaloes-6,6'-dimycolate TDM) which is a potent initiator of the innate and acquired immune response. Specifically, it inhibits the migration of neutrophils and is involved in the depression of microsomal enzymes and glycogen synthesis. It also induces granuloma formation, inhibits phagosome-lysosome fusion, decreases TNF secretion, increases IL1-β secretion, induces leakage of liposomes and is leukotoxic

iii. **sulfatides** are the component of the mycobacterial wall which promotes survival of virulent tubercle bacilli within macrophages by inhibiting phagoloysosme formation. Sulfatides have also been found to inhibit phosphorylative oxidation in mitochondria (Thoen and Himes, 1986; Brubaker, 1985)

iv. **mycobactins** and **exochelins** are iron chelators. It had been proposed that exochelins bind iron in the extracellular aqueous environment and transport

the metal ion to mycobactin, another molecule in the mycobacterial cell wall, which facilitates transport of iron into the cell cytoplasm

v. **lipoarabinomannan (LAM)** is a potent oxygen radical scavenger, increases secretion of TNF and inhibits protein kinase C. Activation of macrphages by IFN-γ and induction of nitric oxide are inhibited by treatment with LAM which results in decreased microbicidal activity. LAM also induces IL-8, a chemotactic factor for neutrophils that may be involved in the initiation of granuloma formation (Quinn et al., 1996).

vi. **macrophage inhibitory factor A3 (MIF-A3)** scavenges oxygen radicals and induces interleukin 6 mRNA expression

Research is ongoing to identify the genes and proteins involved in the virulence of mycobacteria as in hopes of providing new bacterial targets which can be utilized in creating vaccines and chemotherapeutic agents, as well as for use in the development of more selective diagnostic reagents (Smith, 2003). Such studies are *in vitro* utilizing tissue culture (macrophages, dendritic cells and pneumocytes) or *in vivo* where the three main animal models used are mice, guinea pigs and rabbits.

Although several genes have been identified as being important in the pathogenesis of tuberculosis, their role in virulence is less well known. The slow generation rate of *M. tuberculosis* and *M. bovis* is identified as a limiting factor in the elucidation of the role of such genes in the virulence of tuberculosis. The BCG vaccine strain of *M. bovis* is however known to lack several virulence genes. The Rv3875 gene which encodes ESAT 6 in RD1 of this particular strain is implicated in virulence. Mutations in this gene reduce virulence and specifically reduce the ability of the organism to spread

locally and to lyse pneumocytes (Hsu et al., 2003). Transgenic strains of BCG in which the RD1 is reconstituted regain virulence.

**Immunity**

The interacting network of immune responses to mycobacteria is complex. The macrophage is the principal effector cell of protective innate immunity whereas the T lymphocyte of various subtypes is the major inducer of the protective acquired immune response (Kaufmann, 2002; Skinner et al, 2001). Cross talk between T cells and macrophages is achieved by various cytokines (IFN-γ, TNF-α and LT-α). This cross talk leads to macrophage activation and granuloma formation (Kaufmann, 2002).

**Macrophages**

*Mycobacterium bovis* can multiply in resting macrophages but is killed in activated macrophages. Activation of macrophages is effected by cytokines and results in increased size, increased subcellular organelles, ruffled cell membranes, and enhanced phagocytic and microbicidal activities including production of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and lysosomal enzymes (Monack et al., 2004; Cosma et al., 2003; Dannenberg 1999 and 1991). Activated macrophages are able to destroy small numbers of bacilli but are overpowered by bacilli in large numbers. Thus, macrophages are involved in both the control (destruction) and dissemination of tubercle bacilli.

In the mouse model of tuberculosis, IFNγ and TNF-α act synergistically in the production of nitric oxide (NO) and RNI by macrophages via the action of the inducible form of nitric oxide synthase (NOS2 or iNOS) (Flynn and Chan, 2001; Stenger and

Modlin, 1999) However, mycobacteria may inhibit NOS2 recruitment to phagosomes during macrophage infection (Miller et al., 2004). Nitric oxide in association with IFNγ can limit damaging immunopathological consequences of chronic mycobacterial infection (Copper et al, 2002). In humans, NO is one of the few host products which is known to be directly toxic to mycobacteria at clinically significant concentrations (Cosma et al, 2003; Long et al., 1999; Nozaki et al., 1997). However, the role of NO in humans affected with tuberculosis remains unclear (Rook et al., 2001).

Tubercle bacilli produce large amounts of ammonia within lysosomal vacuoles which limits phagolysosome fusion and diminishes the potency of the intralsosomal enzymes via alkalinization. In this way mycobacteria are able to evade killing in lysosomal vacuoles (Russell et al, 1996; Schaible, 1998; Flynn and Chan, 2001). Additionally, it has been found with in vitro studies of mouse macrophages that tryptophane aspartate-containing coat protein (TACO), a protein found in phagosomes containing living mycobacteria, also facilitates the interruption of the fusion of phagososme with lysosomes (Flynn and Chan, 2001; Ferrari et al, 1999).

Cell surface reactors present on macrophages also influence the fate of engulfed mycobacteria. In *M. tuberculosis* infection interactions with the constant regions of immunoglobulin receptors (Fc receptors- FcRs) and Toll-like receptors stimulate the host defense mechanisms whereas those with complement receptors (CR3) promote mycobacterial survival (Kaufmann, 2001; Thoma-Uszynski, 2001; Brightbill, 1999)

**T cell response**

The various types of T lymphocytes which are implicated in the induction of the protective acquired immune response in tuberculosis include CD4, CD8, γδ and CD1

restricted T cells. The role of the various T cell populations in control of mycobacterial infection may vary in different animal species (Wedlock, 2002).

**CD4**

The importance of this T cell subset in controlling acute mycobacterial infection was confirmed with numerous experimental models utilizing antibody depletion and knockout mouse strains deficient in CD4 or MHC class II (Collins and Kaufmann, 2001). Two functionally different subsets of CD4 T helper cells are recognized. The Th1 subset produces the cytokines interleukin-2 (IL-2) and IFN-γ whereas the Th2 subset produces Il-4 and Il-5. The Th1 subset is associated with CMI and is therefore important in immunity to tuberculosis. Mycobacterial antigens are presented to CD4 T cells via MHC class II. Under the influence of IL-12 and Il-18 from the antigen presenting cells (APCs) (e.g. macrophages and dendritic cells), CD4 T cells producing a Th1 type immune response are stimulated (Skinner et al., 2001). CD4 T cell then produce IFNγ in response to the wide variety of mycobacterial antigens. IFNγ produced by these cells activates the macrophage to enhance the ability to kill the mycobacteria that they harbor (Stenger and Modlin, 1999).

Recent research has indicated that CD4 T cells also mediate IFN-γ independent control of *M. tuberculosis*. Such processes occur via a NO-dependent mechanism and are important during secondary response to *M. tuberculosis* and in preventing the reactivation of tuberculosis (Cowley and Elkins, 2003).

Another important function of CD4 T cells (the long-lived portion) is to mediate immunological memory (Skinner et al., 2001).

**CD8/CD 1/ γδ T cells**

The role of CD8 cells in tuberculosis is less well understood, however, such cells appear to be more involved in controlling the chronic phase of the disease or latent infection (Skinner et al, 2001). In spite of the intracellular location of mycobacteria within phagosomes, it is known that CD8 T cells are required for a successful immune response against the organism (Collins and Kaufmann, 2001).

CD8 T cells are split into Tc1 and Tc2 subsets based on the cytokines they secrete. Tc1 cells secrete Il-2, IFNγ and TNF-α, whereas Tc2 cells secret Il-4, IL-5 and Il-10. The Tc1 phenotype, which is similar to a Th1 phenotype, is the predominant type of CD8 T cell (Smith and Dockrell, 2000).

CD8 T cells in mycobacterial infections are both classically (MHC I) and nonclassically (CD1) restricted. MHC class I molecules present peptide antigens, whereas CD1 presents lipids or glycolipids which are abundant in the mycobacterial cell wall to T cells (Schaible and Kaufmann, 2000). The mechanism by which mycobacterial proteins gain access to the MHC class I molecules is not fully understood as this pathway is usually reserved for those pathogens that reside in the cytoplasm of affected cells (Kaufmann, 2002; Flynn and Chan, 2001). However, classically presented antigens result in the activation of antimycobacterial effects such as IFNγ production and lysis of infected cells by perforin and granzymes or via the Fas/FasL pathway (Stenger and Modlin, 1999). Likewise, the way in which mycobacterial glycolipids are presented by CD1 molecules is also not entirely known (Kaufmann, 2002). However, saposins which are cofactors for enzymatic sphingolipid

hydrolysis have recently been shown to promote the loading of glycolipids onto CD1 molecules (Kaufmann, 2004).

CD1 molecules are divided into two groups. Group I includes CD1a, b and c, whereas CD1d is the only member of group II currently identified. Mycobacterial lipid containing antigens that have been reported to be presented by group I CD1 include mycolic acid, LAM, phosphatidylinositol mannoside, glucose monomycolate and isoprenoid glycolipids. CD1b surface expression is downregulated in cells infected with *M. tuberculosis* (Collins and Kaufmann, 2001; Kaufmann, 2001). To date, no bacterial antigens presented by group II CD1 molecules have been identified. In mycobacterial infections, several different T-cell subsets have been found to interact with CD1, including $CD4^-$ $CD8^-$ (double-negative) T cells, $CD4^+$ or $CD8^+$ single positive cells and γδ T cells (van Crevel et al., 2002)

Gamma delta (γδ) T cells are primarily localized to mucosal surfaces and are therefore most prominent in organs directly exposed to antigens from outside environments e.g. lungs, intestine and skin. Although their precise role in mycobacterial infections is not completely known such cells are capable of producing IFN-γ and are also a rich source of cytokines (IL-2). The γδ cells are thought to be involved in influencing the local cellular traffic of lymphocytes and monocytes and limiting access of inflammatory cells that may cause tissue damage (Skinner et al., 2001). Recently it was proposed that γδ T cells may have little to no effect in immunity to tuberculosis, as gene-deleted mice were identical to wild type mice in their ability to control infection and survive (Mouges et al., 2001).

The higher proportion of circulating γδ cells in ruminants is suggestive of a greater role in the protective immune response against bovine tuberculosis than that seen in other species (Wedlock et al, 2002)

The role of humoral immunity (antibodies, B cells) in the control of infection with *M. bovis* is not well defined at present it may be of limited importance. Antibodies develop at a late stage in bovine tuberculosis and are associated with disease progression (Pollock and Neill, 2002)

**Cytokines**

As previously stated the cytokines which are of primary importance in immunity to tuberculosis are those secreted by Th1 type $CD4^+$ T cells and activated macrophages. Although both NK cells and $CD8^+$ T cells are also capable of secreting these cytokines, they are of lesser importance. Cytokines of importance include interleukin-1 (IL-1), Il-2, Il-12, Il-18 and IFNγ. IL-1 is produced by activated macrophages and many other cell types. It is important in the acute phase response- (e.g. fever and cachexia) and also facilitates T lymphocyte expression of IL-2 receptors and IL-2 release.

The type 1 cytokines (IL-2, IL-12, IL-18 and IFNγ) all have essential roles in cell mediated immunity (CMI). Il-2 has a role in inducing expansion of the pool of lymphocytes specific for an antigen and aids in the multiplication of T cells and the generation of memory cells. Interleukin-12 which is produced by antigen-presenting cells (macrophages and dendritic cells), is the principle mediator of cell-mediated immunity (CMI) and DTH. It can augment both the granulomatous response and protection against mycobacterial infection. Additionally, IL-12 can stimulate production of IFNγ, TNF-α and GM-CSF which activate macrophages, NK cells, and

naïve Th cells into Th1 cells (van Crevel et al., 2002; Kobayashi et al., 2001; Jullien et al, 1997).

IFNγ is central to many of the effector functions which are activated upon mycobacterial infection. Its many functions include stimulation of the production of ROI and RNI by macrophages, increasing the expression of class II molecules resulting in increased antigen presentation and upregulation of the expression and secretion of TNF from macrophages. IFNγ is essential to the host as it controls bacterial growth. Thus once macrophages are activated with IFN-γ they negate the block that the bacterium exerts over phagosome maturation and acidify the vacuoles to ~ pH 5.2 (Russell, 2001). IFNγ can also mediate anti-inflammatory effects by preventing excessive cellular influx and T cell proliferation and/or activity at the site of antigen deposition (Copper et al, 2002).

Humoral immunity is not protective against tuberculosis. However three of the cytokines produced by Th2 cells do have an important regulatory role in immunity to tuberculosis. IL-10 which is produced by macrophages and T cells down regulates IL-12 production, which leads to a decrease in IFNγ production. It also down regulates production of TNF-α. IL-10 also directly inhibits $CD4^+$ T cell responses and inhibits the APC function of cells infected with mycobacteria. Il-4 and Il-13 also down regulate the Th1 response.

In mycobacterial infections TNF-α is important for walling off infection and preventing the dissemination of mycobacteria as it affects cell migration to and localization within the granuloma. It is therefore effective in the acute response (Skinner et al., 2001). Thus as with many other infections, the production of TNF-α

must be finely balanced, as its overproduction leads to increased cellular accumulation which compromises lung function and exacerbates tissue damage (Collins and Kaufmann, 2001)

**CMI**

Cell mediated immunity may be defined as a beneficial host response characterized by an expanded population of specific T lymphocytes (Th1) that, in the presence of microbial antigens, produce cytokines locally. Interferon gamma (IFN-γ) and IL-2 produced by these Th1 lymphocytes are critical in the production of CMI. In CMI, activated macrophages are able to kill and destroy bacilli which were ingested, that is the bacilli are destroyed intracellularly (Dannenberg, 1993; Danneberg, 1999). The macrophages which are activated are the surrounding or perifocal macrophages. The now activated perifocal macrophages ingest and destroy the bacilli escaping from the edges of the caseous center and released from the dead bacilli-laden macrophages. Cell mediated immunity is indicated in tuberculosis as the pathogen is intracellular, primarily residing in macrophages, thus humoral immunity mediated by antibody production would be ineffective.

Since the main function of CMI is to activate the surrounding uninvolved macrophages, it serves a protective function in immunity to tuberculosis by limiting the spread of infection in the involved tissue, usually the lung. Thus, CMI limits the spread of tubercle bacilli by activating macrophages to kill the bacilli that they ingest (Dannenberg, 1999)

**DTH**

Like CMI, tissue damaging delayed type hypersensitivity (DTH) is also mediated by Th-1 type lymphocytes. Unlike CMI however, macrophages in DTH remain inactivated. Delayed type hypersensitivity is of importance in tuberculosis as it is the means whereby bacilli-laden macrophages, which multiply during the log phase of bacterial growth, can be destroyed. Killing of bacteria in this way results in the formation of the caseous center of the tubercle. Thus in DTH, bacilli are inhibited extracellulary within the necrotic debris (Dannenberg, 1993 and 1991). The destruction of tubercle bacilli via DTH occurs at the expense of host tissues. In a susceptible host, bacilli escaping from the edge of the lesion are again ingested by nonactivated macrophages where they are able to multiply. The DTH response will again destroy the bacilli-laden macrophages and the adjacent tissues. If prolonged, this cycle will ultimately result in destruction of so much viable lung tissue that it results in death of the host (Dannenberg and Rook, 1994)

Delayed type hypersensitivity leads to pathologic responses such as granulomatous inflammation, calcification, caseous necrosis and cavity formation (Kobayashi et al, 2001). Release of cytotoxic factors and hydrolytic enzymes from macrophages in combination with the release of tuberculin-like antigens from the bacillus in excessive concentrations is principally responsible for the caseation necrosis characteristic of many tuberculous lesions (Dannenberg, 1999; Dungworth, 1993).

The currently approved *in vivo* diagnostic skin test for tuberculosis is based on the DTH reaction (Adams, 2001). This antigen specific test is facilitated by memory T cells. In the caudal fold test purified protein derivative from *M. bovis* (PPD-B) is

injected subcutaneously. Results are read after 48 to 72 hours. In any positive animals or 'suspects' the comparative cervical test is done within 7 days where PPD-B and PPD-A (*M. avium*) are both injected subcutaneously (Adams, 2001).

Due to the limitations of the standard skin test and the comparative cervical research is ongoing to identify in vivo tests with better sensitivity and specificity. BOVIGAM™ an in vitro cellular diagnostic is the best known. BOVIGAM™ is based on the detection of IFN-γ. Whole blood from suspect cattle is incubated overnight with bovine PPD, avian PPD or negative control antigens. Interferon γ in the supernatant plasma is then measured by enzyme immunoassay (EIA) (Wood and Jones 2001). Development of ancillary tests is also encouraged.

A major drawback of both DTH and CMI in immunity to tuberculosis is that they can exert their influence only locally and not systemically. This is of importance in the development of new vaccines for tuberculosis.

**Apoptosis**

The role of apoptosis in mycobacterial infections remains controversial. Although some researchers hold the view that macrophages infected with mycobacteria can be removed via apoptosis (Kaufmann, 2001; Oddo et al, 1998) others have queried whether apoptosis is indeed required (Stenger et al, 1997; Palmer et al., 2002[a]). Currently research indicates that apoptosis may play a role in the immunity to tuberculosis via CD8 T cell activation. Apoptotic blebs from infected macrophages containing pathogen-derived antigens are subsequently engulfed by uninfected professional APC or dendritic cells. The dendritic cells utilizing MHC1 and CD1 on

their surface can then present microbial antigens to CD8 T cells (Winau et al., 2004; Schaible et al., 2003).

**Mycobacteriosis in birds**

Historically avian tuberculosis is the term used to refer to infection with *Mycobacterium avium* in birds. However, mycobacteriosis is the term now preferred to refer to disease in birds infected with members of the genus *Mycobacterium*. There is also the risk of zoonosis as MAI and *Mycobacteriun genavense* can cause disease in humans, usually immunocompromised patients (AIDS, young children).

The identity and function of avian cytokines involved in mycobacterial immunity remain undetermined (Tell et al., 2001). As with lesions seen in mammals with mycobacterial infection, the granuloma is also present in birds with mycobacteriosis (Montali, 1988).

***Mycobacterium avium-intacellulare* complex (MAIC)**

This complex includes *M. avium* subspecies *avium, M. avium* subsp. *paratuberculosis, M. avium* subsp *silvaticum* and *M. intracellulare. Mycobacterium avium* subsp *paratuberculosis* the causative agent of Johne's disease in ruminants and *Mycobacterium avium* subsp. *silvaticum*, also known as the wood-pigeon bacillus, will not be discussed further here. *Mycobacterium avium* and *Mycobacterium intracellulare* share several growth characteristics and some species-specific agents therefore these species are often grouped together and termed *M. avium-intracellulare* complex. The classical *M. avium* serotypes 1-3 are far more pathogenic for birds than are those of *M.*

*intracellulare* (Montali et al., 1976). *M. intracellulare* appears to be minimally pathogenic for birds (Tell et al 2001). Interestingly, whereas birds are susceptible to *M. avium* serovars 1-3, they are very resistant to infection with *M. bovis* and *M. tuberculosis* (Thoen and Chiodini, 1993).The orders of birds that appear to be most susceptible to mycobacteriosis in zoological collections are Anseriformes, Gruiformes and Galliformes(Tell et al, 2001)

In any avian species, susceptibility to mycobacterial infection probably increases as the intensity or number of stressors increases. Stressors include but are not limited to malnutrition, overcrowding, adverse environmental conditions, pinioning and concurrent disease (Tell et al, 2001)

Infected birds, which are shedding *M. avium* via the feces, are a principle source of infection for other birds. Clinically affected pet birds can present with weight loss, depression, diarrhea, polyuria, poor feathering and lameness. Abdominal distention, subcutaneous and conjunctival masses, along with dull and damaged feathers is detected on physical examination (VanDerHeyden, 1997). Lesions are usually limited to the alimentary tract, liver and spleen. Bone marrow and joint involvement is also reported. Birds do not usually have pulmonary lesions with tuberculosis. Mineralization rarely occurs but caseating granulomas are usually present in the liver. Histologically lesions consist of multiple granulomas with central caseous necrosis.

In Ziehl-Neelsen stained sections large numbers of acid-fast bacilli occur in the central necrotic area as well as in the adjacent epithelioid macrophages (Fulton and Theon, 2003, Tell et al 2001, Hines et al 1995).

In domestic mammals, pigs are the most susceptible species to *M. avium* infection. Such animals are thought to be infected via the oral route and infected swine can exhibit clinical disease. Lesions occur primarily in mandibular and mesenteric lymph nodes, the intestinal mucosa, tonsils and liver. Such lesions are generally proliferative, caseogranulomas and tubercles are rare in swine with MAIC infection (Hines et al. 1995, Dungworth 1993, Timoney et al.1988)

The economic impact of MAI infection in domestic animals can be indirect due to transmission of mycobacteria from infected birds to swine and cattle. In cattle, MAI organisms can cause non-specific positive reactions to tuberculin, thus compounding problems in eradication and surveillance programs for bovine tuberculosis (Tell et al 2001).

***Mycobacterium genavense* in birds**

*Mycobacterium genavensae* is a very fastidious organism which does not grow well on conventional solid media but can be isolated in liquid media (Middlebrook 13 or BACTEC 13A) culture (Preheim, 1999). The optimal solid medium for primary cultures is Middlebrook 7H11 acidified to pH 6 and supplemented with charcoal and sheep blood. Colonies are observed within six to twelve weeks of incubation. Added blood and charcoal were not as essential for subcultures (Realini et al 1999).

Sequencing of 16S RNA revealed a specific sequence for *M. genavense* that was used for the formal description of the species. The phylogenetic tree based on 16S RNA sequences showed that *M. genavense* and *M. simiae* are closely related, on the same branch, rooted deeply from the basis of slowly growing mycobacteria. Because of the

fastidious growth of *M. genavense,* molecular methods are especially useful in the identification of the species. In particular, efforts have been made to develop specific probes (Bercovier and Vincent, 2001).

*Mycobacterium genavense* was first identified in 1990, in a human patient with acquired immunodeficiency syndrome (AIDS) (Bottger et al 1992, Hirschel et al 1990). This mycobacterium appears to have a wide geographic distribution and has been isolated from patients in Europe, the United States and Australia (Pechere et al 1995). The source of *M. genavense* infection in humans is unknown, although an intestinal source has been proposed since a variety of uncharacterized, fastidious mycobacteria have been isolated from intestinal tissue and feces. Because the growth requirements of *M. genavense* are unknown, it is not possible to exclude any environmental source (Falkinham, 1996)

*Mycobacterium genavense* is now recognized as the most frequent etiologic agent of avian mycobacteriosis (cutaneous and disseminated) in pet birds, especially in Passeriformes and Psittaciformes (Bercovier and Vincent, 2001; vanDerHeyden, 1997; Hoop et al., 1996).

In birds, the clinical signs associated with the disease due to infection with *M. genavense* are similar to any mycobacteriosis and are not specific i.e. sudden death without any symptoms or following a wasting syndrome and emaciation (severe muscular wasting), acute respiratory distress and sometimes diarrhea (Bercovier and Vincent, 2001; vanDerHeyden, 1997). In general, the evolution of disease is more rapid than that of *M. avium* infection (Bercovier and Vincent, 2001)

Gross post mortem findings are nonspecific. In birds, *M. genavense* infection is associated with an enlarged spleen and liver, and thickening of the intestinal wall, trachea and lungs. If present on internal organs, inflammatory nodules are non-caseous and infiltrated by large macrophages. In the intestine, the mucosal area is generally heavily infiltrated, suggesting an intestinal origin of the infection. Severe muscular wasting, cutaneous nodules and subcutaneous granulonmas can also be observed (Bercovier and Vincent, 2001; Hopp et al., 1996; Kiehn et al., 1996; Portaels et al., 1996; Hoop et al., 1993).

As it was originally isolated from HIV-infected patients, *M. genavense* typically causes disseminated disease in those with advanced AIDS. Clinically the disease mimics that caused by MAI. Spiking fevers, weight loss, diarrhea, anemia, splenomegaly and lymphadenopathy are common, whereas pulmonary symptoms are rare (Preheim, 1999).

### *Mycobacterium tuberculosis* in birds

In contrast to *M.* bovis, *M. tuberculosis* has a more limited host range and does not appear to have an indigenous animal host or reservoir. This conclusion is primarily based on the facts that *M. tuberculosis* does not appear to exist in the wild as an animal pathogen and that animals that do become infected appear to be accidental hosts (Montali et al., 2001). Humans and primates are the only known reservoir hosts for *M. tuberculosis*. Spread of *M. tuberculosis* from humans to psittacines was first described in the 1920's and 1930's (Hinshaw, 1933). As infections with *M. tuberculosis* are reported sporadically in pet psittacines they should always be considered a differential

for masses involving the face and head of a bird (Tell et al., 2001; Washko et al, 1998; Hoefer et al., 1996; Woerpel and Rosskopf, 1983; Thoen et al., 1977; Ackerman et al, 1974). In addition to psittacines, *M. tuberculosis* infection has been reported in Passeriformes (Hoop, 2002). Histologic lesions of *M. tuberculosis* in birds typically include caseous granulomas with giant cells and small numbers of acid-fast bacilli (Montali et al, 2001, Montali et al, 1976). It is unlikely that *M. tuberculosis* infection in birds is of great zoonotic potential (Montali et al, 2001).

Nonhuman primates are also susceptible to infection with *M. tuberculosis*. Infection with this agent has also been reported in captive elephants, dog, cats, swine and rarely cattle (Michalak et al., 1998; O'Reilly and Daborn, 1995).

### *Mycobacterium bovis* in birds

There are no reported cases of naturally acquired infection with *M. bovis* in birds (Pillai et al., 2000; Thoen et al., 1977). However, in experimental studies pigeons, crows and starlings were found to be somewhat susceptible (Fitzgerald et al., 2003[b]; Butler et al., 2001) whereas mallard ducks are resistant (Fitzgerald et al., 2005).

## *Mycobacterium* species infection in rodents

### Voles

Susceptibility of voles to mycobacteria is recorded from the inception of studies with this family of bacteria, as the field mouse (*Arvicola [Microtus] arvalis)* described by Koch in 1884 are, in fact, "Southern field" or "common voles". Voles are naturally susceptible to infection with *Mycobacterium microti* the so-called vole bacillus. Experimental infection with *M. tuberculosis* and *M. bovis* is also reported. There is a single retrospectively confirmed report (spoligotyping) of *M. bovis* infection in three voles from southern England but there are no documented cases of voles naturally infected with *M. tuberculosis* (Delahay et al., 2002; Little et al, 1982; Francis, 1958).

Griffith (1937&1939) confirmed that voles are susceptible to infection with *M. bovis* and *M. tuberculosis* when inoculated subcutaneously (SQ) and orally. Lesions were more severe with *M. bovis*, with generalized tuberculosis seen. Caseous lymphadenitis was a prominent feature in addition to granulomatous pneumonia and splenomegaly. Large numbers of bacilli were present in affected tissues when evaluated histologically and in culture. Wells in 1938 used the same two species of *Mycobacteria* but inoculated them intraperitoneally (IP). He obtained similar results and deduced "that the vole is at least 100,000 times as sensitive to bovine tubercle bacilli as it is to human tubercle bacilli". Griffith (1941) in further experiments with voles inoculated subcutaneously with bovine and human strains, the so called 'vole test', determined that these animals could not be used to differentiate between ordinary *M. tuberculosis* strains and *M. bovis* strains which were avirulent for the rabbit and guinea pig.

Jespersen (1975[a]) repeated the experiments conducted by Koch in *Microtus arvalis,* the common vole. Contrary to Koch's report that common voles are susceptible to both *M. bovis* and *M. tuberculosis* Jespersen determined that the common vole is susceptible to *M. bovis* only. Jespersen again demonstrated the lethality of *M. bovis* versus *M. tuberculosis* in *Arvicola terrestris*, the vole rat (1974) and in *Microtus agrestis*, the field vole (1976). Additionally, Jespersen conducted a series of experiments in red mice or bank voles *Clethrionomys glareolus*. Bank voles (1975 [b]) were inoculated IP with *M. bovis*, *M. tuberculosis*, *M. bovis* BCG and *M. avium*. Bacteremia was most pronounced in voles that received *M. bovis*. Comparative studies with white mice showed less severe bacteremia with *M. bovis*. In another series of experiments bank voles were inoculated via the subcutaneous, peritoneal and intravenous routes respectively (1977[a, b, and c]). In all instances, *M. bovis* was universally fatal whereas the voles were resistant to *M. tuberculosis*.

*Mycobacterium microti* was first described by Wells in the 1930's [formerly known as *Mycobacterium muris* and *Mycobacterium tuberculosis* var. *muris*] to cause tuberculosis in field voles (*Microtus agrestis)* (Wells and Oxon, 1937). This bacillus is a member of the *Mycobacterium tuberculosis* complex and differs from other members in its curved morphology, very slow growth *in vitro* and a predilection for infecting laboratory animal species. *M. microti* is identified by PCR-based spoligotyping and/or *IS6110* restriction fragment length polymorphism (RFLP) typing. To date, *M. microti* infection has also been reported in wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*), shrews (*Sorex araneus*) and rarely in llamas (*Lama vicugna*

*molina*), cats, pigs and humans (Frota et al., 2004; Oevermann et al., 2004; Cavanaugh et al., 2002, Neimann et al., 2000; Foudraine et al., 1998; Kremer et al., 1998).

Common routes of infection in voles are by ingestion of diseased voles or their excreta and by infection of wounds received in fighting (Rankin and McDiarmid, 1968). Initially Wells and Oxon suggested that gross lesions observed in affected voles are indistinguishable from those due to *M. bovis* and include caseous granulomas in the lung and lymph nodes and splenomegaly. However, Griffith in 1939 in experimentally infected voles did not detect 'tubercles' but there were subcutaneous masses of necrotic tissue admixed with acid-fast bacilli.

Due to the fastidious growth of this bacillus with routine mycobacterial culture, the prevalence of infection in animals and humans may be underestimated (vanSoolingen, 2001). Although *M. microti* was first described over 70 years ago, few studies have been undertaken to better understand its transmission between animals, zoonotic potential or pathogenesis (Cavanagh et al, 2002).

As the vole bacillus is of lower virulence when compared to other mammalian tubercle bacilli it was proposed as an alternative to *M. bovis* BCG for use in vaccination. However, the use of this vaccine in humans did result in clinical disease in some immunocompetent individuals (van Soolingen et al., 1998). Research is ongoing with use of *M. microti* in vaccination (Brodin et al., 2004; Manabe et al., 2002; Dannenberg et al., 2000).

**Rats**

Results of inoculation studies in rats consistently report high resistance to infection with human, avian and bovine forms of tubercle bacilli (Francis 1958, Koch 1882 and 1932).

Griffith (1907[a]) conducted a series of experimental inoculations with *M. bovis* in rats. Large doses ranging from 1.0g to 10.0g of bacilli were used. Significant findings were that rats subjected to IP inoculation were more susceptible compared to those subjected to SQ inoculation. Although doses in excess of 5.0g did result in death from tuberculosis in some of the animals, there was no gross or histologic evidence of classic tubercles. Large numbers of bacilli were often cultured from lymph nodes, liver, spleen, kidneys, lungs, omentum and bone marrow. Bacilli were occasionally detected in the blood. Rats inoculated orally with tuberculous milk or tissues failed to develop tuberculosis. On histology, foci of necrosis with intralesional bacilli were seen in two out of 26 rats. Tissue smears revealed acid fast bacilli in the mesenteric lymph node in 17 of the rats and in the celiac lymph node in one rat.

The majority of experimental studies in the rat were performed on albino or laboratory strains. Evidence for strain differences in susceptibility to *M. bovis* infection is therefore equivocal. Thus, Cobbett (1917) mentioned that the brown rat might be more susceptible than the white rat while results of Schalk et al in 1935 indicated that the brown rat is not easily infected with mycobacteria. However, as Schalk and associates used *M. avium* a valid comparison cannot be made (cited in Francis 1958).

Hehre and Freund in 1939 reported their findings of intravenous inoculation of *M. bovis* Ravenel strain in albino rats. Rats received 1mg and occasionally 10mg doses. Of

the eight rats inoculated, only one survived up to eighteen months. However as there were no control animals used it was uncertain if tuberculosis did indeed contribute to the demise of the seven rats that succumbed. Gross lesions indicative of tuberculosis were minute tubercles in the lung and enlargement of the spleen. On histology, small tubercles with intralesional bacilli were in the lung, spleen, liver and kidney. Caseation was not present. The authors observed that these results supported the great resistance of the rat to tuberculosis as 0.00001 mg doses of the same Ravenel strain given IV to rabbits resulted in their demise within three to six months and at necropsy, there were massive lesions in the lungs and kidneys.

Wessels (1941[a]) in studies on Wistar albino rats given 0.1mg doses of *M. bovis* intravenously (IV) found tuberculos lesions in visceral organs [lungs, liver, spleen and tracheobronchial lymph nodes] except the kidneys. The rats however did not exhibit any clinical signs of tuberculosis. Furthermore deposition of tubercle bacilli 24 hours after IV inoculation was greatest in the liver followed in decreasing order by the lung, spleen, tracheobronchial lymph nodes and kidney (Wessels 1941[b]). However, the overall maximum rate of bacterial replication occurred in the lungs. Histologic evaluation (Wessels 1941[c]) confirmed the presence of immature epitheliod cells in the lung that were incapable of destroying tubercle bacilli. In addition, caseation was not a feature of microscopic lesions.

Ratcliffe (1953, 1952) in a series of aerosol inoculation studies concluded that Long-Evans rats developed pulmonary tubercles very slowly. There was no evidence of spread to thoracic lymph nodes or other organs.

Thorns et al 1982 found that inbred Fisher F-344 rats when inoculated with *M. bovis* IP, harbored small numbers of the organism in their tissues (lungs) while producing very little or undetectable CMI. It is probable that rats and other species that are more resistant to disease are able to modulate the cell-mediated immune response so that the protective Listeria-like reaction is not overwhelmed by the more harmful necrotic Koch-type reaction. It may be significant that degraded acid-fast bacilli were seen associated with macrophages in many sections of infected rat tissues. The growth of mycobacteria in rats was therefore controlled by the host, which resulted in a persistent subclinical infection with no mortality.

Recently Sugawara and associates (2004) in a study with Lewis female rats which were infected aerially with *M. tuberculosis* report that granulomatous lesions were detected in the lungs, spleen, lymph nodes and liver. Additionally, expansion of IFN-γ, TNF-α and iNOS mRNA increased strongly over time but decreased after 12 weeks of infection.

Apart from the experimental studies done in laboratory strains environmental surveys also evaluated wild type or pigmented strains of rat. In 1982, Little et al summarized their findings of a survey of wild animals in an area of Dorset, Great Britain where *M. bovis* infection is widespread in cattle and badgers. They stated that *M. bovis* was isolated from the lymph nodes in two out of 90 rats (*Rattus norvegicus*) but there were no lesions observed. They postulated that these rats might become infected by eating undigested grains of maize that were sometimes observed in badger feces on the particular farm or from eating the carcasses of dead tuberculous badgers. They concluded that the non-progressive nature of the disease in rats, however, makes

them unlikely to transmit infection to other rats or other species of mammals. Excretion is unlikely to occur and the rat may be regarded as a dead-end host. Hancox (1996) however thinks this view may be erroneous as there may be passive transmission of *M. bovis* by 'healthy' rats given prior reports of culture of bacteria from mesenteric lymph nodes in animals with no gross or microscopic lesions.

Bosworth (1940) reported the incidental finding of *M. bovis* in two wild rats in a survey that he conducted to determine if these rats were carriers of *Brucella abortus*. There were no gross lesions consistent with tuberculosis in either of these naturally infected rats.

Pillai and associates (2000) initiated a study on the premises of 14 dairy herds in the El Paso milkshed area in the state of Texas, USA. Texas had the most (cumulative) tuberculous cattle herds of any state during the decade ending in 1997. Three roof rats (*Rattus rattus*) were sampled [mandibular, prescapular, popliteal, parotid, suprapharyngeal and bronchial lymph nodes, tonsil, lung, spleen, liver, mesenteric and supramammary glands] for mycobacterial culture but *M. bovis* was not detected.

Wilesmith and associates (1986) conducted a similar study in East Sussex, Great Britain during a three-year period from 1981 to 1983. Of the103 rats (*Rattus norvegicus*) captured all were negative for *M. bovis* on mycobacterial culture [spleen, kidneys, liver, lung, heart and any visible lymph nodes].

It should be noted that rats in addition to cats and mice are naturally susceptible to *Mycobacterium lepraemurium* the so-called rat leprosy bacillus. In rats, the disease presents in two forms glandular and musculocutaneous. The acid-fast bacillus occurs in infected tissues in large numbers but there is no associated caseation or necrosis

(Rankin and McDiarmid, 1968). Natural infection likely occurs via cutaneous abrasions and respiratory mucocsal surfaces. Ingestion is another possible route of infection (Rojas-Espinosa and Lovik, 2001). Although a separate species from *Mycobacterium leprae* (the cause of leprosy in humans), *M. lepraemurium* in rodents was used historically as a model of the human disease.

**Mice**

Much of the early research conducted with *Mycobacterium* spp. in mice utilized white or albino mice that frequently were not classified to the strain level (Table 1:2). This made it challenging at best to advance laboratory studies with mice from the late 1890's up to the 1940's. During this period, it was generally accepted that mice, like rats, were resistant to tuberculosis. However, Koch did propose differences in the susceptibility of mice of varying strains with white or albino mice being resistant to inoculation with bovine bacilli and pigmented strains being susceptible (Smith, 1898). It is now suspected that variability in resistance to tuberculosis in mice is dependent on their genotype (Kramnik and Boyartchuk, 2002; Chackerian and Behar, 2003).

The mouse has largely replaced guinea pigs and rabbits as the animal model of choice in experimental tuberculosis studies (primarily those of *M. tuberculosis* and, to a lesser extent, *M. bovis*). The vast majority of studies are concerned with the immunology of mycobacterial infection, vaccine development and, more recently, genetics of host resistance and susceptibility. Mice are suited to use in such research as they are inexpensive, can be easily housed in biosafety level three (BL3) facilities and an extensive immunological database is available. In addition, the importance and role

of T cell subsets in mice is consistent with observations in humans, and inbred strains including knockout mice show a range of resistance or susceptibility to infection (North and Jung, 2004; Orme, 2003; Orme et al., 2001; Glickman and Jacobs, 2001; Griffin et al., 1995; Leeford, 1984). However, a major disadvantage to the use of mice in the study of tuberculosis is that delayed type hypersensitivity (tuberculin skin test) response is poor. Caseation and liquefaction of pulmonary tubercles is also not a feature in murine tuberculosis (McMurray, 2001). Laboratory strains of mice currently used in tuberculosis research, primarily *M. tuberculosis* are listed in Table 1:3.

Several routes of inoculation have been utilized in studies of tuberculosis in the mouse model including but not limited to SQ, IP, IV, oral, intranasal (IN), inhalation/ intratracheal (IT) and intracerebral (Francis, 1958). However, IV and aerogenic routes are the more common experimental routes. Irrespective of the route used in experimental studies, the lungs are the most severely affected in susceptible mice as evidenced by gross and histologic lesions. Gross pulmonary lesions observed are enlargement of the lungs up to four or five times the normal size which are firm to consolidated with multifocal to coalescing granulomas/tubercles. Histologic lesions range from interstsitial aggregates of foamy macrophages with abundant lymphocytes in resistant animals to a more florid granulomatous and necrotizing pneumonia in susceptible mice. Large foamy and epitheliod macrophages with moderate to abundant numbers of neutrophils are reported in pulmonary lesions of susceptible strains, but the numbers of lymphocytes are reduced when compared to resistant strains. Acid-fast bacilli accumulate intracellularly within macrophages as well as extracellularly in the necrotic debris (Turner et al. 2003, Turner et al. 2001, Raleigh and Youmans, 1948). It

is proposed that lymphocyte (CD4+) migration to the site of inflammation and the associated cytokines (IFN-γ), which they release, serve to modulate the severity of pulmonary lesions in mice due to tuberculosis (Chackerian and Behar, 2003; Turner et al,. 2001; Dunn and North, 1995). Unlike tuberculosis in humans, typical tubercles, cavitation and Langhans giant cells are not evident in mice (Francis 1958, Raleigh and Youmans 1948). Other organs affected less consistently are the spleen, liver and various lymph nodes (Griffin et al.1995).

Although laboratory strains of mice are derived from the domestic or house mouse (*Mus musculus*) documented instances of natural/environmentally acquired infection with *M. bovis* in the wild type mouse were not encountered in a review of the literature. Little and associates (1982) evaluated 14 house mice, Wilesmith and associates (1986) examined 58 house mice, Fischer and associates (2000) examined 33 house mice and Pillali and associates (2000) examined 54 house mice. In each of the environmental surveys, all mice evaluated had no gross or microscopic evidence of tuberculosis and tissue cultures for *M.bovis* were consistently negative.

Natural infection with *M. avium* is, however, reported in mice. deJong in 1903 reported such an occurrence in the white laboratory mouse and Hemmert-Halswick in 1934 documented a similar occurrence in the gray mouse (cited in Raleigh and Youmans 1948).

**Nramp/Bcg**

The *Bcg/Ity/Lsh* locus in mice is located on chromosome 1 and is thought to control the innate resistance to infection of certain intracellular pathogens, namely *Mycobacterium* spp., *Salmonella typhimurium* and *Leishmania donovani*. The *Bcg* gene has subsequently been renamed *Nramp1* as it was found to be allelic with the gene that encodes the natural resistance associated macrophage protein (Nramp). Recently this gene has been renamed solute carrier family11 member 1(*Scl11a1*), OMIM [Online Mendelian Inheritance in Man] #600266. Thus, *Nramp1* and *Scl11a1* are used interchangeably. The *Nramp* family of proteins is highly conserved from bacteria to man. Homologues have been identified in the fruit fly *Drosophila* (70% identity), the worm *Caenorhabditis* (*C. elegans*, 68%), plants (*OsNramp* family, 50-60%) and the yeast *Saccharomyces cerevisiae* (40-45%) (Skamene et al.1998).

In mice, the Nramp-1 protein is an integral membrane protein expressed exclusively in the lysosomal compartment of monocytes/ macrophages. In human leukocytes, however NRAMP1 is primarily expressed in polymorphonuclear leukocytes (Buschman and Skamene 2001). This membrane protein is highly hydrophobic with a predicted molecular mass of 60kD and has twelve transmembrane (TM) domains, a glycosylated extracellular loop and several phosphorylation sites. After phagocytosis, Nramp1 is targeted to the membrane of the microbe-containing phagosome (late endosome/lysosome) in the macrophage (Gruenheid et al., 1997).

Initially it was thought that Nramp1 and its human homologue NRAMP1within the phagosome may influence the intracellular concentration of $Fe^{2+}$ and/or other divalent cations (Bellamy 1999, Canonne-Hergaux et al 1999, Zwilling et al 1999, Govoni and

Gros 1998). Divalent cations are essential cofactors for many microbial metabolic enzymes, and alterations in their availability could have pleiotropic effects on antigenically unrelated microbes included in the phagosome (Govoni and Gros 1998). Now it is known that Nramp1 (Slc11a1) is a highly pH-dependent antiporter that can flux bivalent cations in either direction depending on the pH on either side of the membrane. As Nramp1 (Slc11a1) is localized to the late endosomal/lysosomal membrane delivery of cations from the cytosol to the mycobacteria containing phagosomes is associated with bacteriostasis, bacterial damage and can also inhibit the ability of mycobacteria to block phagolysosomal fusion and acidification (Goswami et al. 2001).

Sequencing of the *Nramp1* mRNA in mice revealed the difference in the *Bcg* locus of resistant (dominant) mouse strains ($Bcg^r$) when compared to susceptible (recessive) ($Bcg^s$) was a single glycine (G) to aspartate (D) substitution at amino acid position 169 in Nramp molecules. Thus, $G^{169}$ in resistant mouse strains is replaced by $D^{169}$ in susceptible strains (Nakanaga et al 1999, Govoni et al 1996, Vidal et al, 1995, Sakmene 1994). However, it should be noted that these initial studies utilized the attenuated *M. bovis* (BCG) strain, *M. lepraemurium*, *M. intracellulare*, *M. smegmatis* and *M. avium* (Skamene et al. 1998).

Other researchers have suggested that it may be too simplistic to associate resistance to mycobacterial infection in mice with a single genetic locus, namely *Nramp1*. North and Medina (1998, 1996[a and b]), in experimental studies with the virulent H37Rv strain of *M. tuberculosis* in mice found that although the infection in the lung was initially controlled, some strains namely DBA/2, A/J and CBA/J, were unable to

maintain this control. Tuberculosis in these strains was later reactivated which led to their demise. C57 background mice were however able to limit bacterial growth. Confirmation of the lack of *Nramp1* to influence infection in mice with virulent *M. tuberculosis* was revealed in studies of gene knockout mice on a 129/J background. *Nramp1*-resistant and susceptible mice each had comparable susceptibility in all of the organs evaluated (North et al. 1999).

In addition to humans and mice, *NRAMP* genes are documented in cattle (Barthel et al.2000, Feng et al. 1996). Primary studies in rhesus macaques and deer with *M. tuberculosis* and *M. bovis* respectively however failed to identify an association between resistance to infection and *NRAMP* genes (Deinard et al 2002, Mackintosh et al. 2000).

It is likely that genetic resistance to mycobacterial infection is multifactorial and therefore not related to just one gene. Genetic 'systems' and loci other than *NRAMP* which have been implicated are interferon-γ-receptor, vitamin D receptor (VDR), interleukin 1 gene cluster, specific HLA alleles and the *sst1* (*susceptibility to tuberculosis 1*) locus (Kramnik et al. 2000, Wilkinson et al. 2000, Wilkinson et al. 1999, Goldfeld et al. 1998, Newport et al. 1996).

**Surveillance for bovine tuberculosis in white tailed deer and other wildlife in Michigan, 2000-2003**

Initially *M. bovis* was thought to be maintained only in cattle (Dungworth, 1993). However, the presence of maintenance or reservoir host of bovine tuberculosis in some countries threatens to disrupt efforts to eradicate this disease from domestic animals. Indeed Cosivi and associates (1998) state "wild animal TB represents a permanent reservoir of infection and poses a serious threat to control and elimination programs". Maintenance hosts of *M. bovis* include the brushtail possum (*Trichosurus vulpecula*) in New Zealand, Eurasian badger (*Meles meles*) in Ireland and the United Kingdom, bison (*Bison bison*) in Canada, African buffalo (*Syncerus caffer*) in Uganda, white-tailed deer (*Odocoileus virginianus*) in Michigan, USA and lechwe antelope (*Kobus leche*) in Zambia. Spillover hosts are numerous (de Lisle et al. 2002, de Lisle et al. 2001, O'Reilly and Daborn 1995)

*Mycobacterium bovis* infection in various species of deer including fallow deer (*Dama dama*), axis deer (*Axis axis*), sika deer (*Cervus nippon), red deer* (*C. elaphus*), roe deer (*Capreolus capreolus*) moose or elk (*Alces alces*) and white-tailed deer *(Odocoileus virginianis*) is sporadically reported. Countries where this disease has been detected include but are not limited to the United Kingdom, New Zealand, the Republic of Ireland, Denmark, Switzerland, Germany, Sweden, the USSR, India, Canada and the United States of America (Clifton-Hadley and Wilesmith 1991). Tuberculosis due to *M. bovis* affects both free-living wild and captive deer (Kaneene et al., 2002; de Lisle et al. 2001).

Gross lesions in tuberculous deer usually present as thin walled abscess filled with cream colored purulent material. Less frequently, the 'classical granuloma' common in cattle with caseation and calcification may occur in deer. Tissues most often affected are retropharyngeal, submandibular, mediastinal and mesenteric lymph nodes and the lungs. Histologically, acid-fast bacilli occur in small numbers or are rare to nonexistent. Lesions in affected cervids range from granulomatous to necrotizing or suppurative with partial mineralization (Schmitt et al 1997, Rhyan and Saari 1995). In white - tailed deer, liquifaction of tuberculous lesions over time is common (Palmer et al 2002[b]). Similar to cattle, deer are infected via the respiratory and gastrointestinal tract (O'Reilly and Daborn 1995).

Tuberculosis due to *M. bovis* is endemic in white-tailed deer (WTD) in Michigan. The genesis of this outbreak was recognized in 1994 with the discovery of an infected four and a half year old hunter harvested male WTD from the northeastern area of the lower peninsula of Michigan. Other WTD were identified the next year in a follow up study of animals within a ten mile radius (Schmitt et al, 1997). *Mycobacterium bovis* infection in deer in Michigan was reported sporadically prior to this; a single WTD from Alpena county in 1975 and in 1962 an outbreak at a deer park involving exotic fallow deer (Quinn and Towar, 1963).

As it was realized that WTD in Michigan were a maintenance host for *M. bovis,* the only known occurrence of this phenomenon, annual surveillance of the disease in deer was initiated in 1995 and is ongoing. The total number of deer evaluated at the Diagnostic Center for Population and Animal Health (DCPAH) (formerly the Animal Health Diagnostic Laboratory (AHDL), Michigan State University (MSU) from 1995

through fall 2003 was 107, 791. The number of deer positive for *M. bovis* during this interval was 455. . The prevalence of bovine TB in WTD in the state of Michigan is highest in the counties of Oscoda, Alcona, Montmorency, Alpena and Presque Isle (5-county area). Cumulative data on the WTD hunter harvest surveillance from the inception through 2003 are presented in Table 1: 4 and Figure 1:1.

The establishment of white-tailed deer as a maintenance host for *M. bovis* was thought to be related to the focal density of the population and utilization of supplemental feeding (Schmitt et al, 1997). *M. bovis* could, therefore, be transmitted directly from deer-to deer as well as via contamination of feedstuff not fully ingested by infected animals (Palmer et al. 2004[b], Palmer et al. 2001). Thus, a voluntary ban on supplemental feeding was instituted by the Michigan Department of Natural Resources (MDNR) in the fall of 1998 with a mandatory ban introduced in the fall of the following year in addition to restrictions on baiting. A retrospective study conducted by Miller et al. (2003) showed that an increase in the prevalence of *M. bovis* in WTD was associated with an increase in the percentage of supplemental feeding sites that provided fruits and vegetable and this provided evidence to support the feeding ban.

In conjunction with feeding restrictions, there was concomitant increase in the number of antlerless deer permits issued and extension of hunting seasons (firearm/muzzleloader, archery, and youth) to increase the numbers of WTD harvested which consequently drives the population down. This would limit congregation of deer in any given location at any given time that would translate into reduced potential for the spread of *M. bovis* from animal to animal.

Infection in deer likely occurred from spill over from infected cattle [the state was declared TB- free in 1979]. Subsequently infected deer transmitted *M. bovis* back to cattle (Palmer et al. 2004[a]). Up to October 2004, 32 bovine tuberculosis infected herd have been identified in Michigan, 26 beef herds and six dairy herds. Three of the affected herds are not confined to the 5-county area but were still found in counties where bovine TB positive deer were found (Figure 1:2).

The protocol for evaluation of WTD for bovine TB at the DCPAH, MSU entails examination of the cranial lymph nodes (medial retropharyngeal, parotid, submandibular) in deer heads submitted through the Michigan Department of Natural Resources (MDNR). The basis for submission of the head only is founded in a review of the literature which documents the retropharyngeal lymph node as the most commonly affected site in cervidae (Griffin and Mackintosh 2000, O'Reilly and Daborn 1995, Griffin and Buchan 1994, Whiting and Tessaro 1994). Although less frequently entire carcasses and other tissues, usually the lungs, are also submitted when displaying gross lesions suggestive of tuberculosis. Samples of grossly visible lesions are then collected for histopathology (routine H&E and acid-fast) and for submission to the Michigan Department of Community Health (MDCH) Tuberculosis laboratory for mycobacterial isolation and identification.

As only lesioned deer are sampled, there was concern that this protocol may result in an underestimation of the prevalence by as much as 57% (Palmer et al. 2000). However, a more extensive study in the free ranging deer population in the fall of 2001 where all negative deer, that is those having no grossly visible lesions, taken in six townships within the core (DMU 452) (Figure 1:3) were sampled. The estimated

apparent and true prevalence were determined to be 2.7% and 3.6%, respectively (O'Brien et al. 2004)

In addition to white-tailed deer, *M. bovis* infection has also spilled over to other carnivore/omnivore wildlife species in Michigan. In response to the discovery of endemic disease in the deer population, surveillance of other noncervid wildlife was initiated in 1996 (Bruning-Fann et al., 2001). To date 16 different species have been tested. Forty-two positive animals were identified from 1997 thru 2003 in six of the species (black bear, bobcat, coyote, opossum, raccoon and red fox) (Table 1:5). The majority of affected animals are confined to the 5 - county area which supports the transmission of infection from WTD. In contrast to the deer surveillance study, in other species, the entire carcass is submitted except for those of black bears which were evaluated (necropsied) in the field. In addition to cranial lymph nodes thoracic (mediastinal, tracheobronchial) and abdominal (mesenteric, hepatic, ileocecal) are collected and pooled for histopathology (H&E and AF) and mycobacterial culture.

Gross lesions suggestive of tuberculosis detected in five animals were predominantly confined to abdominal lymph nodes [enlarged mesenteric lymph node in four; focal pulmonary granuloma in one]. This is supportive of infection via ingestion of contaminated tuberculous material. On microscopic evaluation, 10 of the 42 culture positive animals had low or even rare numbers of acid-fast bacilli. These 10 animals also had histologic lesions [caseogranulomas] suggestive of tuberculosis. However, in an additional four culture positive animals with histologic lesions, acid-fast bacilli were not detected. As spillover hosts are implicated in the dissemination of bovine tuberculosis experimental studies are ongoing to better define pathogenesis and

attendant risk factors in such species. It is anticipated that elimination or decrease in the prevalence on *M. bovis* infection in cattle and WTD will preclude persistence of infection in carnivore/ omnivore species in Michigan.

In experimental studies the North American opossum was found to develop lesions primarily in the lung resulting from IM, oral, and aerosol inoculation. Thus, dissemination of bacteria via aerosol in naturally infected opossums is possible. An orally inoculated animal also shed mycobacteria in the feces. However, unlike their New Zealand relative the brushtail possum, maintenance of bovine tuberculosis in North American opossums in the lower peninsula of Michigan does not appear to be a major factor in the transmission of organisms to cattle and deer (Fitzgerald et al. 2003[a], Diegel et al. 2002)

Palmer and associates (2003[c]), in studies with raccoons inoculated with varying doses of *M. bovis* and via varying routes, concluded that large doses were required to establish infection and low numbers of bacilli were excreted in nasal secretions and saliva. It is, therefore, unlikely that tuberculosis in raccoons is widespread. Dissemination of tubercle bacilli from infected raccoons into the environment may be unlikely as *M. bovis* was not isolated from urine or feces in any of the raccoons in this study. Two of the IV inoculated raccoons did, however, develop fistulous tracts in infected superficial lymph nodes similar to lesions that develop in naturally infected brushtail possums.

In addition to the unique occurrence of maintenance of *M. bovis* infection in WTD in Michigan bovine tuberculosis is also reported in North America in a related cervid species the elk (*Cervus elaphus*). These animals either were in a captive herd or

infected by close contact with infected cattle (Lees et al. 2003, Thoen et al. 1992, Rhyan et al. 1992). Since the geographic location of the reintroduced herd of elk in Michigan overlaps that of the *M. bovis* infected WTD, surveillance of elk commenced in 1996 on a voluntary basis. Mandatory testing of all elk killed/ hunted commenced in 1998. Four elk positive for *M. bovis* have been identified from a total of over 1200 elk evaluated from 1996 thru December 2003 (Figure 1:4). Histologically, lesions were detected in three of the four elk [suppurative tonsillitis; caseogranulomas in medial retropharyngeal LN; multinucleated giant cells in LN] and further, acid-fast bacilli were seen in two of these three elk.

It is challenging to propose a mode of infection for the elk in the northern Lower Peninsula as only the head is submitted for evaluation for tuberculosis. However, evaluation of farmed elk involved in an outbreak of bovine TB in Alberta, Canada in 1991 suggested that aerosol transmission was the most significant means of spread within the herd. Similar to WTD in Michigan, gross lesions were predominantly in the lymph nodes and were of a suppurative nature. Retropharyngeal lymph nodes were the most consistently affected lymph node in the head. Other visceral lesions consistent with tuberculosis were observed only in the lung and pleura of affected elk (Fitzgerald et al. 2000, Whiting and Tessaro 1994). The prevalence (apparent) of gross lesion in these elk was determined to be 39.8%. This high prevalence is likely indicative of the increased population density and management factors in these farmed animals. The apparent prevalence of bovine Tb in free-roaming elk near Riding Mountain National park in Manitoba, Canada is approximately 1 %, which is nearer to that currently detected in Michigan elk (~ 0.31 %) (Lees 2004).

**Objectives for research**

Given the wide host range of *M. bovis*, and the identification of WTD in Michigan as a reservoir host, it was realized that there is insufficient information on the transmission and pathogenesis of infection by this bacterium in many of the wildlife species in the state which are in contact with cattle as well as free-ranging and captive cervidae. Thus the global objective of this collection of studies was to experimentally introduce *M. bovis* into select wildlife species in order to provide necessary data on the transmission and pathogenesis in such species and ultimately to assess the attendant risk associated with them serving as reservoir or spillover hosts

The four species which were experimentally inoculated with *M. bovis* were wild turkeys, meadow voles, house mice and Norway rats. For each species, a route of experimental exposure was chosen which would most closely mimic that to which they would potentially be exposed to *M. bovis* if it were present in their natural habitat. Thus, turkeys were inoculated via the trachea and orally. The voles were exposed via the nasal and oral routes, whereas the mice and rats were orally inoculated. In each of these studies, the stated hypothesis was that such synanthropic species can be infected with *M. bovis* and would thereafter shed bacilli in the environment and act as a source of infection in cattle and humans.

Additionally, in the house mice another experiment was performed to determine their phenotype for the *Bcg/Nramp-1* gene in order to determine if this gene is associated with their susceptibility to infection with *M. bovis*. Mice were selected as the sequence for this particular gene is well documented in this species.

In addition to studies evaluating transmission of bovine tuberculosis in wildlife another area of research which is lacking is a method to diagnose mycobacterial infection which is relatively fast, inexpensive and reliable. Mycobacterial culture is still upheld as the 'gold standard' in diagnosis although it can take up to eight to twelve weeks for a definitive diagnosis. Consequently, in this body of work a novel experimental technique for the identification of *M. bovis* in formalin fixed paraffin embedded tissues which utilized laser capture microscopy and PCR was investigated in an attempt to improve turn around time for diagnosis.

**Figure 1: 1** Map of the lower peninsula of Michigan which depicts the total number of deer positive for bovine tuberculosis from 1995 through 2003 and the counties where they were captured. Also included are the deer with tuberculosis detected in 1975 and 1994 as well as a positive captive deer herd diagnosed in 1997.

Images are presented in color

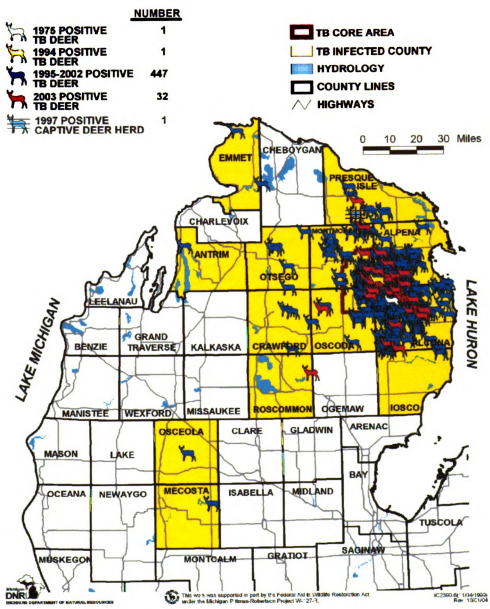

BOVINE TUBERCULOSIS SURVEY RESULTS
NUMBER
1975 POSITIVE TB DEER 1
1994 POSITIVE TB DEER 1
1995-2002 POSITIVE TB DEER 447
2003 POSITIVE TB DEER 32
1997 POSITIVE CAPTIVE DEER HERD 1
TB CORE AREA
TB INFECTED COUNTY
HYDROLOGY
COUNTY LINES
HIGHWAYS
0 10 20 30 Miles
CHEBOYGAN
EMMET
PRESQUE ISLE
CHARLEVOIX
MONTMORENCY
ALPENA
ANTRIM
OTSEGO
LEELANAU
LAKE MICHIGAN
LAKE HURON
BENZIE
GRAND TRAVERSE
KALKASKA
CRAWFORD
OSCODA
ALCONA
MANISTEE
WEXFORD
MISSAUKEE
ROSCOMMON
OGEMAW
IOSCO
OSCEOLA
CLARE
GLADWIN
ARENAC
MASON
LAKE
BAY
OCEANA
NEWAYGO
MECOSTA
ISABELLA
MIDLAND
TUSCOLA
MUSKEGON
MONTCALM
GRATIOT
SAGINAW
DNR
This work was supported in part by the Federal Aid in Wildlife Restoration Act under the Michigan Pittman-Robertson Project W- 27-R

**Figure 1:1**

**Figure 1:2** Map of the lower peninsula of Michigan which depicts the total number of beef and dairy herds in which animals were diagnosed with bovine tuberculosis from 1998 through October 2004.

Images are presented in color

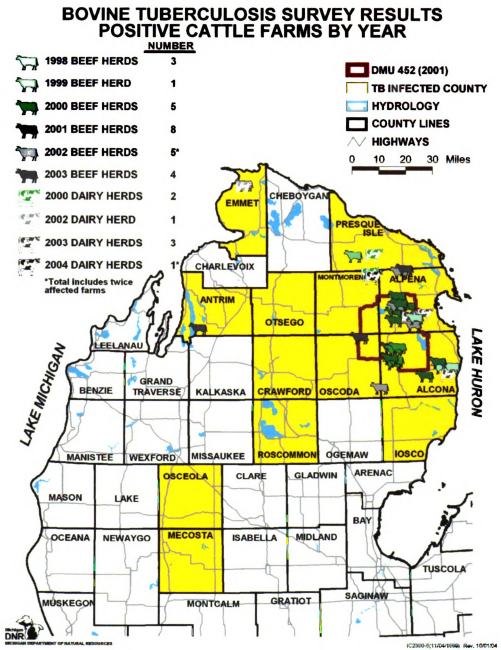

BOVINE TUBERCULOSIS SURVEY RESULTS
POSITIVE CATTLE FARMS BY YEAR
NUMBER
1998 BEEF HERDS 3
1999 BEEF HERD 1
2000 BEEF HERDS 5
2001 BEEF HERDS 8
2002 BEEF HERDS 5*
2003 BEEF HERDS 4
2000 DAIRY HERDS 2
2002 DAIRY HERD 1
2003 DAIRY HERDS 3
2004 DAIRY HERDS 1*
*Total includes twice affected farms
DMU 452 (2001)
TB INFECTED COUNTY
HYDROLOGY
COUNTY LINES
HIGHWAYS
0 10 20 30 Miles
LAKE MICHIGAN
LAKE HURON
EMMET
CHEBOYGAN
PRESQUE ISLE
CHARLEVOIX
MONTMORENCI
ALPENA
ANTRIM
OTSEGO
LEELANAU
BENZIE
GRAND TRAVERSE
KALKASKA
CRAWFORD
OSCODA
ALCONA
MANISTEE
WEXFORD
MISSAUKEE
ROSCOMMON
OGEMAW
IOSCO
OSCEOLA
CLARE
GLADWIN
ARENAC
MASON
LAKE
BAY
OCEANA
NEWAYGO
MECOSTA
ISABELLA
MIDLAND
TUSCOLA
MUSKEGON
MONTCALM
GRATIOT
SAGINAW

**Figure 1:2**

**Figure 1:3** Map of the lower peninsula of Michigan which depicts the location of deer management unit (DMU) 452 and the counties of the lower peninsula of Michigan where animals (all species) with bovine tuberculosis were detected. Images are presented in color

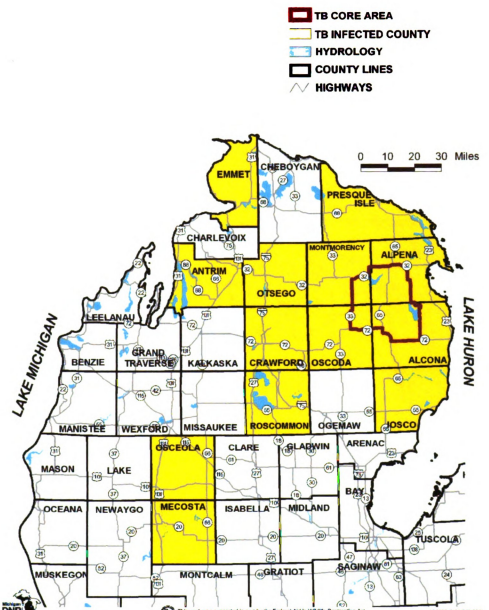

TB CORE AREA
TB INFECTED COUNTY
HYDROLOGY
COUNTY LINES
HIGHWAYS
0 10 20 30 Miles
EMMET
CHEBOYGAN
PRESQUE ISLE
CHARLEVOIX
MONTMORENCY
ALPENA
ANTRIM
OTSEGO
LEELANAU
LAKE MICHIGAN
LAKE HURON
BENZIE
GRAND TRAVERSE
KALKASKA
CRAWFORD
OSCODA
ALCONA
MANISTEE
WEXFORD
MISSAUKEE
ROSCOMMON
OGEMAW
IOSCO
OSCEOLA
CLARE
GLADWIN
ARENAC
MASON
LAKE
BAY
OCEANA
NEWAYGO
MECOSTA
ISABELLA
MIDLAND
TUSCOLA
MUSKEGON
MONTCALM
GRATIOT
SAGINAW
MICHIGAN DEPARTMENT OF NATURAL RESOURCES
This work was supported in part by the Federal Aid in Wildlife Restoration Act under the Michigan Pittman-Robertson Project W-127-R
IC2306-6(11/04/1999) Rev 02/24/05

**Figure 1:3**

**Figure 1:4** Map of the lower peninsula of Michigan which depicts the total number of elk positive for bovine tuberculosis from 1996 through 2003 and the counties where they were captured.

Images are presented in color

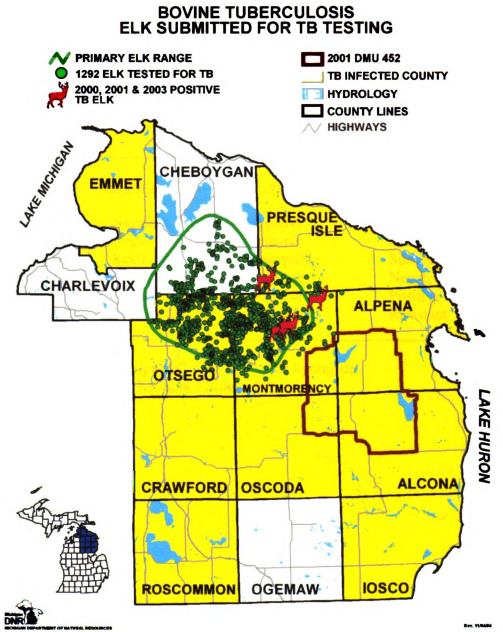

BOVINE TUBERCULOSIS
ELK SUBMITTED FOR TB TESTING
PRIMARY ELK RANGE
1292 ELK TESTED FOR TB
2000, 2001 & 2003 POSITIVE TB ELK
2001 DMU 452
TB INFECTED COUNTY
HYDROLOGY
COUNTY LINES
HIGHWAYS
LAKE MICHIGAN
EMMET
CHEBOYGAN
PRESQUE ISLE
CHARLEVOIX
ALPENA
OTSEGO
MONTMORENCY
LAKE HURON
CRAWFORD
OSCODA
ALCONA
ROSCOMMON
OGEMAW
IOSCO

**Figure 1:4**

**Table 1:1 A summary of *in vitro* biochemical tests used to identify *M. bovis*, *M. avium* and *M. tuberculosis***

| | M. bovis | M. avium | M. tuberculosis |
|---|---|---|---|
| Nitrate | Negative | Negative | Positive |
| Niacin | Negative | Negative | Positive |
| PZA[1] | Resistant | Susceptible | Susceptible |
| Catalase | Negative | Negative | Negative |
| Urease | Positive | Negative | Positive |
| TCH[2] | Susceptible | Resistant | Resistant |

[1] pyrazinamide, [2] thiophen-2-carboxylic acid hydrazide
After Witebsky and Kruczak-Filipov 1996

**Table 1:2 A summary of pertinent findings of research (1907 through 1953) in various strains of mice experimentally infected with *Mycobacterium bovis* via different routes**

| Route/Investigator SQ/IP | Year | Strain | Results |
|---|---|---|---|
| Griffith, AS[3] | 1907[b] | not stated | IP mice death due to Tb; SQ no deaths due to Tb. Mice less resistant to growth of Tb bacillus than rats |
| Griffith, AS[1&3] | 1911, 1937 | white & field | White mice more susceptible than rats. Field voles more susceptible than field or house mice. |
| Gerstl & Thomas[1] | 1940-41 | dilute brown albinos, ABC black/ white | Lesions regressed 80 days PI. |
| **IV** | | | |
| Gunn, Nungester [1] & Hougen | 1933-34 | white | No observable differences in lesions with bovine and human Tb strains |
| Schwabcher & Wilson[1] | 1937 | white | Similar survival with bovine and human Tb strains [22-33 days] |
| Kirchheimer et al[3] | 1950 | Strong A | Bacilli shed in feces |
| **IN/ aerosol** | | | |
| Schwabacher & Wilson[2] | 1937 | white | Lesions similar to IV infection and were dose-dependent |
| Glover [3] | 1944 | not stated | Lesions primarily in the lungs, lacked caseation |
| Ratcliffe[3] | 1952 | albino | Lung lesions 4-8 weeks PI |
| Ratcliffe[3] | 1953 | albino | Death from bronchial spread in < 60 days |

[1] After Darzins 1958
[2] After Francis 1958
[3] See references
SQ- subcutaneous; IP- intraperitoneal; IV-intravenous; IN-intranasal; Tb- tuberculosis; PI- post-inoculation

**Table 1:3 Classification of inbred strains of mice based on their susceptibility to infection with *M. tuberculosis***

| Resiatant | Intermediate | Susceptible |
|---|---|---|
| A/Sn | BALB/c | 129/Sv |
| BALB/c | | A/J |
| C57BL/10 | | $C3H^c$ |
| C57BL/6 | | CBA |
| | | DBA/2 |
| | | I/St |
| | | SWR[1] |

After Chackerian and Behar, 2003; Medina and North, 1998
[1] Turner et al. 2003

**Table 1:4** A summary of the surveillance for *Mycobacterium bovis* infection in white-tailed deer in the state of Michigan, USA for 1995-2003

**Core area also referred to as deer management unit (DMU) 452** is an area of approximately 1561 square km as defined by administrative boundaries of the Michigan Department of Natural Resources after the 1994 tuberculous WTD was found

**Apparent prevalence (AP)** calculated as the number of deer exhibiting gross lesions consistent with TB that are mycobacterial culture positive divided by the total number of deer submitted for testing multiplied by 100

**True prevalence (TP)** is the total number of diseased deer in the population. TP was calculated from the sensitivity and specificity using the formula of Rogan and Gladden (1978) as referenced by O'Brien et al. in 2004.

$$\hat{p} = \frac{\hat{t} + \beta - 1}{\alpha + \beta - 1}$$

$\hat{p}$ = true prevalence, $\hat{t}$ = apparent prevalence, $\beta$ = specificity and $\alpha$ = sensitivity

**Five county area (CO)** – The five counties with the highest prevalence of *M. bovis* infection in WTD are Presque Isle, Montmorency, Alpena, Oscoda and Alcona

**Table 1:4 A summary of the surveillance for *Mycobacterium bovis* in white- tailed deer in the state of Michigan, USA for 1995-2003**

| | Total # deer | #TB Pos | Core AP (%) | Core TP (%) | 5-CO AP (%) | 5-CO TP (%) |
|---|---|---|---|---|---|---|
| 1995 | 386 | 18 | 5.1 | 6.8 | NA | NA |
| 1996 | 3,690 | 47 | 2.5 | 3.3 | 0.17 | 0.23 |
| 1997 | 3,518 | 67 | 4.4 | 5.9 | 0.43 | 0.57 |
| 1998 | 7,915 | 75 | 2.7 | 3.6 | 0.28 | 0.37 |
| 1999 | 17,502 | 56 | 2.3 | 3.1 | 0.19 | 0.25 |
| 2000 | 22,011 | 53 | 2.6 | 3.5 | 0.35 | 0.47 |
| 2001 | 20,461 | 56 | 1.8 | 2.4 | 0.55 | 0.73 |
| 2002 | 16,137 | 51 | 2.8 | 3.7 | 0.48 | 0.64 |
| 2003 | 16,171 | 32 | 1.7 | 2.3 | 0.22 | 0.29 |

**Table 1: 5** Summary of carnivore and omnivore species in Michigan that were tested for *M. bovis* infection for the years 1997 through 2003

* Total number of this species of animal tested

† Total number of animals positive for *M. bovis* on culture

‡ Prevalence is the total number of positive animals of a given species divided by the total number of animals in that group multiplied by 100

**Table 1:5 Summary of carnivore and omnivore species in Michigan that were tested for *M. bovis* infection for the years 1997 through 2003**

| SPECIES | TESTED* | POSITIVE† | PREVALENCE‡ |
|---|---|---|---|
| Badger (*Taxidea taxus*) | 46 | 0 | 0 |
| Black bear (*Ursus americanus*) | 214 | 7 | 3.27 |
| Bobcat (*Felis rufus*) | 58 | 4 | 6.90 |
| Coyote (*Canis latrans*) | 379 | 18 | 4.75 |
| Feral cat (*Felis silvestris catus*) | 35 | 0 | 0 |
| Feral dog (*Canis familiaris*) | 1 | 0 | 0 |
| Gray fox (*Urocyon cinereoargenteus*) | 5 | 0 | 0 |
| Mink (*Mustela vison*) | 5 | 0 | 0 |
| Opossum (*Didelphis virginiana*) | 381 | 2 | 0.52 |
| River otter (*Lutra canadensis*) | 10 | 0 | 0 |
| Porcupine (*Erethizon dorsatum*) | 1 | 0 | 0 |
| Raccoon (*Procyon lotor*) | 335 | 8 | 2.39 |
| Red fox (*Vulpes vulpes*) | 29 | 3 | 10.3 |
| Skunk (*Memphitis memphitis*) | 21 | 0 | 0 |
| Snowshoe hare (*Lepus americanus*) | 1 | 0 | 0 |
| Long-tailed weasel (*Mustela frenata*) | 1 | 0 | 0 |
| **TOTAL** | **1,522** | **42** | **2.76** |

## CHAPTER TWO

### Experimental Inoculation of Wild Turkeys (*Meleagris gallopavo*) with *Mycobacterium bovis*


**Abstract**

Although avian species are known to be susceptible to infection with *Mycobacterium* spp. organisms much remains unknown about the susceptibility of birds to infection with *M. bovis*. The objective of this current study was to determine if wild turkeys (*Meleagris gallopavo*) can be infected with *M. bovis* when inoculated by the oral or intratracheal route. Six turkeys were orally inoculated and another six were inoculated via the trachea with a high dose of *M. bovis*, 1 x $10^5$ CFU/ml. Six turkeys were sham inoculated controls. Two turkeys from each treatment group were sacrificed on day 30, 60 and 90 post-inoculation (PI). There were no gross or microscopic lesions consistent with mycobacteriosis in the twelve inoculated turkeys over the 90 day duration of this study. Fecal cultures were also consistently negative for *M. bovis* when sampled prior to inoculation and on days 1, 30 and 60 PI. Two intratracheally inoculated turkeys were positive for *M. bovis* in visceral tissues at 30 days post-inoculation. However, this finding was only indicative of passive persistence of mycobacteria in the tissues and not of infection as there were no attendant lesions or clinical compromise to support infection. Thus, it can be concluded that wild turkeys are

resistant to infection with *M. bovis* and, therefore, pose minimal threat as reservoir or spillover hosts for this organism.

**Introduction**

Free-ranging white-tailed deer (WTD) (*Odocoileus virginnianus*) in the Lower Peninsula of Michigan are known to be reservoir hosts for *Mycobacterium bovis*, the causative agent for bovine tuberculosis (deLisle et al., 2001; Schmitt et al., 1997). Spillover from deer has resulted in infected dairy and beef cattle in the state.

A need for determining the ability of wild turkeys to serve as reservoir or spillover hosts of *M. bovis* organisms was kindled by the observation that in the area of the lower peninsula where bovine TB is endemic, that the greatest number of wildlife/cattle interactions with other species is with wild turkeys (Hill, 2005). Anecdotal evidence suggests that wild turkeys are frequently observed on farms in this area of Michigan, in their and in cattle feeders. The number of wild turkeys in Michigan has recovered from near extinction of the species [in the US in the early 1900's] and can now support two hunting season per year in the spring and fall (CNN Interactive, 1998). Turkey hunting brings millions of dollars to states' Department of Natural Resources, public, and private organizations (McCullough, 2001).

The diet of wild turkeys varies according to season and habitat but they forage mostly on the ground for seed, nuts, acorns, buds, berries and insects. In the fall they form flocks with several males accompanying several females. Consequently

these birds could be exposed to grains and pasture contaminated by tuberculous cattle as wild turkeys do occasionally ingest manure or pick undigested corn out of manure. Given that such birds can fly and are able to cover more than a mile when airborne they could potentially disseminate infectious material to adjoining uninfected premises. Additionally, the interaction of hunters and other naturalists with wild turkeys is indicative of potential zoonosis.

Such concern about wild turkeys disseminating *M. bovis* is warranted as wild turkeys have been found to be carriers of select avian pathogens and parasites in environmental studies done in the United States. Peterson and associates (2002) found that wild turkeys in the Edwards Plateau of Texas were seropositive for *Mycoplasma gallisepticum* and *M. synoviae*. Furthermore, some of theses birds tested positive for reticuloendotheliosis virus by PCR. Hopkins and associates (1990) in a survey of wild turkeys in Arkansas found that some birds were positive for *Pasteurella multocida*, *Bordatella avium* and Newcastle disease virus. Avian species are known to be susceptible to infection with *Mycobacterium* spp. organisms, and interspecies transmission of mycobacteria in birds is well documented. Several species of birds including those of the order Galliformes which embraces domestic chickens and turkeys are known to be susceptible to infection with *Mycobacterium avium*. Wild birds (sparrows, starlings, pigeons) can spread *M. avium* to domestic poultry, pet birds and captured exotic birds (Fulton and Thoen, 2003; Tell et al., 2001). Other species of birds, particularly in the order Psittaciformes, are susceptible to infection with *M. tuberculosis* (Hoop, 2002; Tell et al., 2001; Washko et al, 1998; Hoefer et al., 1996; Woerpel and Rosskopf, 1983; Thoen et al., 1977; Ackerman et al, 1974).

Recently, infection of pet birds with *M. genavense* has been documented (Bercovier and Vincent, 2001; vanDerHeyden, 1997; Hoop et al., 1996). However, much remains unknown about the susceptibility of birds to infection with *M. bovis*. Additionally, birds are implicated as vectors in the transmission of zoonoses such as West Nile, influenza type A, chlamydiosis, and salmonellosis.

Given the presence of bovine tuberculosis in white-tailed deer in Michigan which serve as a reservoir host it is important to determine the potential of other wildlife species to serve as reservoir hosts as this could delay or hinder efforts to eradicate this disease. Therefore there was a need to determine via experimental infection if wild turkeys are indeed susceptible to bovine tuberculosis. The objective of this pilot study was to ascertain whether wild turkeys are susceptible to infection with *M. bovis* via oral or intratracheal routes. Further, this study also determined the feasibility of infected turkeys disseminating this organism in the environment via fecal shedding. The hypothesis for this study is that wild turkeys can be infected with *M. bovis* when exposed via oral and intratracheal routes. Infected birds can then disseminate mycobacteria via their feces to the environment.

## Materials and Methods

### Animals

Eighteen wild turkey poults (ten male; eight female) were obtained from Murray McMurray Hatchery, Webster City IA. Twelve of the birds were inoculated and the remaining six were sham inoculated controls. Inoculated and

control birds were housed in separate rooms. Inoculated birds were housed individually in biosafety cabinets in a high efficiency particulate air (HEPA)-filtered room (biolevel-3 conditions) at the Michigan State University Containment Facility.

**Inocula**

Inocula used were prepared by the tuberculosis laboratory, Michigan Department of Community Health (MDCH). The *M. bovis* isolate was from a positive WTD identified by annual surveillance and was confirmed as the strain typical to all cases by restriction fragment length polymorphism (RFLP). Seven-day growth in Middlebrook 7H9 broth was adjusted to 0.5 McFarland turbidity standard, diluted to 1:100 with sterile water and tested by plate counts to determine the colony-forming units (CFU) per unit of volume. The dose was confirmed again at the time of inoculation. A single 1 ml dose containing approximately 1 x $10^5$ CFU/ml was administered orally via a tomcat catheter. Intratracheal (IT) inoculates had a 0.5 ml dose injected into the trachea via a 1- cc tuberculin syringe.

**Study design**

All experimental procedures were approved by the All-University Committee on Animal Use and Care at MSU. Seven-day old turkey poults were randomly separated into six oral inoculates, six IT inoculates and three sham inoculates for each route and dose. Fecal samples were collected from each bird on the day prior to inoculation and on day 1 post inoculation (PI). Fecal samples were obtained from surviving birds on

days 30 and 60 PI. All fecal samples were submitted to the tuberculosis laboratory, MDCH, for mycobacterial isolation and identification. Birds were fed Purina® turkey grower W/O (Purina Mills Inc., St Louis, MO, see appendix V) and water *ad libitum*. Birds were evaluated daily for food and water consumption and general physical condition. Each bird was weighed on the day of inoculation and every two weeks thereafter. Two birds from each inoculation group and one control bird from the corresponding sham inoculates were sacrificed on days 30, 60 and 90 PI. Turkeys were euthanized by intracardiac injection of sodium pentobarbital solution (Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI). At necropsy the total body weight in grams (g) as well as the weight of the liver, lung and spleen was recorded for each bird. Tissues collected at necropsy were preserved in 10% neutral buffered formalin (NBF) and included brain, lung, trachea, heart, liver, kidney, spleen, gonad, adrenal gland, proventricular-ventricular junction, pancreas, small intestine (SI) and cecal tonsil. Tissues were routinely processed and sectioned (5μm) for staining with hematoxylin and eosin (H and E). All tissues were also stained with Ziehl-Neelsen (acid fast).

Tissues for mycobacterial culture were collected using sterile instruments and were grouped into three pools; pool A: lung and trachea, pool B: liver, kidney and spleen, pool C: small intestine and cecum.

**Mycobacterial isolation and identification (refer to index IV)**

Mycobacterial cultures were performed at the Tuberculosis Laboratory, MDCH. Briefly the tissue specimens were homogenized digested and

concentrated (Kent and Kubica, 1985). Fecal samples did not require homogenization and were cultured after digestion and concentration. One each of a Lowenstein-Jensen medium slant (Becton-Dickinson, Cockeysville, MD), Middlebrook 7H11S medium slant (Becton-Dickinson) and a BACTEC 12B broth vial (Becton-Dickinson, Sparks, MD) was inoculated with the resulting concentrated material. Media were examined weekly for mycobacterial growth up to 8 weeks. Cultures determined to consist of acid fast organisms by slide examination (Kent and Kubica, 1985) were tested by nucleic acid probe (Accuprobe®, Gen-Probe® San Diego, CA) to ascertain if they were members of the *M. tuberculosis* complex (Reisner et al., 1994). Biochemical testing and high-performance liquid chromatography was performed for complete speciation of mycobacteria that is to differentiate *M. bovis* from other members of the *M. tuberculosis* complex (Reisner et al., 1994; Butler, 1991; Kent and Kubica, 1985).

**Statistical analysis**

SAS version 9.1.3 statistical software was used for all calculations (SAS Institute, Inc., Cary, NC). The Wilcoxon rank-sum test was used to ascertain statistical significance ($P < 0.05$) of changes in total body weight and organ weights within treatment groups and between groups. The association between routes of exposure and outcomes of interest (gross lesions, microscopic lesions and mycobacterial culture) were compared with the two-tailed Fischer's exact test.

## Results

### Weight change data

There was no significant change in the total body weight in any of the turkeys from the three treatment groups for the duration of the study ($P > 0.05$). Additionally, none of the birds exhibited any adverse clinical signs or symptoms of inappetence.

### Gross lesions

There were no gross lesions suggestive of mycobacteriosis detected in any bird from the three treatment groups on post mortem evaluation.

### Microscopic lesions

In all of the turkeys there were no microscopic lesions consistent with or suggestive of mycobacteriosis detected in any of the tissues evaluated. Acid fast stained sections were negative. Non-tuberculosis associated lesions detected in wild turkeys included a single foreign body granuloma at the proventricular-ventricular junction in an oral inoculate at day 60 PI and focal superficial mucosal ulceration within the proventriculus in an oral inoculate at 90 days PI. Nonspecific findings detected in some birds were prominent lymphoid aggregates surrounding secondary bronchi in the lung and randomly disseminated in the parenchyma of liver, kidney, adrenal gland and testis in male birds (total number of turkeys affected; oral 6, IT 6, controls 5).

**Mycobacterial isolation/identification**

Visceral tissues in pool B (liver, lung and spleen) in two IT inoculated turkeys were positive for *M. bovis* on day 30 PI. Tissues from oral inoculates and control birds were consistently negative for *M. bovis*. Tissues in pool C (small intestine and cecum) in two turkeys, one IT inoculate and the other oral were positive for *M. avium* on day 60 PI. A *Mycobacterium* spp. organism other than M TB complex or *M. avium* complex was cultured from pool C of an oral inoculate on day 90 PI. Fecal samples in all birds were consistently negative for *M. bovis* prior to inoculation and on days 1, 30 and 60 post-inoculation

**Statistical Analysis**

Changes in total body weight (Wilcoxon rank-sum $\chi^2$= 2.63; P = 0.2316) and organ weights (Wilcoxon rank-sum $\chi^2$ liver =0.8889, P =0.6412; lung = 0.1520, P = 0.9268; spleen = 0.5395, P = 0.7636) in turkeys in the three treatment groups were not statistically significant. As there were no gross or histologic lesions consistent with tuberculosis detected in any of the turkeys this nullified the necessity for statistical analysis. Similarly, statistical tests were not required for the consistently negative fecal cultures.

**Discussion and conclusion**

Results of this study indicate that wild turkeys of the order Galliformes are highly resistant to infection with *M. bovis*. This organism was only cultured from

the visceral pool of tissues in two birds 30 days PI that were inoculated intratracheally. However, there were no gross or microscopic lesions detected in these two birds to suggest that this was indeed an active infection (Table 2:1). The only conclusion that can be derived from this occurrence is that such birds were able to disseminate the organism from the site of inoculation but remained physiologically nonresponsive to the presence of *M. bovis* organisms.

Further support for turkeys being resistant to *M. bovis* is that they were inoculated at 7 days of age when their immune system should be still immature but in spite of this they were still resistant. In a similar challenge study in chickens with *M. avium* birds as young as eight weeks of age were highly susceptible to mycobacterial infection (Tell et al., 2001).

The wild turkeys in this study were not observed to shed *M. bovis* in their feces on any of the days selected for sampling post-inoculation. Again this supports their high resistance to bovine tuberculosis as fecal shedding is the principal way in which *M. avium* is spread from infected to susceptible birds (Tell et al., 2001).

Exposure of WTD to feed material contaminated with *M. bovis* is a proven means of transmission of bovine tuberculosis in this species (Palmer et al., 2004[b]; Palmer et al., 2001). Results of environmental contamination in cattle-to-cattle spread and cattle to other species is more equivocal (Phillips et al. 2003).

Additionally, the results of this study in conjunction with the previous inoculation studies done in pigeons, crows and starlings suggests that avian species are variably susceptible to *Mycobacterium* spp. organisms. Pigeons are

reportedly highly resistant to *M. avium* but are, however, moderately susceptible to *M. bovis* (Fitzgerald et al., 2003[b]). The reverse of this is true in wild turkeys which are highly resistant to *M. bovis* but moderately susceptible to *M. avium* (Tell et al., 2001). To summarize results in the series of experimental studies on susceptibility to infection with *M. bovis* in avian species known to frequent the area of endemic bovine tuberculosis in Michigan pigeons are susceptible, starlings and crows are partially susceptible, and wild turkeys and mallard ducks are highly resistant (Fitzgerald et al., 2005; Fitzgerald et al., 2003 [b]; Butler et al., 2001). Hence, the results of this study and previous studies would suggest that it is imprudent to extrapolate the effects of infection with a particular *Mycobacterium* spp. organism across various avian species. Moreover, further research may be required on the physiology of avian species susceptible to infection with *Mycobacterium* spp. organisms as well as on their immunological response to such infection.

In conclusion, the results of this study in which *M. bovis* was experimentally inoculated in wild turkeys via oral and intratracheal routes demonstrate that such birds are resistant to infection with this organism. They were no gross or microscopic lesions consistent with mycobacteriosis detected in any inoculated turkey for the duration of the study. Consistently negative PI fecal cultures also indicate that these birds are unlikely to contribute to the retention or spread of this organism on infected farm premises.

**Table 2:1** Summary of research findings in wild turkeys (*Meleagris gallopavo*) inoculated with *M. bovis*

* - Number of birds expressing this feature/ total number of birds tested

† - Histologic lesions

‡ - Intratracheal

§ - Includes two IT inoculates positive for *M. bovis* on day 30 post-inoculation (liver, kidney and spleen)

‖ - An IT inoculate positive for *M. avium* 60 days post-inoculation in the intestine

¶ - Includes an oral inoculate positive for *M. avium* in the intestine on day 60 post-inoculation and another oral inoculate positive for *Mycobacterium* spp. other than *M. tuberculosis* complex or *M. avium* complex in the intestine on day 90 post-inoculation

**Table 2:1 Summary of research findings in wild turkeys (*Meleagris gallopavo*) inoculated with *M. bovis***

| Treatment Group | Number of turkeys | Gross Lesions* | Histo Lesions*† | Mycobacterial Culture Tissue* *M. bovis* | Mycobacterial Culture Tissue* Non *M. bovis* | Fecal* |
|---|---|---|---|---|---|---|
| IT‡ | 6 | 0/6 | 0/6 | 2/6§ | 1/6‖ | 0/6 |
| Oral | 6 | 0/6 | 0/6 | 0/6 | 2/6¶ | 0/6 |
| Control | 6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |

# CHAPTER THREE

## *Mycobacterium bovis* Experimental Inoculation in Three Wild Rodent Species: Meadow Voles (*Microtus pennsylvanicus*), House Mice *(Mus musculus)* and Norway Rats *(Rattus norvegicus)*


### Abstract

*Mycobacterium bovis* has a wide host range and is known to infect several wildlife host species, which effectively limits attempts to eradicate bovine tuberculosis from livestock. Therefore the purpose of this current study was to determine if several rodent species, namely meadow voles (*Microtus pennsylvanicus)*, house mice *(Mus musculus)* and Norway rats *(Rattus norvegicus)* which are known to frequent the area of the lower peninsula of the state of Michigan where bovine tuberculosis is endemic, can be experimentally infected with *M. bovis*. The objectives of the study were to 1) determine if these rodents can be infected, and if so, to document attendant pathological processes/pathogenesis; 2) detect any fecal shedding of *M. bovis*; 3) evaluate the relative susceptibility of the three species and 4) determine if genotype is a factor in susceptibility in house mice. Thirty-six animals of each species were used and studies were run for 60 days. In each study there were three treatment groups with 12 animals per group. The voles received high dose inocula via oral and intranasal routes where as the mice and rats were given high dose and low dose oral inocula. Meadow voles were the most susceptible to *M. bovis* infection. In intranasal inoculates all 12 voles were affected (gross and microscopic lesions, positive tissue and fecal mycobacterial

cultures). Seven of the oral inoculates were similarly affected. House mice were also susceptible to infection (14/24 animal were culture positive). Thus, although house mice were of a resistant phenotype for the *Bcg* gene, this alone did not protect them from infection. Rats were essentially resistant as there was a single positive culture in a high dose inoculate animal with minimal attendant lesions detected in this animal and no lesions or positive mycobacterial cultures in the other 23 inoculated rats. Results of this study indicate that meadow voles and house mice can be infected with *M. bovis* and can potentially serve as spillover hosts. Concerted efforts should, therefore, be made to reduce or eliminate these rodents on premises where domestic livestock with bovine tuberculosis are found.

**Introduction**

*Mycobacterium bovis* is able to infect a wide range of species including humans (O'Reilly and Daborn, 1995; Grange and Yates, 1994). Thus much interest is generated in cross-species infection and reservoir hosts. Reservoir hosts identified include badgers in Great Britain, brush-tail possum and ferrets in New Zealand and white tail deer (WTD) in Michigan, USA (deLisle et al., 2002; deLisle et al., 2001; Morris et al., 1994).

Subsequent to the discovery in 1994 of WTD in Michigan serving a reservoir host for *M. bovis*, surveillance has been ongoing to identify any other species that may act as reservoir hosts. Several carnivore and omnivore species such as coyotes, raccoons, bobcats, opossums and red foxes were identified as spill-over hosts but to date WTD

are the only known reservoir host in this outbreak (Bruning-Fann et al., 2001; Schmitt et al, 1997)

A continuing obstacle in the control or eradication of *M. bovis* from infected premises is the presence of these wildlife reservoirs. If infection is uncontrolled in these species, then domestic livestock and indeed humans are at constant risk for infection or reinfection (Bengis *et al.*, 2002; Wedlock *et al.*, 2002; Cousins, 2001; Nelson, 1999; O'Reilly and Daborn, 1995).

Prior to this study, research on tuberculosis in mice and rats has primarily been limited to laboratory/albino strains. These laboratory strains are derived from house mice and Norway rats respectively (National Wildlife Federation, enature). Voles, house mice and brown rats are all known to be prolific breeders. The diet of voles and house mice consists of grasses, seed grain, bark and some insects. Norway rats are omnivorous and feed on meat, insects, wild plants, seed and stored grains. In all instances due to the close association of these rodents with man and livestock, they can potentially be exposed to undigested grain in fecal material from tuberculous cattle. Rats can potentially feed on a tuberculous carcass and contaminated eviscerated material (gut piles). Consequently concern is expressed about such species potentially disseminating mycobacteria on farm premises. Such concern is warranted as mice and rats are known to spread diseases such as typhus, spotted fever, tularemia, bubonic plague, Hantavirus, Lyme disease, toxoplasmosis and leptospirosis. Voles can also be infected with *Francisella tularensis*, the causative agent of tularemia (Sainsbury, 2003; Wallach and Boever, 1983). Furthermore these rodent species are themselves preyed upon by birds, carnivore and omnivore species such as owls, hawks, dogs, house cats,

skunks, weasels, foxes and coyotes and could also potentially spread *M. bovis* to these predators. Infection in these species is also of concern as they have the potential of infecting humans and initiating a public health crisis.

In inbred laboratory strains of mice it has been suggested that the *Bcg* gene (also known as *Nramp1* and *Scl11a1*), located on chromosome one, may have some function in protecting animals with the homozygous/dominant phenotype ($Bcg^r$) from infection with intracellular organisms namely *Mycobacterium* spp., *Salmonella typhimurium* and *Leishmania donovani*. Conversely, mice with the heterozygous/recessive phenotype ($Bcg^s$) are susceptible to intracellular pathogens (Skamene et al., 1998). Nucleotide sequencing has revealed that a single nucleotide change substituting A for G, yielding a change in amino acid sequence from nonconservative glycine to aspartate at position 169 is predicted in mice with the $Bcg^s$ phenotype (Nakanaga et al 1999, Govoni et al 1996, Vidal et al, 1995, Sakmene 1994). Yet, there are no published studies on the effect of the *Bcg* gene in infections with the aforementioned pathogens in the wild type house mouse from which today's laboratory strain were derived.

As a continuation of experimental inoculation studies in various species [North American opossums, crows, starlings, pigeons, mallard ducks] (Fitzgerald et al., 2005; Fitzgerald et al, 2003 [a]; Fitzgerald et al., 2003 [b]; Diegel et al, 2002; Butler et al., 2001) known to frequent farms in the northeastern section of the lower peninsula of Michigan which is the nidus of the current *M. bovis* outbreak in this state, it was realized that little was known about the pathology and pathogenesis of bovine tuberculosis in the three most common rodent species which are often recognized infesting such premises. If

such species can be infected, and if infection is uncontrolled in these species, then they could potentially place domestic livestock at constant risk. To date there are no reported studies of *M. bovis* infection and attendant risk factors in the three wild rodent species evaluated in this current study meadow voles (*Microtus pennsylvanicus*), house mice (*Mus musculus*) and brown rats (*Rattus norvegicus*).

The stated hypotheses of this study were 1) free living wild rodent species indigenous to Michigan can be infected with *M. bovis* and serve as reservoir or spillover hosts. Dissemination of mycobacteria from these rodents to the environment places livestock at risk, and 2) in house mice the *Bcg*$^r$ resistance phenotype protects these animals against infection with *M. bovis*.

The objectives of this study were to determine i) if the rodents studied can become infected with *M. bovis* when experimentally inoculated and further if they can disseminate mycobacteria into the environment via fecal shedding following inoculation, ii) the relative susceptibility to infection with *M. bovis* in the three species, iii)specifically in the house mouse, what role if any does genotype play in susceptibility, and iv) documentation of any variation in the pathology of *M. bovis* infection in the three rodent species (gross and microscopic lesions, variations in total body weight and organ weights, mortality, dissemination and maintenance of infection).

## Materials and Methods

### Inocula

Inocula used were prepared by the tuberculosis laboratory, Michigan Department of Community Health (MDCH). The *M. bovis* isolate is from a positive WTD identified

by annual surveillance and was confirmed as the strain typical to all cases by restriction fragment length polymorphism (RFLP). Seven-day growth in Middlebrook 7H9 broth was adjusted to 0.5 McFarland turbidity standard, diluted to 1:100 with sterile water and tested by plate counts to determine the colony-forming units (CFU) per unit of volume. The dose was confirmed again at the time of inoculation. Voles and house mice were anesthetized prior to inoculation with isoflurane (IsoFlo®, Abbott Animal Health, North Chicago, IL) administered in an inhalation chamber attached to a gas anesthesia machine with a precision vaporizer. Animals were dosed orally via a tomcat catheter gavage. Voles were inoculated intranasally in each nostril via a micropipette.

**Study design**

Each animal was weighed prior to inoculation (day 0) and at weekly intervals there after. They were evaluated daily for signs of respiratory distress, weight loss or other signs of ill health (bristled hair, hunched posture, reluctance to move). Animals were euthanized with an overdose of isoflurane (IsoFlo®, Abbott Animal Health, North Chicago, IL) to minimize any suffering. All experimental procedures were approved by the All-University Committee on Animal Use and Care at MSU.

**Meadow voles**

Thirty-six meadow voles (19 male; 17 female) were sourced from the MSU meadow vole colony in Giltner Hall. All animals tested negative for five specific pathogens prior to inoculation (listed in appendix I). To assess fecal shedding, fecal samples for mycobacterial culture and isolation were obtained from each vole on the

day prior to oral inoculation, day 1 post inoculation (PI), and from surviving animals on day 30 PI. The voles were randomly assigned to one of four groups: 12 received 5 x $10^3$ CFU of *M. bovis* orally in a total volume of 0.5 ml, 6 oral sham inoculates were given a similar volume of sterile water, 12 were given 1 x $10^5$ CFU intranasally, a total volume of 20µl in each nostril, and 6 IN were sham inoculates were given a similar volume of sterile water. The voles were housed in a secure BSL-3 facility in rodent cages placed in Horsfal units. Rodent chow (Teklad 22/5 rodent diet (W) 8640, Harlan Teklad, Troy IL, see appendix VI) and water were supplied *ad libitum.* Voles were sacrificed at 30 and 60 days post inoculation (PI) or earlier if they exhibited marked weight loss or signs of illness.

Each animal was weighed prior to inoculation (day 0) and at weekly intervals thereafter. At necropsy, total body weight (TBW) in grams (g) was obtained for each animal. The weight of the lung, liver and spleen was also recorded. Tissues harvested at necropsy were preserved in 10% neutral buffered formalin (NBF) and included brain, nasal turbinates, trachea, lung, heart, liver, kidney, spleen, gonad, adrenal gland, small intestine (SI), large intestine (LI), cranial, thoracic and abdominal lymph nodes. Tissues were routinely processed and sectioned (5µm) for staining with hematoxylin and eosin (H and E). All tissues were also stained with Ziehl-Neelsen (acid fast).

Tissues for mycobacterial culture were collected using sterile instruments and were grouped into three pools: pool A (lung, tracheobronchial lymph nodes and cranial lymph nodes); pool B (liver, kidney, spleen); and pool C (SI, LI and mesenteric lymph nodes).

**House mice**

Thirty-six house mice (18 male; 18 female) were sourced from The Geriatrics Center, University of Michigan, Ann Arbor. All mice tested negative for the 14 specific murine pathogens listed in appendix II. Fecal samples were procured from each mouse as described for the voles on day 0 and day 1 PI, and from surviving animals on days 20 and 40 PI. The mice were randomly assigned to one of three groups: 12 received a high dose of *M. bovis* (1x $10^4$ CFU), 12 received a lower dose (1x $10^2$ CFU) and 12 were sham inoculated controls. Each mouse received 0.25ml total volume orally. The mice were housed and fed in a manner identical to the voles. Mice were sacrificed at 20, 40 and 60 days PI or earlier if they exhibited marked weight loss or signs of illness.

The necropsy protocol for the mice was identical to that previously described for the voles however nasal turbinates were not included in the tissues harvested. Histopathologic processing and staining in addition to the tissues harvested and pooled for mycobacterial culture were identical to that in the voles.

**Norway rats**

Thirty-six male brown Norway rats were obtained from Charles River Laboratories, Portage, MI. All rats tested negative for the specific pathogens listed in appendix III. Fecal shedding was assessed in each rat as previously described for house mice. The rats were randomly assigned to one of three groups: 12 received a high dose of *M. bovis* (5 x $10^3$ CFU), 12 low dose (1 x $10^2$ CFU) and 12 were sham inoculated controls. The

rats were housed and fed in a manner identical to the voles. Rats were sacrificed at 20, 40 and 60 days PI.

The necropsy protocol, histopathologic processing and staining as well as mycobacterial culture were identical to that in the mice.

**Mycobacterial isolation and identification (refer to index IV)**

Mycobacterial cultures were performed at the Tuberculosis Laboratory, MDCH. Briefly the tissue specimens were homogenized digested and concentrated (Kent and Kubica, 1985). Fecal samples did not require homogenization and were cultured after digestion and concentration. One each of a Lowenstein-Jensen medium slant (Becton-Dickinson, Cockeysville, MD), Middlebrook 7H11S medium slant (Becton-Dickinson) and a BACTEC 12B broth vial (Becton-Dickinson, Sparks, MD) was inoculated with the resulting concentrated material. Media were examined weekly for mycobacterial growth up to 8 weeks. Cultures determined to consist of acid fast organisms by slide examination (Kent and Kubica, 1985) were tested by nucleic acid probe (Accuprobe®, Gen-Probe® San Diego, CA) to ascertain if they were members of the *M. tuberculosis* complex (Reisner et al., 1994). Biochemical testing and high-performance liquid chromatography was performed for complete speciation of mycobacteria that is to differentiate *M. bovis* from other members of the *M. tuberculosis* complex (Reisner et al., 1994; Butler, 1991; Kent and Kubica, 1985).

**Bcg gene protocol in house mice**

**DNA extraction**

Briefly, approximately 100mg of fresh frozen liver from each mouse was homogenized in 1mL of TRIzol® reagent (GIBCO BRL®, Grand Island, NY, USA) and then incubated at room temperature for 5 minutes. Next 0.2mL of chloroform per 1 mL of trizol was added, tubes capped and shaken vigorously by hand for 15 seconds. Samples were then incubated at room temperature for an additional 2 to 3 minutes. The solutions were centrifuged at 12, 000 x g for 15 minutes at 4° C. The upper aqueous phase containing RNA was removed and discarded. Next 0.3mL of 95% ethanol was added per 1mL of TRIzol and samples mixed by inversion. Samples were again stored at room temperature for 2 to 3 minutes and then the DNA sediment was pelleted by centrifugation at 2000 x g for 5 minutes at 4° C. The phenol-ethanol supernatant was carefully removed. The DNA pellet was washed twice in a solution containing 0.1 M sodium citrate in 10% ethanol. At each wash the DNA pellet was stored in the washing solution for 30 minutes at room temperature, with periodic mixing, and then centrifuged at 2,000 x g for 5 minutes at 4° C. Following the two washes the DNA pellet was suspended in 75% ethanol, stored for 10-20 minutes at room temperature with periodic mixing and centrifuged at 2,000 x g for 5 minutes at 4° C. The supernatant was removed and the DNA pellet was air dried for 5-15 minutes. The DNA was dissolved in 100µl of 8mM NaOH and 1/10 volume of 10x TE buffer added. The mixture was centrifuged at 12,000 x g for 10 minutes to remove insoluble material. The purified DNA in the supernatant was transferred to a micocentrifuge tube and stored at 4° C or -20 ° C

**Amplification, sequencing and detection procedures**

The variant codon 169 lies near the 5 prime end of exon 6 of the mouse NRAMP gene. To facilitate amplification of this region from genomic DNA, we placed amplification primers in the introns flanking exon 6. The mouse mRNA (L13732) was aligned to the mouse genome using the Genome Browser (available through University of California, Santa Cruz) and the intronic regions identified.

The figure below shows the sequence amplified: Primer targets are in bold, intronic sequences are in lower case and exonic sequences are in uppercase

**caagctat ttgggttcct gac** tccccag atctgggtca ggctgattga
tctgccctac ttggtttcag ATCCCGCTG TGGGaCGGTG TACTGATCAC CATTGTGGAC
ACCTTCTTCT TCCTCTTCTT GGATAACTAT gtgagagaa agcccctcat cttgggggat
aaaagtaggc

agggctgccc ttggggagcc ttggcttctc ctagggctgt gggtcccccc

ccatccagct atatggtgtg a **gcaacttac tcctgtttcc gtt**

**cgttgaatc aggacaaagg caa**

PCR with a final volume of 25 µl was set up using 10 pM primers, 0.635 U DNA polymerase (Gibco BRL, Gaithsersburg, MD) and final concentrations of 100µM nucleotides, 1.5 mM $MgCl_2$, 50mM Tris CL (pH 8.3) and 10 mM KCl. A 1.5 µl DNA test sample was added to a 23.5 µl volume of master mix.

Amplification conditions were as follows: 94 ° C for 4 minutes, 94 ° C for 1 minute, 67 ° C for 1.5 minutes and 72 ° C for 2 minutes repeated 40 times with a final 10 minute extension at 72° C (MJ Research PTC-100 Peltier Thermal Cycler, Watertown MA, USA). The amplification products were separated on 2% agarose gels in TBE buffer (90 mm Tris-borate, 2mm EDTA). A 6 µl aliquot of the PCR product

was restricted with *Aci* I overnight at 37° C The samples were then separated on 2% agarose gels in TBE buffer. The amplification product from the dominant allele produces a 200 base pair (bp) restricted fragment whereas the amplification product from the resistant allele produces a 271 bp fragment. The bands were excised and purified using the QIAEX II kit (Qiuagen, Valencia, CA) following the manufactures directions with each sample resuspended in a final volume of 30 µl of 10mM Tris, pH 8. Manual sequencing was performed using the Thermosequence Sequenase radioloabeled terminator cycle sequencing kit (USB Corporation, Cleveland, OH). Samples were sequenced using the forward primer (5'-CAA GCT ATT TGG GTT CCT GAC) used in PCR amplification. Sequencing followed the manufactuer's standard protocol for dGTP termination. The reaction products were separated on 6% denaturing polyacrylamide gels and visualized by exposure to x-ray film.

**Statistical analysis**

SAS version 9.1.3 statistical software was used for all calculations (SAS Institute, Inc., Cary, NC). The Wilcoxon rank-sum test was used to ascertain statistical significance ($P < 0.05$) of changes in total body weight and organ weights within treatment groups and between groups. The association between routes of exposure and outcomes of interest (gross lesions, microscopic lesions and mycobacterial culture) were compared with the two-tailed Fischer's exact test. The significance of differences between survival times of the rodents with variables of species and treatment was computed with the non-parametric Kaplan-Meier estimate of the survivor function.

## Results

### Animal Health

Ten of the voles exhibited respiratory distress arising from tuberculosis and either died 'naturally' or were euthanized prematurely. This included a total of six oral inoculates and four IN inoculates. Deaths in voles occurred earlier in those that received an oral inoculum (between days 12 and 21 PI) when compared to the IN inoculates (death in three of the four occurred after day 45 PI) (Figures 3:1a and b). Four of the mice in the HD group died prematurely as a result of tuberculosis. Deaths in these mice occurred between days 28 and 35 PI (Figures 3:1a and b). All of the rats remained clinically healthy throughout the study.

### Weight change data

Although the voles were severely clinically affected with tuberculosis (lack of appetite, marked decrease in activity/moribund, respiratory distress/ abdominal breathing) they did not exhibit statistically significant total body weight loss (Wilcoxon rank-sum $\chi^2 = 5.1481$; $P = 0.0795$). The increase in the weight of lungs in voles affected with bovine tuberculosis (intranasal and oral inoculates) when compared to the weight of the lungs in control animals was statistically significant (Wilcoxon rank-sum $\chi^2 = 11.8528$; $P = 0.0027$). However, there was no difference in the weights of the liver and spleen between inoculated and sham inoculated control voles. Four of the HD mice lost weight (ranged from 1.7g to 5.4g lost) during the first 30 days of the study (two of which were euthanized and two died from TB) but after this time period the mice were

able to regain and maintain their body weight. Mice also had no statistically significant body weight loss over the duration of the study (Wilcoxon rank-sum $\chi^2 = 0.3865$; P = 0.8243). As in the voles the increase in the weight of the lungs in inoculated mice (HD and LD) when compared with control mice was statistically significant (Wilcoxon rank-sum $\chi^2 = 9.4977$; P = 0.0087). Inoculation in the Norway rats (HD and LD) caused these animals to have a lower mean total body weight when compared to the sham inoculated controls (Wilcoxon rank-sum $\chi^2 = 22.3844$; P = <0.0001). There was no observable differences in any of the organ weights between inoculated and control rats.

**Gross lesions**

Gross lesions suggestive of tuberculosis were detected in 19 of the 24 inoculated voles and included; pneumonia (severe, focally extensive to diffuse, firm and consolidated), hepatitis and hepatomegaly, splenitis and splenomegaly and lymphadenopathy (submandibular. parotid, cervical, traceobronchial, and mesenteric lymph nodes affected) (Table 3:1 and Figures 3:2 and 3:3). NonTB associated lesions were detected in two voles; severe cecal dilation and an ovarian mass in one vole and a severe urogenital infection in another (cystitis with enteritis in the adjoining intestine, both transmural). In addition two of the voles had cutaneous mites.

Twelve of the 24 inoculated mice exhibited gross lesions suggestive of tuberculosis. Gross lesions of caeogranulomatous multifocal to diffuse and coalescing pneumonia which ranged in severity from mild to severe was present in eight of the mice (all HD days 20 and 40 PI) (Table 3:2 and Figure 3:4). One

mouse in the LD group (day 40 PI) had congested lungs which were mottled red and tan. Enlarged lymph nodes were detected in four mice (superficial cervical, tracheobronchial, mesenteric, inguinal, lumbar) (one HD day 20 PI; two HD day 40PI and one LD day 60PI). Splenomegaly was present in two mice day 60 PI (one HD; one LD). A nonTb associated lesion was present in one mouse a 4mm diameter focal pulmonary mass (HD day 20PI).

There were no gross lesions suggestive of *M. bovis* infection in any of the rats on post mortem evaluation.

**Microscopic lesions**

Histologic lesions in the voles were the most extensive and severe of the three rodent species (Table 3:3 and Figure 3:5). In each of the affected organs lesions were moderate to severe, multifocal to coalescing, caseogranulomatous and granulomatous to pyogranulomatous. Numbers of acid fast bacilli ranged from abundant to rare (Figures 3:6 and 3:7). Of note in the microscopic lesions seen in the voles was partial mineralization which was often evident in areas of caseogranulomatous lymphadenitis (Figure 3:8). One IN inoculate (day 30 PI) had multinucleate giant cells in the liver (Figure 3:9). Moderate to severe granulomatous and necrotizing rhinitis was a prominent feature in the IN inoculates (11 of 12) and in a single oral inoculate (Figure 3:10). Non Tb associated lesions detected in meadow voles on microscopic evaluation included colonic nemotodiasis in five animals, an ovarian teratoma in one vole (#26/ IN), cystitis (transmural

chronic fibrosing, pyogranulomatous and necrotizing with intralesional bacteria) and adjacent transmural enteritis in one vole (# 31).

In house mice histologic lesions consistent with tuberculosis were present most frequently in the lungs and were seen in 11 mice; seven HD and four LD (Table 3:4 and Figure 3: 11). Acute to subacute lesions were seen in mice sacrificed on days 20 and 40 PI and presented as severe coagulative necrosis (sequestrum) with moderate to massive numbers of acid fast bacilli (+ macrophages on day 40 PI). Chronic granulomatous pneumonia with infiltrates of macrophages, epitheliod cells and rare multinucleated giant cells were evident in mice sacrificed at day 60 PI. Acid fast bacilli were present in macrophages in small numbers or were rare. Other organs affected were lymph nodes (three tracheobrochial and one mesenteric) (Figure 3:12) and spleen in four mice, and liver in one animal. In each of these tissues acid fast bacilli were present in macrophages, but tissue architecture was otherwise unaffected. The only nonTb lesion detected microscopically was a focal pulmonary bronchiogenic adenocarcinoma in one mouse day 20 PI.

In a single Norway rat (HD 20 days PI) there was focal aggregation of small numbers of multinucleate giant cells which contained a few acid fast bacilli within a tracheobronchial lymph node (Figure 3:13). In another rat, LD day 60 PI, in sections of cerebrum a single blood vessel (vein) contained a few acid fast bacilli. NonTB associated lesions seen in the Norway rat included a meningeal granular cell tumor (control rat day 40 PI) and a focal sperm granuloma in the testis in another rat (day 40 PI).

**Mycobacterial isolation and identification**

In the voles all fecal cultures were negative prior to inoculation and the sham inoculated controls remained negative on days 1 and 30 PI. Eight of the 12 IN voles and 9 of 12 oral inoculates had positive fecal cultures on day 1 PI. Two of the five surviving IN inoculates had positive fecal cultures on day 30 PI whereas the five surviving oral inoculates all had negative fecal cultures when sampled 30 days PI. All fecal cultures taken prior to inoculation and on days one, 20 and 40 PI for the house mice and rats were consistently negative.

Positive tissue cultures were obtained in all 12 of the IN inoculated voles and in seven of the oral inoculates. Tissues from sham inoculated voles were all negative on culture. Fourteen of the 24 inoculated mice had positive tissue cultures, nine HD and five LD. All tissues from sham inoculated mice were negative. A single HD rat had a positive culture for tissues in pool A on day 20 PI. Tissues in pool B and C were negative. All other rats were consistently negative for all tissue sampled.

**Mouse *Bcg* study**

All of the house mice were of the resistant phenotype and therefore the amplified nucleotide sequence was cut with *Aci I* to give 200 and 71 bp segments (Figure 3:14). Sequencing revealed a guanine nucleotide at position 596 (*Bcg*$^r$) as opposed to adenine which is seen in mice with the susceptible phenotype and this translates to a glycine residue (G) at amino acid position 169 in the Nramp molecule (Figure 3:15).

**Statistical analysis**

There was a statistically significant difference seen between animals of the three different species that were positive for *M. bovis* on mycobacterial culture (Fisher's exact test, $P = 2.927 \times 10^{-24}$). Thus being a vole or mouse was consistent with being highly susceptible to developing bovine tuberculosis. Additionally susceptibility to infection with *M. bovis* is true with both routes of inoculation in meadow voles (Fisher's exact test, $P = 2.123 \times 10^{-13}$).

In voles there was a significant difference between the gross lesions in intranasal versus orally inoculated animals (Fisher's exact test, P =0.0046). A similar association was obtained for histologic lesions in voles (Fisher's exact test, P =0.0046).

With the mice there was a significant difference between the gross lesions in high dose and low dose animals (Fisher's exact test, P =0.0391). However, with microscopic lesions HD inoculation in mice did not indicate greater probability of having lesions when compared to low dose animals (Fisher's exact test, P =0.2203).

**Discussion and Conclusion**

The results of this study indicate that meadow voles are very susceptible to infection with *M. bovis* via oral and intranasal routes and house mice are also susceptible to infection via the oral route. Conversely, Norway rats appear to be very resistant to experimental infection with high doses of *M. bovis* when given via the oral route. Respiratory/ inhalation and oral routes of experimental infection were chosen as they represent the routes of infection known to occur in established wildlife reservoirs

(brush tail possums, badgers, and whitetail deer) and the several spillover hosts of *M. bovis* (de Lisle et al, 2001; O'Reilly and Daborn, 1995). The doses selected were chosen to be of sufficient magnitude to accelerate any expected pathological responses in these species. Additionally similar doses have been used in other inoculation animal studies both within this laboratory and by other researchers (Diegel et al., 2002; Buddle et al., 1994; Corner and Presidente, 1981; Corner and Presidente, 1980).

Evidence in support of meadow voles being the most susceptible to experimental inoculation with *M. bovis* of the three rodent species studied include deaths due to tuberculosis in 10 of the 24 inoculated animals, extensive gross and histologic lesions (19 animals) and positive mycobacterial cultures (19 animals). Sensitivity of voles to *M. bovis* seen in this study supports the work of Jespersen (1974, 1975, 1976, 1977[a, b and c]) in common (*Microtus arvalis*), field (*Microtus agrestis*) and bank (*Clethrionomys glareolus*) voles as well as in the vole rat (*Arvicola terrestris*), all of which developed lethal infections following experimental inoculation of *M. bovis*. Similar results were obtained earlier by Griffith (1939 and 1937) and Wells (1938). Gross lesions of caseous lymphadenitis in addition to granulomatous pneumonia, splenomegaly and hepatitis similar to what obtained in this study were seen in these earlier studies.

Further, the results in this current study demonstrate that infected voles are capable of disseminating *M. bovis* via their feces as there were 19 positive fecal cultures out of the 24 inoculated animals. Seventeen of these occurred on day one post-inoculation and are likely indicative of passive transit through the gastrointestinal tract. The majority of these (nine) were seen in voles that were inoculated orally. However, the eight positive fecal cultures in intranasally inoculated voles suggest that the inoculum was coughed

up from the respiratory tract and subsequently swallowed in these animals. In addition, two of the intranasal inoculates had positive fecal cultures at 30 days post-inoculation which indicates both dissemination of *M. bovis* and a state of infection in these animals. Support for voles having the ability to disseminate *Mycobacterium* spp. organisms via the feces is seen with the closely related *M. microti*, or vole bacillus which like *M. bovis* is a member of the *M. tuberculosis* complex. *Mycobacterium microti* can be spread from vole to vole via the ingestion of the excreta from diseased voles (Rankin and McDiarmid, 1968). Griffith (1939) also detected acid-fast bacilli microscopically (acid-fast smears) in the feces of field voles experimentally inoculated with *M. microti*. However feces in this study were not cultured, so other acid fast mycobacteria/ organisms should be included in a differential.

The meadow voles were more susceptible to infection via the oral route in comparison to the intranasal route as six of these animal died prematurely or were euthanized in less than 21 days (Figure 3:1a) compared to death of a single intranasal inoculate at day 19 with three others occurring within days 46 to 52 post-inoculation.

Positive fecal cultures in the meadow voles also indicate potential to serve as a reservoir host. Although the high doses of *M. bovis* used in this study resulted in the demise of some animals the dose to which these animals would be exposed to in the environment would be much lower. Thus the voles could become infected either orally or via the IN route (both equally can lead to fecal shedding) and survive long enough to further disseminate *M. bovis* via fecal shedding.

The results of this study are the first to document the susceptibility of house mice to infection with *M. bovis* via the oral route. Contrary to Lefford (1984) who states that adult mice cannot be infected by virulent tubercle bacilli via the gastrointestinal tract results of this current study clearly demonstrate that adult house mice can in fact be infected orally. Pierce and associates (1947) did, however, demonstrate that immature house mice (four to six weeks old) were susceptible to oral infection with *M. tuberculosis*. Extensive gross and micoscopic lesions in affected mice as well as positive mycobacterial cultures in the lungs, liver and spleen are indicative of dissemination of *M. bovis* from the site of inoculation, that is the gastrointestinal tract. Both gross and microscopic lesions in mice following oral inoculation with *M. bovis* were most severe in the lung, which is similar to previously published studies in laboratory strains of mice experimentally infected with *M. bovis* (Gunn, Nungester and Hougen, 1933-34, cited in Darzins 1958; Ratcliffe, 1953; Ratcliffe 1952, Glover, 1944; Griffith 1907[b]). Similar to what has previously been reported in mice experimentally infected with *M. tuberculosis*, caseation or calcification of pulmonary granulomas was not evident in the lungs of affected house mice in this study (Cosma et al., 2003). There is however no report of mice naturally infected with *M. bovis* following several surveillance/ environmental studies (Pillali et al., 2000; Fischer et al., 2000; Wilesmith et al., 1986; Little et al., 1982), which is possibly due to their exposure to lower *M. bovis* inoculation doses.

Greater severity of infection was evident in mice that received the higher dose inoculum. A total of eight HD mice (67%) had gross and microscopic lesions consistent with tuberculosis compared to four (33%) of the LD mice. The results in house mice

also suggest that those animals receiving the HD oral inoculum of *M. bovis* suffered adverse effects up to day 40 PI. By this time severe pulmonary disease had occurred in seven of the eight animals in this group which were affected, all eight animals had positive mycobacterial cultures and four deaths due to bovine tuberculosis occurred between days 28 and 35 post-inoculation. However after day 40 it appears that the immune system, likely cell-mediated immunity (CMI), is activated which allowed surviving mice in this group to control or maybe even reverse their infection (based on only one positive mycobacterial tissue culture in the four mice sampled).The converse of this was seen in mice receiving the low dose oral inoculum as chronic pulmonary disease was detected in these animals at day 60 post-inoculation (three of the four animals in this group with positive mycobacterial tissue cultures). This suggests that the initial LD inoculum received by these mice was not as effective in stimulating a CMI response which therefore allowed *M. bovis* organisms to persist in the pulmonary tissues. Thus house mice might serve as an ideal animal model for tuberculosis in humans infected with *M. tuberculosis* and *M. bovis* as there is evidence that some of these mice were able to recover from their infection. However, this would require an in depth study of their immune system.

Although fecal cultures in the house mice were consistently negative, in six of the 14 mice with positive tissue cultures the intestinal pool was positive for *M. bovis*. This indicates that the mice were able to retain (become infected with) *M. bovis* following oral exposure and could possibly have shed mycobacteria sporadically in the feces which was not detected by the sampling methods used in this current study.

Results of the mouse/Bcg study indicate that genetics are not a reliable indicator of susceptibility to infection with *M. bovis* in this wild type strain, since all house mice were determined to be of the resistant phenotype but were relatively susceptible to oral infection. Genotypically house mice conform to the DBA/2 and C57BL/6-Bcg$^r$ strains which are all resistant (Nakanaga et al., 1999). Medina and North (1996) also demonstrated that the Bcg gene was unable to protect resistant strains of mice namely DBA/2, from infection (intavenous) with *M. tuberculosis H37RV*.They concluded that although the resistant allele of the *Nramp1* gene can provide mice with natural resistance to *Salmonella typhimurium* , *Leishmania donovani*, and BCG there was no measurable influence on resistance to infection with virulent *M. tuberculosis*. Likewise the presence of a dominant/resistant *Bcg* in wild type house mice does not afford them any resistance to infection with *M. bovis*.

The lack of gross lesions and consistently negative fecal cultures in rats is indicative of inherent resistance to oral infection with *M. bovis* in this species. However, there was one positive tissue culture (lung, tracheobronchial and cranial lymph nodes) is a single HD rat on day 20 PI. This rat also had histological lesions consistent with mycobacterial persistence/colonization as multinucleated giant cells with acid fast bacilli were present in the tracheobronchial lymph node. Moreover, this also indicates that Norway rats are capable of disseminating mycobacteria following oral inoculation. Lack of lesions (gross and microscopic) and negative mycobacterial cultures in the Norway rats in this study beyond 20 days PI is supportive of prior reports of rats having the ability to modulate their cell-mediated immune response to *Mycobacterium* spp. infection which essentially renders them resistant (Thorns et al., 1982). In a recent

study with female Lewis rats, Sugawara and associates (2004) report that animals infected aerially with *M. tuberculosis* developed granulomatous lesions in the lungs, spleen, lymph nodes and liver. This begs the question of rats being susceptible to *Mycobacterium* spp. organisms via a respiratory route. However, wild type rats are unlikely to be exposed to the large dose of *M. tuberculosis* used in this study ($2 \times 10^6$ CFU) in their natural environment.

Of concern, however, is the finding in this study of decreased total body weight in inoculated rats when compared with controls although none of the rats exhibited any other adverse clinical signs (respiratory distress, bristled hair, reluctance to move etc.). A similar lack of clinical signs in rats affected with bovine tuberculosis following experimental inoculation is also reported by Griffith (1907[a]) with intraperitoneal (IP) and subcutaneous (SQ) routes as well as by Wessels (1941[a]) who utilized the IV route. These other researchers also report widespread replication of *M. bovis* in several tissues (lungs, liver, spleen, lymph nodes, kidneys, omentum, and bone marrow) which was not a feature in this present study. Accordingly, one can conclude that Norway rats are essentially resistant to infection with *M. bovis* at high oral doses and are incapable of disseminating this organism laterally via fecal shedding. Support for rats being dead end hosts for *M. bovis* is gleaned from previously reported environmental survey studies in which rats were negative on culture (Pallai et al., 2000; Wilesmith et al., 1986) or lacked lesions in the face of positive cultures (four animals total) (Little et al., 1982; Bosworth, 1940).

In conclusion, the results of this study indicate that meadow voles are highly susceptible to infection with *M. bovis* via both oral and intranasal routes. Further,

infected voles can disseminate mycobacteria via their feces. In voles, lesions consistent with tuberculosis occurred in several tissues including the lungs, liver and spleen, various lymph nodes and in the intranasal inoculate the nasal turbinates. House mice are also susceptible to infection with *M. bovis* via the oral route but are apparently less efficient in transmitting mycobacteria via fecal shedding when compared to the vole. Affected mice suffered most lesions in the lungs. The rat is the most resistant of the three rodent species to oral infection with *M. bovis*.

In the house mouse *Bcg* genotype alone was found to be ineffective in predicting susceptibility to infection with *M. bovis* as these animals were all of the resistant phenotype ($Bcg^r$; glycine residue at position 169 in the nucleotide sequence) but did in fact become infected with *M. bovis*.

Although meadow voles and house mice are unlikely to encounter the high dose inocula of *M. bovis* used in this study in their natural environment, it is recommended that appropriate measures be taken to eliminate these animals or at least control their numbers on premises where bovine tuberculosis positive animals are present. Further, it would be strongly advised that access of voles and mice to food and water sources of domestic animals be restricted wherever possible.

**Table 3:1 Gross lesions consistent with tuberculosis detected at necropsy in voles experimentally inoculated with *Mycobacterium bovis***

| **Voles: # affected/ total # in group** | **Route** | **Lung** | **LN*** | **Liver** | **Spleen** |
|---|---|---|---|---|---|
| **Day 30** | | | | | |
| 7/7 | IN† | 5 | 6 | 2 | 1 |
| 7/7 | Oral | 7 | 3 | 0 | 2 |
| **Day 60** | | | | | |
| 5/5 | IN† | 3 | 5 | 1 | 5 |
| 0/5 | Oral | 0 | 0 | 0 | 0 |
| **Total # affected/ total # inoculated = 19/24** | | **15** | **14** | **3** | **8** |

* Lymph node
† Intranasal

**Table 3:2 Gross lesions consistent with tuberculosis detected at necropsy in mice experimentally inoculated with *Mycobacterium bovis***

| **Mice: # affected/ total # in group** | **Dose** | **Lung** | **LN*** | **Liver** | **Spleen** |
|---|---|---|---|---|---|
| **Day 20** | | | | | |
| 4/4 | HD† | 3 | 1 | 0 | 0 |
| 1/4 | LD‡ | 1 | 0 | 0 | 0 |
| **Day 40** | | | | | |
| 4/4 | HD† | 4 | 2 | 0 | 0 |
| 1/4 | LD‡ | 1 | 0 | 0 | 0 |
| **Day 60** | | | | | |
| 1/4 | HD† | 0 | 1 | 0 | 1 |
| 1/4 | LD‡ | 0 | 0 | 0 | 1 |
| **Total # affected/ total # inoculated = 12/24** | | **9** | **4** | **0** | **2** |

* Lymph node
† High dose
‡ Low dose

**Table 3:3 Microscopic lesions consistent with tuberculosis detected in voles experimentally inoculated with *Mycobacterium bovis***

| Voles: # affected/ total # in group | Route | Lung | LN* | Liver | Spleen | Turbinates | Other |
|---|---|---|---|---|---|---|---|
| **Day 30** | | | | | | | |
| 7/7 | IN† | 4 | 5 | 7 | 6 | 6 | Encephalitis (1) |
| 7/7 | Oral | 7 | 3 | 7 | 7 | 1 | None |
| **Day 60** | | | | | | | |
| 5/5 | IN† | 4 | 5 | 5 | 5 | 5 | Encephalitis (1) Thyroiditis (1) |
| 0/5 | Oral | 0 | 0 | 0 | 0 | 0 | None |
| **Total # affected/ total # inoculated = 19/24** | | **15** | **13** | **19** | **18** | **12** | **3** |

* Lymph node
† Intranasal

**Table 3:4 Microscopic lesions consistent with tuberculosis detected in mice experimentally inoculated with *Mycobacterium bovis***

| **Mice: # affected/ total # in group** | **Dose** | **Lung** | **LN*** | **Liver** | **Spleen** |
|---|---|---|---|---|---|
| **Day 20** | | | | | |
| 4/4 | HD† | 3 | 1 | 0 | 1 |
| 0/4 | LD‡ | 0 | 0 | 0 | 0 |
| **Day 40** | | | | | |
| 4/4 | HD† | 4 | 1 | 1 | 1 |
| 1/4 | LD‡ | 1 | 0 | 0 | 1 |
| **Day 60** | | | | | |
| 0/4 | HD† | 0 | 0 | 0 | 0 |
| 3/4 | LD‡ | 3 | 2 | 0 | 1 |
| **Total # affected/ total # inoculated = 12/24** | | **11** | **4** | **1** | **4** |

* Lymph node
† High dose
‡ Low dose

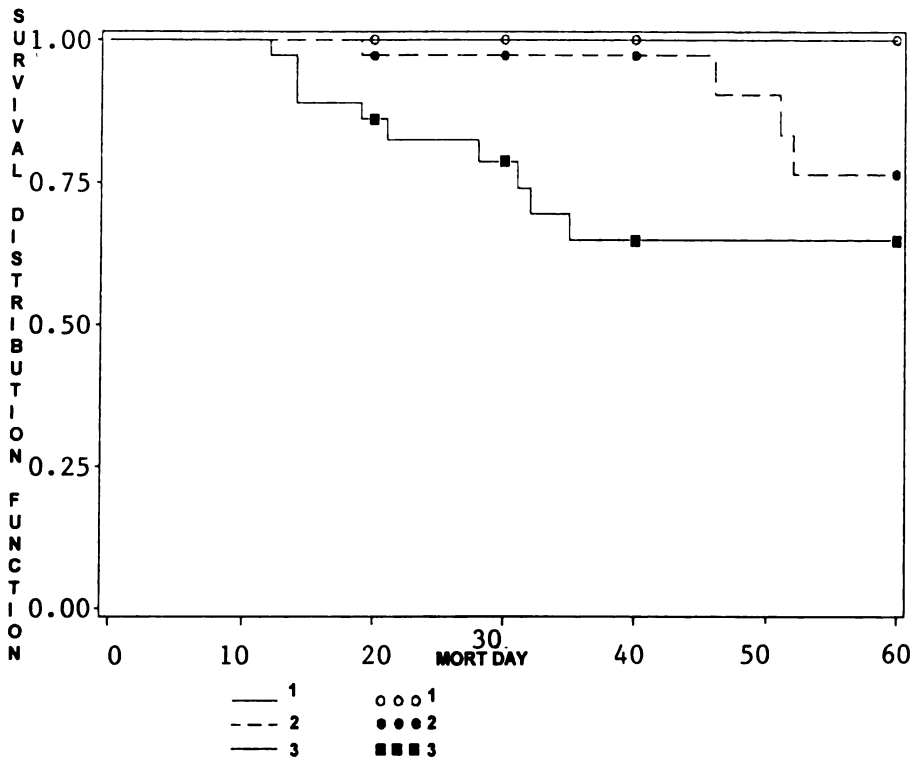



**Figure 3:1a** Survival analysis for the rodent species according to treatment. The treatment codes are as follows; 1 = controls, 2 = low dose/intranasal, 3 = high dose/oral

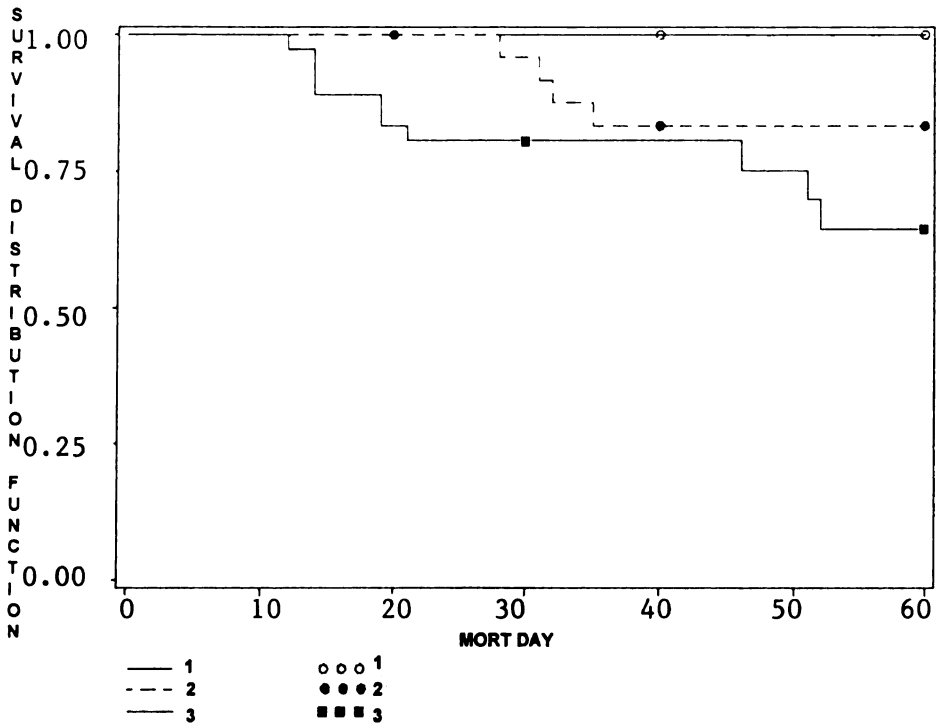



**Figure 3:1b** Survival analysis for the rodent species according to species.

The species codes are as follows; 1 = rat, 2 = mouse, 3 = vole

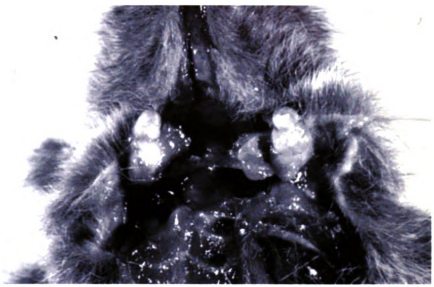


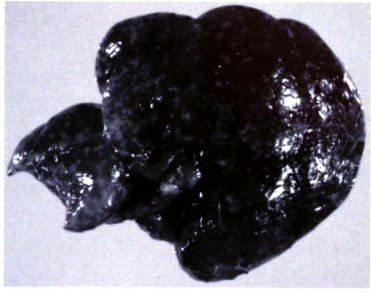
**Figure 3:2** Vole # 18 IN 51 days PI

Bilaterally enlarged submandibular and superficial cervical lymph nodes

**Figure 3:3** Vole # 14 IN 52 days PI with *M. bovis*

The liver (top) contains several (more than 100) randomly disseminated pale tan slightly raised granulomas which ranged from 1 mm to 2 mm in diameter.

The lungs (bottom) were diffusely firm, non-collapsing, mottled pale tan and red and contained similar raised granulomas which ranged from 1mm to 5 mm in diameter



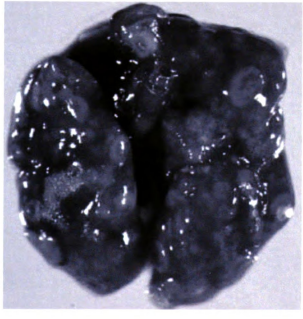


**Figure 3:3**

**Figure 3:4** Bottom- House mouse # 8 HD (died) 30 days PI with *M. bovis* The lungs were diffusely firm, non-collapsing, mottled pale tan and red and contained raised granulomas which ranged from 2mm to 6 mm in diameter admixed with foci of necrosis

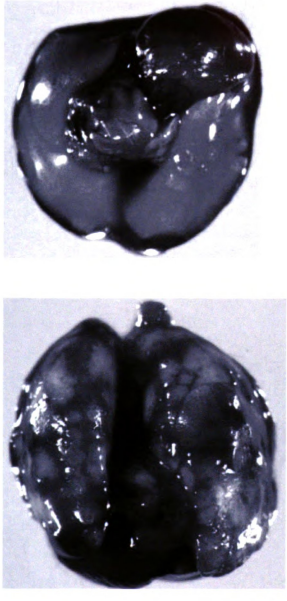


**Figure 3:4**

**Figure 3:5** Top - Vole- normal lungs sham inoculated control, H&E X 6.25

**Figure 3:5** Bottom - A section of lung from vole # 16 IN day 19 PI with *M. bovis*, H&E X 12.5

The lungs have severe, diffuse granulomatous and necrotizing pneumonia which has almost totally effaced the pulmonary parenchyma

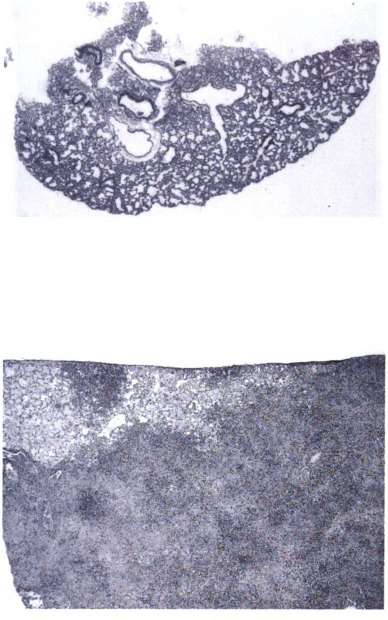


**Fig 3:5**

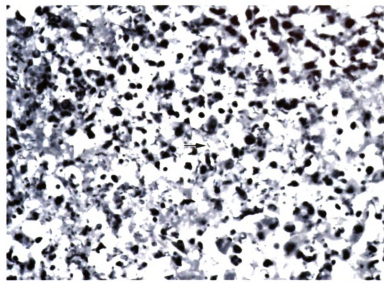


**Fig 3:6** Photomicrograph of a section of lung from a vole inoculated with *M. bovis* IN, Ziehl-Neelsen acid-fast stain

A section of lung from vole # 16 IN day 19 PI with *M. bovis*, X 200

The necrotic debris within the pulmonary parenchyma contains numerous acid-fast bacilli (arrow), Ziehl-Neelsen acid-fast

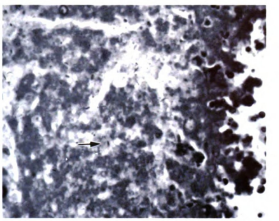


**Figure 3:7** Photomicrograph of a section of nasal turbinate from a vole inoculated with *M. bovis* IN, Ziehl-Neelsen acid-fast stain

A section of nasal turbinate from vole # 13 IN day 60 PI with *M. bovis*, H&E X 200

The necrotic debris adhered to the nasal turbinate surface contains moderate number of acid-fact bacilli (arrow), Ziehl-Neelsen acid-fast

**Figure 3:8** Section of mesenteric lymph node from a vole inoculated with *M. bovis* IN

A section of mesenteric lymph node from vole # 19 IN day 46 (euthanized prematurely) PI with *M. bovis*, H&E X 6.5

The mesenteric lymph node has severe, focally extensive caseogranulomatous lymphadenitis

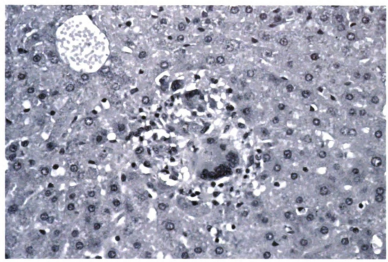


**Fig 3:9** Section of liver from a vole inoculated with *M. bovis* IN

A section of liver from vole # 25 IN day 30 PI with *M. bovis*, H&E X 100

The liver contained a few (less than 10) randomly disseminated multinucleated giant cells (one shown) [mild multifocal granulomatous hepatitis]

**Figure 3:10** Top - A section of nasal turbinate from vole # 13 IN day 60 PI with *M. bovis*, H&E X 6.25

There is moderately severe, focally extensive granulomatous rhinitis

**Figure 3:10** Bottom - A section of nasal turbinate from vole # 13 IN day 60 PI with *M. bovis*, H&E X 50

Necrotic cellular debris is adhered to the epithelial surface of the nasal turbinate (arrow)

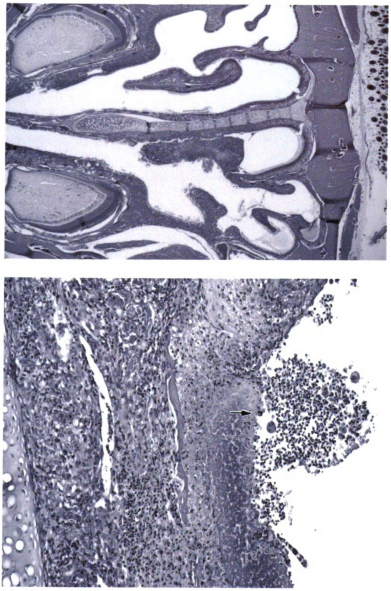


**Figure 3:10**

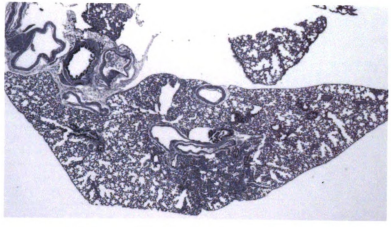
**Figure 3:11** Top - House mouse- normal lungs sham inoculated control, H&E X 7.5

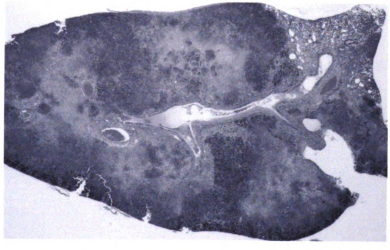
**Figure 3:11** Middle - A section of lung from house mouse # 16 HD day 40 PI with *M. bovis*, H&E X 7.5

The normal lung parenchyma is almost totally effaced by severe extensive coagulative necrosis. Admixtures of degenerate neutrophils, lymphocytes, plasma cells and macrophages surround the necrotic foci.

**Figure 3:11** Bottom - A section of lung from house mouse # 16 HD day 40 PI with *M. bovis*, X 150

The necrotic pulmonary parenchyma contained abundant acid-fast bacilli (arrow), Ziehl-Neelsen acid-fast





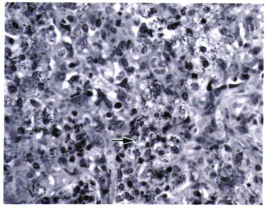


**Figure 3:11**

**Figure 3:12** Top - A section of tracheobronchial lymph node from mouse #3 LD day 60 PI with *M. bovis*, H&E X 50

The medullary sinuses of the tracheobronchial lymph node are infiltrated by moderate numbers of histiocytes

**Figure 3:12** Bottom -A section of tracheobronchial lymph node from mouse #3 LD day 60 PI with *M. bovis*, X 250

The histiocytes which have infiltrated the medullary sinuses have small numbers of acid-fast bacilli within the cytoplasm (arrow), Ziehl-Neelsen acid-fast

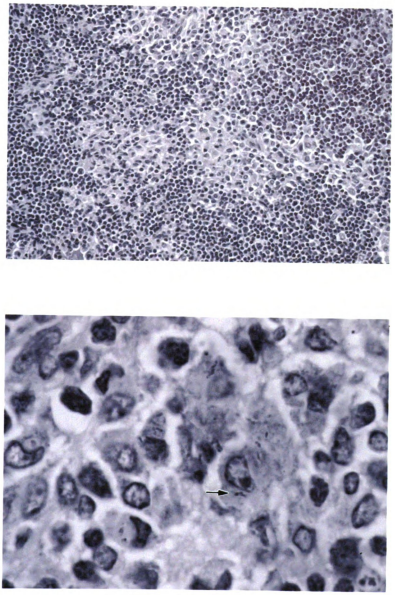


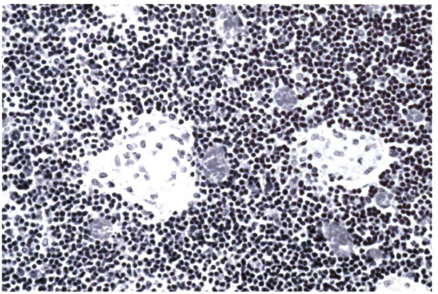
**Figure 3:12**

**Figure 3:13** Top - A section of tracheobronchial lymph node from Norway rat #29 HD day 20 PI with *M. bovis*, H&E X 100

The tracheobronchial lymph node had randomly disseminated multinucleated giant cells (less than 10)

**Figure 3:13** Bottom -A section of tracheobronchial lymph node from Norway rat #29 HD day 20 PI with *M. bovis*, H&E X 500

The multinucleated giant cells contained a few acid-fact bacilli (arrow), Ziehl-Neelsen acid-fast



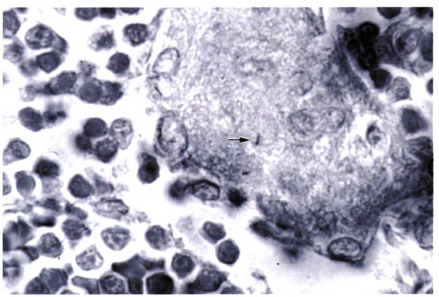


**Figure 3:13**

**Figure 3:14** PCR amplification of *Bcg* DNA.

M – molecular weight marker 100-bp ladder

U – amplified Bcg DNA which is uncut

Mouse samples- all other lanes are DNA from the 36 mice that were amplified and then cut with *Aci I* which show the 200 bp cut segment

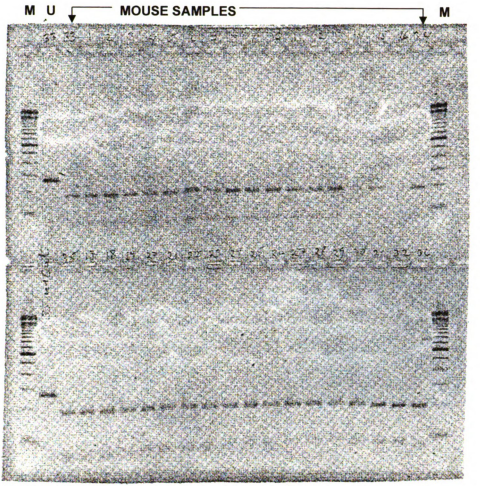



**Figure 3:14**

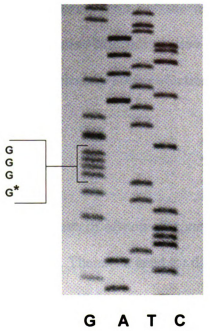



**Figure 3:15** Nucleic acid sequence of *Nramp 1 Bcg* DNA in house mice

The sequence indicated in the gel is as follows (top to bottom)

GTGTTACCATAGTCATGTGGCGGGG*TGTCGCCCTACGA

*Indicates the guanine nucleotide at position 596

# CHAPTER FOUR

## A Comparison of Mycobacterial Culture with Polymerase Chain Reaction on Granulomas Isolated by Laser Capture Microdissection in the Diagnosis of *Mycobacterium bovis* Infection


### Abstract

A major impediment to the diagnosis of *Mycobacterium bovis* infection is the organism's fastidious growth in culture. There is a need for diagnostic techniques which have improved sensitivity and specificity compared to mycobacterial culture, particularly those that are more cost effective with reduced turnaround times. In this study laser capture microdissection (LCM) was used to procure granulomas from formalin-fixed, paraffin-embedded lung tissues from house mice (*Mus musculus*) inoculated with *Mycobacterium bovis*. Thirty-six lung samples were evaluated. PCR - DNA amplification of a 123-bp segment of IS 6110 which is specific for organisms of the *M. tuberculosis* complex was used to detect *M. bovis* in the microdissected granulomas. LCM/PCR results were compared with the gold standard of mycobacterial culture. Cohen's kappa (κ) values for complete agreement between mycobacterial culture and LCM/PCR in diagnosing *M. bovis* infected house mice was 0.68 (95 % CI 0.425- 0.941) which is classified as 'good' agreement. The LCM/PCR method has a faster turnaround time of three to four days when compared to 8-12 weeks for culture. Additionally, the LCM method was more cost effective at $55 per sample, compared

with $100 for mycobacterial culture. Thus, the LCM method appears to be inferior to mycobacterial culture in detecting true positives or infected individuals and would, therefore, not be adequate for use as a screening test. Yet a specificity of 95.6% may be indicative of this procedure having some usefulness as a confirmatory test.

**Introduction**

In 1993 the World Health Organization (WHO) eclared that tuberculosis in humans due to *M. tuberculosis* was a global health emergency (Ainsa et al., 2001). Rapid and accurate diagnosis of a disease outbreak is an essential first step in an epidemiologic outbreak investigation, and subsequent control/ management as antibiotic treatment differs according to the species of mycobacterium (Soini and Musser, 2001; Watterson and Drobniewski, 2000). A chief goal in tuberculosis research, that is primarily evident with *M. tuberculosis* infections but is no less important for *M. bovis*, is the development of improved diagnostic and therapeutic intervention strategies (Garg et al., 2003).

Although mycobacterial culture using a combination of solid and liquid media is accepted as the 'gold standard' for isolation of *Mycobacterium* spp. organisms prior to identification, it is very time consuming. These bacteria have slow generation times on the order of 15 to 20 hours and visible bacteria are detected only after several weeks of incubation. It may take as long as six to eight weeks to identify mycobacteria in clinical specimens (Butcher et al., 1996). Since the discovery of mycobacteria by Koch in 1882, the acid-fast smear technique is still the most rapid method for detection of *M. tuberculosis* (Caws and Drobniewski, 2001). However, this test is neither sensitive nor

specific for tuberculosis (Woods, 2001). Sensitivity for this technique is reported to range from 61% to 90% (Ulukanligil et al., 2000; Fitzgerald et al., 2000). Therefore, there is a need for techniques that are more sensitive and specific than acid-fast bacteria (AFB) smear techniques and more rapid than culture. In an effort to expedite the final diagnosis of *Mycobacterium* spp. several molecular methods have been developed within the past decade for direct detection, identification, and susceptibility testing. Diagnostic laboratories are increasingly favoring such methods since biochemical testing is slow, cumbersome, and may yield ambiguous results. Molecular methods can reduce the turnaround time from weeks to days (Soini and Musser, 2001; Caws and Drobniewski, 2001).

Amplification of nucleic acids is a common starting point for molecular diagnostic methods. Polymerase chain reaction (PCR) is the most widely recognized and utilized of these techniques. Two nucleic acid based tests have been approved by the Food and Drug Administration for direct detection of *M. tuberculosis* from clinical specimens. The Amplicor Mycobacterium tuberculosis Test (Amplicor; Roche Diagnostic Systems, Inc., Branchburg, NJ) is a PCR based test and the Enhanced Mycobacterium tuberculosis Direct Test (E-MTD; Gen-Probe, San Diego, CA) is based on the transcription amplification system (TAS) where target rRNA is converted into cDNA isothermally. A drawback of these tests is that they are only approved for *M. tuberculosis* complex organisms (*M. tuberculosis*, *M. bovis*, attenuated *M. bovis* BCG strains, *M. africanum*, *M. microti and M. canettii*). Culture is still required to identify nontuberculous mycobacteria and for drug susceptibility testing (to determine drug/antibiotic sensitivity; response to therapy). Moreover the tests do not differentiate

between live and dead mycobacteria and, therefore, are unsuitable for monitoring TB therapy. Assays based on PCR using formalin-fixed paraffin-embedded sections with typical tuberculous lesions are being used increasingly by diagnostic laboratories since primers are available which are highly specific for *M. tuberculosis* complex mycobacteria. Such assays are attractive as they are more rapid than bacterial culture by three to four weeks (Adams, 2001)

Other tests which are routinely used are nucleic acid analysis without amplification e.g. nucleic acid probes (GenProbe) which can identify mycobacteria to the species level. DNA microarrays are the latest molecular method with potential to provide reliable results on mycobacterial speciation in a rapid and reliable manner (Soini and Musser, 2001).

Sensitive and specific confirmatory tests for bovine tuberculosis samples collected at post mortem enhance depopulation procedures in a herd situation. Such tests would also be able to detect subclinical infection in animals presented for slaughter and would prevent meat or any other byproducts being passed for food consumption or further processing. Timely and accurate test results would reduce the cost incurred by the owner/producer and regulatory agencies (federal and state) when herds are held in quarantine awaiting confirmation of the herd's bovine tuberculosis status. In addition, the availability of such test results could limit further transmission to other animals as well as humans.

The aim of this study was to evaluate the novel diagnostic procedure of laser capture microdissection (LCM) in conjunction with PCR for the identification of *M. bovis* from formalin- fixed paraffin-embedded pulmonary tissues from experimentally

infected house mice. It is our hypothesis that the LCM/PCR method is comparable to mycobacterial culture, the gold standard, in both sensitivity and specificity while having an improved turnaround time and reduced cost.

## Materials and Methods

### Inoculum

Inocula used were prepared by the tuberculosis laboratory, Michigan Department of Community Health (MDCH). The *M. bovis* isolate is from a positive white-tailed deer (WTD) identified by annual surveillance and was confirmed as the strain typical to all cases by restriction fragment length polymorphism (RFLP). Seven-day growth in Middlebrook 7H9 broth was adjusted to 0.5 McFarland turbidity standard, diluted to 1:100 with sterile water and tested by plate counts to determine the colony-forming units (CFU) per unit of volume. The dose was confirmed again at the time of inoculation of the house mice. The oral inocula were administered via tomcat catheter gavage.

### Inoculation of house mice

Thirty six adult house mice were sourced from The Geriatrics Center, University of Michigan, Ann Arbor. All animals were known to be free from common murine pathogens tested for at Charles River Diagnostics, Wilmington, MA prior to inoculation (listed in appendix II). To assess fecal shedding fecal samples for mycobacterial culture and isolation were obtained from each mouse on the day prior to oral inoculation, day 1 post inoculation (PI) and from surviving animals on days 20 and 40 PI. The mice were

randomly assigned to one of three groups: 12 received a high dose of *M. bovis* (1x $10^4$ CFU), 12 low dose (1x $10^2$ CFU) and 12 were sham inoculated controls. Each mouse received 0.25ml total volume orally. Inoculated and control mice were housed individually in microisolator cages in a HEPA-filtered room (biolevel-3 conditions) at the Michigan State University Containment Facility. Rodent chow and water were supplied *ad libitum.* Four mice from each group were sacrificed at 20, 40 and 60 days post inoculation (PI).

Each animal was weighed prior to inoculation (day 0) and at weekly intervals thereafter. All animals were euthanized with an overdose of isoflurane (IsoFlo®, Abbott Animal Health, North Chicago, IL). At necropsy, total body weight in grams (g) was obtained for each animal. The weight of the lung, liver and spleen was also recorded. Tissues harvested at necropsy were preserved in 10% neutral-buffered formalin (NBF) and included brain, trachea, lung, heart, liver, kidney, spleen, gonad, adrenal gland, small intestine (SI), large intestine (LI), cranial, thoracic and abdominal lymph nodes. Tissues were routinely processed and sectioned for staining with hematoxylin and eosin (H and E). All tissues were also stained with Ziehl-Neelsen (acid fast).

Tissues for mycobacterial culture were collected using sterile instruments and were grouped into three pools; pool A: lung, tracheobronchial lymph nodes and cranial lymph nodes, pool B: liver, kidney and spleen, pool C: SI, LI and mesenteric lymph nodes

**Preparation of lung tissue and laser capture microdissection**

Five-μm sections of lung on uncoated, uncharged glass slides were deparaffinised in xylene and rehydrated by immersion in absolute and 95% ethanol. Sections were then stained with hematoxylin. To prevent cross contamination the microtome blade was cleaned with absolute alcohol between each block. With the laser capture microscope (Pix-Cell II, Arcturus Engineering, Mountain View, CA) a 7.5 μm laser beam was used to procure selected granulomas. The amplitude range was 60-70 mW for 2 ms.

**DNA extraction**

The microdissected granulomas adhered to the thermosensitive membranes were peeled from the caps and transferred into a 2 ml microcentrifuge tube containing 50μl of tris-ethylenedaiminetetraacetic-acid (TE) buffer (10mM tris-HCl 1 mM EDTA, pH 8.4) (modified after Zhu et al. 2003). Mycobacterial cell lysis was achieved by placing the tubes in an ultrasonication water bath for 5 minutes (80W) (model # 2510, Branson, Danbury CT). Samples were then boiled for 10 minutes and snap frozen (ethanol on dry ice). After freezing the sample was centrifuged at 16,000 x g for 10 minutes and 5 μl of the supernatant was used for each PCR test (modified after Eing et al., 1998).

**Amplification and detection procedures**

Samples were examined with primer sets for amplification of a 123 base-pair (bp) segment of insertion sequence (IS) 6110 as follows; the forward primer sequence was

5'-CTCGTCCAGCGCCGCTTCGG and the reverse primer sequence was 5'-CCTGCGAGCGTAGGCGTCGG (Eisenach et al., 1990).

A hot-start method which utilized heat activated DNA polymerase was selected for amplification (TakaRa Taq™ HS 10x PCR buffer dNTP mixture, Takara Mirus Bio, Madison, WI). The final reaction volume of 25 µl contained 10 pM primers, 0.635 U DNA polymerase and 0.2 nM nucleotides. A 5 µl DNA test sample was added to a 20 µl volume of master mix. Positive control DNA was obtained from *M. bovis* cultures. Each PCR run included a negative control (dd $H_2O$) and a reagent only negative control.

Amplification conditions were as follows: 94 ° C for 30s and 72 ° C for 75s repeated 50 times with a final 10 minute extension at 72° C (MJ Research PT-200 Peltier Thermal Cycler, Waltham, MA, USA). 12 µl of the amplification products were analyzed by gel electrophoresis [1.5% agarose, SB buffer (5 mm disodium borate decahydrate, pH8.5)] and stained with ethidium bromide. Electrophoresis was performed for 20 minutes at 200V. Ultraviolet transillumination was used to delineate base pair fragments (UVP, Ultraviolet Products, Upland, CA). Images were taken with the Kodak EDAS 290 system (Eastman Kodak Co., Rochester, NY).

**Statistical analysis**

Agreement of LCM/PCR with the 'gold standard' of mycobacterial culture was calculated with Cohen's kappa statistic (κ). Kappa values of < 0.4 are considered poor; 0.40 – 0.60 fair; 0.60 – 0.80 good; > 0.80 is excellent. Validity was assessed by sensitivity, specificity, accuracy and predictive value measures. Sensitivity is defined as

the percentage of true positive samples that are so indicated by the test whereas specificity is the percentage of true negative samples that are so indicated by the test. The positive predictive value (PPV) is the probability that a test positive sample is a true positive. Conversely the negative predictive value (NPV) is the probability that a test negative sample is a true negative. Accuracy is defined as the degree of agreement between the estimated value (teat value or measurement) and the true value and is determined by a+d/ (a+b+c+d) x 100.

**Results**

Of the thirteen mycobacterial culture positive lung samples (pool A) from *M. bovis* infected house mice, nine of them were also positive by the LCM/PCR technique (Tables 4:1 and 4:2). The LCM/PCR technique detected *M. bovis* in one mouse that was culture negative on lung tissue. Yet tissues from this particular mouse were positive on culture in the liver, kidney and spleen.

The sensitivity for the LCM/PCR technique when compared to mycobacterial culture was 69.2%, specificity was 95.6%, positive and negative predictive values were 90% and 84.6% respectively (Table 4:2).

Cohen's kappa statistic (κ) for agreement of LCM/PCR with mycobacterial culture was 0.68 (95 % CI 0.425- 0.941).

**Discussion and Conclusion**

Since its inception in the mid 1990's laser capture microdissection is increasingly being utilized in pathology for the procurement of groups or even individual cells of

interest primarily in cancer studies (Frost et al., 2001; Suzuki et al., 2001; Webb T, 2000). In the laser capture procedure a dehydrated tissue section is placed on the stage of a specially designed microscope. Once the cells of interest are visually selected a thermoplastic membrane is directly opposed to the cells. A low power infrared laser beam is applied to the cells which melts the overlying membrane. The polymers in the membrane surround the cells of interest and hold them tight. Such cells remain embedded in the membrane once it is lifted (Curran et al., 2000; Fend and Raffeld, 2000). The main advantage to LCM is that a relatively pure population of cells can be procured for subsequent analysis of DNA, RNA and protein (Eltoum et al., 2002; Grant and Jerome, 2002; Suarez-Quian et al., 1999; Simone et al., 1998; Bonner et al., 1997; Emmert-Buck et al., 1996).

It is known that culture, the gold standard for mycobacterial isolation and identification, is unable to provide a diagnosis in a timely fashion, although being very specific. Thus, research is ongoing to identify diagnostic tests which have greatly reduced turnaround times while retaining reliability. When employed in the detection of mycobacteria, molecular techniques are successful in reducing turnaround time, but are often less reliable than culture with reduced sensitivity and specificity. Specifically, challenges that occur with DNA amplification procedures are related to the extraction and purification of mycobacterial DNA from a larger mass of tissue. The overall objective of this study, therefore, was to utilize LCM to extract focal granulomas containing mycobacteria from formalin-fixed paraffin-embedded lung tissue in *M. bovis* infected mice in an effort to enhance the purity of DNA extracted for PCR (Figures 4:1 and 4:2). Consequently this method would have equivalent sensitivity and

specificity when compared to the gold standard of mycobacterial culture which utilizes a combination of liquid and solid media. Indeed a Cohen's kappa value of 0.68 indicates there is good agreement for LCM/PCR and mycobacterial culture.

PCR is currently the most widely utilized molecular technique employed by diagnostic laboratories to detect mycobacteria. Advantages are that small quantities of DNA on the order of one to 10 bacilli can be amplified and detected. Ethidium bromide stained gels can resolve 1fg of DNA (Garg et al., 2003). However, a disadvantage resulting from this is that remnant DNA from inactivated mycobacteria can be amplified thus PCR can detect infection but not active disease necessarily. PCR also does not facilitate species identification.

Another disadvantage in the use of PCR is the difficulty in extracting of DNA from *Mycobacterium* spp. organisms due to the presence of a thick lipid-rich cell wall. In this study alone, enzymatic (proteinase K) and chemical (Extract-N-Amp™ Plant PCR kit, Sigma-Aldrich Corporation, St. Louis, MO) extraction methods were attempted (data not presented) without success. Although the physical extraction procedure utilized (sonication/boil and freeze-thaw) was the most successful it still did not produce consistent results. Failure of PCR to identify all of the *M. bovis* infected lung samples can be related to problems previously identified by other researchers when applying molecular techniques to mycobacteria.

Failure to detect all of the culture positive mouse lung samples in this study may be due to incomplete digestion of the mycobacterial cell wall to release the DNA, presence of inhibitors of PCR in the tissues or a dilution of available DNA in the extraction process. Even though the granuloma containing mycobacteria was successfully

harvested, failure to digest the mycobacterial cell wall would manifest as a false negative. Another caveat to be considered in the procurement of granulomas is that the mycobacteria may be unevenly distributed in the tissue and it is possible that this variation in numbers of organisms within and between granulomas could contribute to variation in the amount of DNA procured. This problem is not one that is unique to this study and suggests that further research is need to devise better and more consistent methods of extracting DNA from *Mycobacterium* spp. organisms (Honore-Bouakline et al., 2003).

Another drawback is the presence of contaminants which hinder DNA amplification. Exogenous inhibitors include residual fixative or paraffin, anticoagulants and detergents. Mycobacterial cell wall components contribute to endogenous inhibition (Goldsworthy et al., 1999; Pfyffer, 1999).

False negative results with the LCM-PCR procedure may have occurred due to the uneven distribution of mycobacteria in the 50µl of TE buffer. Mycobacteria are known to aggregate in liquid cultures and solutions.

Additionally the reduced sensitivity of the LCM/PCR technique when compared to mycobacterial culture may be related to fixation of tissues with formaldehyde (10%NBF). Formalin is a cross-linking fixative that is it causes cross-linking between protein and DNA. Formaldehyde initiates DNA denaturation at the AT-rich regions of double-stranded DNA creating sites for chemical interaction. The overall rate of formalin-induced modification of DNA is dependent on the concentration, temperature and pH of the fixative. Formalin can decrease the quality and quantity of DNA which can be extracted from tissues immersed in this fixative. The average size of DNA

extracted from tissues fixed in buffered formalin decreases with increasing fixation time (Serth et al., 2000; Williams et al., 1999). Short-term treatment of sections with formalin have been shown to significantly reduce DNA solubility (Srinivasan et al, 2002)

Cataloluk et al. (2003), in summarizing the prior reports of other researchers as it related to their study on archival paraffin-embedded, *M. tuberculosis* infected tissues, stated that the preservation process or the storage time after preservation of tissue samples may lead to false positive and/or false negative results. Therefore shortening the amount of time that the mouse pulmonary tissue was immersed in formalin before processing for paraffin embedding could improve the quantity and quality of DNA obtained.

Wards and associates in 1994 were able to demonstrate the utility of PCR in detecting *M. bovis* in fresh tissue. They obtained a specificity of 91% but used a different *M. tuberculosis* complex-specific insertion sequence (IS1081). Miller and associates in 1997 used PCR to detect *M. bovis* from archival formalin-fixed paraffin-embedded tissues from various ruminant species. They obtained a sensitivity of 93%. Similar to the conclusions of Miller et al.1997 with a crude freeze-thaw DNA extraction protocol a negative PCR result following sample selection with LCM could not be interpreted to mean that the tissue does not contain *M. tuberculosis* complex bacteria, specifically *M. bovis* in this study.

In a follow up study in 2002 of 102 culture negative samples, Miller and associates obtained a specificity of 59.8% for *M. tuberculosis* complex. The overall specificity for mycobacterial infection was 43.1%. Although the crude PCR procedure used in this

earlier study was able to detect an additional 41 *M. TB* complex positive samples out of a total of 102 that were culture negative but had lesions suggestive of mycobacterial infection, the LCM/PCR technique utilized in this study was superior in this respect as the specificity was 95.6% versus 59.8%. The high false negative rate (low specificity) at the National Animal Disease Center (Miller study) may be due to the decontamination procedure used at this particular center (the tissues were submitted in sodium borate to control contaminating organisms). Sodium borate (bleach) is notorious for killing mycobacteria after prolonged exposure (Miller, 2002).

It should be noted that in this current study the specificity was determined based on culture results in the lung only. However, if specificity was determined using a positive culture result for any of the tissues submitted for a given mouse, then the specificity of the LCM/PCR technique versus culture would be 100%. This is so as the mouse determined to be positive by LCM/PCR but culture negative on the lung was, however, positive on culture in the pooled liver, kidney and spleen.

Selva et al. (2004) reported a sensitivity of 92% and a specificity of 100% in a similar LCM/PCR study for the detection of *M. tuberculosis* in granulomas from formalin fixed tissues. The greater sensitivity in this study is likely as a result of *M. tuberculosis* having up to 20 copies of IS6110 whereas the majority of *M. bovis* isolates from infected cattle have only a single copy of IS6110 (Durr et al., 2000). Similar to bovine isolates, the majority of WTD isolates in Michigan (strain used to inoculate the mice in this study) were found to have a single copy of IS6110 (Whipple et al., 1999).

Further, Selva and associates concluded that LCM did not increase the sensitivity of PCR when compared to that on whole tissue sections. Referring to the study done by

Miller et al. (1997) with crude DNA extracted from formalin-fixed paraffin-embedded tissues with sensitivity of 97%, a sensitivity of 69.2% in this current LCM/PCR study is supportive of the conclusion reported by Selva.

The efficiency of the LCM/PCR technique could be enhanced by increasing the initial sample size (multiple caps per individual slide/animal), using a nested primer method, or increasing the number of amplification cycles (Goldsworthy et al., 1999). Further, the efficiency of this procedure could be improved with the use of an internal control. Apart from increasing the total cost internal controls are not failsafe especially if different primers are used and it involves amplification from a different primer binding site.

The turnaround time for the LCM/PCR method is 3-4 days compared to eight to twelve weeks for mycobacterial isolation and identification (Table 4:3). The laser capture method is also more economical as the total cost was a little over $55 compared to $100 for mycobacterial culture. Sensitivity may be improved by using more than one capful of tissue. Although this would increase the turnaround time by a negligible amount the total cost of this technique would increase precipitously and approach that of mycobacterial culture.

In summary, this study demonstrates that the LCM/PCR test has a sensitivity of 69.2% but costs less and has a more efficient turnaround time when compared to mycobacterial culture. However, a specificity of 95.6% and a $\kappa$ value of 0.68, indicative of good agreement, suggest that the LCM/PCR procedure could with further refinement be utilized as a confirmatory test in the diagnosis of *M. bovis* infections in a clinical setting. Yet, much remains to be achieved in order to attain the ideal typing method

which should be inexpensive, reproducible, and rapid, easily performed and have direct application to clinical specimens (Adams, 2001).

**Table 4:1** A summary of the results (Ziehl-Neelsen acid-fast stained sections, mycobacterial culture, LCM/PCR) in house mice (*Mus musculus*) orally inoculated with *M. bovis*.

* Low dose

† High dose

‡ Controls

§ Hematoxylin and eosin/ Ziehl- Neelsen acid fast

‖ Culture

¶ Laser capture microdissection/ PCR technique

**TABLE 4: 1 Summary of the results in house mice (*Mus musculus*) orally inoculated with *M. bovis*.**

| | LD* | | | | HD† | | | | CON‡ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **DAY 20** | | | | | | | | | | | | |
| **MOUSE #** | **1** | **2** | **13** | **14** | **17** | **18** | **27** | **28** | **19** | **24** | **33** | **34** |
| **HE/AF§** | - | - | - | - | + | + | + | + | - | - | - | - |
| **CUL‖** | - | - | + | - | + | + | + | - | - | - | - | - |
| **LCM¶** | - | - | (-) | - | + | + | + | + | - | - | - | - |
| **DAY 40** | | | | | | | | | | | | |
| **MOUSE #** | **5** | **6** | **11** | **12** | **8** | **15** | **16** | **25** | **20** | **23** | **30** | **36** |
| **HE/AF** | + | - | - | + | + | + | + | + | - | - | - | - |
| **CUL** | - | - | - | + | + | + | + | + | - | - | - | - |
| **LCM** | - | - | - | + | + | + | + | (-) | - | - | - | - |
| **DAY 60** | | | | | | | | | | | | |
| **MOUSE #** | **3** | **4** | **9** | **10** | **7** | **26** | **29** | **31** | **21** | **22** | **32** | **35** |
| **HE/AF** | + | + | + | - | - | - | - | - | - | - | - | - |
| **CUL** | + | + | + | - | - | - | - | + | - | - | - | - |
| **LCM** | + | (-) | (-) | - | - | - | - | + | - | - | - | - |

**Table 4:2 Comparisons between the results of mycobacterial culture and isolation (gold standard) and the results of LCM[a]/PCR[b]**

| | | Mycobacterial culture | | |
|---|---|---|---|---|
| | | + | - | |
| LCM/PCR* | + | 9 (a) | 1 (b) | **10** |
| | - | 4 (c) | 22 (d) | **26** |
| | | **13** | **23** | **36** |

**[a] LCM- laser capture microdissection**
**[b] PCR – polymerase chain reaction**
*** Sensitivity 69.2%; specificity 95.6%; positive predictive value 90%; negative predictive value 84.6%; accuracy 86%**

$$\kappa = \frac{O - E}{1 - E} = \frac{0.86 - 0.56}{1 - 0.56} = 0.68$$

†Chance/ observed agreement [O] = 0.86
‡ Expected agreement [E] = 0.56

$$^{\dagger} O = \frac{a + d}{a + b + c + d}$$

$$^{\ddagger} E = \frac{[(a+c)x(a+b)]+[(c+d)\ x\ (b+d)]}{[a + b + c + d]^2}$$

**Table 4:3** A summary of the total laboratory costs for laser capture microsdissection (LCM) with PCR on pulmonary granulomas from formalin-fixed paraffin-embedded tissues. This cost is compared with for the gold standard, mycobacterial culture. The turnaround time for the LCM/PCR procedure and mycobacterial culture is also displayed

**Table 4:3 A summary of the costs and turnaround time for the LCM/PCR procedure and mycobacterial culture**

**Costs:**

| LCM | | Culture | | |
|---|---|---|---|---|
| | | | **Negative smear/ Culture neg** | **Positive smear/ Culture positive** |
| Processing/labor | $ 20 | Processing/labor | $25 | $25 |
| Cap | $ 20 | AF smear/slide | $4.50 | $10 |
| Slide | $ 8 | Culture | $5.50 | $10 |
| TaKaRa Taq™ | $0.33 | Prelim ID/probe | NA | $25 |
| PCR run | $7.00 | | Confirmatory/ | |
| | | Biochem tests | NA | $30 |
| **Total** | **$55.33** | | **$35** | **$ 100** |

**Turnaround time:**

| | | | |
|---|---|---|---|
| Fixation/ slide | 2-3 days | AF stain smear | 1 day |
| Laser capture | 30 mins | | |
| DNA extraction | 30 mins | Preliminary report | 2 weeks |
| PCR set up | 1.5 hrs | | |
| Cycle run | 1.40 hrs | | |
| Gel/ photo | 30 mins | *Final report | 3 - 8 weeks |
| | | * 12 weeks if smear pos/culture neg at 8 weeks | |
| **Total** | **3-4 days** | | **8- 12 weeks** |

**Figure 4:1** Laser capture microdissection (LCM) of a pulmonary granuloma from a LD mouse (#17) on day 20 PI, X 35

**Top panel**: before LCM.

**Middle panel**: After LCM.

**Lower panel**: Captured granuloma.

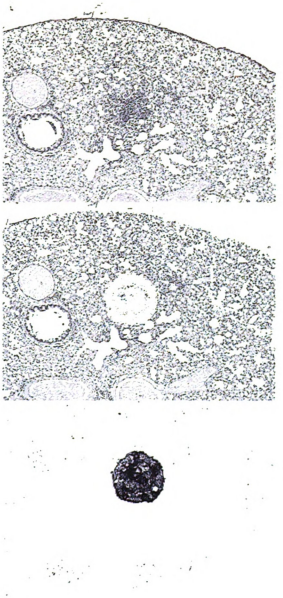


**Figure 4:1** Laser capture microdissection of pulmonary granulomas from a house mouse inoculated with *M. bovis*

**Figure 4:2** PCR amplification from formalin-fixed paraffin-embedded microgranulomas in lung tissues of house mice inoculated with *M. bovis* day 20 PI.

**MW:** molecular weight marker 100-bp ladder

**Lane 1**- mouse # 27 HD culture positive

**Lane 2**- mouse # 28 HD not culture positive on pool A

**Lane 3** – mouse # 13 LD culture positive but no microscopic lesions detected

**Lane 4**- mouse # 14 LD culture negative

**Lanes 5 and 6** – mice # 33 and # 34 respectively sham inoculated controls

**Lane7** – Negative control dd $H_2O$

**Lane 8** - PCR reagent only negative control

**Lane 9** – Positive control

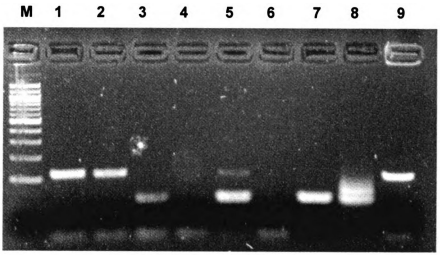



**Figure 4:2**

# CHAPTER FIVE

## Conclusions and Further Research

Since the discovery in 1882 by Robert Koch of *Mycobacterium bovis* as the cause of bovine tuberculosis, this disease remains today as one of considerable importance. Unlike the closely related human pathogen *M. tuberculosis*, *M. bovis* has a broad host range and an ability to survive, produce disease and be transmitted apparently with ease from one host to another, posing significant challenges to the control and understanding of the pathogenesis of *M. bovis* infection. Even after more than a century of recognizing its role in causing disease in animals and often in humans, much still remains unknown about this formidable pathogen.

In the state of Michigan there has been resurgence of bovine tuberculosis since the state was declared free of this disease in 1979. Following the discovery of *M. bovis* in white-tailed deer (*Odocoileus virginianus*) in 1994, it has become accepted that white-tailed deer are a maintenance host for this disease. Given this unprecedented occurrence of white-tailed deer maintaining *M. bovis* and serving as a source of infection for the disease in cattle, there is now a concerted effort to identify any other wildlife species which may serve as reservoir or spillover hosts for this organism. In addition to the test and slaughter technique performed in domestic animals which includes captive cervidae (Michigan Department of Agriculture, USDA, there is also ongoing surveillance of wildlife which are found in the area of the state where the disease is endemic (MDNR, MSU researchers).

Complementary to these surveillance studies it was appreciated that little was known about the transmission, pathogenesis and pathology of *M. bovis* infection in such wildlife. Indeed if members of these various species were to be infected with *M. bovis* would the disease be recognized? To this end, experimental inoculation studies were begun in these animals notably in avian species such as pigeons (*Columba livia*), starlings (*Sturnus vulgaris*), American crows (*Corvus brachrhynchos*) and mallard ducks (*Anas platyrhynchos*). The North American opossum (*Didelphis virginiana*) was also studied as it is related to the brushtail possum (*Trichosurus vulpecula*) in New Zealand that is recognized to be a significant reservoir host in that country.

As a continuation of these inoculation studies for determining what effect, if any, does *M. bovis* have on wildlife which interact with cattle and humans in the state, this current study examined the disease (transmission, pathogenesis, pathology) in three rodent species (meadow voles, house mice, Norway rats) and also in wild turkeys, all of which are known to frequent infected farm premises in the northeastern area of the lower peninsula where bovine tuberculosis is endemic.

The voles (*Microtus pennsylvanicus)* received oral and intranasal inocula whereas the house mice *(Mus musculus)* and rats *(Rattus norvegicus)* received high and lower dose oral inocula. The results of this study showed that the rats were resistant to oral infection with *M. bovis* as they failed to develop extensive lesions pathognomonic for bovine tuberculosis. Fecal cultures were consistently negative and there was a single positive tissue culture in an animal that receive a high dose inoculum. Consequently, Norway rats appear to play no role in endemic bovine tuberculosis in Michigan.

Conversely, the meadow voles were found to be highly susceptible to bovine tuberculosis via both of the inoculation routes to which they were exposed. This was evidenced by the development of extensive lesions consistent with tuberculosis in several tissues and positive mycobacterial tissue and fecal cultures. House mice were also found to be susceptible to oral infection with *M. bovis* as they too developed lesions consistent with tuberculosis and had positive tissue cultures. The response in mice was dose dependent as animals that received high dose inocula were more severely affected with a third of these animals actually succumbing. Hence, it was concluded that meadow voles and house mice are capable of acquiring bovine tuberculosis via the oral route and can potentially disseminate mycobacteria in their feces. It is, therefore, recommended that the numbers of these rodent pests be controlled on farm premises in Michigan where bovine tuberculosis is endemic, especially where herds have been depopulated and prior to repopulation, as there is a real risk of these rodents spreading *M. bovis* via their feces into feed and water sources and in so doing introducing or reintroducing bovine tuberculosis.

As an adjunct to the inoculation study in the house mice, further experimentation was conducted to elucidate their phenotype for the *Bcg/Nramp-1/ Scl11a1* gene which is thought to confer some resistance to infection by *Mycobacterium* spp. organisms. However, it was found that although the house mice studied were of the resistant phenotype (*Bcg*$^r$) this did not prevent them from being infected with *M. bovis*. This suggests that in house mice, a *Bcg* resistance phenotype alone does not predict their response to infection with *M. bovis*.

Similar to what was found in Norway rats, wild turkeys were also determined to be resistant to infection with *M. bovis* when inoculated with high doses via oral and intratracheal routes. So although wild turkeys are found in large numbers on farms in the area where bovine tuberculosis is endemic, results of this study demonstrate that there may be minimal risk of such birds serving as maintenance or spillover host of *M. bovis*.

The final study of this dissertation dealt with another aspect of tuberculosis where there is limited advancement, namely the development of a diagnostic test which is accurate, relatively inexpensive and has a rapid turnaround time. Currently mycobacterial culture (isolation and identification) is the 'gold standard' for diagnosis. Although this test is very specificity it can take up to twelve weeks to obtain the final result. Techniques incorporating PCR are increasingly being used in the diagnosis of mycobacterial infections in an effort to improve the turnaround time but this is often at the expense of sensitivity and specificity. To this end, a novel technique with the use of laser capture microsdissection to select granulomas from formalin- fixed paraffin-embedded tissues for evaluation by PCR to diagnose *M. bovis* was developed. However, this technique had a sensitivity of 69% when compared to the gold standard of mycobacterial culture. The turnaround time was considerably reduced, however, at three to four days compared to eight to twelve weeks for culture. The LCM/PCR procedure may have some usefulness as a confirmatory test if further refined and tested on a larger number of samples or even in granulomatous lesions of other species (e.g. cattle). Such studies were precluded by the limitations of time and cost in this present body of work.

Arising out this body of work are other interesting questions relating to *M. bovis* infection in the rodent species evaluated. Specifically, in the voles and house mice it would be of interest to determine their response to lower dose inocula via the inhalation route. It is well accepted that cattle as well as humans are usually infected with mycobacteria via this route. As these rodents are found associated with both cattle and humans, it should be determined if they can be infected via this route as well.

Additionally, in the rodent species evaluated reports in the literature of environmental surveys indicate that both Norway rats and meadow voles can be infected naturally with *M. bovis*, although in very small numbers. It would, therefore, be of interest to do similar live trapping studies on farms in Michigan where tuberculous cattle have been detected to determine if, indeed, the mice, rats and voles exposed to *M. bovis* do become infected.

The results of the *Bcg* study in house mice are also of interest as they indicate that much remains to be learned about the way in which genotype is related to infection with mycobacteria, specifically in this case *M. bovis*. The satellite DNA sequence of *Bcg* (*Nramp1*) in rats is also known (Ge et al., 1996), thus a study similar to the one in mice but performed on rats would serve to provide much needed information about the way in which genotype may or may not relate to tuberculosis in these species. Ultimately such research may be extrapolated to what has been obtained in man.

As the lungs were the principal organ affected in the house mice and this is similar to what occurs in humans this suggests that this species could reasonably be used as an animal model of the human disease for the development of vaccine and

chemotherapeutic agents for the treatment of tuberculosis in humans. The hope of eradication of tuberculosis, both human and bovine, rests with the development of improved diagnostics and new vaccination strategies. Vaccination of wildlife reservoirs initially is not focused on preventing infection in such animals but seeks to prevent the shedding of mycobacteria once these animals are infected. Although encouraging results have been realized in preliminary vaccine trials in badgers in the United Kingdom and Ireland much remains to be done. Many of the prior studies of vaccination in animals relied on *M. bovis* BCG but it is realized that, as in humans, this particular vaccine does not offer reliable protection in animals. Also it introduces problems in differentiating vaccinated cattle from infected animals. Further research is need to develop novel adjuvants for such vaccines and an effective way in which repeated doses of vaccine can be administered to wildlife. Experimental studies to determine the preferred route of inoculation and dosages of vaccine required will therefore be necessary in white-tailed deer once such a vaccine is identified. Given that the complete genome sequence of *M. bovis* is known (Garnier et al., 2003) it is hoped that such knowledge will be translated into the development of effective vaccines for the prevention of infection in both animals and humans. Moreover, this knowledge should assist in the development of better and more specific molecular based techniques for diagnosis of *M. bovis* infection.

Although the LCM/PCR study ultimately proved to be of low sensitivity (69.2%) the fact that it did reduce the turnaround time for a final diagnosis to three to four days suggests that with further modifications in the extraction of mycobacterial DNA

from formalin-fixed paraffin-embedded tissues this method may ultimately be further applied.

In summary, this body of work provided essential information on the transmission and pathogenesis of *M. bovis* infection in several wildlife species. Specifically, wild turkeys (*Meleagris gallopavo*) were found to be resistant to infection via both the oral and intratracheal route which is supportive of them having no role in the maintenance of bovine tuberculosis in Michigan. Norway rats were also found to be resistant to bovine tuberculosis and similar to the wild turkeys pose no threat. However, results of this study did indicate that meadow voles and house mice if infected with *M. bovis* in their natural environment can potential spread this organism via their feces. Finally application of a novel diagnostic technique, namely LCM/PCR, failed to improve on the sensitivity of the gold standard of mycobacterial culture and at best this new technique could only be applied as a confirmatory test.

# Appendices

# APPENDIX I

The meadow vole (*Microtus pennsylvanicus*) colony was tested and negative for:

| | |
|---|---|
| SEND | Sendai virus |
| PVM | Pneumonia virus of mice |
| MHV | Mouse hepatitis virus |
| Reo-3 | Reovirus type 3 |
| LCM | Lymphocytic choriomeneigitis virus |

The colony tested positive for Helicobacter (Helicobacter spp. not bilis or hepaticus) in August 2000 but was negative at the time animals were sourced for this inoculation study

## APPENDIX II

The house mouse (*Mus musculus*) colony was tested and negative for:

| | |
|---|---|
| SEND | Sendai virus |
| PVM | Pneumonia virus of mice |
| MHV | Mouse hepatitis virus |
| GDV II | Theiler's mouse encephalitis virus (es) |
| REO | Reovirus |
| MPUL | *Mycoplasma pulmonis* |
| LCMV | Lymphocytic choriomeningitis virus |
| ECTRO | Ectromelia virus |
| K | Mouse pneumonitis virus |
| POLY | Polyoma virus |
| MAV 1 & 2 | Mouse adenovirus |
| EDIM | Epizootic Diarrhea of Infant Mice virus |
| MPV | Mouse parvovirus |
| PARV NS1 | Mouse parvovirus (ELISA) |

The colony tested positive for Syphacia spp. and was treated with fenbendazole prior to the inoculation study.

# APPENDIX III

## SEROLOGY

The Norway rat (*Rattus norvegicus*) colony was tested and negative for:

| | |
|---|---|
| SEND | Sendai virus |
| PVM | Pneumonia virus of mice |
| SDAV | Sialodacryoadenitis |
| KRV | Kilham rat virus |
| H1 | Toolan's H-1 virus |
| REO | Reovirus |
| REV | Rat enterovirus |
| MPUL | *Mycoplasma pulmonis* |
| LCMV | Lymphocytic choriomeningitis virus |
| HANT | Hantaan virus |
| MAV | Mouse adenovirus |
| ECUN | *Encephalitozoon cuniculi* |
| CARD | Cilia-associated respiratory bacillus |
| RPV | Rat parvovirus |

## PARASITOLOGY

The colony was tested and negative for numerous but un-named ectoparasites, endoparasites and enteric protozoa

Norway rats cont'd

**BACTERIOLOGY**

Rats were tested and negative for *Bordatella bronchioseptica*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Salmonella* spp., *Streptobacillus moniliformis*, *Helicobacter hepaticus*, *Helicobacter bilis*, *Helicobacter* spp., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pasteurella multocida*, *Pasteurella pneumotropica*, *Pasteurella* spp., *Streptococcus pneumoniae*, Beta *Streptoccus* groups B & G, Beta *Streptococcus* spp.

A few rats tested positive for *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

# APPENDIX IV

Mycobacterial isolation and identification procedure as practiced at the Michigan Department of Community Health (MDCH), Tuberculosis Laboratory Lansing, Michigan (Kent & Kubica, 1985, Reisner et al. 1994, Butler et al. 1991).

Tissue samples are ground using a Kendall™ sterile disposable 50 cc tissue grinder. . Decontamination is accomplished using the mucolytic agent N-Actely-L-cysteine (NALC) along with a high pH sodium hydroxide/tri-sodium citrate decontaminant. The digested specimen is concentrated by centrifugation at 5000 g for 15 minutes. The supernatant is poured off leaving a spun sediment for subsequent testing.

Smears for microscopic evaluation are prepared from the concentrated sediment and stained using an Auramine-O (AO) fluorescent acid-fast staining method. Smears found AFB positive by AO are confirmed using the Ziehl-Neelsen acid-fast staining method.

Preliminary cultures are made as follow (0.5 ml inoculum/ each):

i. One Lowenstein-Jensen (LJ) medium slant (egg based) (Becton-Dickinson, Cockeysville, MD)

ii. One Middlebrook 7H11S medium slant (agar based) (Becton-Dickinson)

These are incubated at 37°C with 5% $CO_2$

iii. One BACTEC 12B broth vial (containing $^{14}C$ for radiometric detection of growth) (Becton-Dickinson, Sparks, MD)

Bactec 12B vials are evaluated twice a week for two weeks then once a week for a further two weeks.

If growth suggestive of *Mycobacterium* spp. is detected then subcultures (for identification and susceptibility testing) are inoculated into Middlebrook 7H9 broth (bead bottles) (Becton-Dickinson) and are incubated for 3-4 days for subsequent inoculation to a panel of media.

Subcultures are inoculated as follows:

- Four LJ slants; two are inoculated with 6 drops (~ 0.3 ml) from the 7H9 broth, the other two are inoculated with a 1:100 dilution from the broth
- One LJ deep tube inoculated with 4 drops (~ 0.2 ml) from the 7H9 broth
- One 5% NaCl tube made with 0.3 ml inoculum
- One 7H11 slant made with 0.3 ml inoculum

Antibiotic susceptibility is a concern, usually only for human isolates. Mycobacterial isolates may be tested for susceptibility to rifampin, streptomycin, isoniazid, ethambutol and pyrazinamide.

Subcultured plates and slants are evaluated weekly for eight weeks. BACTEC 12B vials are read twice weekly for two weeks and thereafter at weekly intervals up to eight weeks. Cultures may be read for up to 12 weeks, if the smear of the specimen is positive and the culture is negative at 8 weeks of incubation.

Speciation of mycobacteria is accomplished using three methods of identification:

1. Biochemical tests- a major draw back to such tests is that they are very time consuming
2. Nucleic acid probe utilizing the Accuprobe® *Mycobacterium tuberculosis* complex culture identification test (Gen-Probe® San Diego, CA). Samples for Accuprobe® can be obtained from the 7H11 medium, Lowenstein-Jensen

medium or BACTEC vials. The fluorescently labeled DNA probe (for the M Tb complex) forms a hybrid with the ribosomal RNA from the mycobacteria (test sample). The DNA-rRNA hybrid fluorescence is detected and then recorded

3. High-performance liquid chromatography (HPLC): Long chain fatty acids/ mycolic acids are digested into shorter fragments, extracted and then passed through a column with a stationary phase. As these fatty acids migrate at different rates the number, height and pattern of the peaks is used to identify the mycobacteria. The rate of migration through the column is directly related to differences in the mycolic acid carbon chain fragment length resulting from unique fragmentation of different *Mycobacterium* spp.

If strain differentiation is a concern for molecular epidemiologic purposes then the specimen is submitted to the National Veterinary Services Laboratory, Ames, Iowa for restriction fragment length polymorphism (RFLP) testing. This is necessary to detect insertion sequence (IS) 6110 the most important insertion sequence detected in the M Tb complex.

## APPENDIX V

# PURINA® BRAND TURKEY GROWER W/O

8386

**A complete feed for growing turkeys**
**CAUTION:** Use Only As Directed.

GUARANTEED ANALYSIS

| | |
|---|---|
| Crude protein (Min) | 20.0000% |
| Lysine (Min) | 1.0000% |
| Methionine (Min) | 0.4200% |
| Crude fat (Min) | 3.0000% |
| Crude fiber (Max) | 5.5000% |
| Calcium (Ca) (Min) | 1.1000% |
| Calcium (Ca) (Max) | 1.6000% |
| Phosphorus (P) (Min) | 0.8000% |
| Salt (NaCl) (Min) | 0.2000% |
| Salt (NaCl) (Max) | 0.7000% |

INGREDIENTS

Grain products, plant protein products, processed grain by-products dicalcium phosphate, calcium carbonate, animal protein products, salt, magnesium sulfate, potassium sulfate, DL-methionine, L-lysine, choline chloride, calcium lignin sulfonate, niacin supplement, calcium pantothenate, pyridoxine hydrochloride, biotin, riboflavin supplement, vitamin E supplement, vitamin $B_{12}$ supplement, menadione dimethylpyrimidinol bisulfite (source of vitamin K), folic acid, vitamin A supplement, vitamin $D_3$ supplement, manganous oxide, zinc oxide, copper sulfate, calcium iodate, sodium selenite.
08BH G 8386-

DIRECTIONS

Feed as sole ration following Turkey Starter W/O Poultry Feed to turkey toms a total of 23 pounds per bird or between 8-13 1/2 weeks of age and to turkey hens a total of 13.5 pounds per bird or between 7 1/2-11 1/2 weeks of age.

**Important:**

A feeding program is only as effective as the management practices and disease control measures followed.

# APPENDIX VI

Rodent Diet

Teklad *2.2/5* Rodent Diet (W) ..8640

Guaranteed Analysis:

Crude Protein, not less than. ..... 22.0%

Crude Fat, not less than ..... 5.0%

Crude Fiber, not more than ..... 4.5%

Ingredients:

Soybean meal, ground com, wheat middlings, com. flakes, fish meal, dried molasses, soybean oil, ground wheat, dried whey, dicalcium phosphate, dried brewers yeast, calcium carbonate, iodized salt, choline chloride, vitamin A acetate, vitamin D3 supplement, vitamin E supplement, niacin, calcium pantothenate, riboflavin, thiamine mononitrate, pyridoxine hydrochloride, menadione sodium bisulfite complex (source of vitamin K activity), folic acid, biotin, vitamin B12 supplement, magnesium oxide, calcium iodate, cobalt carbonate, chromium potassium sulfate.

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