DISSERTATION

VACCINATION OF WHITE-TAILED DEER WITH *MYCOBACTERIUM BOVIS* BACILLE CALMETTE-GUERIN: EFFICACY, IMMUNOLOGY, AND MOLECULAR DETECTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PAULINE NOL ENTITLED VACCINATION OF WHITE-TAILED DEER WITH *MYCOBACTERIUM BOVIS* BACILLE CALLMETTE-GUERIN: EFFICACY, IMMUNOLOGY, AND MOLECULAR DETECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

VACCINATION OF WHITE-TAILED DEER WITH *MYCOBACTERIUM BOVIS* BACILLE CALLMETTE-GUERIN: EFFICACY, IMMUNOLOGY, AND MOLECULAR DETECTION

Wildlife reservoirs of *Mycobacterium bovis* are believed to play very important roles in the epidemiology of bovine tuberculosis in many countries throughout the world. In the United States, a free-ranging white-tailed deer (*Odocoileus virginianus*) population in northeastern Michigan serves as such a reservoir. Although changes in management have decreased prevalence of the disease, additional tools, such as vaccination, are needed to achieve eradication of bovine tuberculosis from Michigan deer. In this project, the efficacy of oral and parenteral *Mycobacterium bovis* bacille Calmette-Guerin Danish strain 1331 (BCG) was evaluated for its ability to protect white-tailed deer against disease caused by *M. bovis* infection. In addition, cellular and humoral immune responses in deer to BCG vaccination and *M. bovis* challenge were examined, and molecular detection techniques were developed to monitor shedding of BCG and *M. bovis* in vaccinated and infected animals. Results indicate that white-tailed deer vaccinated with BCG both orally and parenterally were protected from development of severe disease after experimental infection with virulent *M. bovis* when compared to unvaccinated deer. Antibody responses to *M. bovis* antigens by the deer in

this study were evaluated over time using multiantigen (multiantigen print immunoassay, rapid test, and immunoblot to whole-cell sonicate) and single antigen tests (lipoarabinomannan [LAM] ELISA and immunoblot to MPB83). Multiantigen tests detected minimal to no antibody responses in vaccinated deer after challenge, whereas antibody responses were more readily detectable by these tests in unvaccinated deer with more advanced disease. The ELISA results indicated an overall decrease in detectable antibodies produced against LAM-enriched mycobacterial antigen in vaccinated animals as compared to unvaccinated animals after challenge. Few trends could be determined from the immunoblot results. Cellular immunity was measured via interferon gamma production and lymphocyte proliferation in response to mycobacterial antigens. Findings in regards to cellular immunity were inconclusive. Molecular techniques developed to detect *M. tuberculosis* complex in cervid feces, nasal and pharyngeal swabs, soil, feed, and hay produced data indicating that deer shed *M. bovis* and BCG only intermittently at 1-3 months after vaccination and 1-4 months after *M. bovis* challenge. The findings from these studies strongly support further research that could lead to the eventual use of BCG in wild white-tailed deer herds affected by bovine tuberculosis. The data also encourage the improvement and potential use of antibody-based assays, such as the multiantigen tests and the LAM-ELISA as ante-mortem tools to assess disease progression in white-tailed deer in both experimental and field vaccine trials. Finally, further improvements in the molecular detection of *M. bovis* could enable more effective monitoring of shedding of *M. bovis* by deer in both experimental and free-ranging environments.

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CHAPTER 1: GENERAL INTRODUCTION

Bovine tuberculosis (BTb), caused by the bacterium, *Mycobacterium bovis*, is a disease of worldwide and historic importance. *Mycobacterium bovis* is a member of the *Mycobacterium tuberculosis* Complex (MtbC), which harbors all slow-growing mycobacteria causing tuberculous disease in mammals, including humans. Other members of this group include *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. microti*, and *M. pinnipedi*, also including a few not yet speciated members such as dassie bacillus and oryx bacillus (4, 11, 21). Bovine tuberculosis affects a wide variety of species as *M. bovis* has one of the largest host distributions of all bacterial pathogens (6, 9, 16). *Mycobacterium bovis*, like all members of the MtbC, is an acid fast, gram positive bacillus that resides in the intracellular environments of macrophages and to a lesser extent, dendritic cells (18, 21). *Mycobacterium bovis* causes a progressive granulomatous disease usually in the lymph nodes of the thorax, head, and neck as well as in the lungs; however, all tissues of the body are susceptible to infection. Protection against disease within the host is dependent on a competent cellular immune response (1, 18). Animals affected by BTb usually experience asymptomatic disease; however, in later stages of disease, clinical signs may include weight loss, coughing, dyspnea, lymphadenitis, draining lesions and alopecia (3, 21).

Throughout the twentieth century extensive campaigns to eliminate BTb from humans, cattle, and other livestock have been carried out in the United States, the United Kingdom, Europe, Australia, and New Zealand (18). These campaigns

involved rigorous test and slaughter efforts using tuberculin skin testing, as well as implementing pasteurization (9, 12, 18). Although, great advancements have been made regarding this endeavor, BTb has managed to evade eradication in many countries due to its maintenance in wildlife reservoirs (9, 10). One example of this is the reason for this dissertation work. Wild white-tailed deer (*Odocoileus virginianus*) in the northern lower peninsula of Michigan, USA are a reservoir host for *M. bovis* (17, 20, 23). Although recent changes in management practices applied to this free-ranging population have decreased the prevalence of the disease to approximately 1%, infection in local domestic cattle herds continue to occur (15). Also in North America, white-tailed deer are a spillover host for *M. bovis* in cattle herds in northern Minnesota, and BTb is found in deer in Riding Mountain National Park in Manitoba, Canada, where it is endemic in the park's wild elk herd. Other examples of wildlife reservoirs of BTb are the brushtail possum (*Trichosaurus vulpeca*) in New Zealand, the European badger (*Meles meles*) in the United Kingdom and Ireland, the African buffalo (*Syncerus caffer*) in South Africa, wood bison (*Bison bison athabascae*) in Canada, and wild boar (*Sus scrofa*) in Europe (5, 13, 14, 24).

Vaccination of this deer population and other wildlife populations has been proposed by managers to be an added tool to be used to reduce disease and transmission of *M. bovis* to a point where it can finally be eradicated from affected areas (2). Oral vaccination is being evaluated in a number of wildlife species such as brushtail possums and European badgers (20, 22, 25). Oral vaccination has been implemented in the United States and Europe in order to control rabies in red fox (*Vulpes vulpes*), raccoon dog (*Nyctereutes procyonoides*) and raccoon (*Procyon lotor*) (7, 8).

This dissertation describes studies conducted to assess the efficacy of vaccination with *M. bovis* bacilli Calmette-Guerin (BCG), both orally and parenterally, of a captive herd of white-tailed deer and subsequent experimental challenge with *M. bovis*. The basic study design is as follows: thirty yearling female white-tailed deer were obtained in January, 2005 from four BTb-free deer farms throughout the state of Iowa, USA and were housed in an outdoor facility at USDA/ARS National Animal Disease Center (NADC) in Ames IA, USA. At time of challenge, deer were transferred to a biosafety level (BL)-3 animal building. All deer were housed and cared for according to the Association for Assessment and Accreditation for Laboratory Animal Care International and institutional guidelines.

Mycobacterium bovis BCG Danish strain 1331 in culture and in lipid-formulated pellets were prepared at the University of Otago, Immune Solutions Ltd. as described in Aldwell et al., 2003. Eight deer were orally vaccinated with approximately 1×10^9 colony forming units (cfu) BCG via lipid formulated bait. Another group of 8 deer were orally vaccinated with approximately 1×10^9 cfu BCG in 1 ml liquid medium via syringe and catheter. Seven deer were vaccinated subcutaneously with approximately 3.4×10^6 cfu BCG in 1 ml liquid medium. Seven deer received 1 ml liquid medium orally directly into the back of the mouth via syringe and catheter and served as the control group. Control and vaccinated deer were housed together in an outdoor paddock until challenge in an indoor BL3 facility.

At 12 weeks post-vaccination, all animals were anesthetized, moved into a BL3 facility also at NADC, and immediately challenged with a total of 228 cfu *M. bovis* strain 9839 (a strain originating from an infected Michigan white-tailed deer) directly into the

palatine tonsillar crypts (114 cfu/tonsil) via pipette, the dose being divided between the two tonsils. All deer were euthanized and necropsied five months post-challenge.

Humoral and cellular immunological responses to vaccination and infection were measured in the deer and are reported here. In addition, molecular techniques were developed in order to detect *Mycobacterium tuberculosis* Complex in cervid biological and environmental samples so that shedding of *M. bovis* and BCG could be monitored during these experiments.

This dissertation is organized into six chapters and includes this introduction, a literature review, three chapters formatted as manuscripts for submission to three different journals, and finally a general discussion and overall conclusions. Chapter 2 is a review of the literature pertaining to bovine tuberculosis, its history, epidemiology, immunopathogenesis, vaccines, and presence in wildlife, particularly in white-tailed deer. Chapter 3 is a manuscript published in the *Journal of Wildlife Diseases*. It describes the evaluation of orally- and parenterally-administered BCG Danish strain 1331 for its ability to protect white-tailed deer from disease induced by experimental infection with virulent *M. bovis*. Chapter 4 is a manuscript published in *Clinical and Vaccine Immunology*. It describes humoral immune responses of white-tailed deer to vaccination with BCG and experimental challenge with *M. bovis*. Chapter 5 is a manuscript submitted to *Applied and Environmental Microbiology* and describes the development, validation, and application of molecular techniques used to detect MtbC in deer feces, oropharyngeal and nasal swabs, soil, hay, and feed in order to monitor for the presence of *M. bovis* in these samples throughout the study. Chapter 6 includes discussion and general conclusions.

Chapters 3-5 are formatted according to the guidelines of the journals in which they are published and to which they were submitted.

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CHAPTER 2: BOVINE TUBERCULOSIS IN CATTLE, WHITE-TAILED DEER, AND OTHER WILDLIFE: A LITERATURE REVIEW

INTRODUCTION

The following is a review of the relevant literature on the topic of bovine tuberculosis. Topics to be covered are etiology, history, epidemiology, pathology, and management. Although other species will be addressed, emphasis will be placed on two principal species: the domestic cow (*Bos taurus*) and the white-tailed deer (*Odocoileus virginianus*).

ETIOLOGY OF BOVINE TUBERCULOSIS

Bovine tuberculosis (BTb) is caused by the bacterium *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MtbC). Over 150 species exist in the *Mycobacterium* genus, only a few of which are pathogenic to vertebrates, including those of the *M. avium-intracellulare* complex, which contains *M. avium avium* and *M. avium paratuberculosis,* the agent of Johne's disease. Another historically well-known *Mycobacterium* is the bacillus that causes leprosy, *M. leprae*. Other members of the MtbC are *M. tuberculosis, M. africanum, M. canetti, M. caprae, M. microti,* and *M. pinnipedi*, also including a few not yet speciated members such as dassie (hyrax) bacillus and oryx bacillus (79, 108, 131). *Mycobacterium tuberculosis* is the principal agent of tuberculosis in humans and occurs worldwide, although *M. africanum, M. canetti, M. bovis, and M. caprae* cause significant disease in humans as well.

Mycobacterium africanum is most prevelant in western and equitorial Africa and *M. canetti* is found in the horn of Africa (74). *Mycobacterium caprae*, recently given its own speciation, is found in central and southern Europe and can infect a wide variety of species including humans and wildlife (7, 8, 58, 134). *Mycobacterium microti* is mainly a bacillus of small rodents and *M. pinnipedi* is predominantly found in seal species although both organisms are capable of infecting other hosts including humans (24, 39, 57, 72, 78, 126). *Mycobacterium bovis* has one of the widest host ranges of all MtbC pathogens and, like *M. tuberculosis*, is found throughout the globe (46, 90). Bovine tuberculosis has been reported in a variety of mammals including cattle, bison, buffalo, marsupials, hares, equines, camels, pigs, sheep, goats, deer, antelope, elephants, cats, dogs, fox, mink, badgers, moles, ferrets, rats and primates, including man (37, 38, 46, 54, 63, 90).

HISTORICAL PERSPECTIVE

Much controversy surrounds the actual age and ancestry of modern tuberculous mycobacteria (27). Gutierrez and others hypothesize that a progenitor of *M. tuberculosis* was present in East Africa as early as 3 million years ago, and they suggest that it may have infected early hominids at that time (50). It is likely, however, that all modern members of the MtbC, except perhaps *M. canettii* (which by some is thought to be the actual common ancestor or alternatively, to have coevolved with the other species), had a common African ancestor (named *M. prototuberculosis* by various researchers) about 35,000–15,000 years ago that affected humans and then transitioned to animal hosts (55, 148). This transition may be linked to plant and animal domestication that took place in

the Fertile Crescent around 13,000 years ago (148). Through the strong link between humans and cattle, it is known that *M. bovis* specifically has caused tuberculosis in both humans and cattle for thousands of years (28, 50, 76, 119).

In more recent history, BTb has been reported in slaughterhouses of Europe from the early 1800's (28). Philipp Klenke in 1843 became the first to experimentally demonstrate the transmissibility of TB and, in 1846, established that there was a relationship between tuberculous cervical adenitis (known as scrofula) and drinking milk from diseased cows (21, 46, 51, 76). In 1865 Jean Villemin showed that the cause of tuberculosis (or pthisis) was an actual agent that could be transferred between animals and humans (21, 51, 82). Robert Koch finally and famously isolated the organism in 1882 from both bovine and human tissues; and in 1890, Koch developed the first tuberculin (called "old tuberculin") from a concentrated sterile filtrate of autolysed heat-killed liquid cultures of *M. tuberculosis* (46, 82, 113, 122). However, it was Theobald Smith who demonstrated in 1898 that strains of Koch's tubercle bacilli from the two hosts differed in cell morphology, cultural characteristics, and virulence in rabbits (38, 46). Based on previous epidemiological and experimental evidence, it was still generally, and correctly, accepted that tuberculous cattle could transmit the disease to humans. Interestingly, in 1901, Koch presented a momentous paper at the British Congress on Tuberculosis in London stating that the tubercle bacillus of bovine origin was of minimal concern to man. His argument was based on the low incidence of intestinal tuberculosis in German hospitals (38, 44, 92). The impact of this statement was significant in the scientific community, considering the great esteem in which it held Dr. Koch. As a result, Koch's opinions caused notable setbacks in the advancement of tuberculosis

control in cattle. Fortunately, there was much opposition to Koch's views by other prominent scientists including Bernhard Bang, Edmond Nocard, and Robert McFaydean (21). Eventually, after the formation of a King's Commission on Tuberculosis by Edward VII of England, which instituted a number of elegant and thorough experiments, enough evidence was accumulated in support of *M. bovis'* virulence to humans, so that Koch conceded (44, 92).

Over the next several decades, with the implementation of tuberculin testing of cattle with Koch's Old Tuberculin and later PPD's, and the introduction of milk pasteurization, prevalence of *M. bovis* in cattle and humans decreased significantly in Europe, United Kingdom, United States, and Australia. More details regarding use of tuberculin as a diagnostic tool for bovine tuberculosis control and eradication can be found later in this chapter.

MICROBIOLOGICAL CHARACTERISTICS

Mycobacterium bovis is a slow growing, gram positive, thicked walled, acid-fast bacterium whose cell surface consists of a diffuse capsule, a two-layered wall and a plasma membrane (113). These features enable mycobacteria to resist hostile intracellular environments as well as those outside a host (113). The cell wall of this family of bacteria is of high lipid content, which, particularly due to mycolic acids, is responsible for the organisms' ability to retain basic dyes such as carbol fuchsin and resist decolorization with dilute acids. This "acid-fastness" is an important feature in the identification of mycobacteria, although this trait is not entirely unique to mycobacteria (113). *Mycobacterium bovis* differs from the human strain in being microaerophilic,

nitratase-negative, sensitive to thiophen-2-carboxylic acid, resistant to pyrazinamide, and requiring pyruvate to grow in culture (47). Until 1970, the bovine tubercle bacillus was regarded as being a type or variant of *Mycobacterium tuberculosis* and was termed *M. tuberculosis* var. bovis or *M. tuberculosis* subsp. *bovis*. The separate species name *Mycobacterium bovis* was proposed by Karlson and Lessel in 1970 (56).

EPIDEMIOLOGY AND INFECTION

The epidemiology of bovine tuberculosis is unique to the environment in which it is found and the ecology of its host(s) within that environment, driving the mechanisms of infection, maintenance of the disease, and transmission within a population and among populations.

The most common routes of *M. bovis* infection are via aerosol and oral routes (22, 23, 37, 109). Other routes that occur less frequently are cutaneous, congenital, and genital (85, 113). Infections thus can occur through exposure to aerosols, secretions, excretions, or tissues of other infected animals. Indirect exposure can also occur through contaminated materials, such as feed, contaminated pastures, water, and fomites (143). Wells and others found that successful infection of the respiratory tract via inhalation required exposure to single cells of *M. bovis* or droplet nuclei 1-5 μm in diameter (60, 77, 144, 145). The minimum infective dose by the aerosol route is very low, perhaps just one organism when delivered to the correct location, compared with the oral route (109).

Transmission via the respiratory route due to direct contact among animals plays a significant role in the epidemiology of bovine tuberculosis in many species; however, infection through contact with contaminated substrates can be important as well

depending on many factors, as stated above. Infection via bite wounds can occur in species such as badgers (43). Historically, reported infection in humans has most frequently been reported to be associated with the consumption of infected milk products, although pulmonary disease was not uncommon among cattle farmers. Excretion of bacilli onto feed via saliva, nasal secretions, lesion secretions, urine, or feces is implicated as a source for transmission among livestock and wildlife species and this type of transmission has been demonstrated in controlled settings (43, 77, 101, 103, 106). Interestingly, there are examples of humans transmitting the infection to cattle via urine contamination of the feed source for the animals (63). Also, in certain environments favorable to survival of *M. bovis* on pasture or in soil, animals using these pastures could feasibly be exposed (62). Favorable conditions include high humidity, low temperatures, low levels of ultraviolet light, and many others (77). The bacilli have been shown to remain viable under ideal conditions for up to 15 months (32, 77, 128, 151).

Francis, in 1947, concluded that the respiratory route was responsible for 80 to 90 per cent of infections in cattle through exposure to droplet nuclei and dust, and possibly to a much lesser degree, exposure to rumenal bacteria in eructated gases (37, 43, 80). At the time of Francis' writing, bovine tuberculosis seemed to be much more prevalent in dairy cows than in beef cattle (37). Dairy cattle were intensively managed, especially those dairies that supplied large cities. It is in these closely contained environments that aerosolization of *M. bovis* probably played a significant role in the transmission of tuberculosis. This mode of transmission is also likely to predominate in today's industries where infected cattle are the known source of *M. bovis*, although in well ventilated conditions *M. bovis*-infected cattle do not readily infect other cattle (22). In

addition, it is believed that only certain tuberculous individuals act as effective disseminators and these do so probably intermittently and only under certain circumstances (60). Other mechanisms of transmission of *M. bovis* from cattle to cattle, much less common than aerosolization, involve contaminated milk fed to calves, congenital infections, venereal transmission, and contaminated pastures under certain environments (37, 62, 77).

As stated above, the current environments in which *M. bovis* exists in ecosystems that are shared between and among cattle, other species of livestock, and wildlife are more likely conducive to a combination of aerosol and oral routes of infection in the various species, although aerosol is still likely the main mode of transmission (88, 100-102, 105, 123). Concerning the oral route of infection, greater numbers of organisms are needed to infect cattle via this route in comparison to the aerosol route, findings ranging from 5×10^3 CFU to 1×10^7 CFU (101, 109, 124). However, direct oral inoculation of only several thousand organisms can successfully lead to infections and indirect transmission has been demonstrated between *M. bovis*-infected deer and cattle that consumed contaminated feed and were exposed to pen waste material (101).

CLINICAL DISEASE AND PATHOLOGY

Infected animals are often asymptomatic; however, in animals in later stages of disease, notable clinical signs may include weight loss, coughing, dyspnea, lymphadenitis, draining lesions and alopecia (6, 131). In humans, lymphadenitis of the head and neck is seen most often , as these lymph nodes receive bacilli after uptake by the tonsils in the event of consumption of infected dairy products.

Grossly, one may observe pathological changes involving the formation of granulomas in tissues of *M. bovis*-infected cattle which most frequently occur in the lymph nodes of the thorax and the lungs, and somewhat less frequently in lymph nodes of the head (13, 46, 77, 81, 82, 127, 146). Primary lesions in cattle are generally the source for subsequent formation of lesions via the lymphohematogenous route, and the progressive development of disease. At times, when the primary lesion can be arrested, the disease is categorized as latent; however, latency cannot be clearly defined among researchers of mycobacteria (37). Many experimental infection studies have been carried out in cattle and the patterns of lesions noted have been very similar to those observed in naturally infected cattle, although experimentally-induced disease may be influenced by a number of variables, including the strain of organism, dose of organism, route of inoculation, culture conditions for growth of the organism, as well as host factors (13, 19, 27, 129). In experimentally infected animals, early granulomas in the upper respiratory tract tend to contain large numbers of acid-fast organisms within aggregates of neutrophils and macrophages in sub-epithelial lymphoid tissue and within the surface epithelial cells in the upper respiratory mucosa. Early lung lesions are similar to lymph node lesions but also contain multinucleated giant cells (19). Gross lesions can be seen as early as 14 days postchallenge as was observed by Cassidy and others (19). More advanced lung lesions appear as multifocal aggregates of neutrophils with a distinct mantle of macrophages. Large numbers of acid-fast organisms are usually seen within the central neutrophil aggregates and in the cytoplasm of the surrounding macrophages. In later stages of disease, extensive central necrosis of neutrophil and macrophage aggregates can be seen surrounded by a mantle of intact and degenerate neutrophils,

macrophages, macrophage giant cells, and lymphocytes. Fibrosis circumscribing the lesions may also be present as well as mineralization of the core (19). Grossly, the majority of advanced lesions in cattle are caseous or caseo-mineralized in nature, and about 16% of them become heavily mineralized and coarse so that they feel gritty upon incision (49).

In white-tailed deer the majority of lesions in naturally infected animals tend to be located in the medial retropharyngeal lymph nodes, although they are also commonly found in the thoracic lymph nodes, lungs and mesenteric lymph nodes. It has been suggested that tonsils and other oropharyngeal lymphoid tissues play a significant role in white-tailed deer, from which efferent lymphatics drain to the retropharyngeal lymph nodes (99, 102, 121, 129). This would suggest that there may be more of an oral component than is seen in cattle. It could also be that inhalation of organisms which do not penetrate to the lower respiratory tract can initiate multiplication at a site in the oropharyngeal area, which drains to the retropharyngeal lymph nodes. From there, infection may spread to additional sites (77). Clinical signs and appearance of gross lesions in white-tailed deer are very similar to those in cattle. However, lesion distribution and structure of granulomas in this species are somewhat different.

Extensive studies regarding lesion development in white-tailed deer experimentally infected with *M. bovis* have been carried out by Palmer and his colleagues (99, 102). In a study in which deer were inoculated intratonsilarly with approximately 300 colony forming units *M. bovis* (102), microscopic lesions could be observed as soon as 28 days in the medial retropharyngeal lymph nodes, and lesions in the lung tissue were seen at 42 days postinoculation. Granulomas are composed of aggregates of

macrophages, Langhans-type multinucleated giant cells, and few lymphocytes. Early granulomas contain small, centrally located foci of neutrophils in which moderate to large numbers of acid-fast bacilli are present. In the early stages, there is no mineralization or evidence of collagen deposition associated with the granulomas. In later stages, lesions progress to coalescent granulomas which develop central areas of necrosis and mineralization. Acid-fast bacteria are present in low numbers and only within the necrotic cores (102). The areas of necrosis are surrounded by variable thick bands of cellular infiltrate containing macrophages, Langhans-type multinucleated giant cells, and lymphocytes. Thin bands of collagen surround the granulomas separating them from unaffected tissue. The cellular constituents of lesions seen in deer in the present study are similar to those seen in cattle (19, 93, 94). However, in contrast to cattle, deer experience only mild fibrosis surrounding granulomas, and these lesions tend to be of a more liquefactive nature than is seen in cattle (94, 102). These abscess-like lesions are thought to contribute to increased dissemination of the bacterium within the host and also to increased transmissibility among individuals of this species (18, 102).

IMMUNOLOGY/IMMUNOPATHOLOGY

Since the discovery of the tubercle bacillus, researchers have strived to understand the complete immunopathogenesis of the MtbC; however, this has yet to be fully achieved. On the cellular level, the organism's main mode of entry into the host is via the macrophage, although dendritic cells become infected as well. The macrophage is both a key target of invasion for mycobacteria and the primary effector cell for control of mycobacterial infections (143). The majority of exposure events do not lead to infection,

and the innate immune response of the host eliminates the bacteria (18). A successful infection occurs when virulent bacilli are able to evade destruction after ingestion by macrophages or by dendritic cells. Multiple factors allow the bacteria to do this, and it is believed that the process is dependent on lipid complexes in the mycobacterial cell wall, such as lipoarabinomannan and phosphatidyl inositol mannoside (131). With the help of these waxy components as well as proteins, the bacilli are able to prevent the lysosomal fusion with the phagosome, thus limiting acidification of that structure and enabling mycobacterial survival inside the macrophage (131).

Upon interaction of the mycobacteria with macrophages and dendritic cells, copious production of cytokines and chemokines occurs through toll-like receptor signaling. These cytokines, including interleukin-12 (IL-12), tumor necrosis factor-alpha (TNF-alpha), and chemokines induce a rapid, non-specific influx of macrophages and neutrophils to the site in infection, representing the initial formation of the granulomatous lesion as described above (10, 19). It is generally accepted that protective immunity after infection is dependent on the acquired cell-mediated immune (CMI) response of the host (2, 113). In 1942, Lurie first demonstrated that in reinfected rabbits, mononuclear phagocytes could engulf and kill tubercle bacilli at an increased rate compared with normal phagocytes (70). It was Mackeness in 1968 who first suggested that the activation of macrophages required substances (lymphokines) produced by certain subsets of lymphocytes, which were eventually determined by North (1973) and Lefford (1975) to be T lymphocytes or T cells (61, 73, 87). The T cell is crucial for acquired protection involving activation of macrophages, as seen in Lurie's rabbits (17). Specifically, CD4+ T cells are thought to play a crucial role in macrophage activation by

producing interferon-gamma (IFN-gamma) and by acting as cytotoxic cells, thus being capable of lysing heavily infected macrophages to enable antigen presentation (18, 67). Antigen presentation through class II (CD4+ T cells) and class I (CD8+ T cells) major histocompatibility complex processes, are essential in orchestration of the type 1 (Th1) adaptive immune response. Other types of T cells, such as gamma-delta T cells, also play important roles as immunoregulatory cells; however the exact nature of these roles is less clear (67).

The control or containment of infection is dependent on the maintenance of an extremely complex balance of immune responses with strong bias toward a Th1 CMI response. This state is generally thought to allow the establishment and maintenance of the organisms within the granuloma, although, in the case of *M. bovis* infection, eventually this balance is lost and there is progression towards advanced disease in the animal. Increased pathology is associated with an immune profile that biases a humoral immune response. Increases in anti-inflammatory cytokines, such as IL-10 and IL-4, are observed in advanced disease, in addition to increases in IgG1 antibody production. The importance of these dynamics in immunopathology to diagnostic testing will be addressed later in this chapter. There is also contrasting belief by some that mycobacteria can exploit the granuloma for local expansion and systemic dissemination of bacteria, therefore arguing against the granuloma having an exclusively protective role in mycobacterial disease (29).

DIAGNOSTICS

Ante-mortem Diagnostics

Tuberculin skin testing

Tuberculin tests have been used to diagnose tuberculosis in cattle for over a century (75). These tests rely on the measurement of delayed type hypersensitivity (DTH) response after injection of an antigen, usually in the form of tuberculin. Delayed-type hypersensitivity is dependent on the presence of the CMI response described earlier in this chapter. In *M. bovis*-infected cattle, injected tuberculin elicits a reaction associated with an influx of sensitized T-lymphocytes and monocytic cells into the site of inoculation (139). The DTH was first described by Koch and can be identified in the form of a febrile response or development of skin swelling upon injection of mycobacterial products into a sensitized subject (Koch, 1890, as reviewed by Pritchard, 1988) (113). Robert Koch's "old tuberculin", as described earlier in this chapter, was first intended to be used as a curative agent. It was soon discovered, however, that, unlike non-tuberculous people, tuberculous individuals injected with old tuberculin developed a febrile illness which lasted several hours. This diagnostic method was then adapted to cattle in the form of a subcutaneous injection followed up with serial measurements of body temperature, and was successfully utilized at the end of the 19th century and during the first 2 decades of the 20th century, championed by John McFadyean in the United Kingdom (44). Obtaining multiple temperature readings was very laborious and time consuming, and so alternatives were sought.

The intradermal skin test, described by Moussu and Mantoux in 1908, was determined to be just as sensitive as the subcutaneous test by Ernest in 1920, and it was

soon adopted by many countries as the official test in the form of either the caudal fold test, the single intradermal test administered to the skin of the neck, or the double intradermal test (75). The caudal fold test was adopted in the United States for its test and slaughter campaign. In the 1930's and 1940's researchers (Seibert in 1934 and Green in 1946) began producing tuberculins in standardized ways which we know as purified protein derivatives (PPDs) (75). In addition, it became apparent that a certain percentage of skin test positive cattle did not show any evidence of *M. bovis* infection at post-mortem. From this came the development of comparative skin tests, which used PPD derived from mammalian (later bovine) and avian tuberculin. Thus intradermal skin testing was the key component of bovine tuberculosis control programs throughout Europe, Australia, and the United States during the 19th century and is still the official testing technique currently used in the United States in the form of a single intradermal test followed up with the comparative test.

Estimates of the sensitivity of tuberculin tests in cattle range from 32-99%, while specificity is estimated to be 75.5%-99.9% (30, 75, 91). Palmer et al. reported that in white-tailed deer of known *Mycobacterium bovis* infection status, the sensitivity and specificity of the comparative cervical skin test were 97 and 81% respectively (107). The sensitivity of the test is affected by the interpretation key used, by the potency and dose of tuberculin administered, disease status, status in relation to parturition, observer/operative variation, and other factors. The test specificity is influenced by the tuberculin(s) and type of tuberculin test; also by the interpretation key used, and by sensitization as a result of exposure to *M. avium, M. paratuberculosis,* or environmental mycobacteria (91). Animals experiencing advanced pathology tend to be anergic to skin

testing due to an increasing bias toward an antibody-based immune response as described previously. False positives can occur when animals are exposed to non-MtbC mycobacteria, especially when using the single tests. Production and potencies of PPDs are variable and inconsistent throughout the world, even today (113). Efforts are currently being made to evaluate the differences in PPDs manufactured worldwide and recommendations are being brought forth to standardize these products and to investigate alternative antigens. All of the factors mentioned above account for the wide ranges of sensitivities and specificities of this test.

Interferon gamma test

In the face of these issues, there is demand for development of alternative methods of testing for bovine tuberculosis. The interferon gamma assay for cattle (Bovigam®, Prionics AG, Schlieren-Zurich, Switzerland) is one which has become widely used and is approved as a complementary test to the skin test in many countries. This test, essentially an *in vitro* version of the comparative cervical test, relies on active cellular immune function (111). It requires the collection of whole blood which is aliquotted and placed in culture with certain test antigens such as bovine and avian PPD's as well as a negative control. After a 16-48 hour incubation, plasma supernatant is harvested and subjected to an enzyme-linked immunosorbent assay (ELISA) which quantifies interferon-gamma produced in the sample (30). The production of interferon-gamma by the cultured lymphocytes exposed to *M. bovis* antigen is increased if the animal is infected with *M. bovis*. Increased production of interferon-gamma can be detected as early as 1 to 4 weeks post-infection with this test (30). As summarized by Wood and Jones in 2001, the sensitivity of the interferon-gamma test in cattle varied

between 81.8% and 100% for culture-confirmed animals and specificity ranged between 94% and 100% (149). Some animals can be positive by both tests, but others may be positive to only skin test or IFN-g test (84). Therefore, these two tests used in parallel could increase the overall sensitivity of diagnosis (84, 111). Although there are conflicting reports, there is also evidence that administering the interferon-gamma test 3-7 days after single intradermal skin test will elicit a boost in interferon-gamma production by T cells of infected animals, thus also increasing the sensitivity of the interferon-gamma assay (14, 45, 98, 120, 132). Lastly, as with the skin test, alternative antigens, such as ESAT-6, are being investigated for use with the interferon-gamma test in order to increase specificity (111). In white-tailed deer and other cervids, the interferon-gamma test used is Cervigam® (Prionics AG). Although early research initially showed promise in diagnosing BTb-infected white-tailed deer (104), subsequent studies indicated that this commercial assay does not reliably detect interferon-gamma production by this species in up to 66% of the deer tested (141). Reevaluation of this assay needs to be performed in order to correct these problems and produce an effective interferon-gamma test for cervid species.

Serologic/antibody-based assays

An accurate serologic test for tuberculosis has been sought for many years. Antibody-based assays are easy to perform relative to other assays such as interferon gamma or skin testing. This is due to the fact that one blood draw is required, serum can be stored over time prior to analysis if necessary, and the tests are easily carried out in the lab. In contrast, skin testing requires two to four animal handling events. Although the gamma-interferon test only requires one animal handling event, reliable results depend on

overnight delivery of whole blood to the laboratory and skillful handling of the specimen for cell culture. Unfortunately, although there have been great advances in tuberculosis serology, the assays have yet to equal or surpass in accuracy the other two types of tests. The sensitivities of antibody-based tests generally fall short of the CMI-based tests, especially in early stages of infection (30, 35, 110). One of the reasons for this is the ongoing need to identify immunodominant antigens that can be used to correctly diagnose infected animals.

Until recently, antigens used for diagnostics have been poorly defined and consisted of whole-cell preparations and culture filtrates, such as tuberculin (97). Extensive research has been carried out to evaluate various antigens in order to develop antibody-based assays that are more sensitive and specific than the skin test or the interferon-gamma test. Among these antigens are MPB83, MPB70, ESAT-6, and CFP-10. These antigens and others have succeeded in increasing specificity of these assays, but sensitivity has been little improved (16, 20, 30, 42). Antibody development to MtbC-specific antigens is highly dependent on many factors. These factors include the animal species affected, duration of infection, extent of pathology, and individual animal variations. This has led to the conclusion by some that including multiple antigens in serologic testing provides the greatest chance of identifying an infected animal (71, 137, 140). In addition, it has been shown that cattle and deer with multiple lesions tend to have greater antibody levels than animals in earlier stages of infection and that antibody-based tests are more effective in these animals, especially those that have become anergic to CMI-based tests and would thus not be detected unless there were visible clinical signs (64, 71, 112, 117). Furthermore, in trials where the interferon-gamma assay was

compared with serological assays, there appeared to be an inverse relationship between interferon-gamma responses and antibody production in cattle (117, 149, 150). It should be noted, however, that it is possible to detect experimentally infected cattle as early as four weeks after inoculation via serology depending on antigens used (140). Serologic assays have also been shown to be useful when performed in series with the tuberculin skin test due the induction of anamnestic antibody responses following skin testing (68, 69, 71, 112), thus increasing detection rate of suspect infected animals.

In practice, although the sensitivity and specificity of certain ante-mortem tests are reported here based on data reported in the literature, it should be emphasized that the ability to predict the presence or absence of disease from test results is dependent on the prevalence of the disease in the population tested, in addition to the sensitivity and specificity of the test. The higher the prevalence the more likely it is that a positive test is predictive of the disease; this measure is referred to as the positive predictive value of the test. Thus the positive predictive value of tuberculin tests at the beginning of an eradication program is likely to be high whereas, as programs make progress in reducing the disease prevalence, the positive predictive value decreases and the negative predictive value increases (75).

Post-mortem Diagnostics

Bacteriological culture and histopathology

Unlike in humans where sputum from a patient is routinely cultured to test for mycobacterial infection, bacteriological culture is mainly a post-mortem diagnostic tool in animals suspected of *M. bovis* infection. Tissues undergo a fairly intensive decontamination process involving NaOH before culturing in order to reduce the

numbers of fast-growing competitive organisms that may impede growth of MtbC bacteria. Cultures are observed daily for up to 60 days before a negative reading can be made. Tissue culture is the gold standard to which all other diagnostic techniques are compared, although the cumbersome nature of this procedure causes some loss of sensitivity, and recovery of specific MtbC bacteria is highly dependent on the individual laboratory methods (130).

Histopathology is a very effective way of detecting evidence of *M. bovis* infection in tissues. The presence in tissues of microscopic granulomatous lesions containing acid-fast organisms, as described earlier in this chapter are generally indicative of mycobacterial infection, and *M. bovis* is confirmed via positive culture or detection of MtbC-specific DNA.

Molecular assays

There have been great advances in the molecular detection and differentiation of *Mycobacterium* spp. Some of these techniques include polymerase chain reaction (PCR), restriction enzyme analysis, restriction fragment length polymorphism, variable number tandem repeat analysis, and spoligotyping. The PCR, usually based on the IS6110 region of the MtbC, is currently used in the United States as a follow up to positive histopathologic diagnosis. The other techniques are used for differentiation of species as an aid to epidemiological investigations. These techniques all have advantages and disadvantages depending on the specific case and are utilized based on the given situation.

BOVINE TUBERCULOSIS IN WILDLIFE

While great advancements have been made over the last century regarding control and eradication of BTb in many parts of the world, the disease has managed to evade eradication in numerous countries due to its maintenance in wildlife reservoirs (54, 77). Wild white-tailed deer in the northern lower peninsula of Michigan, USA are a reservoir host for *M. bovis* (105, 123, 142). Although recent changes in management practices applied to this free-ranging population have decreased the prevalence of the disease to approximately 1%, infections in local domestic cattle herds continue to occur (89). White-tailed deer are also a spillover host for *M. bovis* in cattle herds in northern Minnesota, USA. In addition, BTb is endemic in elk and is found to a lesser extent in white-tailed deer in Riding Mountain National Park in Manitoba, Canada. There are many other examples of wildlife reservoirs for BTb as well. Among them are the brushtail possum (*Trichosaurus vulpeca*) in New Zealand, the European badger (*Meles meles*) in the United Kingdom and Ireland, the African buffalo (*Syncerus caffer*) in South Africa, wood bison (*Bison bison athabascae*) in Canada, and wild boar (*Sus scrofa*) in Europe (31, 83, 86, 143).

Bovine tuberculosis in wild white-tailed deer in North America

In the 1940's Michigan, USA had the highest number of tuberculosis positive cattle of any state in the country. By 1979, Michigan declared itself bovine tuberculosis free (41). In November of 1975, a hunter-killed 9.5 year-old female white-tailed deer was diagnosed with bovine tuberculosis in the northwestern region of the lower peninsula of the state (123). Prior to this, there had only been a handful of diagnoses of bovine tuberculosis in free-ranging deer in North America (9, 40, 66, 116). Most of these cases

were associated with wild deer herds whose ranges overlapped with infected domestic livestock operations (40, 66, 116). As Michigan was on the cusp of BTb freedom, this disease occurrence in a free-ranging white-tailed deer was considered an isolated case and no monitoring was done on the wild deer population or livestock in the area. Accordingly, it was also not reported in the literature. Nineteen years later, in November 1994, a hunter-killed 4.5 year-old male white-tailed deer within 13 kilometers of the 1975 case, was submitted to the Michigan Department of Natural Resources due to the hunter's discovery of lesions in the animal. This deer was confirmed to be infected with *M. bovis*. This time, livestock and deer in the vicinity were examined, including an intensive survey of hunter-killed deer taken during the 1995 season. Results of this 1995 survey revealed that the apparent prevalence of BTb in hunted wild white-tailed deer in northern Michigan was close to 3.5% and that this herd maintained the disease, thus potentially serving as a wildlife reservoir for BTb (123). The strain, as characterized by restriction fragment length polymorphism, is a unique one to the area, and is therefore not believed to have originated from tuberculous cattle brought in from Mexico (89). Most strains involved in herd infections in other states in the United States are related to strains found in Mexican cattle. Currently, an area encompassing four counties in the northwest corner of the lower peninsula of Michigan is considered a BTb-endemic area (Figure 2.1). The prevalence has remained below 2% in the past few years (5).

In 2005, BTb was detected on five farms in extreme northern Minnesota, USA. In response to these findings in domestic cattle, nearly 500 wild white-tailed deer were immediately collected from the area, and two were determined to be positive for *M. bovis*. Several thousand animals have been collected in subsequent years, and the

prevalence is estimated at approximately 0.4%. The strains obtained from both the cattle and the deer were the same and are compatible with strains obtained from strains traced back to Mexico. Bovine tuberculosis is not considered endemic in Minnesota deer; however, if left unchecked, the disease has the potential to establish itself in the herd and pose a long term problem similar to that in Michigan.

MANAGEMENT

Cattle and other captive livestock

Spanning the twentieth century, the United States, Canada, Great Britain, Australia, New Zealand, Denmark, and other countries developed programs aimed at the control and eradication of bovine tuberculosis in their livestock populations. These campaigns relied heavily on tuberculin testing and slaughter of reactor animals. These practices were combined with slaughterhouse surveillance for detection of lesioned animals and tracebacks to farms of origin (63). In 1917, the United States initiated a nation-wide program to eradicate BTb from the country's cattle herds that was dubbed the National Cooperative State-Federal Bovine Tuberculosis program. In this program, the accredited herd plan was established, and herds deemed free of tuberculosis were given an accredited rating. By 1929 the incidence of BTb in the United States had been reduced by 50% (33, 34). By 1940 every state reported a less than 0.5% disease prevalence (37). To achieve this low prevalence, over 200 million cattle were tuberculin tested, and nearly 4 million were sent to slaughter (34, 37). An official test and slaughter program in Canada reduced prevalence to below 1% by 1944 (37). An eradication program in Great Britain did not commence until 1935 and, in addition, was then delayed

by World War II. In 1950, approximately 18% of cattle herds were infected, which was reduced to 1-2% by 1970 (21, 31). The benefits of the bovine tuberculosis eradication programs have been great. Not even taking into account the value of disease prevention in human populations, it has been estimated that such control programs in the United States have an annual economic benefit of between 30 and 300 million dollars (118). At the time of this writing, only four states in the U. S. are not considered free of bovine tuberculosis in their cattle herds, two of which, Minnesota and Michigan, are associated with the disease in wild deer. Great Britain is experiencing a resurgence in BTb in its cattle herds which has been attributed to increased movement of animals after the 2001 foot and mouth disease depopulations, as well as the persistence of disease in the European badger. New Zealand has been very successful in reducing BTb in cattle but is battling a prominent wildlife reservoir in the form of the brushtail possum, as mentioned previously. Australia has successfully eradicated BTb from its domestic and wild populations, last cases in cattle and water buffalo having been detected in 2002 (4). In other developing countries, prevalence of BTb in cattle can vary enormously. In South and Central America, prevalence can range from as low 0.01% to as high as 79%, depending on the regions sampled (131). Similar findings can be obtained throughout the developing world.

Treatment of animals with BTb has been attempted in only a few cases but is largely not done. With the discovery of drugs that are effective against mycobacterial infections, treatment of humans became possible; however, this approach in livestock has never been a viable option. Treatment has been administered to valuable animals, such as those in zoos, although this practice is decreasing. Overall, the high cost of drugs,

antibiotic residues in meat and dairy products, and the potential for development of drug resistance are all factors preventing treatment from being used as a management tool.

Bovine tuberculosis occurs in captive deer sporadically in North America. Farmed deer are managed similarly to cattle via test and slaughter programs and abattoir surveillance; however, existing diagnostic tests for cattle are not as reliable in some deer species making detection of infected animals often problematic, and the multiple handling events required during skin testing create compliance issues in the industry. In North America, BTb emerged as a problem in the captive cervid industry in 1990 when an infected herd in Canada traced back to several farms in the United States (147). Captive cervid herd breakdowns continue to occur; as recently as 2009 elk ranches in the states of Indiana and Nebraska experienced BTb outbreaks

The success of earlier national bovine tuberculosis eradication programs was achieved at a time when herds were smaller, the intensity and demands of production lower, and before the emergence of significant wildlife reservoirs of *Mycobacterium bovis* (21). The presence of wildlife reservoirs has added serious complications to many BTb eradication programs and, unless properly managed, will invariably prevent complete eradication from the domestic species that share an ecosystem with wild populations that are capable of maintaining the disease.

White-tailed deer

Unlike in cattle and other livestock, where control and management programs, when implemented, have been relatively uniform, each reservoir/infected wildlife population requires a unique set of approaches to management. Management strategies should be aided by and adapted to the ecology of the species and the epidemiology of the

disease within the population. There are very few known cases throughout the world where cervid species act as maintenance hosts for bovine tuberculosis, two important examples of which are elk in Riding Mountain National Park in Canada, and white-tailed deer in Michigan. In Michigan, the distribution of BTb in the deer population suggests that unique management and ecological/environmental factors have allowed the persistence of this disease in the affected area. Only rarely are infected deer outside the core area detected, and this is thought to be due to differences in these predisposing factors throughout Michigan (52). Specific unique factors that are believed to have facilitated emergence of a reservoir for BTb in white-tailed deer in northern lower peninsular Michigan are: establishment and maintenance of hunt clubs since the late 1880's, habitat enhancement for deer use, protection from unregulated hunting, and feed supplementation which intensified in the 1980's and 1990's. Other environmental factors influencing the epidemiology of the disease are those that cause congregation of deer in addition to artificial feeding such as water sources, natural cover, and reduced human contact (115). O'Brien and others (2002) noted significantly increased odds of *M. bovis* infection in two-year-old bucks and these odds continue to increase with age. This suggests that, in Michigan, much of the transmission of BTb likely occurs between yearling bucks and may be attributed to their dispersal and sparring behaviors. In contrast to bucks which join all-male groups that travel together throughout the year except during rut, does form permanent matriarchal family groups and only disperse to form new groups (89). In response to the Michigan BTb outbreak, management efforts have focused on population decrease and banning of winter supplemental feeding. Data indicating that prevalence of BTb has decreased in the core area suggest that this has

been an adequate strategy, especially when both does and younger males are targeted for hunting, in order to effectively reduce population densities and reduce transmission respectively. Banning of supplemental feeding prevents abnormal concentration of animals that may be infected and is thus also an important aspect of disease management. Unfortunately, negative social consequences regarding enforcement of bans on supplemental feeding, as well as resistance to increased hunting, have hampered disease management efforts.Ttherefore prevalence of BTb remains low but stagnant, and outbreaks in cattle continue to occur.

Vaccination of cattle

There have been a number of attempts to control cattle tuberculosis by vaccination. Koch attempted to market vaccine prepared from human tubercle bacilli which usually caused only self-limiting disease in cattle; however, the discovery of viable and virulent human tubercle bacilli in the milk of vaccinated cows led to the discontinuation of such vaccination practices (46). In the first two decades of the twentieth century, Albert Calmette and Camille Guerin developed the BCG strain of *M. bovis.* This vaccine has been used to vaccinate millions of people against tuberculosis since that time, and although its efficacy is highly variable, especially in adults, a better vaccine has yet to be developed and BCG remains the vaccine to which all new vaccines are compared (25, 34). In 1940, the vole bacillus, *M. microti*, was discovered to induce protection against MtbC infection that was as good as or perhaps better than BCG, but because its virulence was greater than that of BCG, it was not pursued as an alternative vaccine (37, 125). Numerous trials throughout the world were conducted with BCG in cattle with variable success starting before the 1930's up until 1990, as summarized by

Skinner et al (2001) (125). Due to only partial efficacy of the vaccine, as well as its ability to induce tuberculin reactivity, thus interfering with test and slaughter programs, BCG was never adopted as a vaccine for cattle (12, 37, 125).

Recently, a renewed interest has developed regarding the use of BCG in cattle, as well as in wildlife species. New data suggested that lower doses of BCG than those used in the earlier trials induced better protection and potentially had minimal effect on tuberculin reactivity (11, 125). Trials were conducted in New Zealand with doses of BCG Pasteur ranging from 10^4 to 10^6 CFU (14, 15). These studies demonstrated the protective capabilities of BCG, but in a subsequent study, BCG failed to protect cattle from disease (125). It is thought that animals or people that are exposed to environmental mycobacteria prior to BCG vaccination experience a masking or inhibition of protection or develop immunity to the vaccine itself (36, 133). Subsequent studies have indicated some success in improving upon BCG by administering DNA vaccines in combination with BCG (136). Several other vaccine constructs, such as recombinant *Mycobacteria* and attenuated strains with gene deletions, are also being evaluated (136, 138). Thus far these recombinant strains have not surpassed BCG in efficacy, but efforts will continue towards developing an effective vaccine in cattle that will not interfere with diagnostic testing and control programs.

Vaccination of deer and other wildlife

The development and implementation of a vaccination program for wildlife, which would effectively reduce disease and shedding of *M. bovis* by deer and other affected wildlife species, would significantly aid efforts in eradicating BTb from those countries where the disease is being maintained in both livestock and wildlife

populations. Control of wildlife disease through vaccination is not a new or untried strategy, as oral vaccination programs targeting rabies in red fox (*Vulpes vulpes*), raccoon (*Procyon lotor*), and coyote (*Canis latrans*) have already been proven to be effective in reducing disease in these wild species (3, 53, 135). The option of using vaccination as a management tool for *M. bovis*-infected wild populations is currently being investigated in the context of all of the wildlife reservoirs mentioned previously. The efficacy of BCG has been evaluated to varying degrees in brushtail possum, European badger, European wild boar, white-tailed deer, African buffalo, and other non-reservoir species as well, such as red deer (*Cervus elaphus*) and ferrets (*Mustela furo*) (1, 26, 48, 59, 65, 83, 95, 96, 114). Thus far, it has been determined that vaccination with BCG is protective for all those species mentioned, excepting African buffalo, and would be feasible in those species that maintain BTb in their populations.

Palmer and colleagues have produced a considerable amount of data pertaining to the vaccination of white-tailed deer with BCG and subsequent experimental challenge with virulent *M. bovis* (95, 96). Their studies show that BCG vaccination, both oral and parenteral, successfully protects this species from disease progression over a five-month period after infection when compared with unvaccinated animals. Data obtained from one of these studies will be reported in subsequent chapters of this dissertation. The promising results seen with BCG in white-tailed deer in a controlled setting strongly support further research towards its implementation in the field. Future studies include long-term efficacy trials, safety trials, and exploration of baits and methods of vaccine administration to a large wild deer population. These topics will be addressed in the final chapter of this dissertation.

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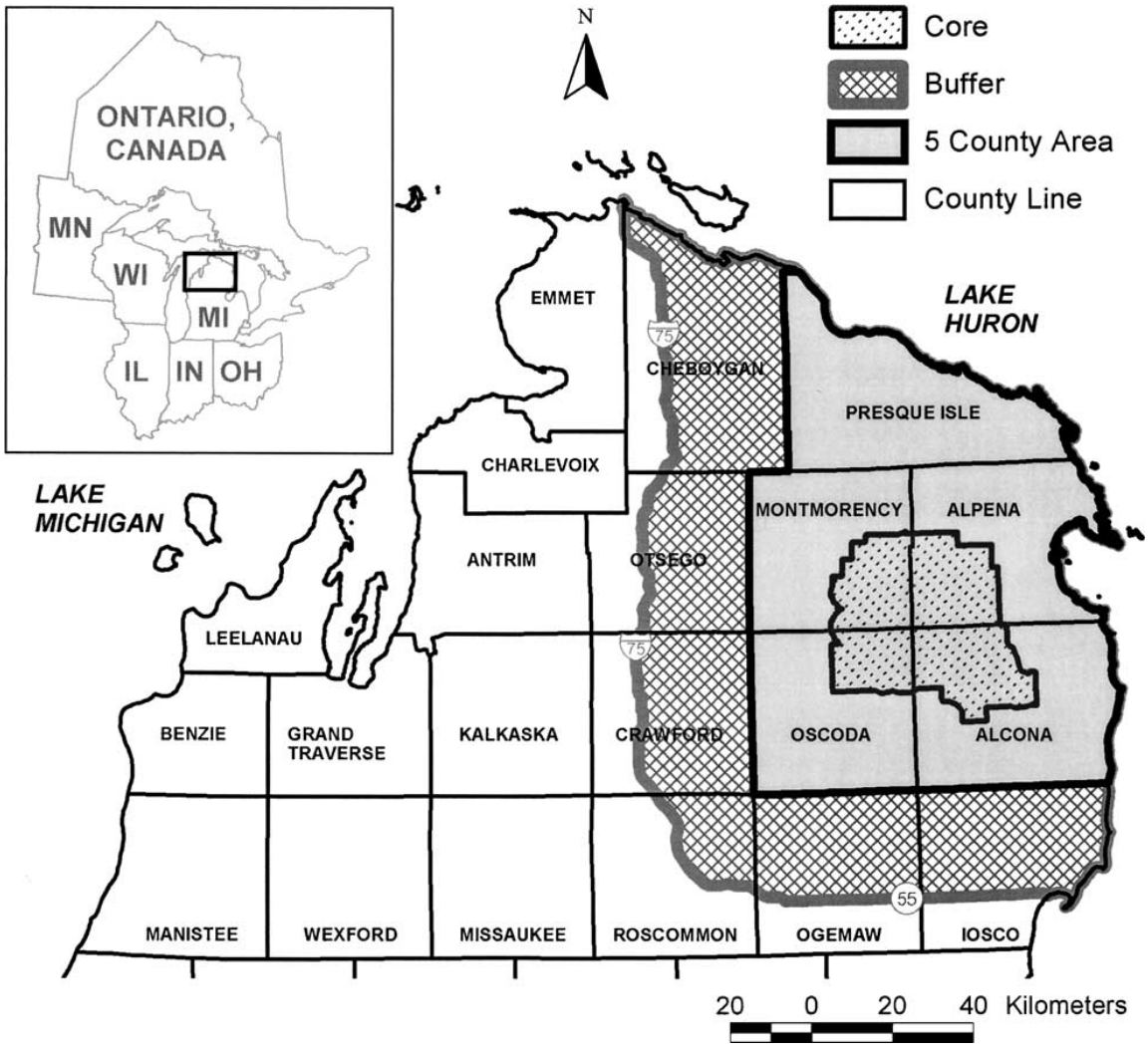

Figure 2.1. Geographic area under surveillance in northern Michigan, USA. From O'Brien et al, 2002 (89)

Chapter 3: EFFICACY OF ORAL AND PARENTERAL ROUTES OF *MYCOBACTERIUM BOVIS* BACILLE CALMETTE-GUERIN VACCINATION AGAINST EXPERIMENTAL BOVINE TUBERCULOSIS IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*): A FEASIBILITY STUDY

ABSTRACT

We investigated the efficacy of oral and parenteral *Mycobacterium bovis* bacille Calmette-Guerin Danish strain 1331 (BCG) in its ability to protect white-tailed deer (*Odocoileus virginianus*) against disease caused by *M. bovis* infection. Twenty-two white-tailed deer were divided into four groups. One group (n=5) received 10^9 colony forming units (cfu) BCG via a lipid-formulated oral bait; one group (n=5) received 10^9 cfu BCG in culture directly to the oropharynx, one group (n=6) was vaccinated with 10^6 cfu BCG subcutaneously, and one group served as a control and received culture media directly to the oropharynx (n=6). All animals were challenged 3 months after vaccination. Five months post-challenge, the animals were examined for lesions. Results indicate that both oral forms of BCG, and parenterally-administerd BCG offered significant protection against *M. bovis* challenge as compared to controls. This study suggests that oral BCG vaccination may be a feasible means of controlling bovine tuberculosis in wild white-tailed deer populations.

INTRODUCTION

Bovine tuberculosis (BTb), caused by *Mycobacterium bovis*, poses a serious continual threat to the health and economic wellbeing of both livestock and humans worldwide. In the developing world, one third of the population lacks effective agricultural and food safety programs, leaving them at substantial risk for zoonotic infection by *M. bovis* (Nelson, 1999). In addition, wildlife reservoirs of BTb are believed to play a very important role in the epidemiology of this disease in many countries. In the United States, a free-ranging white-tailed deer (*Odocoileus virginianus*) population located in northeastern Michigan serves as such a reservoir (Schmitt et al., 1997; Fitzgerald et al., 2000; Palmer et al., 2000; Waters et al., 2004).

In 1994, *M. bovis* was isolated from a hunter-killed white-tailed deer buck from northern Michigan. Subsequent surveillance of the free-ranging herd verified an epidemic presence of the disease. Several other wild mammalian species in the area have also been found to be infected or exposed to *M. bovis* including cervids and carnivores (Schmitt et al., 1997; Brunning-Fann et al., 2001). The self-sustaining presence of bovine tuberculosis in this wild deer population, and possibly other wild populations, poses a significant challenge to Michigan's efforts to reestablish its BTb-free status in domestic livestock. The implementation of an oral vaccination program, that would effectively reduce disease and shedding of *M bovis* by deer and other affected species, would significantly aid efforts in eradicating BTb from Michigan.

We assessed the efficacy of orally-administered *M. bovis* bacille Calmette-Guerin Danish strain 1331 (BCG) in white-tailed deer against infection with virulent *M. bovis*. *M. bovis* bacille Calmette-Guerin is currently the only vaccine licensed for use in humans

against tuberculosis (*M. tuberculosis*), and there are currently no accepted vaccination programs available to livestock. In fact, BCG remains the gold standard against which all experimental tuberculosis vaccines are compared, and despite intense efforts to develop a more effective vaccine, to date there are no new commercially available tuberculosis vaccines. Our study compared two types of oral BCG vaccination, as well as parenteral BCG vaccination, in their ability to protect white-tailed deer from disease caused by experimental infection of *M. bovis*.

MATERIALS AND METHODS

Animals

Thirty yearling female white-tailed deer were obtained from four BTb-free deer farms throughout the state of Iowa, USA and were shipped to USDA/ARS National Animal Disease Center (NADC) in Ames IA, USA in January, 2005 and housed in an outdoor pen facility. Prior to shipment, the animals were socialized as a group on one of the supplying farms for three weeks. After delivery to NADC, animals were acclimated for four weeks prior to start of the study. At time of challenge, deer were transferred to a biosafety level (BL)-3 animal building. All deer were housed and cared for according to the Association for Assessment and Accreditation for Laboratory Animal Care International and institutional guidelines. The available facility will not allow for more animals to be housed. Therefore this study was restricted to use a maximum of 30 animals.

Mycobacterium bovis BCG and *M. bovis* challenge strains

Mycobacterium bovis BCG Danish strain 1331 in culture and in lipid-formulated pellets were prepared at the University of Otago, Immune Solutions Ltd. as described in Aldwell et al., 2003 (Appendix A). Vaccine doses were determined using standard enumeration techniques by serial dilution plate counting on Middlebrook's 7H11 media (Becton-Dickinson, Cockysville, Maryland, USA). *Mycobacterium bovis* (strain 9839 Ames designation) was grown to mid-log phase on Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose complex (OADC) (Difco, Detroit, MI) plus 0.05% Tween 80. Bacilli were harvested from culture media by pelleting the cells by centrifugation at 2000 x g, washing twice with 1 ml of phosphate-buffered saline solution and diluting to the appropriate cell density in 2 ml of PBS. Challenge dose was determined as described above for vaccine doses.

Vaccination and challenge of animals

White-tailed deer were orally vaccinated (n=8) with approximately 1 x 10^9 colony forming units (cfu) BCG via lipid formulated bait (oral bait group). These deer were each offered a one gram, unflavored lipid pellet inside a piece of fresh apple and allowed to voluntarily eat the bait and apple so that they chewed the material before swallowing. Another group of 8 deer were orally vaccinated with BCG in liquid medium (oral liquid group). A 1 ml preparation of approximately 1.9 x 10^8 cfu BCG in 7H9 broth was administered via 3 ml syringe and a 10 French 25 cm sterile urinary catheter (*Self-Cath*®, Mentor, Minneapolis, MN, USA). Care was taken to contact the back of the mouth with the inoculum in order not to bypass the pharyngeal lymphoid tissue. Seven deer were vaccinated subcutaneously in the right caudal cervical area with a 1 ml preparation of

approximately 3.4 x 10^6 cfu BCG (parenteral group). Seven deer received 1 ml 7H9 broth orally directly into the back of the mouth via syringe and catheter and served as the control group. Control and vaccinated deer were housed together in an outdoor paddock until challenge in an indoor BL3 facility.

At 12 weeks post-vaccination, all animals were anesthetized with an intramuscular (IM) injection of ketamine hydrochloride (6 mg/kg) and xylazine (2 mg/kg), moved into a BL3 facility also at NADC, and immediately challenged with a total of 228 cfu *M. bovis* strain 9839 (a strain originating from an infected Michigan white-tailed deer) directly into the palatine tonsillar crypts (114 cfu/tonsil) via pipette, the dose being divided between the two tonsils. Oral liquid and parenteral doses of BCG and challenge dose of *M. bovis* were retrospectively determined via culture as described above. The oral bait dose-as estimated from culture of bacilli prior to lipid formulation was approximately 9.2 x10^8 cfu.

Mononuclear cell culture.

Whole blood was collected by jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were obtained from whole blood collected in 60 cc syringes containing 5ml 2X acid citrate dextrose. PBMC were isolated from buffy coat fractions using a Ficoll-Hypaque (Histopaque-1083, Sigma, St. Louis, MO, USA) technique followed by a lyse and restore step as described (Waters et al., 2004a). PBMC were resuspended in supplemented RPMI 1640 (Sigma) and counted. The RPMI 1640 was supplemented with 25mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 50 μM 2-

mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS; National Veterinary Services Laboratory [NVSL], Ames, Iowa, USA)

Lymphocyte blastogenesis

Lymphocyte blastogenesis assays were performed on days 0, 28, and 83 days post-vaccination. Mononuclear cells (5 x 10^5 cells) were added to wells of 96-well round bottom microtiter plates (Falcon, Becton-Dickinson, Lincoln Park, New Jersey, USA). Wells contained medium plus 10 μg/ml *M. bovis* purified protein derivative (PPDb, Prionics AG, Schlieren, Switzerland), 10 μg/ml *M. avium* PPD (PPDa, Prionics AG), 10 μg/ml rESAT6:CFP10 (E:C, Waters et al., 2004a), 1 μg/ml pokeweed mitogen (PWM, Sigma), or medium alone (no stimulation). Each antigen treatment was run in triplicate. Cells were incubated for 6 days at 37 C in 5% CO_2 in air at which time each well was pulsed with 0.5 μCi methyl-[^{3}H]thymidine (Amersham Life Science, Arlington Heights, Illinois, USA) in 50μl RPMI. Then PBMC were incubated for another 24 hr and harvested onto fiber filters with a 96-well plate harvester (EG&G Wallace, Gaithersburg, Maryland, USA), and the radioactivity levels were measured by liquid scintillation counting. Data are presented as stimulation indices (SI) where:

$$SI = \frac{\text{Mean counts/min (antigen or mitogen-stimulated cultures)}}{\text{Mean counts/min (non-stimulated cultures)}}$$

Interferon-γ enzyme-linked immunosorbent assay

Interferon-γ (IFN- γ) responses to mycobacterial antigens were measured at -77, 0, 28, 49, and 83 days post-vaccination, and at 13, 52, 88, and 119 days post challenge (98, 137, 173, and 204 days post-vaccination, respectively) using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cervigam®, Prionics) modified as described

by Palmer et al., 2004 to determine interferon production. Briefly, heparinized whole blood was collected by jugular venipuncture, of which 1.5 ml was added to 24-well tissue culture plates (Falcon, Becton-Dickinson; Lincoln Park, New Jersey). Treatments administered included PBS (no stimulation), 20 µg/ml PPDb, 20 µg/ml PPDa, 20 µg/ml PWM, or 10 µg/ml E:C. Plates were incubated for 48 hr at 37 C in a humidified chamber with 5% CO_2. Samples were centrifuged at 400 x g and the plasma supernatants harvested and stored at -80 C until analysis using the Cervigam® ELISA according to manufacturer's instructions. Optical density was measured at 450 nm using an automated ELISA microplate reader (Molecular Devices, Menlo Park, CA). Concentrations (ng/ml) of IFN-γ in test samples were determined by comparing absorbances of test samples with absorbances of standards within a linear curve fit (Instat 2.0, GraphPad Software, Inc, San Diego, CA, USA). IFN-γ data (ng/ml) used for statistical analysis were derived from calculating the differences between response to antigen and response to no stimulation (PBS) (antigen stimulation (ng/ml) – no stimulation (ng/ml).

Post mortem examination

Twenty weeks post-challenge, the deer were anesthetized with ketamine hydrochloride (6 mg/kg) and xylazine (2 mg/kg) and euthanized by intravenous administration of sodium pentobarbital (*Sleepaway*®, Fort Dodge, IA, USA). Tissues collected for gross pathology, histopathology, culture, and polymerase chain reaction (PCR) were: palatine tonsils, mandibular lymph nodes (LN), parotid LN, medial retropharyngeal LN, right prescapular LN, tracheobronchial LN, mediastinal LN, right cranial lung lobe, liver, hepatic LN, and mesenteric LN. In the case of bilateral lymph nodes, excepting the prescapular LN, portions of both right and left lymph nodes were

collected for both culture and histopathology. All lung lobes were carefully examined for lesions and each was weighed individually for comparison with those of the other deer in the study. A scoring system based on number and extent of gross lesions was applied to all lymph nodes collected and all lung lobes for statistical comparison (adapted from Vordermeier et al., 2002). For the lung lobes scoring was as follows: a score of "no lesions" meant there were no gross lesions detected. A score of "mild" represented tissues that contained any lesions less than or equal to 5 gross lesions of less than 10 mm in diameter. A score of "severe" was assigned to tissues with greater than or equal to 6 gross lesions of less than 10 mm in diameter or any lesions larger than 10 mm in diameter. For the lymph nodes scoring was as follows: a score of "no lesions" meant there were no gross lesions detected. A score of "mild" represented tissues that contained one small focus 1 to 2 mm in diameter. A score of "severe" was assigned to tissues with several small foci or extensive necrosis. All tissues for culture and PCR were stored in whirlpak bags at –80 C until testing. Tissues for histopathology were fixed in neutral-buffered 10% formalin and processed by routine paraffin embedment techniques. Sections were cut 5-µm thick, stained with hematoxylin and eosin, and examined under light microscopy. Sections containing microscopic lesions compatible with tuberculosis were subsequently stained with Ziehl-Neelsen for identification of acid-fast organisms. A scoring system was applied to all tissues collected for histopathology based on the number and extent of microscopic granulomatous lesions and was conducted as follows: a score of "no lesions" meant there were no granulomatous lesions detected; a score of "mild" represented tissues that had granulomas containing macrophages, giant-cells, lymphocytes, and neutrophils, but had incomplete encapsulation and minimal to no

necrosis present; a score of "severe" was given to any granulomas that displayed encapsulation, caseous necrosis, and mineralization.

Tissue Culture and Polymerase Chain Reaction

We selected right cranial lung lobe, mediastinal LN, and medial retropharyngeal LN for quantitative culture. Tonsil and mesenteric LN were selected for general culture. In a Class 3 biosafety cabinet, a small piece of each tissue was removed and stored at -80 C for fresh tissue PCR analysis. All tissues were then homogenized using a blender (Oster, Shelton, CT) with 50 ml (lymph nodes) or 100 ml (lung) phenol red broth (NVSL). A 7.5 ml aliquot was removed and the remaining sample stored at -80 C for PCR analysis. The aliquot was then subjected to NaOH decontamination as follows. Five ml 0.5M NaOH was added to the 7.5 ml aliquot and allowed to incubate for 10 min. Then 10 N HCl was added dropwise until a yellow color was obtained followed by a dropwise addition of 1.0 N NaOH until a dusty rose color was achieved. Samples for general culture were centrifuged at 3000 x g for 15 min. The supernatant was discarded and the remaining sediment was used to inoculate three tubes of 7H10 media and 3 tubes of 7H11 media (NVSL). Samples for quantitative culture were vortexed at high speed for 5 sec and serially diluted at 1:10 dilutions in PBS out to 10^{-6}, vortexing for 5 sec between each dilution. One hundred microliters of each dilution was used to inoculate 7H11 agar plates (NVSL). The undiluted sample was used to inoculate one tube of 7H10 media. All cultures were placed in an incubator at 37 C. Plates were kept in plastic bags to prevent the agar from drying out. Samples were checked every 30 days, and final counts/assessments were made at 60 days.

Samples that produced growth on 7H10 and 7H11 media and considered suspect for *Mycobacterium* spp. were picked and stored in 1 ml phosphate buffered saline at -80C for follow up PCR to confirm or rule out the presence of *M. bovis*. Extraction of DNA from cell culture samples was performed using the BioRad InstaGene^{TM} matrix (Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's instructions with the following modifications: briefly, each sample was vortexed after which 200ul of cell homogenized sample was transferred into a bead beater tube (Sarstedt, Inc., Newton, North Carolina) containing 0.5 g of 0.5 mm zirconium/silica beads (Biospec Products, Inc., Bartlesville, OK). Samples were bead beated using the MBB-8 Mini Bead Beater (Biospec Products, Inc., Bartlesville, OK) for 2 cycles (4,500 rpm) of 10 sec. After a quick spin, 50 ul of bead beaded cells were transferred to a new tube to which 200 ul of Instagene Chelex matrix was added. Next, 14.2 ul of proteinase K (Amresco, Inc., Solon, Ohio) (20mg/ml) was added to each sample which, after vortexing, was then incubated at 60 C for 30 min. The proteinase K was inactivated by heating the samples to 100 C for 10 min. Finally, the samples were centrifuged at 11,000 x g for 10 min and the resulting supernatants containing DNA were saved. For the purposes of PCR, dilutions were made of the DNA samples ranging from 1/10 to 1/10,000 depending on extent of turbidity. For every extraction, one tube containing 200ul of PBS was included half way between the total sample size and at the end of the extraction samples to serve as appropriate negative extraction controls.

The samples were then analyzed for *M. tuberculosis* complex DNA using a PCR targeting the IS6110 regions of *M. tuberculosis* complex (IS6110 PCR). The IS6110 PCR was performed using a PTC-100 MJ Thermalycler (MJ Research, Inc., Waltham,

Massachusetts, USA) for 50 cycles. The primers used in the IS6110 PCR were as follows: forward primer (5'CTCGTCCAGCGCCGCTTCGG3'), and reverse primer (3'CCTGCGAGCGTAGGCGTCGG5') (Operon Biotechnologies, Inc., Huntsville, Alabama) Each IS6110PCR reaction (25 µL) occurred in a 0.65 µL PCR tube (Sorenson BioScience, Inc., Salt Lake City, Utah) and consisted of 1X GeneAmp Buffer II, 1.5 mM MgCl (Applied Biosystems, Foster City, California, USA), 2.5 mM of each deoxynucleoside triphosphate (Roche Applied Science, Indianapolis, Indiana), 0.4 µM of each primer, 1.875 units of AmpliTaq Gold Polymerase (Applied Biosystems), 10 µL template, and 6.625 uL PCR-grade water (Hyclone, Logan, Utah) covered with a 30 uL layer of Chill-Out wax buffer (Bio-Rad Laboratories). An amplification profile of (1) 94 C for 10 min, (2) 94 C for 45 sec, (3) slope -22 degrees at 1 degree C/1second, (4) 72 C for 2.25 min, (5) slope +22 degrees at 1.5 degrees C/1 sec, (6) repeat 2-5 for 49 cycles, and (7) 72 C for 2.25 min was followed by a final extension of 72 C for 10 min.

A positive control (purified *Mycobacterium bovis* DNA at 0.5-5 fg/uL) and a negative control (water) were included in every experiment. Following the amplification protocol, ten µl of the amplification reactions were size fractionated through 2.5% agarose (Amresco, Inc., Solon, Ohio) gels in 1X TAE buffer (40 mM Tris acetate, 1mM EDTA) (Amresco, Inc., Solon, Ohio). Gels were stained in 0.001mg/ml ethidium bromide (Amresco, Inc., Solon, Ohio) for 15 min and products were then visualized using the BioRad Gel Doc EQ System (Bio-Rad Laboratories, Hercules, California, USA) UV transilluminator. Product size was 123 base pairs.

Medial retropharyngeal lymph node, mediastinal LN, and right cranial lung lobe-derived fresh and homogenized tissues were also directly tested for *M. tuberculosis*

complex by PCR. Frozen homogenates were placed at 4 C overnight to thaw. In a class 2 biosafety cabinet, approximately 1.3 ml homogenate was removed from the bottom of the 50 ml polypropylene centrifuge tube and transferred to a 2 ml polypropylene centrifuge tube. Samples were centrifuged for 10 min at 11,000 x g. Saving the resultant phenol broth supernatant in a new tube, 100 mg of the tissue was weighed out and placed into a 2 ml bead beater tube containing 2.5g of 2.5mm and 0.5g of 0.5mm zirconia/silica beads, to which 100 µl of the supernatant was returned. For every nine samples, a bead beater tube was filled with 200 µl PBS to serve as negative controls. All tubes were quick spun and placed in a heat block for 10 min at 100 C. The samples were allowed to cool for 10 min. Tubes were then bead beated for 3 cycles (4,500 rpm) of 30 seconds. Samples were cooled in an ice block in between cycles to avoid overheating. At this point, samples were considered safe to handle outside the biosafety cabinet. DNA extraction was then accomplished using the Fermentas Genomic DNA Purification Kit® according to manufacturer's instructions (Fermentas Life Sciences, Hanover, Maryland, USA). Following the manufactures' recommended DNA ethanol (ETOH)/salt precipitation, one Breath-Easier® tube membrane (ISC Bioexpress, Kaysville, UT) was placed over each open tube to avoid cross-contamination and allow for proper ETOH evaporation while incubating at 65C under a still-air hood. DNA samples of the homogenized tissue were rehydrated in 100ul of TE buffer (10 mM Tris, 1mM EDTA) (Amresco, Inc., Solon, Ohio) and diluted to 1/60 and 1/80 in PCR-grade water for PCR.

In a class 2 biosafety cabinet, 100 mg of fresh frozen tissue was weighed out from each sample for DNA extraction. Tissues were minced and placed in bead beater tubes, containing 2.5g of 2.5mm and 0.5g of 0.5mm zirconia/silica beads, to which 100 µl of TE

buffer was added. Tissues underwent bead beating homogenization with 2 pulses of 30 seconds at 4,500 rpm. Samples were cooled in an ice block in between cycles to avoid overheating. The Epicentre MasterPure^{TM} DNA Purification Kit was used according to manufacturer's instructions with some modifications (Epicentre Biotechnologies, Madison, Wisconsin, USA). Briefly, samples were quick spun and 500 µl of T and C Lysis Solution with 0.675mg/ml proteinase K was added to each sample. Samples were incubated for 60 min at 56 C and again for 10 min at 100 C to inactivate the proteinase K and render any remaining *M. bovis* non-viable. After the samples cooled, 250 µl of MPC Protein Precipitation Reagent was added and samples were centrifuged at 11,000 x g for 10 min at 4 C. The supernatant was then treated with 750ul of 100% isopropanol. and centrifuged for 15 min at 11,000 x g at 4 C. The isopropanol was discarded and the pellet was washed 2 times with 75% ETOH. Any remaining ETOH was allowed to evaporate by placing a Breath-Easier® tube membrane over each open tube as described above. DNA samples of the fresh tissue were rehydrated in 100ul of TE buffer and diluted to 1:60 and 1:80 for IS6110 PCR.

DNA from fresh and homogenized tissues underwent PCR analysis as described above. Any DNA samples deemed positive were subjected to 2 repeat PCR analyses and corresponding tissue samples were subjected to one additional extraction and PCR analysis to confirm presence of *M. bovis*. Unlike culture, these assays do not reflect the viability of the mycobacteria, but serve as a sensitive method to test for evidence of infection at some time during the experiment.

Statistical analysis

Fisher's exact test (4 x 3; one-sided) was used to compare all groups based on number of animals with gross lesions (none, mild, and severe) and microscopic lesions (none, mild, and severe) (Samuels and Witmer, 1999) (Proc FREQ; SAS 9.1, SAS Institute, Cary, North Carolina, USA). If an association existed, Fisher's exact test (2 x 2; one-sided) was again used to compare the number of animals with lesions within each vaccine group to the number of animals with lesions within the control group or to each other based on no lesions versus lesions (mild and severe combined). Culture and PCR results were compared in the same manner (Fisher's exact test; 2 x 2; one-sided) in order to determine differences in number of culture/PCR positive animals among groups. As this was a resource-intensive pilot study which was limited to a small number of animals, statistical priority was placed on minimizing the chance of type 2 error in order to detect differences if they were biologically valid. It was determined that using 8 animals per group, at an alpha level of 0.1, the test would have a power of 0.8 in detecting a difference when the proportion of animals without lesions in the vaccine groups exceeded the proportion of animals without lesions in the control group by 0.5 (SAS Institute). Therefore, differences determined by the one-sided Fisher's exact test to have p values of less than or equal to 0.1 were considered significant.

We modeled IFN-γ and lymphocyte blastogenesis response variables as functions of treatment group, time point, and group x time point interaction. We coupled this fixed-effects model structure with 3 alternative covariance structures to account for random effects of individual deer, effects of repeated measures within individuals, and unequal variances among treatment groups using the GLIMMIX procedure of SAS

(Littell et. al. 2006, SAS Institute 2006). These models are more robust to nonnormality than they are to nonhomogeneous variances; however, we include model structures that estimated separate variances by group to account for any nonhomogeneity. The most general covariance structure (model 2) estimated separate variance components and autoregressive correlation coefficients by treatment group. Models with reduced covariance structures included one model estimating a common variance component (random effect of individuals) and a common autoregressive correlation among treatment groups (model 1), and one model estimating separate variances by group (i.e., no repeated measures effect; model 3). We used an information theoretic approach to select the most parsimonious form of covariance structure for each response variable (Burnham and Anderson 2002, Littell et. al. 2006). For the selected models, we considered fixed effects as significant for p values of ≤0.1.

RESULTS

Animals

Due to illnesses and injuries, 8 animals were removed at various times throughout the study. Twenty-two animals remained in the study at the time of post-mortem examination. Of these 22 animals, 5 were in the oral bait group, 5 were in the oral liquid group, 6 were in the parenteral group and 6 were in the control group.

IFN-γ and lymphocyte proliferative responses to mycobacterial antigens

Overall there was no detectable interaction between group and sampling time regarding IFN-γ responses to PPDb or E:C (Model 2; p=0.25 and 0.39 respectively); however there was interaction present regarding IFN-γ responses to PPDa (Model 3;

p=0.0001), at three different time points (Appendix B). Least squares means estimates of vaccinates were compared to those of the control group as well (Appendix B). At 28 d post-vaccination and at 13 d post challenge (98 d post-vaccination) the parenteral group produced significantly greater levels of response to PPDb relative to the control group (p=0.09 and p=0.057 respectively). At 52 d post challenge (137 d post-vaccination) the parenteral group exhibited increased reactivity to PPDa in relation to the control group (p=0.060) whereas the oral group exhibited decreased reactivity to PPDa (p=0.002). The control group showed greater reactivity to PPDb and E:C than all three vaccine groups at 52 d post challenge ($p<0.096$) (Appendix B).

There were no detectable differences in lymphocyte blastogenic responses to any of the *M. bovis* antigens after vaccination between vaccine groups and the control group individually or over time ($p\geq0.57$) (Model 3; Appendix C).

Post mortem examination

When present, gross or microscopic lesions consistent with tuberculous lesions were found in the right cranial lung lobe and mediastinal LN. Lesions were not consistently observed in any other tissues, including medial retropharyngeal LN, although they were present. Therefore, only the right cranial lung lobe and mediastinal LN were suitable for statistical analysis.

The number of animals sustaining gross lesions in the right cranial lung lobe and mediastinal LN in the vaccine groups was significantly lower than in the control group (oral bait-0/5; oral liquid-1/5; parenteral-1/6; control-6/6 for cranial lung lobe) (oral bait-0/5; oral liquid-0/5; parenteral-1/6; control-6/6 for mediastinal LN) ($p<0.02$) (Table 3.1).

No differences could be detected among the vaccine groups regarding the number of lesions in either right cranial lung lobe or mediastinal LN (p=1.0).

The numbers of animals with microscopic lesions observed in the right cranial lung lobe and mediastinal LN of the oral liquid and oral bait groups were significantly lower than in the control group (oral bait-0/5; oral liquid-0/5; control-6/6 for cranial lung lobe) (oral bait-0/5; oral liquid-0/5; control-6/6 for mediastinal LN) (p=0.002). In the parenteral group, there were fewer animals having histologic lesions in the right cranial lung lobe compared to the control group (parenteral-1/6; control-6/6) (p=0.03). However, regarding the mediastinal LN, the parenteral group did not appear to differ from the control group (parenteral-3/5; control-6/6) (p=0.18), but did differ from both oral groups, having more animals with microscopic lesions in that tissue compared to the other vaccine groups (p=0.08) (Table 3.2 and 3.3). No differences could be detected among the vaccinee groups regarding the number of microscopic lesions in the right cranial lung lobe (p=0.13). Microscopic lesions in the medial retropharyngeal lymph nodes were found in 2 animals from the oral liquid group, 1 animal from the parenteral group, and 1 animal from the control group. Again, these data were not subjected to statistical analysis and are summarized in Table 3.3.

Presence of *M. tuberculosis* complex in tissues

Considering all five tissues cultured (right cranial lung, mediastinal LN, medial retropharyngeal LN, tonsil, mesenteric LN), the oral bait and parenteral groups, but not the oral liquid group, had significantly fewer culture positive animals than did the control group in which all six animals were positive (oral bait: 0/5 pos., p=0.002; parenteral: 2/6 pos., p=0.03; oral liquid: 3/5 pos., p=0.18). No significant differences were noted among

the vaccine groups (p=0.18). When looking at differences in individual tissues (Table 3.3), there were fewer animals in the oral liquid, oral bait, and parenteral groups that were culture positive for *M. tuberculosis* complex in the mediastinal LN than in the control group (oral liquid-0/5; oral bait-0/5; parenteral-0/6; control-4/6) ($p \leq 0.045$). Regarding the right cranial lung, there were differences, although not statistically significant, detected between the vaccine groups and the control group (oral liquid-0/5; oral bait-0/5; parenteral-0/6; control-3/6) (p=0.18). No differences were observed among the vaccine groups either. No differences were seen in the medial retropharyngeal LN cultures (Table 3.3). Quantitative culture results supported the above findings that *M. bovis* was more readily cultured out of mediastinal LN and right cranial lung lobe collected from unvaccinated animals than in tissues from vaccinated animals. These results are reported in Table 3.3; however, they were not subjected to statistical analysis due to small sample size.

When PCR was applied directly to tissues and tissue homogenates, we observed similar trends but greater differences among the groups as compared to culture regarding presence of *M. tuberculosis* complex in right cranial lung lobe and mediastinal LN (Table 3.3). The number of animals testing PCR positive for *M. tuberculosis* complex in mediastinal LN in the three vaccine groups was significantly fewer than in the control group (oral liquid-0/4; oral bait-0/5; parenteral-0/6; control-4/6) ($p \leq 0.008$). As well, there were significantly fewer animals testing PCR positive in the right cranial lung lobe in the three vaccine groups than in the control group (oral liquid-0/4; oral bait-0/5; parenteral-1/6; control-6/6) ($p \leq 0.072$). Again there were no differences noted among the vaccine groups (p=0.28). Culture, PCR, and histopathologic results in the three tissues are summarized in Table 3.3.

DISCUSSION

The present study indicates that oral BCG Danish 1331 is effective in protecting white-tailed deer against disease caused by experimental *M. bovis* infection. Orally vaccinated deer had fewer tuberculous lesions, both gross and microscopic, than did control deer five months after challenge. To the authors' knowledge, this is the first study reporting efficacy of oral BCG in this species in experimental settings. Waters et al. (2004b) evaluated cellular immune responses in white-tailed deer vaccinated subcutaneously with BCG Pasteur, and demonstrated strong IFN-γ and proliferative responses to PPDb, suggesting that BCG may be protective against *M. bovis* infection in this species. Challenge with *M. bovis* was not performed in this study. Miller and others (1999) orally administered a recombinant BCG expressing *Borrelia burgdorferi* OspA to white-tailed deer and detected both cellular and humoral immune response to *M. bovis* antigen, evidence that BCG was taken up via oral route. However, potential efficacy of the vaccine against *M. bovis* infection was not investigated, as the sole purpose of this study was to test the feasibility of BCG as an oral delivery system for non-mycobacterial antigens. In a New Zealand study investigating BCG (Pasteur 1173P2) in red deer (*Cervus elaphus*), researchers vaccinated animals directly in the tonsilar crypt (Griffin et al., 1999). Similar to oral BCG in white-tailed deer, the New Zealand scientists demonstrated that one dose of 2.5×10^6 cfu BCG into the tonsilar crypt of red deer protected 8 of 10 animals from disease 21 weeks after *M. bovis* challenge, and 5 of 10 animals were culture negative. Red deer that were vaccinated subcutaneously with the same single dose of BCG experienced comparable protection, also similar to the white-

tailed deer in this study. It should be noted that this and subsequent studies also demonstrated that red deer vaccinated with two subcutaneous doses of BCG 4-8 weeks apart fared better than either subcutaneous or intratonsilar single dose groups after challenge (Griffin et al., 1999; Griffin et al., 2006).

The results of the present experiment indicate that white-tailed deer can be vaccinated orally using BCG incorporated in a lipid-formulated bait. This oral bait has successfully induced protection against *M. bovis* and *M. tuberculosis* infection in a number of species, including laboratory mice (Aldwell et al., 2003), brushtail possum (*Trichosurus vulpecula*) (Aldwell et al., 2003), European badger (*Meles meles*) (S. Lesellier, pers. comm), and domestic cattle (Buddle et al., 2005). Buddle and others (2005) first demonstrated that ruminants can be vaccinated using this method. In their study, calves fed 10^9 cfu BCG, Pasteur 1173P2, via oral bait and challenged 21 weeks after vaccination had significantly lower lung and lymph node lesion scores than did non-vaccinated cattle. The current findings are consistent with this previous study, and suggest that white-tailed deer are a good candidate for oral vaccination programs to control bovine tuberculosis in the field.

This study examined the level of protection provided by oral BCG in white-tailed deer 5 months post-challenge. Although the level of protection was significant based on analyses of lung and mediastinal LN, these data do not suggest that a lack of lesions equates to sterile immunity. A few animals still had evidence of infection, based on histologic lesions or positive detection of *M. bovis* in medial retropharyngeal LN and occasionally in other tissues as well (data not shown). For this reason, it was important to test tissues in parallel via histopathology, PCR, and culture, as culture for Mycobacteria

is quite difficult and is therefore relatively insensitive as an independent test. It would be of great interest to evaluate disease progression, if any, in this species in subsequent vaccine trials through extending the time between challenge and post-mortem examination. Griffin and others (2006) addressed this question in red deer and found that animals vaccinated subcutaneously 8 weeks apart remained refractory to disease up to 52 weeks, despite the fact that they harbored low numbers of *M. bovis* in their tissues. Animals with stable, non-progressive lesions are less likely to play an active role in disease transmission (Griffin et al., 2006). Therefore, vaccines that do not produce universal sterile immunity but do significantly limit disease still have great potential to control endemic tuberculosis in wildlife.

The predominant sites of mucosal uptake of oral BCG in ruminants are currently unknown. It has been shown in rabbits that mycobacteria can cross the mucosal barrier through M cells present in gut-associated lymphoid tissues, namely, Peyer's patches (Fujimura, 1986; Tizzard, 2004). In Lagranderie et al. (2000), mice vaccinated orally with 2×10^9 cfu BCG, transiently harbored BCG in both the submaxillary glands and Peyer's patches immediately post-vaccination. However, only the peri-glandular lymph nodes in the submaxillary region maintained high numbers of bacilli 84 days after administration of BCG when compared to the mesenteric lymph nodes, suggesting that primary uptake occurs in the pharyngeal region, where nasopharyngeal lymphoid tissues are present. In contrast, mice which received the same dose intragastrically maintained higher numbers of bacilli in the mesenteric lymph nodes. Aldwell and others (2005) inoculated mice with 5.1×10^7 cfu lipid-microencapsulated BCG and found that the major site of bacterial replication was the mesenteric lymph nodes, indicating that the

lipid encapsulation reduced uptake in the pharyngeal region and protected the attenuated bacillus from destruction in the stomach. However, Dorer et al. (2007) showed BCG to be present in cervical and mesenteric lymph nodes and Peyer's patches of mice 8 weeks after receiving 1-2 x 10^7 cfu lipid-encapsulated BCG. Similar studies in other species corroborate these findings, although they all utilize a monogastric model. It is therefore of vital importance to investigate the potential impact of the complex ruminant gastrointestinal system upon both protected and non-protected BCG in future studies.

Increases in IFN-γ production and proliferation by lymphocytes in the presence of *M. bovis* antigens can be used as indicators that an appropriate cellular immune response to BCG vaccination has occurred. In infected deer, however, robust IFN-γ responses can imply the presence of more severe disease. Thacker et al. (2006) found that PBMC from white-tailed deer with severe pathology due to *M. bovis* infection had greater IFN-γ mRNA expression to PPDb and E:C early on in the infection than did animals with pathology limited to the head lymph nodes. As infection progressed the IFN-γ responses of the low pathology group increased to those of the high pathology group. Although the deer in this study responded well to vaccination, we could not have predicted this based on our IFN-γ or lymphoproliferative data prior to challenge. We also did not observe important differences in IFN-γ production between controls and vaccinates after infection. It is interesting that the group which received subcutaneous BCG vaccination displayed a degree of vacillation in IFN-γ responses not seen in any of the other groups over the course of the study. While this may be due to the difference in the character of the immune response to BCG and *M. bovis* by this group, there may be other factors involved, such as the influence of exposure to antigens of *M. avium*. An important aspect

to consider is that, at multiple time points, samples from up to 20% of the animals did not show an IFN-γ response to any antigen, based on the Cervigam ELISA, including the pokeweed mitogen control. Similar results were reported in a study by Waters et al. (2008) evaluating Cervigam as a diagnostic tool in various deer species, wherein only 44% of white-tailed deer had responses to PWM, calling into question the validity of this test for this species. In addition, the IFN-γ and lymphocyte responses observed in individual deer within groups were extremely variable. It is quite likely that, had our sample sizes been larger, we would have obtained data more consistent with those reported in other studies, or that alternative response markers need to be identified.

Experiments involving large, highly stressed species under BL-3 conditions are typically limited by space and cost. Power and significance levels need to be carefully considered prior to carrying out such a study and analyzing the results. Vaccine studies with low sample sizes are particularly susceptible to producing outcomes that apparently fail to detect differences between or among treatment groups when there actually is a treatment effect. It is therefore justified to increase the alpha level, and hence choose a higher p value in order to increase the power of the test and avoid type 2 error that renders otherwise valuable data meaningless in a practical sense. The purpose of this pilot study was to investigate the feasibility of pursuing further research with oral BCG in white-tailed deer. Despite the fact that our numbers were very small, we were still able to clearly show that BCG does appear to be effective in protecting deer from disease.

In conclusion, the use of BCG for oral vaccination of white-tailed deer shows great potential in controlling disease caused by *M. bovis* infection. Oral vaccination significantly slowed pathological progression of disease in our animals over a five-month

period and thus is a promising candidate for use in field. Oral vaccination of wildlife is a management tool that has been successfully implemented in Europe to control rabies in red fox (*Vulpes vulpes*) (Brochier et al., 1996) and is currently being used in the United States to manage the disease in raccoons (*Procyon lotor*). Oral BCG vaccination of wildlife reservoirs of bovine tuberculosis is being extensively researched for application in many countries, including New Zealand, UK, Ireland, United States, and South Africa. Successful mucosal delivery (intranasal or intraconjunctival) of BCG to a wild population of brushtail possums in New Zealand has already been demonstrated by Corner et al. (2002). The results of this study provide strong support for further research that could ultimately lead to safe and successful field vaccination of white-tailed deer and eventual eradication of bovine tuberculosis in the United States.

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Table 3.1. Gross lesions in the right cranial lung lobe and mediastinal lymph nodes of deer vaccinated with *M. bovis* BCG Danish strain 1331 via oral and parenteral routes versus deer vaccinated with medium alone. All deer were challenged with *M. bovis* strain 9839 three months after vaccination. Tissues were collected 5 months after challenge. For the right cranial lung lobe, scoring was as follows: None=no gross lesions detected; Mild=Five or fewer gross lesions of less than 10 mm in diameter; Severe=Six or more gross lesions of less than 10 mm in diameter or any lesions larger than 10 mm in diameter. For the mediastinal lymph nodes scoring was as follows: None= no gross lesions detected; Mild=Small focus 1 to 2 mm in diameter; Severe=several small foci greater or equal to 5 mm or extensive necrosis.

Vaccine Group	Rt. Cranial Lung Lobe			Mediastinal Lymph Nodes		
	None	Mild	Severe	None	Mild	Severe
Oral Liquid (n=5)	5	0	0	5	0	0
Oral Bait (n=5)	4	1	0	5	0	0
Parenteral (n=6)	5	0	1	5	1	0
Control (n=6)	0	0	6*	0	0	6*

*Significantly different from all three vaccine groups (Fisher's Exact Test; $p<0.1$)

Table 3.2. Microscopic Lesions in the right cranial lung lobe and mediastinal lymph nodes of deer vaccinated with *M. bovis* BCG Danish strain 1331 via oral and parenteral routes versus deer vaccinated with medium alone. All deer were challenged with *M. bovis* strain 9839 three months after vaccination. Tissues were collected 5 months after challenge. None=no granulomatous lesions detected; Mild= presence of granulomas containing macrophages, giant-cells, lymphocytes, and neutrophils, but having incomplete encapsulation and minimal to no necrosis present; Severe=presence of granulomas displaying encapsulation, caseous necrosis, and mineralization.

Vaccine Group	Rt. Cranial Lung Lobe			Mediastinal Lymph Nodes		
	None	Mild	Severe	None	Mild	Severe
Oral Liquid (n=5)	5	0	0	5	0	0
Oral Bait (n=5)	5	0	0	5	0	0
Parenteral (n=6)	5	0	1	2	1	2†
Control n(=6)	0	0	6*	0	0	6†

*Significantly different from all three vaccine groups (Fisher's exact test; $p \leq 0.1$)
†Significantly different from Oral Liquid and Oral Bait groups (Fisher's Exact Test; $p < 0.1$)

Table 3.3. Number of deer having positive culture or positive PCR in selected tissues over total number of animals tested in the treatment group. Deer were vaccinated with *M. bovis* BCG Danish strain 1331 via oral and parenteral routes or received medium alone. All deer were challenged with *M. bovis* strain 9839 three months after vaccination. Tissues were collected 5 months after challenge.

Tissue		Vaccine Group			
		Oral Liquid	Oral Bait	Parenteral	Control
MLN	Culture Pos/Total	0/5	0/5	0/6	4/6*
	(Mean cfu/g[a])	(na)	(na)	(na)	(5.08 ± 1.6) (n=3)
	PCR Pos/Total	0/4	0/5	0/6	4/6*
	Lesions[b] Pos/Total	0/5	0/5	3/5†	6/6†
RCL	Culture Pos/Total	0/5	0/5	0/6	3/6
	(Mean cfu/g)	(na)	(na)	(na)	(3.49 ± 0.16) (n=3)
	PCR Pos/Total	0/4	0/5	1/6	6/6*
	Lesions Pos/Total	0/5	0/5	1/6	6/6*
MRLN	Culture Pos/Total	2/5	0/5	1/6	1/6
	(Mean cfu/g)	4.2 ± 0.5 (n=2)	(na)	(3.4)	(4.61)
	PCR Pos/Total	2/4	0/5	1/6	2/6
	Lesions Pos/Total	2/5	0/5	1/6	1/6

MLN: Mediastinal lymph nodes; RCL: Right cranial lung lobe; MRLN: Medial retropharyngeal lymph node;
[a]Mean bacterial count reported as $\log_{10}$ cfu/g ± Std Dev
[b]Microscopic lesions
*Significantly different from all three vaccine groups (Fisher's Exact Test; $p \leq 0.1$)
†Significantly different from Oral Liquid and Oral Bait groups (Fisher's Exact Test; $p < 0.1$)

CHAPTER 4: HUMORAL IMMUNE RESPONSES OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) TO *MYCOBACTERIUM BOVIS* BCG VACCINATION AND EXPERIMENTAL CHALLENGE WITH *M. BOVIS*[1]

ABSTRACT

Monitoring serum antibody production kinetics to multiple mycobacterial antigens can be useful as a diagnostic tool for the detection of *Mycobacterium bovis* infection as well as for the characterization of disease progression and efficacy of intervention strategies in several species. Humoral immune responses to multiple *M. bovis* antigens by white-tailed deer vaccinated with BCG orally via a lipid-formulated bait (n=5), orally in liquid form (n=5), and subcutaneously (n=6) were evaluated over time after vaccination and after experimental challenge with virulent *M. bovis* and compared to those of unvaccinated deer (n=6). Antibody responses were evaluated using rapid test (RT), multiantigen print immunoassay (MAPIA), lipoarabinomannan ELISA (LAM-ELISA), and immunoblot to whole-cell sonicate and MPB83. The MAPIA and RT detected minimal to no antibody responses over baseline to multiple *M. bovis* antigens in vaccinated white-tailed deer after challenge. This was in contrast to the presence of more readily detectable antibody responses in non-vaccinated deer with more advanced disease. The LAM-ELISA results indicated an overall decrease in detectable antibody produced against lipoarabinomannan-enriched mycobacterial antigen in vaccinated animals as compared to non-vaccinated animals after challenge. Immunoblot data were

inconsistent, but did suggest the occurrence of unique antibody responses by certain vaccine groups to Ag85 and HSP70. These findings support further research toward the improvement and potential use of antibody-based assays, such as MAPIA, RT, and LAM-ELISA, as ante-mortem tools to assess disease progression in white-tailed deer in both experimental and field vaccine trials.

INTRODUCTION

Free-ranging white-tailed deer (*Odocoileus virginianus*) are wildlife reservoirs for bovine tuberculosis (BTb), caused by *Mycobacterium bovis*, in the state of Michigan, USA (15, 18). Current management strategies, such as population reduction and decreased supplemental feeding, have effectively reduced disease prevalence (15). However, BTb continues to maintain a low level presence in the wild deer population. Including effective field vaccination as part of disease management in deer herds with endemic BTb would significantly aid in efforts to eradicate BTb from this potential wildlife reservoir (14, 16).

Vaccination with *M. bovis* bacille Calmette-Guerin (BCG) via oral or parenteral routes is effective in protecting white-tailed deer from disease caused by experimental *M. bovis* infection (14, 16). An important component of evaluating any vaccine candidate is gaining an understanding of the dynamics of a recipient's immunologic response to vaccination and infection over time in comparison with unvaccinated subjects (3, 19, 22). Previous research has shown that monitoring serum antibody production kinetics to multiple mycobacterial antigens is useful in the characterization of disease progression,

efficacy of disease treatment, and as a tool for the diagnosis of *M. bovis* or *M. tuberculosis* infection in several species (11, 12, 21, 23, 24, 25).

In the present study, humoral immune responses to multiple *M. bovis* antigens by white-tailed deer vaccinated with BCG via oral and parenteral routes were evaluated by four different assays over time after vaccination and after experimental challenge with virulent *M. bovis.* This information provides an understanding of the differences in immunologic responses and disease progression in vaccinated and unvaccinated white-tailed deer infected with *M. bovis* and insight regarding use of appropriate diagnostic tests for BTb in a vaccinated population.

MATERIALS AND METHODS

Deer, vaccination, challenge, and necropsy. Serum samples from twenty-two yearling white-tailed deer does were utilized for this study. These animals were part of a larger herd obtained for a vaccine efficacy trial (14). The deer originated from four BTb-free deer farms throughout the state of Iowa, USA and were housed for the vaccination/infection studies at USDA/ARS National Animal Disease Center (NADC) in Ames IA, USA. All deer were housed and cared for according to the Association for Assessment and Accreditation for Laboratory Animal Care International. The Institutional Animal Care and Use Committee approved protocols detailing procedures and animal care prior to initiation of the experiments.

At the beginning of the study, 5 deer voluntarily consumed 1×10^9 colony forming units (cfu) BCG Danish strain 1331 orally via a lipid-formulated bait (Oral Bait) (1, 14), 5 deer received 1.9×10^8 cfu BCG in culture media orally via catheter (Oral

Liquid) as described by Nol and others (14), 6 deer received 3.4 x 10^6 cfu BCG subcutaneously in the right shoulder (Parenteral), and 6 deer received culture media orally via catheter and served as unvaccinated controls (nonvaccinates). *Mycobacterium bovis* BCG Danish strain 1331 in culture and in lipid-formulated pellets was prepared by Immune Solutions Ltd, at the University of Otago, New Zealand, as described by Aldwell and others (1). Vaccine doses were determined using standard enumeration techniques by serial dilution plate counting on Middlebrook 7H11 media (Becton-Dickinson, Cockysville, Maryland, USA). Before vaccination and on a monthly basis throughout the study, blood was collected via jugular vein for serologic analysis of antibody responses. Three months after vaccination, deer were moved from an outdoor facility to a biosafety level 3 animal building. Animals were separated into rooms with 3-4 animals per room, and vaccinated deer were comingled with unvaccinated deer. All deer were then challenged with 228 cfu *M. bovis* strain 9839 (NADC designation) intratonsillarly (114 cfu/tonsil). This strain was originally isolated from a *M. bovis*-infected deer in the state of Michigan, USA. *Mycobacterium bovis* strain 9839 was grown to mid-log phase on Middlebrook 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (OADC) (Difco, Detroit, MI) plus 0.05% Tween 80. Bacilli were harvested from culture media by pelleting the cells by centrifugation at 2000 x g, washing twice with 1 ml of phosphate-buffered saline solution and diluting to the appropriate cell density in 2 ml of PBS. Challenge dose was determined as described above for vaccine doses.

Pathology. Five months after challenge, the deer were euthanized and examined for gross and microscopic lesions, as well as for mycobacterial colonization, via culture and polymerase chain reaction analysis (14). For the present study, the following tissues

were examined for presence of gross lesions consistent with *M. bovis* infection under a scoring system described in detail by Palmer and others (16): palatine tonsil, mandibular lymph node (ln), parotid ln, medial retropharyngeal ln, tracheobronchial ln, mediastinal ln, right cranial lung lobe, right middle and caudal lung lobes, left cranial lung lobe, left caudal lung lobe, accessory lung lobe, liver, hepatic ln, mesenteric ln, and superficial cervical ln. Lymph node lesion scoring ranged from 0 (no lesions) to 3 (most severe). Lung lesion scoring ranged from 0 (no lesions) to 5 (most severe). Each animal received a total pathology score based on the sums of the lesion scores assigned to each tissue evaluated. Additional information on gross and microscopic findings, as well as culture and PCR results, can be found in the paper by Nol et al. (14).

Rapid Test (RT). A rapid immunochromatographic assay (Cervid TB STAT-PAK, Chembio Diagnostic Systems, Inc., Medford, N.Y., USA) was used to detect antibody responses in deer (Appendix D). The test employs a unique cocktailof selected *M. bovis* antigens (proprietary information) and a blue latex bead-based signal detection system. Serum samples were tested as described previously (12). In addition to visual reading, semiquantitative evaluation of test band intensity was performed by an in-house-developed computer-assisted optical reader designed to measure reflectance at 624 nm. The RT densitometry results obtained at 4 months postchallenge were utilized for the purposes of comparison among groups.

Multiantigen print immunoassay. The multiantigen print immunoassay (MAPIA; Appendix E) was performed as described by Lyashchenko et al. (11). Briefly, antigens were immobilized on nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) at a protein concentration of 0.05 mg/ml with a semiautomated airbrush-printing

device (Linomat IV; Camag Scientific, Inc., Wilmington DE, USA). The membrane was cut perpendicular to the antigen bands into 4 mm-wide strips. Strips were blocked for 1 h with 1% nonfat skimmed milk in PBS with 0.05% Tween 20 and then incubated for 1 h with serum samples diluted 1:50 in blocking solution. After washing, strips were incubated overnight with alkaline phosphatase-conjugated antideer IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) diluted 1:500, followed by another washing step. Deer antibodies bound to printed antigens were visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NTB) (Kirkegaard and Perry Laboratories). The MAPIA antigen panel was made up of twelve defined proteins. The following recombinant antigens of *M. bovis* were purified to near-homogeneity as polyhistidine-tagged proteins: ESAT-6 (Rv3875) and CFP10 (Rv3874) produced at the Statens Serum Institut, Copenhagen, Denmark; MPB59 (Rv1886c), MPB64 (Rv1980c), MPB70 (Rv2875), MPB83 (Rv2873) produced at the Veterinary Sciences Division, Stormont, Belfast (7); 16 kDa (Acr1, Rv3391) and 38-kDa (PstS1, Rv0934) were purchased from Standard Diagnostics, Seoul, South Korea. Polyprotein fusions CFP10/ESAT-6 (E6/P10) and Acr1/MPB83 (16/83) were constructed as described in Lyashchenko et al. (12). *M. bovis* culture filtrate (MBCF) was obtained from a field strain of *M. bovis* (T/91/1378; Veterinary Sciences Division, Belfast, UK) cultured in synthetic Sauton's medium for 21 days. Bovine protein purified derivative (PPD-B) was produced by the Veterinary Laboratories Agency (Weybridge, Addlestone, UK) (Appendix E).

Results of the MAPIA were evaluated visually to determine presence or absence of bands corresponding to antigen on the strip. With scanned strips, selected MAPIA

results also underwent semiquantitative densitometry analysis using Scion Image, a public domain NIH Image program (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/). The densitometry software produced arbitrary values of relative absorbance for the purposes of identifying trends among treatment groups. All densitometry values were recorded after subtracting densitometry readings taken from the pre-vaccination strips. Densitometry values obtained at 2 months for MBCF and 4 months for E6P10 and 16/83 were used for comparisons among treatment groups,

Immunoblot assay. Antibody responses of deer were evaluated over time by immunoblot analysis utilizing two sets of antigens: whole-cell sonicate (WCS) of *M. bovis* strain 95-1315, and MPB83 (a kind gift from Jim McNair, AFBI, Belfast, Ireland). *Mycobacterium bovis* whole-cell sonicate was prepared as described in Waters et al. (23). Electrophoresis and immunoblot assays were performed by procedures described by Waters and others (23, 25). Antigen was electrophoresed through preparative 12% (wt/vol) polyacrylamide gels. Electrophoretic transfer of proteins onto pure nitrocellulose was accomplished with the Bio-Rad Trans Blot Cell (Bio-Rad) using sodium phosphate buffer (25 mM, pH 7.8) at 0.8 A for 90 min. After transfer, the filters were blocked with PBST and 2% (wt/vol) bovine serum albumin (PBST-BSA). After blocking, the filters were placed into the slot blot device and individual sera diluted 1:200 in PBST-BSA was added to independent slots. After 2 hr of incubation at room temperature with gentle rocking, blots were washed three times with PBST and incubated with horseradish peroxidase-conjugated anti-goat IgG heavy and light chains (Kirkegaard Perry Laboratories, Gaithersburg, MD, USA) diluted 1:20,000 in PBST-BSA for 1.5 hr. Blots were again washed three times with PBST and developed for chemiluminescence in

SuperSignal detection reagent (Pierce Chemical Co.). Comparisons of the reactivity of serial serum samples against WCS and MPB83 antigens were conducted as described by Waters et al. (23). In addition, Scion Image was used to obtain densitometry readings of individual lanes on each gel's radiographic image. Lane profile plots were generated for each lane. Response to MPB83 antigen was considered positive when a band of the correct size was visually detected.

LAM-ELISA. Lipoarabinomannan-enriched mycobacterial antigen (PK1315) was prepared from *M. bovis* strain 95-1315 (PK1315) as described by Waters et al. (20, 21). Briefly, bacilli harvested from 4-week cultures were sonicated in PBS, further disrupted with 0.1- to 0.15-mm-diameter glass beads (Biospec Products, Bartlesville, OK, USA.) in a bead beater (Biospec Products), centrifuged, filtered (0.22-mpore size), and digested in a 1-mg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, IN, USA) solution (50 mM Tris, 1 mMCaCl2 buffer, pH 8.0) for 1 h at 50°C. Protein concentrations were determined (Bio-Rad, Hercules, CA, USA), and antigens were stored at 20°C. Immulon II 96-well microtiter plates (Dynatech, Chantilly, VA, USA) were used. Each sample was tested in 4 wells, 2 wells of no antigen and 2 wells with antigen. For the no antigen wells, wells were coated with 100 μl/well of .05 M carbonate-bicarbonate coating buffer pH 9.6 (Sigma C3041, Sigma-Aldrich Corp., Mo, USA). Antigen-coated wells received 100 μl/well of PK1315 diluted to 20 μg/ml in coating buffer. Coated plates were incubated overnight at 4°C. Plates were washed three times with 200 μl/well phosphate buffered saline (PBS) containing 0.05% Tween 20 (i.e., PBST; Sigma), and blocked with 200 μl/well commercial milk diluent/blocking solution (diluted 1:20 with distilled H_20, (Kirkegaard and Perry Laboratories). After incubation for 1 h at 37°C in the blocking

solution, wells were washed nine times with 200 µl/well PBST. Test sera were added to wells (100 µl/well). Test and control sera were diluted 1:100 in PBS containing 0.1% gelatin. After incubation overnight at 4°C with diluted test sera, wells were washed nine times with 200 µl/well PBST and incubated for 1 h at 37°C with 100 µl/well horseradish peroxidase (HRP)-conjugated protein G from *Streptococcus* sp. (Sigma P8170) diluted 1:2000 in PBS plus 0.1% fish gelatin. Wells were washed nine times with 200 µl/well PBST and incubated at room temperature with 100 µl/well SureBlue (Kirkegaard and Perry Laboratories). Plates were read kinetically every minute for 15 minutes at 650 nM using an automated ELISA plate reader (FlexStation 3, Molecular Devices, Sunnyvale, CA). For each well, the maximum rate of change for the enzyme (slope of the bell curve) was determined (Vmax). For each sample, the raw Vmax value was calculated by subtracting the Vmax value without antigen from the Vmax value with antigen. The raw Vmax values were also calculated for both the positive control (pc) and negative control (nc). Finally, the Vmax sample/positive (s/p) was calculated by the following formula:

$$\text{Vmax s/p} = \frac{\text{Vmax sample} - \text{Vmax nc}}{\text{Vmax pc} - \text{Vmax nc}}$$

The Vmax s/p was the normalized number for the maximum rate of change for the enzyme and the value used for data analysis. Mean Vmax s/p values for pooled vaccinates was used to compare to mean Vmax s/p for nonvaccinated animals.

Data Analysis. Numbers of vaccinates (pooled) with lesions were compared to number of nonvaccinates with lesions using Fisher's exact test (2 x 2; one-sided) (Proc FREQ; SAS 9.1, SAS Institute, Cary, North Carolina, USA). Differences determined by the one-sided Fisher's exact test to have p-values less than or equal to 0.1 were considered significant. Vaccinated and nonvaccinated animals were also compared based on total

pathology scores derived from lymph node lesions, lung lesions, and lymph node and lung lesions combined using the Kruskal-Wallis non-parametric one-way analysis of variance followed by the Wilcoxon rank sum test (SAS 9.1, SAS Institute). Differences were determined to be significant when p-values were less than or equal to 0.1. Correlations between relative densities obtained from RT bands and pathology scores were analyzed using Spearman's rank test (Proc FREQ, SCORR; SAS 9.1, SAS Institute). Log_{10}-transformed lipoarrabinomannan-ELISA data were analyzed using repeated measures analysis of variance (ANOVA) (Statview software, version 5.0, SAS Institute, Cary, NC). Zero values were replaced by 0.0001 to allow log conversion. A p-value of ≤ 0.1 was considered significant. When significant effects were detected, Fisher's protected-least significant difference (PLSD) method was used to compare treatment groups at specific sampling dates.

RESULTS

Pathology. Five of the 16 vaccinates developed lesions consistent with tuberculosis but none of these animals received a total pathology score greater than 3. All six nonvaccinated animals had lesions in one or more tissues and total pathology scores ranged from 5 to 30 (Table 4.1). The numbers of vaccinated deer with lesions were significantly lower than the number of nonvaccinated deer with lesions (Fisher's exact test; $p<0.0062$). Total lung pathology score, total lymph node pathology score, and lung and lymph node pathology scores combined were all significantly higher in the nonvaccinated group than for vaccinates ($p=<0.001$, $p<0.001$, $p=0.002$, respectively) based on the Wilcoxon rank sum test (Figure 4.1).

Rapid Test. Of the sixteen vaccinated deer, only one animal developed detectable antibodies on RT at any time during the study. One of the six deer in the Parenteral group developed a response on RT starting at 3 months post-vaccination and remained reactive 4 months after challenge. In contrast, five of the six deer in the nonvaccinated group developed detectible antibodies on RT by 4 months post *M. bovis* challenge. Two of those animals developed a response by 2 months post-challenge, and four deer did so by 3 months post-challenge. None of the nonvaccinated deer developed antibodies detectible on RT before *M. bovis* challenge. Relative densities obtained from 4 month postchallenge RT responses from all deer are summarized in Table 4.1. Relative densities from 4-month post-challenge RT responses in nonvaccinated deer were positively correlated (Spearman's $r = 0.60$; $p = 0.003$) with total combined pathology scores from the same group (Figure 4.2). Interestingly, deer 19 and 24, the two animals in the nonvaccinate group with the lowest pathology scores, had the lowest densitometry readings in that group as well. However, these two deer still achieved higher pathology scores than did any of the vaccinated deer (Table 4.1).

MAPIA. The following proteins were recognized by the animals in this study challenge on MAPIA and are reported herein in terms of numbers of deer producing detectable antibodies to these antigens after *M. bovis* challenge: 16/83 (n=19), MBCF (n=17), E6P10 (n=5), CFP10 (n=4), PPDb (n=3), 16kDa (n=2), MPB83 (n=1), and ESAT6 (n=1). Although these data varied on both individual and treatment group bases, the top three antigens (16/83, MBCF, and E6P10) were deemed most useful in evaluating responses by vaccinated and nonvaccinated deer after *M. bovis* challenge. Relative densities measured from responses by deer in the individual treatment groups to 16/83 and E6P10 at 4

months postinfection and for MBCF at 2 months postinfection are summarized in Table 4.1.

The overall numbers of vaccinates and nonvaccinates producing detectable antibodies on MAPIA to the three antigens over the 4-month postinfection period are reported in Table 4.2. Relative to baseline levels (i.e., pre-existing responses prior to vaccination) four of the six deer in the nonvaccinated group had produced detectable antibodies to at least two of the top three antigens at 2 and at 4 months post-infection (Table 4.2). None of the 16 vaccinated deer produced detectable antibodies to more than one of the antigens throughout the postinfection period (Table 4.2). Six of the 8 vaccinates that did produce detectable antibodies to one of the three antigens had also done so before challenge (data not shown).

In the Oral Bait group, 0/5, 1/5, and 3/5 deer had detectable antibodies to E6P10, 16/83 (4 months post-challenge), and MBCF (2 months post-challenge), respectively (Table 4.1). In the Oral Liquid group, 1/5 deer had detectable antibodies to E6P10 post-challenge. However, none of the deer in the Oral Liquid group had detectable antibodies to 16/83 or MBCF over baseline levels at any time postinfection (Table 4.1). In the Parenteral group, 0/6, 1/6, and 2/6 deer responded to E6P10, 16/83, and MBCF, respectively (Table 4.1). In contrast, 3/6, 4/6, and 5/6 animals in the nonvaccinated group developed antibodies to E6P10, 16/83, and MBCF respectively (Table 4.1, Table 4.2) (Refer to Appendix E for visual example).

Immunoblots. Specific bands of reactivity at ~10 kDa, ~15 kDa, ~20-25 kDa, ~32 kDa, ~34-35 kDa, ~42 kDa, ~60 kDa, ~68-70 kDa, ~74-76 kDa, ~90 kDa, and ~125 kDa to *M. bovis* whole cell sonicate (WCS) were detected by immunoblot throughout the study.

The most reactive protein(s) were at the ~20-25 kDa level, as all the deer produced antibodies to these antigens at some time during the study, including before vaccination.

Differences in antibody responses to proteins within specific size ranges were noted among vaccine groups. During the first three months after *M. bovis* challenge, 4/5 animals in the Oral Bait group, 2/5 in the Oral Liquid group, 5/6 in the Parenteral group, and 5/6 in the nonvaccinated group responded above baseline to antigen at approximately the 32kDa level. However, by 4 months postchallenge, 5/6 nonvaccinates continued to produce antibody responses to these proteins, whereas 2/5, 2/5, and 3/6 deer maintained an antibody response in the Oral Bait, Oral Liquid, and Parenteral groups respectively (data not shown). Of the 2 deer in the Oral Bait group that maintained antibody to 32kDa proteins, one deer produced a very weak response. One of the 2 deer in the Oral Liquid group had a weak response whereas the other deer (#84) had a very strong response and was the only deer in that group to have lesions. Unlike in the other vaccine groups, the three deer in the Parenteral group all produced strong antibody responses to 32kDa proteins. Two of the three did not sustain lesions by the end of the study, and the third had a very low pathology score. The deer in the nonvaccinated group that did not respond to 32kDa proteins (#24) was one of two deer that had the lowest pathology score of 5. The other nonvaccinated deer with a low pathology score (#19) had only a weak response to the 32kDa protein relative to the other 4 animals who responded.

All of the deer in the Oral Bait and the Parenteral groups showed antibody responses to proteins at the 68-70 kDa level at some time in the four months post-challenge. At 4 months post-challenge, 4/5 of the deer in the Oral Bait group and 4/6 deer in the Parenteral group still showed antibody production to these proteins. Only 1

animal in each of the other 2 groups developed a response to these antigens. All of the Parenteral group deer and 2 of the Oral Bait group deer had already developed antibody to these proteins after vaccination.

Regarding the MPB83-specific immunoblot assay, there were no patterns in antibody response to this antigen among the vaccine groups. Three of five deer in the Oral Bait group became positive for this antigen at different time points after vaccination, of which only one developed an increased response at 1 month post-challenge which persisted to 4 months post-challenge. None of the deer in the Oral Liquid group developed detectible antibodies to MPB83 on immunoblot after vaccination or challenge; however, one of the animals did have a response on pre-vaccination serum but was negative on every test thereafter. One of 5 deer in the Parenteral group became weakly positive to MPB83 at 1 month post-vaccination, and this response disappeared by 2 weeks after challenge. One of 6 deer in the nonvaccinated group developed detectible antibodies to MPB83 on immunoblot 2 weeks after challenge and maintained this response until 4 months post challenge.

LAM-ELISA. Mean $\log_{10}$ Vmax s/p values obtained from pooled vaccinates and nonvaccinates indicated a post-vaccination trend of increasing detectible antibodies to LAM-enriched mycobacterial antigens in the vaccinated group compared to the nonvaccinated group, ultimately becoming significantly different at 2 weeks post-challenge ($p=0.019$). This is immediately followed by an increase in antibody production in the nonvaccinates and a simultaneous decrease in the vaccinates; the vaccinates produced significantly fewer antibodies than the nonvaccinates at 2 and 4 months after *M. bovis* challenge ($p=0.015$ and 0.003 respectively; Figure 4.3).

DISCUSSION

Our MAPIA, RT, and LAM-ELISA results indicate that vaccinated white-tailed deer which developed fewer and less severe lesions than nonvaccinated animals after *M. bovis* infection, as described here and by Nol et al. (14), generally harbor less and/or diminishing detectable circulating antibody to *M. bovis* antigens compared to nonvaccinated deer with more advanced disease. This finding is consistent with previous studies in which associations were noted between severity of disease caused by *M. bovis* infection and levels of antibody production (5, 10, 12, 23, 26).

Using RT and/or MAPIA, we were generally able to distinguish vaccinated/*M. bovis*-challenged deer with minimal pathology from nonvaccinated/challenged deer with more advanced lesion development. In the present study, with one exception, none of the vaccinated deer responded to RT or produced detectible antibodies to more than one of the three top antigens in MAPIA. One of the two nonvaccinated animals that did not develop a detectible response after infection to at least two of the three antigens analyzed on MAPIA did react on RT postinfection. The single nonvaccinated animal that tested negative in both assays was one of the two animals with the lowest lesion scores. It is possible that given time, this animal would have reacted to these tests as disease increased in severity; it is also possible that this animal had some resistance to *M. bovis* infection and may not have experienced a rapid development of pathology. These tests could serve as ante-mortem tools to aid researchers in evaluating vaccine efficacy in deer against *M. bovis* infection. With this scenario, detection of high-level antibody production to *M. bovis* antigens would be indicative of vaccine failure.

Antibody-based tests could be used to evaluate the status of bovine tuberculosis in a free-ranging, vaccinated herd through live-animal testing. The tests could identify severely affected animals to be removed from the population to prevent shedding of the organism and further spread of infection. Vaccine-protected animals with no or minimal development of lesions would remain in the herd, as it has been suggested that such vaccinated deer experience minimal pathologic changes postinfection and, although infected, are not considered an important source of *M. bovis* to the environment for transmission to other animals (4).

The present study was limited to humoral immune responses of small numbers of vaccinated and unvaccinated white-tailed deer in the first 4-5 months after challenge with *M. bovis*. High biosafety level studies involving large wildlife species often suffer from low power and short duration due to great expense, logistical issues, and animal welfare considerations. Although obstacles exist, it would be extremely useful to conduct follow-up experiments with greater sample sizes and longer duration that monitor postchallenge antibody responses in both vaccinated and unvaccinated deer. BCG-vaccinated white-tailed deer may acquire long-term protection (>1 year) from the development of severe disease, as has been shown in red deer (4). Whether white-tailed deer continue producing similar results on MAPIA, RT, and LAM-ELISA after a year or longer following vaccination and *M. bovis* challenge would be of great interest and utility to a field vaccination program.

Immunoblot results with the whole-cell sonicate antigen suggest that the captive deer used in this study had prior exposure to environmental mycobacteria, although we did not isolate these organisms from the tissues. On-going studies evaluating responses

of captive and free-ranging deer to various WCS antigens (i.e., from *M. kansasii, M. avium, M. avium subsp. paratuberculosis,* and *M. bovis*) indicate that deer are constantly exposed to antigens, presumably ubiquitous mycobacteria present in their environment, that elicit antibody reactive to mycobacterial WCS (6, Waters, personal observation). Obviously, pre-existing responses complicate the analysis of specific responses to vaccination/infection and likely impact vaccine efficacy and disease progression, both in experimental and field situations (2, 22).

Proteins at the 32kDa and 68-70kDa levels elicited relatively unique antibody response patterns among the treatment groups as detected by immunoblot. The 32kDa proteins most likely correspond to the immunodominant antigen 85 complex (Ag85). The Ag85 complex is a member of the mycolyltransferase family found in all mycobacteria and represents a major fraction of the secreted proteins in culture filtrates of *M. bovis*, including BCG (17, 27). Antigen 85 has been used effectively in the form of a DNA vaccine to boost the protective affects of BCG vaccination in mice and cattle. Although it is unclear why the non-vaccinates had prolonged responses to Ag85 relative to the other groups, particularly the orally vaccinated groups, it is possible that mucosal exposure to this antigen prior to challenge leads to a reduction in antibody response over time after challenge and may correspond to a lack of lesion development. Alternatively, effective mucosal vaccination may limit antigen load detected by the host in the form of antibody production. That the animals in the Parenteral group did not respond in this manner could be a result of more effective sensitization to Ag85 afforded by the parenteral route as compared to oral routes of vaccination. The protein(s) in the 68-70kDa range probably corresponds to a heat shock protein (HSP70) that has been shown

to be immunogenic (8, 9). It is unclear why the Parenteral and Oral Bait groups responded more consistently to this antigen when compared to the other groups. More research needs to be conducted in order to understand the significance of the responses to both sets of proteins by BCG-vaccinated white-tailed deer

The animals in this study did not produce readily detectable antibody responses to MPB83 on immunoblot or on MAPIA. This is in contrast to observations in previous studies in cervids wherein MPB83 was found to be immunodominant in these tests (6, 23). However, this antigen when combined with 16KDa protein (also known as Acr1) was the most readily detected antigen on MAPIA in this study and one of the most readily detected antigens in other studies as well (6, 23, 24). These results highlight the variability of responses by deer, as well as by many other host species, to mycobacterial antigens on both an individual and herd basis. These types of findings are not limited to *M. bovis* infections and are observed in the face of a variety of mycobacterial infections. This could be due to genetic differences among individuals and herds, as well as environmental factors. The results of this study and others highlight the importance of a multiantigen approach, and a continued search for effective antigens is needed to develop more sensitive and specific serologic assays for the evaluation of vaccines, disease progression, as well as general diagnosis of *M. bovis* infection in all species. (6, 21, 23).

In conclusion, white-tailed deer that were protected from severe disease due to vaccination with BCG, either orally or parenterally, produced either no detectible antibody responses or decreasing antibody levels on MAPIA RT, and LAM-ELISA, compared to non-vaccinated deer inoculated with *M. bovis*. These results support the potential of antibody-based assays as useful indicators of vaccine efficacy in

experimental vaccine trials as well as for disease monitoring in vaccinated free-ranging deer populations. In addition, although immunoblot data provided limited insight regarding vaccine-induced protection against disease, they do encourage the further investigation of immune responses to Ag85 and HSP70 in deer vaccinated with BCG or similarly attenuated *M. bovis* constructs. More research is needed regarding the development of serologic assays to detect immune responses to vaccination and *M. bovis* infection. In addition, more expansive, longer term studies on the safety and efficacy of BCG in white-tailed deer against *M. bovis* infection are imperative, so that vaccination may ultimately be used for the management of wild deer populations affected by bovine tuberculosis.

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TABLE 4.1. Antibody responses over baseline to *M. bovis* measured by MAPIA and RT densitometry in vaccinated and nonvaccinated deer after challenge. Measurements were made 4 months after intratonsillar infection with 228 cfu *M. bovis* for E6P10, 16/83, and RT, and at 2 months postinfection for MBCF.

Vaccine Group	Animal ID	MAPIA			RT	Total Gross Lesion Score (average)
		E6/P10	16/83	MBCF		
Oral Bait	402	0	0	41*‡	0	0
	404	0	15*	0‡	0	0
	406	0‡	0‡	5*‡	0	0
	410	0‡	0‡	0‡	0	2
	418	0‡	0‡	13‡	0	1
		(0/5)	(1/5)	(3/5)	(0/5)	3 (0.6)
Oral Liquid	14	0	0	0	0	0
	20	38*	0	0	0	0
	81	0	0	0	0	0
	84	0	0	0	0	1
	416	0	0	0	0	0
		(1/5)	(0/5)	(0/5)	(0/5)	1 (0.2)
Parenteral	1	0	0‡	0*‡	87*	0
	23	0‡	0‡	63*‡	0	0
	26	0‡	0‡	0*‡	0	1
	59	0‡	0	83*	0	0
	73	0‡	16‡	0	0	3
	91	0	0	0	0	0
		(0/6)	(1/6)	(2/6)	(1/6)	4 (0.7)
Nonvaccinate	3	94	16	96	32	16
	19	10‡	3‡	45†‡	20	5
	22	12	28	96	39	11
	24	0‡	0‡	0†‡	0	5
	57	0	0	90	27	19
	422	0	45	85	90	30
		(3/6)	(4/6)	(5/6)	(5/6)	86 (14)

* Antibody over baseline 1-3 months post-vaccination, prior to *M. bovis* challenge

† Antibody detected in unvaccinated animals 1-3 months prior to *M. bovis* challenge

‡ Antibody detected in baseline sample

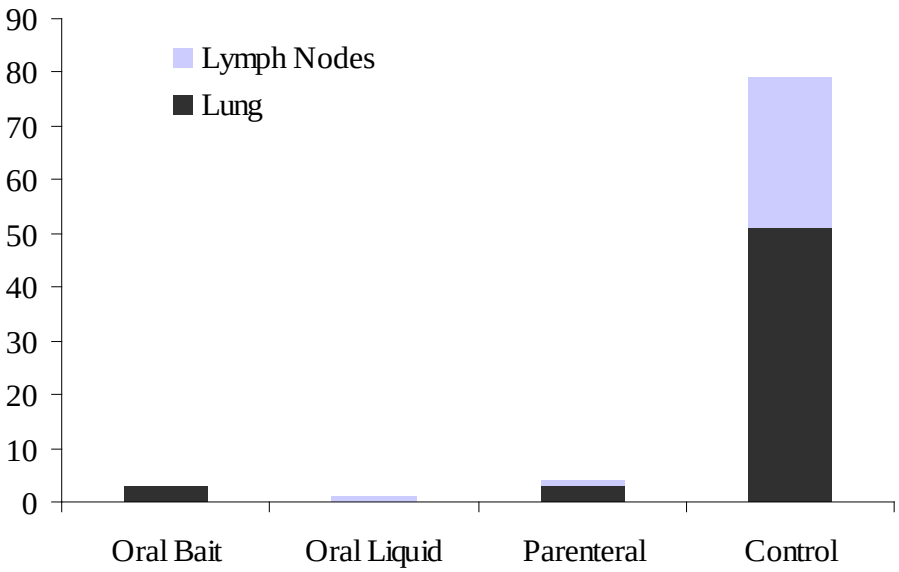
TABLE 4.2. Numbers of vaccinates and nonvaccinates having detectible antibodies to E6P10, 16/83, and MBCF on MAPIA 1-4 months after intratonsillar infection with 228 cfu *M. bovis*

		Time Point (months postinfection)			
Group	Antigen	1	2	3	4
Vaccinates*	E6P10	1/16	1/16	1/16	1/16
	16/83	1/16	2/16	1/16	2/16
	MBCF	5/16	5/16	5/16	5/16
Nonvaccinates	E6P10	0/6	2/6†	2/6‡	3/6†
	16/83	0/6	4/6†	3/6‡	4/6†
	MBCF	3/6	5/6†	4/6‡	4/6†

* None of the vaccinates responded to more than one antigen throughout the 4-month postinfection period.

† Four of six nonvaccinates produced detectable antibodies to more than one antigen at 2 and 4 months postinfection.

‡ Two of six nonvaccinates produced detectable antibodies to more than one antigen at 3 months postinfection.



*Total combined pathology score significantly greater than that of pooled vaccinates

Figure 4.1. Gross pathology scores for selected lymph nodes and lung for individual treatment groups. Scoring took place 5 monthss after intratonsillar infection with 228 cfu *M. bovis* Lymph node lesion scoring ranged from 0 (no lesions) to 3 (most severe). Lung lesion scoring ranged from 0 (no lesions) to 5 (most severe). Each animal received pathology scores based on the sums of the lesion scores assigned to each tissue evaluated.

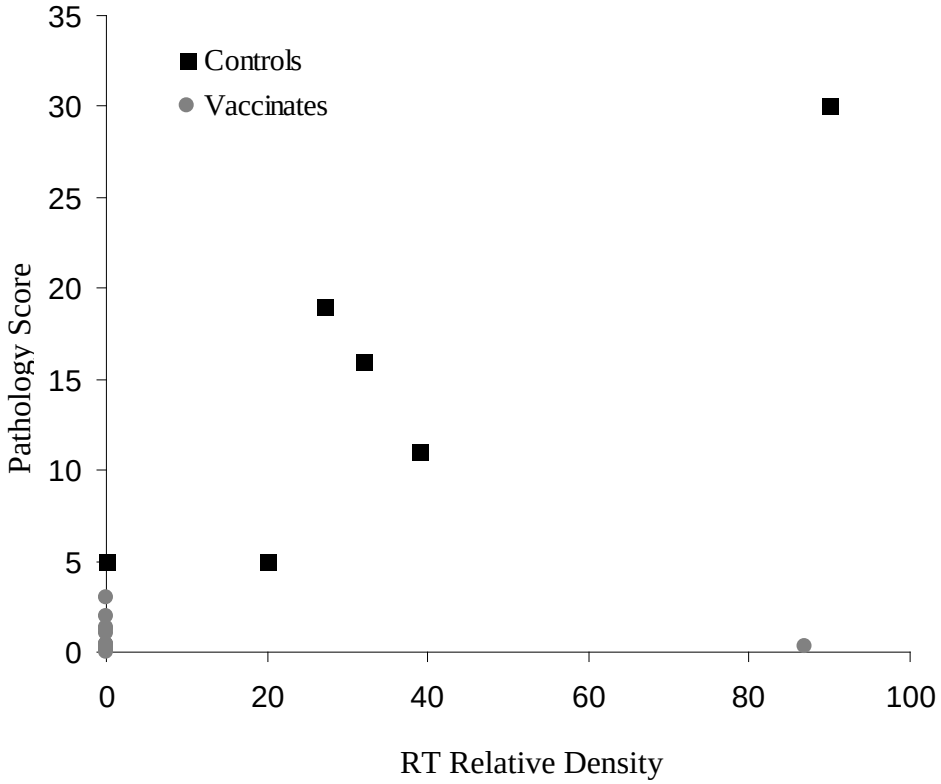
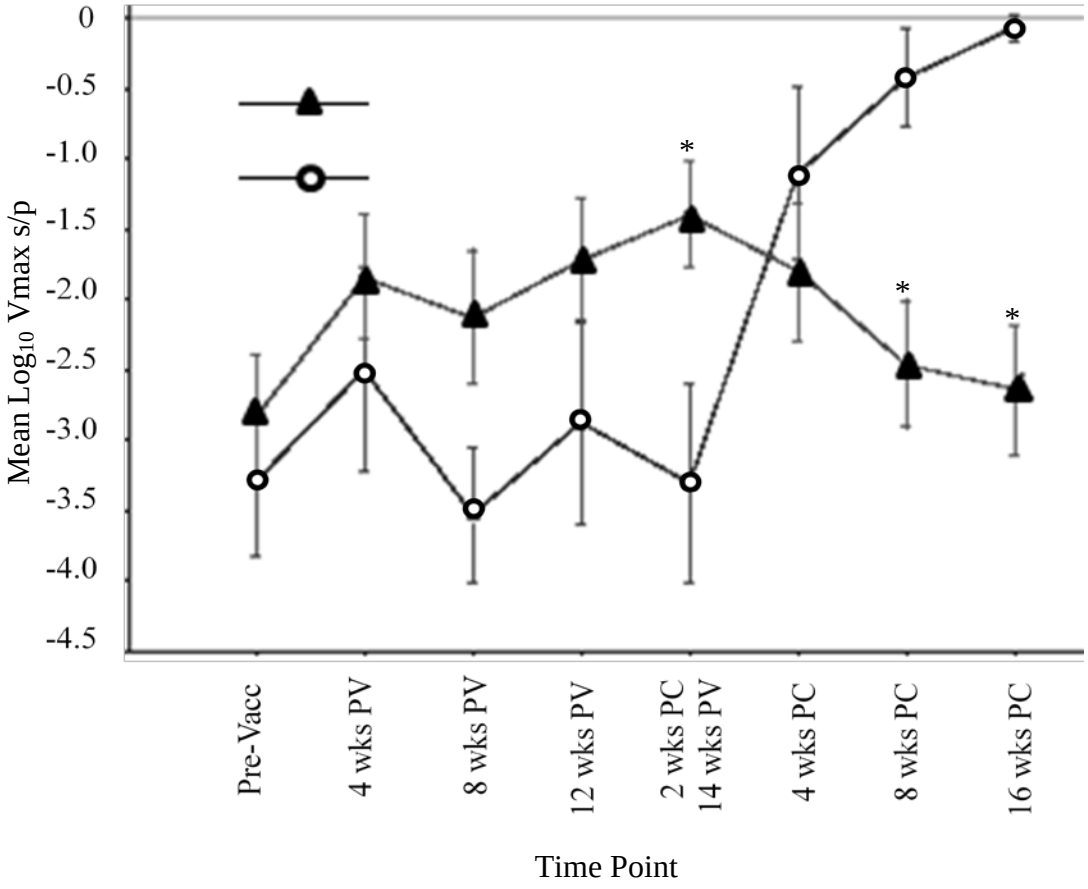


Figure 4.2. Positive correlation between Rapid Test relative densities and disease severity in Vaccinates and Controls shown in terms of pathology scores. Sera were collected at 4 months post *M. bovis* challenge with a total of 228 cfu *M. bovis* strain 9839. Statistical analysis was conducted using Spearman's Rank Test. R=0.60; p=0.0031.



*Mean Vaccinate Log_{10} Vmax s/p value significantly different than mean Control Log_{10} Vmax s/p

Figure 4.3. LAM-ELISA: Mean Log_{10} Vmax s/p Controls vs mean Log_{10} Vmax s/p Vaccinates. Mean Vmax s/p were calculated for Controls and Vaccinates at each timepoint relative to vaccination and challenge. The Vmax s/p was the normalized number for the maximum rate of change for the enzyme. PV = post-vaccination; PC = post-challenge

CHAPTER 5: ANALYTICAL SENSITIVITY AND SPECIFICITY OF A MOLECULAR TECHNIQUE FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX IN SAMPLES FROM BCG-VACCINATED AND *M. BOVIS*-CHALLENGED WHITE-TAILED DEER AND THEIR ENVIRONMENT[1]

ABSTRACT

We developed highly sensitive and specific molecular techniques to detect *Mycobacterium tuberculosis* complex (MtbC) in cervid biological samples and environmental samples, in order to monitor shedding of MtbC by deer in experimental and wild settings. We developed extraction techniques for cervid feces, nasal and pharyngeal swabs, soil, feed, and hay and used an established PCR assay that targets the IS6110 regions of *M. tuberculosis* complex. We applied these techniques to samples collected from white-tailed deer that were orally or parenterally vaccinated with *M. bovis* BCG and experimentally infected with virulent *M. bovis*. Our data indicate that deer shed *M. bovis* and BCG only intermittently at 1-3 months after vaccination and 1-4 months after *M. bovis* challenge. These data will aid future research in vaccination and management of bovine tuberculosis in deer.

INTRODUCTION

Bovine tuberculosis (BTb), caused by the bacterium *Mycobacterium bovis*, is a globally important disease that affects numerous mammalian species including humans. Wildlife reservoirs of BTb exist throughout the world and play significant roles in the

epidemiology of this disease as it relates to the livestock that share living space with these reservoir species (2). Consequently, management of BTb in domestic animal populations becomes significantly more difficult when the disease in free-ranging wildlife is not being controlled. Oral vaccination of wildlife is a management tool that is being explored by many countries with wildlife reservoirs of BTb, including New Zealand (brushtail possum [*Trichosaurus vulpeca*]), United Kingdom and Ireland (European badger [*Meles meles*]), South Africa (African buffalo [*Syncerus caffer*]), and the United States (white-tailed deer [*Odocoileus virginianus*]) (3, 4, 12, 18, 19). A recent study in white-tailed deer has shown that orally-administered *M. bovis* bacille-Calmette-Guerin (BCG), a tuberculosis vaccine for human use, is effective in protecting white-tailed deer from development of lesions cause by *M. bovis* infection (15-17). The concept of orally vaccinating wild deer with BCG raises questions concerning the ability of white-tailed deer to shed BCG after vaccination as well as whether a vaccinated deer will shed virulent *M. bovis* at lower levels after infection when compared to the level of shedding of an unvaccinated deer. The ability to detect *M. bovis* shedding in various biological samples as well as environmental samples through molecular means, without having to perform cumbersome and insensitive *M. bovis* culturing, provides managers and researchers a much more efficient option for monitoring animals, their feces, as well as their habitats for evidence of infection in a particular wild population, and locates possible sources of *M. bovis* for other animals that could come into contact with those areas and substrates (25).

We developed specific techniques to extract mycobacterial DNA from cervid feces, nasal and pharyngeal swab samples, soil, feed pellets, and hay in order to detect *M.*

tuberculosis complex (MtbC) in those samples using polymerase chain reaction (PCR). We applied these techniques to samples collected from BCG-vaccinated and experimentally infected deer.

MATERIALS AND METHODS

Animals. All samples used in this study were obtained from white-tailed deer participating in a BCG vaccine efficacy trial (17, 18). Briefly, yearling female white-tailed deer were allocated into four vaccine groups and were vaccinated as described by Nol et al. (17, 18). One group (oral bait; n=8) received consumed 1 x 10^9 colony forming units (cfu) BCG Danish strain 1331 orally via a lipid-formulated bait (1); the second group (oral liquid; n=8) received 1.9 x 10^8 cfu BCG in culture media orally via catheter; a third group (parenteral; n=7) received 3.4 x 10^6 cfu BCG subcutaneously in the right shoulder; the fourth group (nonvaccinates; n=7) received culture media orally via catheter and served as unvaccinated controls. Through the course of the experiment, 3 deer in the oral bait group, 3 deer in the oral liquid group, 1 deer in the parenteral group, and 1 deer in the nonvaccinate group were removed due to injury or illness. The deer were housed for the vaccination/infection study at USDA/ARS National Animal Disease Center (NADC) in Ames IA, USA. After vaccination, deer were housed together in two outdoor pens with access to both pens at all times. These pens had soil substrate. After challenge, deer were housed in a biosafety level 3 animal facility. All deer were housed and cared for according to the Association for Assessment and Accreditation for Laboratory Animal Care International. The Institutional Animal Care and Use

Committee approved protocols detailing procedures and animal care prior to initiation of the experiments.

***Mycobacterial* Cell Stocks.** A cell stock of rinsed, killed *M. bovis* cells (strain 846146) was kindly provided by Dr. Ian Orme, Colorado State University. The cell stock was at a concentration of 5×10^6 colony forming units (CFU) per ml, as determined by cell plate counts. On the day of each extraction, *M. bovis* cells were placed into a Beadbeater machine (BioSpec Products, Inc., Bartlesville, OK) and subjected to mixing for 15 seconds at 3200 oscillations/minute in order to reduce cell clumping. Depending on the extraction technique employed, cells were then diluted to appropriate concentrations in the resuspension solution (TE Buffer with 0.02% Tween 80) for spiking into the corresponding sample matrix.

Preparation of Fecal, Swab, Soil, Hay, and Feed Spikes and Negative Extraction Controls. Negative deer fecal stock was collected and stored at –70ºC. Samples were thawed and brought to room temperature. One gram of feces was weighed into a sterile 50 ml conical centrifuge tube (VWR, Westchester, PA, USA) and a positive displacement pipette was used to add 250 µl of the appropriate *M. bovis* dilution (100, 50, and 10 cells). A fecal sample spiked with 250 µl of the dilution buffer served as a negative extraction control (0 cells) to verify lack of cross contamination between extraction samples. Four replicates of this trial were performed for estimation of variability. Detection limits for each extraction technique and corresponding treatments were defined as the lowest concentration of mycobacteria detectable in all four replicate trials.

Nasal swabs were collected using a cytology brush (Puritan Medical Products, Guilford, ME, USA) from animals in an unrelated experiment and were stored at –70ºC.

Spiking consisted of thawing each nasal swab sample to room temperature and using a positive displacement pipette to add 250 µl of the appropriate *M. bovis* dilution (10, 5, and 1 cell) to each swab held over a 15 ml conical centrifuge tube (VWR, Westchester, PA, USA). A swab sample spiked with 250 µl of the dilution buffer served as a negative extraction control (0 cells) to verify lack of cross contamination between extraction samples. The validation trial was performed with six replicate sets. Detection limits for each extraction technique and corresponding treatments were defined as the lowest concentration of mycobacteria detectable in all six replicate trials.

Negative soil was collected and stored at −70ºC. Spiking consisted of thawing the collected negative soil stock to room temperature, weighing 0.5 grams soil into a sterile bead beater tube and using a positive displacement pipette to add 125 µl of the appropriate *M. bovis* dilution (5, 2.5, and 1.25 cells). A soil sample spiked with 125 µl of the dilution buffer served as a negative extraction control (0 cells) to verify lack of cross contamination between extraction samples. The validation trial was performed in triplicate. Detection limits for each extraction technique and corresponding treatments were defined as the lowest concentration of mycobacteria detectable in all three replicate trials.

Negative hay and feed stock were stored at −70ºC. Spiking consisted of weighing 2 grams hay into a 4 ounce Whirl-Pak® bag (eNasco, Fort Atkinson, WI, USA) and using a positive displacement pipette to add 250 µl of the appropriate *M. bovis* dilution (10, 5, and 2.5 cells). To prepare feed samples for spiking, 4 grams of feed pellets where weighed and transferred to a 50 ml conical centrifuge tube, and 250 µl of the appropriate *M. bovis* dilution (25, 10, and 5 cells) was added using a positive displacement pipette. A

hay or feed sample spiked with 250 µl of the dilution buffer served as a negative extraction control (0 cells) to verify lack of cross contamination between extraction samples. The validation trial for hay was performed in triplicate and the validation trial for feed with four replicate sets. Detection limits for each extraction technique and corresponding treatments were defined as the lowest concentration of mycobacteria detectable in all replicate trials.

Sample collection from experimental animals. Feces, nasal swabs, and pharyngeal swabs were collected before vaccination, 1, 2, and 3 months after vaccination, and then at 1, 2, 3, and 4 months after challenge. These samples were digitally extracted from the animals as they were manually restrained in a deer handling chute. Fecal samples were stored in Whirl-Pak® bags (eNasco, Fort Atkinson, WI, USA) at –70ºC until testing. Swabs were collected using a sterile cytology brush (Puritan Medical Products, Guilford, ME, USA). Nasal swabs were collected by inserting one brush into each nasal cavity (2 brushes/animal) to a depth of approximately 10 cm. Pharyngeal swabs were collected by sweeping two brushes simultaneously to the back of the oral cavity. Swabs were stored in culture tubes (Falcon, BD Biosciences, San Jose, CA, USA) at –70ºC until testing. Soil, hay, and feed pellet samples were collected at 1, 2, and 3 months after vaccination. Soil was collected in the same four locations in each of two pens at each time point (east pen, near east corner; east pen, far west corner; west pen, near east corner; west pen, far west corner). Pellets and hay were taken directly from all feed bunks or trays regardless of location in pens. Soil, hay, and feed pellets were stored in Whirl-Pak® bags at –70ºC until testing. No soil, feed, or hay was collected after challenge.

Extraction of *M. tuberculosis* complex DNA from deer feces. For each sample, one gram of feces was placed into a 50 ml conical centrifuge tube and incubated with 3.0 ml 11.66% sodium dodecyl sulfate (SDS) for 15 min to allow particulates to settle. The supernatant was then collected and centrifuged at 4280 x g for 20 min. The supernatant was discarded and the resulting pellet was resuspended in 650 µl phosphate buffered saline (PBS), transferred to a bead beater tube (Sarstedt, Inc., Newton, North Carolina) containing 32 x 2.5 mm beads and 0.5 g of 0.5 mm zirconium/silica beads (Biospec Products, Inc., Bartlesville, OK) and centrifuged at 11,000 x g for 10 min. The supernatant was again discarded and the pellet was resuspended via vortexing in 213.5 µl PBS/protein kinase (PK) buffer (Amresco, Inc., Solon, Ohio) (213.5 µl PBS containing 1.265 mg of PK). The sample was then subjected to mechanical and enzymatic disruption through the use of the MBB-8 Mini Bead Beater (Biospec Products, Inc., Bartlesville, OK) via 2 pulses (4,500 rpm) of 30 seconds, with icing (approx 1 min) in between bead beating cycles. The sample was then centrifuged briefly to bring components to the bottom of the tube and heated for 20 min at 72ºC and then for 10 min at 100ºC. After cooling the sample was again briefly centrifuged and applied to the Roche High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The DNA was collected in 200 µl elution buffer. The extracted DNA was concentrated by precipitation with 15 µl 3M Sodium Acetate and 200 µl ice cold (-20ºC) 100% isopropanol. The precipitate was transferred to a new tube to which an additional 200 µl ice cold 100% isopropanol was added and the sample was placed at -80ºC for a minimum of 10 min. The sample was then centrifuged at 11,000 x g for 10 min, at 4ºC, and the pellet resuspended in 300 µl ice

cold 70% ETOH, gently inverted once and centrifuged at 11,000 x g for 10 min at 4ºC. Ethanol was removed, carefully avoiding aspiration of the pellet. Residual ethanol was evaporated from the pellet by use of a microcentrifuge tube membrane (Breath-Easier®, Diversified Biotech, Boston, MA, USA) placed over the opening of the open tube to prevent cross-contamination, and heating the for 10-15 minutes at 65ºC, being careful to avoid over-drying the pellet. The pellet was resuspended in 50 µl Tris-EDTA (10 mM Tris-HCl, 10 mM EDTA, pH 8) and resolubilized by heating at 72ºC for 20 min. The resulting sample was stored at 4ºC until testing via PCR and resultant DNA preparations were diluted 1:10 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

Extraction of *M. tuberculosis* complex DNA from nasal and pharyngeal swabs. Swab samples were thawed, and each swab was handled with a fresh plastic bag to avoid cross-contamination. Each swab was rinsed twice with 6mls cold RBC lysis buffer (155mmol/L NH4Cl, 10mmol/L KHCO3, 1mmol/L EDTA, pH 7.4) into a 15 ml centrifuge tube (Fisher Scientific, Pittsburg, PA, USA). Each sample tube was vortexed and then incubated at 20ºC for 5 min and the procedure repeated once more to ensure complete lysis of red blood cells. Sample tubes were centrifuged at 4,280 x g for 20 min at room temperature to pellet the cells and the supernatant was discarded without disturbing the pellet. The pellet was resuspended in 500 ul PBS and transferred to a bead beater tube containing 15 x 2.5 mm and 0.5g of 0.5 mm beads zirconium/silica beads. The original 15cc tube was rinsed a second time with 500 µl PBS and this rinse was also transferred to the bead beater tube. The bead beater tube was then centrifuged at 11,000 x g for 10 min at room temperature. All except approximately 50 µl of the resulting

supernatant was discarded. One hundred microliters lysis buffer (50 mM Tris-HCl, pH 8; 50 mM KCl, 2.5 mM $MgCl_2$; 0.45% Nonidet P-40; 0.45% Tween 20) with 1.265 mg/ml PK buffer was added to the tube, and the tube was vortexed. The sample was then subjected to mechanical and enzymatic disruption by bead beating using the MBB-8 Mini Bead Beater with 2 pulses (4,500 rpm) of 30 seconds, with 1 min icing in between, followed by incubation at 72ºC for 20 min. The tube was then incubated for 10 min at 100ºC to inactivate the PK. The sample was allowed to cool for 10 min before transferring the lysate to a 1.7 ml low binding tube (Axygen Scientific Inc., Union City, CA) and centrifuged at 11,000 x g for 5 min. The supernatant was transferred a 0.65 ml low binding tube (Axygen Scientific Inc., Union City, CA), and the pellet discarded. The resulting cellular lysate was then ready for PCR testing diluted 1:5 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

Extraction of *M. tuberculosis* complex DNA from soil. Extraction of MtbC from soil was performed using a protocol that incorporated the ZR Soil Microbe DNA Extraction Kit (Zymo Research Corp., Orange, CA, USA). Soil samples were allowed to thaw overnight at 4ºC. Contents and beads from a ZR Bashing Bead Lysis Tube were transferred to a Sarstedt bead beating tube. Five hundred milligrams of soil sample was then added to the bead beating tube. The cells in the soil were subjected to mechanical disruption by bead beating the tube with 2 pulses (4,500 rpm) of 30 seconds using the MBB-8 Mini Bead Beater followed by incubation for 10 min at 100ºC and cooling for 10 min on ice. After cooling the extraction was continued using the ZR Soil Microbe DNA Extraction Kit according to the manufacturer's instructions, including a final filtration with an HRC filter column. All DNA elutions were performed in 1.7 ml low binding

tubes with a 100 µl elution volume. Filtered DNA was then ready for IS6110 PCR testing and resultant DNA preparations were diluted 1:5 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

Extraction of *M. tuberculosis* complex DNA from feed pellets and hay. Extraction from hay and feed pellets was performed using a protocol that incorporated the ZR Soil Microbe DNA Extraction Kit. Beads from a ZR Bashing Bead Lysis Tube were transferred to a Sarstedt bead beating tube. For hay, 2 g of sample was weighed out and placed into a Whirl-Pak® bag. Sixteen mililiters buffered peptone water (Himedia Laboratories, Mumbai, India) containing 0.02% Tween 80 (BPW/Tween) was added and the hay samples were incubated for 15 min with gentle agitation. The liquid BPW/Tween rinse and any hay particles were then transferred to a 15 ml conical tube and centrifuged at 4280 x g for 20 min at room temperature. For feed pellets, 4 grams of sample was weighed out and placed into a Whirl-Pak® bag and 13 ml BPW/Tween was added. The liquid BPW/Tween rinse and any pellet particles were transferred to a 15 ml tube just before the feed pellets began to break up. The remainder of the protocol was the same for both hay and feed. The supernatant was removed from the resulting pellet and 750 µl of Lysis buffer was added to resuspend. The pellet re-suspended in Lysis Buffer was transferred to a bead beater tube containing the ZR Bashing Beads. The cells in the sample were then mechanically disrupted by bead beating with 2 pulses (4,500 rpm) of 30 seconds using the MBB-8 Mini Bead Beater machine. The sample was then incubated at 100ºC for 10 min and allowed to cool for 10 min after which the extraction was continued using the ZR Soil Microbe DNA Extraction Kit according to the manufacturer's instructions, including a final filtration with an HRC filter column. All

DNA elutions were performed in 1.7 ml low binding tubes with a 100 µl elution volume. Filtered DNA was then ready for IS6110 PCR testing and resultant DNA preparations were diluted 1:2 and 1:5 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

Polymerase chain reaction. The samples were then analyzed for *M. tuberculosis* complex DNA using a PCR as described by Nol and others (16, 17) targeting the IS6110 regions of *M. tuberculosis* complex (IS6110 PCR) (10, 24). Briefly, the IS6110 PCR was performed using a PTC-100 Thermal cycler (MJ Research, Inc., Waltham, Massachusetts, USA) for 50 cycles. The primers used in the IS6110 PCR were as follows: forward primer (5'CTCGTCCAGCGCCGCTTCGG3'), and reverse primer (3'CCTGCGAGCGTAGGCGTCGG5') (Operon Biotechnologies, Inc., Huntsville, Alabama). A positive control (purified *Mycobacterium bovis* DNA at 0.5-5 fg/uL) and a negative control (water) were included in every experiment. Following the amplification protocol, 10 µl of the amplification reactions were size fractionated through 2.5% agarose (Amresco, Inc., Solon, Ohio) gels in 1X TAE buffer (40 mM Tris acetate, 1mM EDTA) (Amresco, Inc.). Gels were stained in 0.001mg/ml ethidium bromide (Amresco, Inc.) for 15 min and products were then visualized using the BioRad Gel Doc EQ System (Bio-Rad Laboratories, Hercules, California, USA) UV transilluminator. Product size was 123 base pairs.

Analytical specificity testing of PCR with non-tuberculous mycobacteria and soil microbes. DNA was extracted from cultures of multiple species of non-tuberculous mycobacteria and other soil microbes, namely *M. abscessus* (ATCC 19977), *M. avium* (*Hominissus*, Str 2151), *M. chelonae* (ATCC 35752), *M. fortuitum* ATCC19542), *M. intracellulare* (ATCC 13950), *M. marinum* (ATCC 927), *M. paratuberculosis* (ATCC 19698), *M.*

phlei (ATCC 11758) , *M. simiae* (ATCC 25273), *M. smegmatis* (ATCC 23011), *M. szulgai* (ATCC 35799), *M. terrae* (ATCC 15755), *M. kansasii* (ATCC 12478) and *Nocardia asteroids* (ATCC 3308). DNA was quantified using fluorometry and 10 pg of DNA of each of the species was tested in the IS6110-targeted PCR protocol.

Criteria for positive samples. The following guidelines were observed for reporting positive results in samples. Fecal samples that were positive based on PCR of one DNA extraction event were extracted 2 additional times. If at least 2/3 extractions were positive on PCR then the sample was considered positive. For nasal and pharyngeal swabs, if a swab was positive, the second swab of the same type was extracted and subjected to PCR. To be deemed a positive sample, both extractions had to have been positive. Only two extractions could be performed on swabs as there were only two swabs collected of each type. Soil, hay, and feed samples initially underwent 2 extractions per collection location, and if positive in either, samples were extracted 2 additional times for a total of 4 extractions per collection date/location. At least 2 or 4 extractions had to have been positive for the sample to be considered positive.

RESULTS

Analytical Specificity testing of PCR. The IS6110 PCR produced 123 bp bands only when from DNA originating from *M. bovis* cells, and no banding patterns were visible when DNA from all fourteen of the non-tuberculosis mycobacterial cultures. These results indicate that the analytical specificity of the IS6110-targeted PCR is 100%.

Feces. After evaluating the analytical sensitivity and specificity associated with the fecal extraction procedure, this extraction protocol demonstrated the ability to detect 10-50 *M. bovis* CFU per 1 gram of feces using a 1:10 dilution with PCR testing (Table 5.1 and 5.2).

Of deer tested in the nonvaccinated group for evidence of *M. tuberculosis* complex bacteria in the feces, none of the samples collected from these animals in the first three months after vaccination was positive, and only 1/6 was positive 4 mos after challenge. All fecal samples collected from vaccinated deer at all time points were negative for MtbC.

Nasal and pharyngeal swabs. The nasal/pharyngeal swab lysate DNA extraction method routinely demonstrated the ability to detect 5 *M. bovis* CFU per swab using an optimal 1:5 dilution with PCR testing (Tables 5.1 and 5.2).

Of the nasal and pharyngeal swabs sets collected from the nonvaccinated deer group and tested for MtbC, 1/7 pharyngeal swab sets and was positive prior to vaccination. At no other time points were either nasal or pharyngeal swabs positive for MtbC in nonvaccinates. One of five pharyngeal swab sets collected from deer in the oral liquid group 4 mos postchallenge was positive. At no other time points were either nasal

or pharyngeal swabs sets positive for MtbC in the oral vaccine groups. None of the swab sets from deer in the parenteral group tested positive for MtbC throughout the study.

Soil, feed, and hay. The spiked ZR Soil Microbe DNA extraction method has routinely demonstrated the ability to detect 1.25 *M. bovis* CFU per 500 mg of soil using 1:5 dilution with PCR testing. The protocol described for feed has demonstrated the ability to detect 5-25 *M. bovis* CFU per 4 grams of pellets using 1:2 and 1:5 dilutions with IS6110 PCR testing. The protocol described for hay has demonstrated the ability to detect 5 *M. bovis* CFU per 2 grams of hay using 1:2 and 1:5 dilutions with IS6110 PCR testing (Tables 5.1 and 5.2). None of the soil (n=13), feed pellet (n=6), or hay (n=6) samples (n=6) collected over the three monthss after vaccination tested positive for MtbC.

DISCUSSION

We developed highly sensitive molecular methods for detecting *M. tuberculosis* complex in cervid biological samples as well as environmental samples. These methods allow relatively rapid analysis of such samples when compared to culture and could be very useful assays for evaluating individual animals for shedding of MtbC as well as for monitoring sites subject to heavy animal use for presence of MtbC in soil and feed in both experimental and field settings. Young and others (2005) were successful in detecting as few as 10 *M. bovis* cells in environmental samples (25). Our detection limits in the various types of samples were very comparable to those of Young and colleagues (25).

Based on the results obtained from the BCG-vaccinated and *M. bovis*-infected deer, shedding of vaccine and *M. bovis* appeared to be very limited during the post-vaccination and postchallenge time courses examined in this study. *Mycobacterium tuberculosis* complex was noted only in samples collected from 2 of 22 deer tested at 4 mos postchallenge by either swab or feces, and not at any other time point examined. Therefore, no differences in shedding rates could be determined among vaccine groups or between vaccinates and nonvaccinates. It is possible that evidence of vaccine shedding may have more likely been detected had samples been more frequently collected during the first 30 days after oral vaccination, a window of time during which collection did not occur.

This study and others have shown that fecal shedding of *M. bovis* continues to be very difficult to detect in white-tailed deer, the reason for which has generally been attributed to contaminants in feces, in addition to the likely possibility that its occurrence is infrequent (20, 22, 23). *Mycobacterium bovis* in nasal and oral secretions is generally detected in approximately 20% of experimentally inoculated cattle in the first 60 days after infection, and only intermittently thereafter (13, 14). In white-tailed deer, Palmer and colleagues have been successful in isolating *M. bovis* at a rate ranging from 3-50% of deer in a given experiment (21, 22, 23). The data from these past studies represented isolations through culture of *M. bovis*, a method which is widely known to be very insensitive, especially when culturing contaminated samples such as feces and swabs. We believed that based on the results of our sensitivity testing of the molecular methods reported here, we would see evidence of shedding of *M. bovis* in a higher percentage of swab and fecal samples than seen in previous studies. Our results probably do reflect a

lack of shedding of the experimental animals in our study, although the question can be raised as to whether we could have isolated the bacillus more frequently through culture, which was not done in this case. Subsequent experiments will include culture to rule out this possibility.

The lack of detection of MtbC in soil 1-3 months post-vaccination, as with the biological samples, may also indicate that BCG was not being shed by the deer during this time or that bacilli were missed in the face of sporadic shedding. Three of the soil samples and one of the feed samples did test positive on one extraction/PCR event but none of the results were repeatable on subsequent extractions and thus were judged to be negative. More intense environmental sampling for future studies is warranted in order to minimize the chance of not detecting MtbC when it is actually present but in low numbers. Determining extent of vaccine and *M. bovis* contamination of the environment is important as Young et al. found that *M. bovis* BCG remains viable in soil for more than 15 months, dependent on ambient conditions, and that significant levels of *M. bovis* DNA and RNA persist in the field, indicating the presence of viable cells as an environmental reservoir for infection (25).

It is likely that the pharyngeal swab collected from one of the deer prior to vaccination was positive for MtbC due to contamination by the vaccine preparation. These samples were collected while animals were being vaccinated with BCG. There was therefore a chance for accidental contamination of the sample during the vaccination process. In light of this finding, a more stringent approach to sample handling should be utilized in future trials to avoid chances of such contamination. Another possible, but less likely explanation of the prevaccination positive swab is the possibility that the PCR

we used in this study produced false positive results for MtbC in the presence of certain environmental mycobacteria. The specificity of PCR targeting IS6110 has been questioned due to the presence of regions of homology between DNA of other *Mycobacterium* species and a portion of the IS6110 target (5, 8, 9). However, it has been contended that the 763 – 884 nucleotide region amplified by the IS6110 PCR in this study avoids the homologous region and has been reported to be suitable as a specific MtbC target (6, 7, 11). Furthermore, none of the 14 non-tuberculous *Mycobacterium* species tested in this study and 27 tested by Hellyer et al. produced 123 bp products by PCR (6).

In conclusion, the methods we developed for detection of *M. tuberculosis* complex DNA in cervid and associated environmental samples are very sensitive based on our validation procedures. They can be used to test deer and their environment for evidence of shedding of *M. bovis* in a rapid manner when compared to other means of *M. bovis* detection. Although this study and others continue to emphasize the fact that *M. bovis* is very difficult to detect in biological and fecal samples, more targeted and intense use of the procedures described here may allow improved evaluation of shedding in vaccine studies where sampling can occur within a month after vaccination and where disease progression can be monitored very closely over a longer period of time. In addition, testing of soil and feed with these methods would be very helpful when premises or particular areas are being studied for concentrated sources of *M. bovis* or BCG to resident livestock or wildlife.

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Table 5.1. Proportion of samples spiked with *M. bovis* that had a 123-base pair product following the appropriate DNA extraction method procedures. Results are expressed as the proportion of positive product extracted and detected from each sample type within a set of replicate trials conducted on samples spiked with the indicated number of *M. bovis* cells.

	Spiked *M. bovis* Cell Concentration								
Sample Type	100 cells	50 cells	25 cells	10 cells	5 cells	2.5 cells	1.25 cells	1 cell	0 cells
Soil	*NT	*NT	*NT	*NT	4/4	4/4	3/3	*NT	0/3
Feed Pellets	*NT	*NT	2/4	1/4	2/4	*NT	*NT	*NT	0/4
Hay/Alfalfa	*NT	*NT	*NT	2/3	3/3	0/3	*NT	*NT	0/3
Swabs	*NT	*NT	*NT	6/6	6/6	*NT	*NT	1/6	0/6
Feces	4/4	4/4	*NT	2/4	*NT	*NT	*NT	*NT	0/4

NT: Not tested

Table 5.2. Detection limit of spiked *M. bovis* cells from each sample type, corresponding to the appropriate extraction procedure. Results are expressed as the lowest number of *M. bovis* cells detected from the set of replicate trials conducted per sample type at the indicated optimal DNA dilution.

Limit of Detection of *M. bovis* Cells per Extraction Type			
Sample Type (optimal dilution)	Zymo Method	Lysate Method	Roche Method
Soil (1:5)	1.25	---	---
Feed Pellets (1:2 and 1:5)	5 - 25	---	---
Hay/Alfalfa (1:2 and 1:5)	5	---	---
Swabs (1:5)	---	5	---
Feces (1:10)	---	---	10 - 50

CHAPTER 6: DISCUSSION AND CONCLUSIONS

Wildlife reservoirs of important domestic animal diseases pose significant challenges to management of wildlife and livestock alike. Conventional approaches to animal disease management are exceedingly difficult in the context of a free-ranging wild population. Implementation of test and removal procedures, perhaps applicable to a small, geographically confined population, is often not possible in abundant, widely distributed, or elusive species. Aggressive management procedures or interventions, such as local depopulation of wild species, are common tools used for control of disease but are often not easy to carry out and are frequently not acceptable to the public arena (1, 16). In addition, in what might seem to be a logical approach, culling of wildlife to control disease prevalence and transmission should not be the only management tool used and may not be at all appropriate in certain populations. Disease transmission rates are not necessarily linearly related to density in many populations due to ecological factors such as social organization, movement patterns, and compensatory reproduction (10). Therefore, depopulation efforts can cause social perturbations that actually increase instead of decrease disease prevalence in the long term (4, 10, 24). Examples of controversial culling operations include the management of European badgers (*Meles meles*) in the United Kingdom and Ireland to decrease the densities of this wild reservoir of bovine tuberculosis, and white-tailed deer (*Odocoileus virginianus*) in Wisconsin, USA where an unsuccessful attempt was made at total depopulation within a

limited area where chronic wasting disease was discovered (4, 14). In addition, introduction of regulations intended to reduce disease impact or prevalence, that negatively affects traditional activities of private citizens, tends to face serious opposition and problems with compliance (16). An example of this type of activity is supplemental feeding of wildlife which will be addressed later in this chapter. In light of these issues, wildlife disease managers must be professionally equipped with several tools, not only in order to have the flexibility to adapt to the many challenges inherent in manipulating wild populations, but also to those inherent in human populations.

Vaccination is an important component of most livestock health management programs; and although logistically challenging, vaccination of wildlife can be a vital tool in the kits of wildlife disease managers as well. In situations where population reduction and other forms of management are either not appropriate, cannot be adequately implemented, or are simply not enough when used alone, administration of a protective vaccine to wildlife could potentially augment existing management practices or even replace them.

There are many difficulties and complexities in the realm of developing and implementing a wildlife vaccination program. The process of acquiring an effective vaccine is often a very challenging endeavor and is perceived by many to be the most difficult hurdle to clear. In reality, once a vaccine is determined effective and considered a viable candidate to take in to the field, much of the work still needs to be done (2). The vaccine must be rigorously tested for safety and long term efficacy in the target species as well as non-target species that share the same environment. If the vaccine is to be administered orally, a bait system must be developed and tested. The vehicles and

methods used to administer vaccine to wildlife must be capable of effectively exposing to the vaccine a critical proportion of animals in the target population. This critical proportion can be calculated using simple models based on the density of animal required for endemic persistence of the disease and the carrying capacity of the population in the absence of disease (2). Once this central question is addressed, more complex models can be utilized in order to not only determine how many should be vaccinated but also where, when, and how to do it. This model needs to incorporate information such as the vaccine's actual ability to reduce or prevent transmission, the prevalence of the disease in the wildlife population, the size and demographics of the disease-susceptible population, and the spatial distribution of the susceptible population. Factors specific to the environment, such as geography and climate, as well as others pertaining to the ecology of the species, such as social behaviors, and feeding and migration patterns, are very important in determining how the vaccine should be distributed in the field. Vaccinating the greatest possible number of reservoir species while simultaneously excluding all other species is an enormous challenge.

As mentioned previously, the state of Michigan harbors a wild white-tailed deer population in the northeastern portion of its lower peninsula, that has maintained bovine tuberculosis for possibly many decades within a five-county area, and some suspect BTb became endemic in the herd as early as the 1930's (11). Population reduction and restriction of supplemental feeding has contributed to the decrease in prevalence of BTb in deer since its discovery in the mid 1990's(16) . Hunter harvest has reduced the deer population in the affected area by 51% since 1995 (16). However, resistance and noncompliance with regards to the banning of artificial feeding, a strong tradition in the

state, as well as reluctance towards continued increased deer harvest, have hampered any further advances in disease control efforts. Although apparent prevalence of BTb is relatively low (approximately 1-2%) in the deer herd of the core area, outbreaks in cattle continue to occur and are, with a few exceptions, being attributed to direct or indirect contact with infected deer (15, 16). Proper management of cattle farms in the affected area is also essential for BTb control. In fact, Kaneene et al. (2002) showed that on farm practices regarding exclusion of deer from cattle use areas were the main predictors of BTb status of the farm (8).

Mycobacterium bovis bacille Callmette-Guerin (BCG), the vaccine used in human populations for nearly 70 years to protect against tuberculosis, has been shown to be effective in protecting white-tailed deer against disease caused by experimental infection with virulent *M. bovis* (12, 13, 17, 18) . This vaccine also appears to be effective in other BTb reservoir species such as the brushtail possum (*Trichosurus vulpeca*), the European badger, and wild boar (*Sus scrofa*) (1, 9). This vaccine has great potential for use in the Michigan wild deer population to aid in the reduction and eventual eradication of BTb. As has been shown in red deer (*Cervus elaphus*) and is believed also to apply to white-tailed deer, animals that do not progress to severe disease due to the protective effects of BCG will have a reduced tendency toward shedding bacteria and thus will not play a significant role in transmission (5). Some vaccinated individuals seem to be able to resist infection with *M. bovis* entirely thus suggesting that a subset of vaccinated animals will experience sterile immunity and not participate at all in the transmission of BTb (17). If this reduction in transmission can be achieved in a large enough segment of the

population, it will greatly benefit the existing BTb control program and may allow prevalence to decrease to undetectable levels and eventual eradication.

In order to gain official clearance to use BCG in wild white-tailed deer, much follow-up work needs to be done in the form of larger scale trials evaluating safety and long term protection against *M. bovis* infection. These trials should investigate efficacy of the vaccine if infection were to occur over one year after vaccination and would also study the progress of lesion development for more than one year after infection. Further experiments should look at the safety of multiple doses of BCG and the effects of multiple doses on its protective capabilities. These safety studies should also include a component investigating the effects of BCG vaccination on deer already infected with *M. bovis,* as infected deer may be adversely affected by vaccination. Additional studies must also be carried out looking at the possibility of shedding of vaccine from deer to cattle that could be inadvertently exposed to excreted or secreted BCG and thus potentially test positive on skin test. Many of these proposed experiments have not yet been done due to the inherent difficulties in housing deer species under biosafety level 3 conditions as well as the enormous expense of such trials. Studies addressing the potential for shedding to cattle are underway (M. Palmer-pers comm.; P. Nol). Investigations into various baits specific for white-tailed deer are also being carried out, including the lipid-formulated bait, originally developed for brushtail possums, that successfully delivered BCG to the deer as described in chapter 3 (M. Dunbar-pers comm.) (13).

In the event that data support further use of the vaccine, a controlled field trial may be initiated. A field trial would ideally involve two geographically or artificially confined areas with the same habitat characteristics, comparable and sufficient deer

densities, and similar levels of BTb within their deer populations. Strategies for vaccine distribution (volume and placement) in this field trial as well as for the eventual application to the entire endemic area may be developed through simulation models. Parameters for these models would be installed based on data obtained from the Michigan deer population, including apparent overall prevalence of disease, total numbers and overall deer densities in the five-county region, known areas of disease clustering and deer densities in those particular areas, feeding habits, social behaviors, and deer migration patterns during the late winter when vaccination would likely be most effective and beneficial. Other data to be included would be vaccine-based, such as overall vaccine efficacy, effect (if any) of revaccination in subsequent years, and if known, estimated consumption rate of baits depending on whether the baits are scattered over large areas, placed in a more concentrated manner, or even presented at bait stations.

Once in the field, the ability to specifically monitor vaccination rates in the target population is essential. A good vaccine marker is needed that is easily detected in blood or tissues, is be correlated with actual vaccination, and is long lasting. Chemicals frequently used that can be incorporated into baits are rhodamine B and tetracycline. However, rhodamine B, like many other chemicals, only persists for several weeks and is thus not adequate for long term monitoring. Tetracycline has been a very effective to determine adequate vaccine uptake and distribution by wild populations over the long term and has specifically been used for evaluating oral rabies vaccine programs (3, 6). Unfortunately, tetracycline is toxic to most live bacterial vaccines such as BCG, and therefore can only be used for virally-vectored and protein-based vaccines. Another alternative is to measure immune responses to mycobacterial antigens produced by

virulent field strains of *M. bovis* but are not produced by the attenuated strain BCG. Examples of proteins that are not produced by BCG are ESAT6 and CFP10. However, the measurement of antibodies and/or cellular immune responses developed against mycobacterial antigens is not an adequate indicator of vaccine exposure in wild species due to lack of specificity in the face of probable co-infection with non-tuberculous mycobacteria as well as *M. bovis* in BTb endemic areas. In addition, levels and nature of antibody development to mycobacterial antigens can vary extensively depending on the individual animal (age and species) and the status and extent of infection (12, 27, 29). A potential solution to this problem is the use of positive markers. Proteins, or plasmids expressing proteins to which a target species is very unlikely to be naturally exposed, can be incorporated into a vaccine and induce a unique antibody response with minimal chance of cross-reaction in the immunized animal (20, 25). Research is currently underway investigating an experimental molecular marker expressed by BCG in its ability to elicit long term antibody production in ruminants in order to identify vaccinated animals.

Highly accurate and preferably animal-side diagnostic tests to detect *M. bovis*-infected deer and to assess immune responses in research settings and disease status in field settings continue to be sought after by both managers in the captive cervid industry as well as wildlife researchers and managers. Skin testing remains the official diagnostic test for cervid species, which, because of its requirement for at least two handling events, is extremely inconvenient and not without risk in captive cervids, and is not at all feasible in free-ranging animals. A reliable, commercial interferon gamma test, while currently available for use in cattle, had also been developed for cervids but was not reliable in

white-tailed deer and most other deer species, as indicated in chapter 3 and also by Waters and his colleagues (28). Efforts are being made to develop a better blood test to measure interferon gamma responses in deer, but they are not available at this time.

As described in chapter 4, antibody-based serologic tests, such as the Cervid TB STAT-PAK or Rapid Test (RT) and the multiantigen print immunoassay (MAPIA), (Chembio Diagnostic Systems, Inc., Medford, NY), the lipoarabinomannan enzyme-linked immunosorbent assay (LAM-ELISA), and the western blot are very useful in complementing other diagnostics testing, exploring trends in immune responses of deer to vaccination and experimental *M. bovis* challenge, as well as for evaluating vaccine efficacy (12, 26, 29, 30). The RT, which can be performed animal-side, is also capable of distinguishing vaccinated from unvaccinated animals after *M. bovis* infection, as unvaccinated animals with more severe pathology produce more robust antibody responses detectible by this test (12). Currently available serologic tests, however useful and convenient, are still not very sensitive relative to the skin test in detecting infected animals, especially animals in early stages of disease. Many companies have entered the race to develop a serologic test, using the most optimal antigens, for BTb diagnosis that can replace the skin test. New tests, such as Enfer's multiplex immunoassay, that incorporate the multiantigen concept into increasingly efficient and sensitive technologies, are being evaluated (31). Continued discovery of *M. bovis* antigens that elicit consistent antibody production in multiple species occurs using techniques such as liquid chromatography, mass spectrometry, and microarray analysis (22).

In a research context, other methods used for evaluating cellular and humoral immune responses to *M. bovis* infection or vaccination are being used. They include

lymphocyte blastogenesis assays, which measure general proliferation of lymphocytes exposed to certain *M. bovis* antigens, as well as flow cytometry, which can be used to measure proliferation, due to exposure to select antigens, of individual types of lymphocytes labeled with antibodies to specific activation markers or receptors (30). Molecular methods focusing on gene expression are also used to measure production of cytokines and chemokines, such as interferon gamma, interleukin (IL)-12, IL-10, and IL-4, and CXCL10, to name a few, in order to monitor leukocyte activity in research subjects (7, 23, 32). While these tests are useful for research purposes, they are not practical for use as diagnostic tools in their current form, as they require advanced technical skills and specialized equipment for their execution. Even in research, the data obtained from these methods can be difficult to interpret, as was evident in the work described in chapter 3. Reasons for the inconclusive results obtained from the lymphoblastogenesis assay may be due to the relatively non-specific antigens used in the form of PPDs, small sample sizes, and few time points. Despite the many challenges, as more knowledge is gained and technology improves, diagnostics for use in the field can eventually develop from these techniques

Alternative technologies for BTb diagnosis are also being explored. Use of instrumentation such as mass spectrometry to detect metabolites or volatile compounds uniquely produced by *Mycobacteria*-infected animals, or by the organisms themselves *in vivo*, may in the future allow for diagnosis of tuberculosis via urine, saliva, or exhaled breath (19, 21). But for now, the skin test, a tool that has been used since the beginning of the last century, remains unsurpassed, just like its 100 year-old vaccine counterpart, BCG.

Molecular tools are an efficient way to test biological and environmental samples for the presence of *M. bovis*. The ability to determine the timing and extent of shedding of *M. bovis* or BCG by vaccinated and infected deer is very useful for evaluation of vaccine efficacy in terms of reduction of disease transmission. Although the results of the study presented in chapter 5 showed very little evidence of shedding by vaccinated or *M. bovis*-infected deer, further work will be done in order to determine if the schedule of sample collection inadvertently missed the time periods when shedding of *M. bovis* or BCG might have occurred, or if shedding is truly exceptionally sporadic and/or is associated with only a few individuals in a population.

In conclusion, based on the results of this research, orally-administered BCG holds great promise for use in wild white-tailed deer in Michigan. Much work, in the form of additional controlled experiments, still needs to be done in order to be able to test the vaccine in a field situation. To aid in the continued evaluation of BCG in these trials, serologic and molecular tools will be useful in predicting efficacy of the vaccine after challenge with *M. bovis* but before termination of studies. The further improvement of diagnostic tests will greatly facilitate the management of BTb in domestic cattle and captive cervids. In addition wildlife researchers and managers will be able to monitor with more accuracy, the status of disease in live-captured animals, and the efficacy of a field vaccination trial when it is eventually implemented in the state of Michigan and perhaps elsewhere as well. Successful field evaluation of BCG in wild white-tailed deer will hopefully lead to comprehensive vaccination programs targeting the affected free-ranging deer herds of North America that will ultimately result in eradication of the disease.

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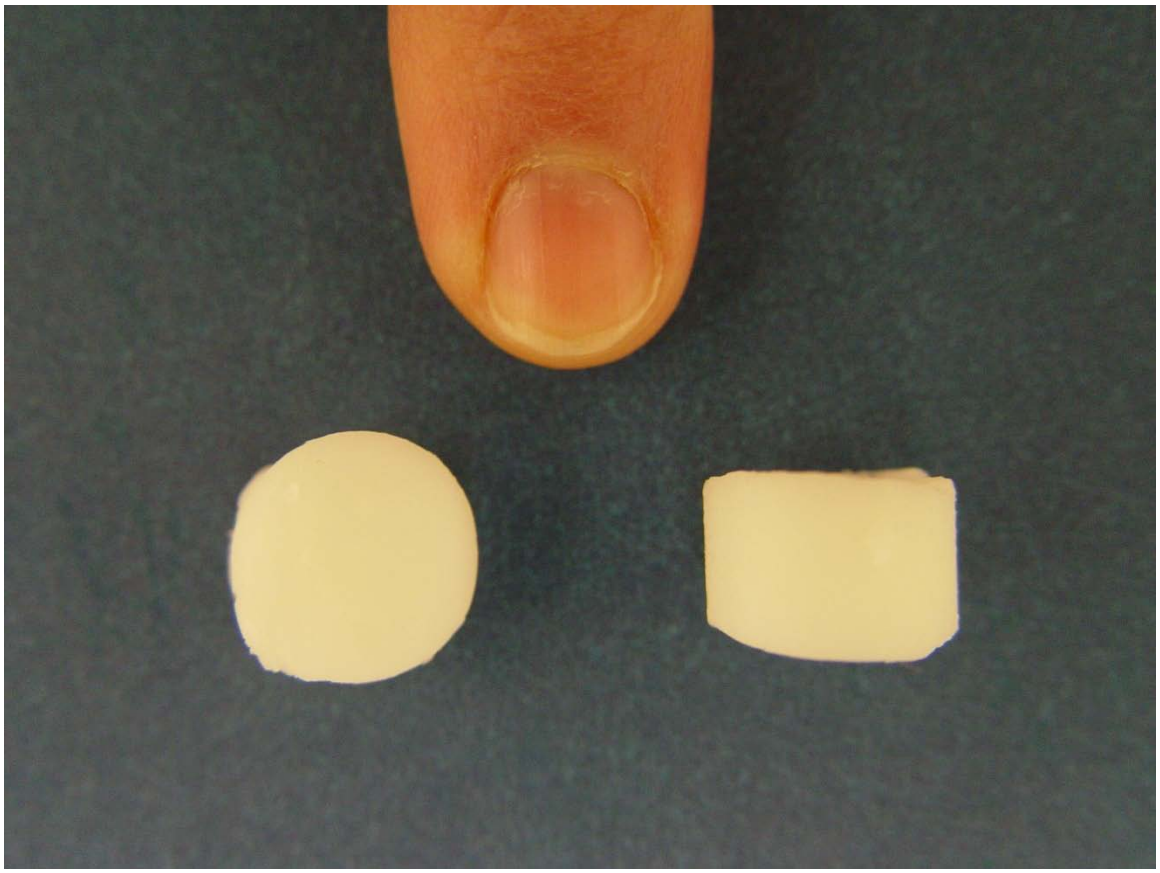
APPENDIX A



Lipid-formulated baits provided by Immune Solutions, Ltd, University of Otago, Dunedin, NZ

APPENDIX B

Three models evaluated for the analysis of interferon gamma data obtained from responses to each of the following antigens: bovine PPD (DeltaB), avian PPD (DeltaA), and ESAT6:CFP10 (DeltaEC)

<u>BOVINE PPD</u>
(Model 2 was utilized based on lowest AICC value)

The GLIMMIX Procedure
Response Distribution Gaussian
Link Function Identity
Variance Function Default
Estimation Technique Restricted Maximum Likelihood
Degrees of Freedom Method Kenward-Roger
Fixed Effects SE Adjustment Kenward-Roger

Model 1 Information
DeltaB repeated measures data
DeltaB = group|timepoint
Random animalid(group)
Random timepoint/sub=animalid(group) ar(1) residual
Variance matrix not blocked
AICC (smaller is better) 1318.92

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	15.40	1.13	0.3675
Timepoint	8	102.90	1.77	0.0905
Group*Timepoint	24	105.30	1.25	0.2153

Model 3 Information
DeltaB = group|timepoint
Random animalid(group) / group=group
No residual correlation structure (no repeated measures effect)
Variance matrix diagonal
AICC (smaller is better) 1296.57

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	73.76	1.59	0.1997
Timepoint	8	108.1	2.05	0.0469
Group*Timepoint	24	103.3	1.21	0.2514

Model 2 Information
Predicted DeltaB by group
DeltaB = group|timepoint
No Random animalid(group)
Random timepoint/sub=animalid(group) group=group ar(1)
residual
Variance matrix blocked by AnimalID(Group)
AICC (smaller is better) 1286.97

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	24.83	1.78	0.1773
Timepoint	8	80.59	2.00	0.0573
Group*Timepoint	24	1.22	1.22	0.2497

Simple Effect Comparisons of Group*Timepoint
Least Squares Means By Timepoint

Simple Effect Level	Group	Group	Est.	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 1	parenteral	placebo	-1.3009	15.9411	68.59	-0.08	0.9352	0.9994	-33.1060	30.5041	-38.6619	36.0601
Timepoint 1	oral bait	placebo	4.1581	12.7493	54.84	0.33	0.7456	0.9664	-21.3937	29.7099	-25.7223	34.0385
Timepoint 1	oral	placebo	-1.4700	11.8406	44.71	-0.12	0.9018	0.9979	-25.3226	22.3825	-29.2208	26.2807
Timepoint 2	parenteral	placebo	0.02000	15.1396	67.01	0.00	0.9989	1.0000	-30.1986	30.2386	-35.6502	35.6902
Timepoint 2	oral bait	placebo	8.8550	11.7317	58.09	0.75	0.4534	0.7530	-14.6278	32.3378	-18.7859	36.4959
Timepoint 2	oral	placebo	-0.7700	10.5329	44.68	-0.07	0.9420	0.9996	-21.9884	20.4484	-25.5863	24.0463
Timepoint 3	parenteral	placebo	31.7540	15.1396	67.01	2.10	0.0397	0.0901	1.5354	61.9726	-3.9162	67.4242
Timepoint 3	oral bait	placebo	7.7271	11.7317	58.09	0.66	0.5127	0.8157	-15.7556	31.2099	-19.9138	35.3680
Timepoint 3	oral	placebo	0.5920	10.5329	44.68	0.06	0.9554	0.9998	-20.6264	21.8104	-24.2243	25.4083
Timepoint 4	parenteral	placebo	4.3925	15.1396	67.01	0.29	0.7726	0.9787	-25.8261	34.6111	-31.3628	40.1478
Timepoint 4	oral bait	placebo	8.1785	11.9037	60.36	0.69	0.4947	0.8030	-15.6294	31.9865	-19.9344	36.2915
Timepoint 4	oral	placebo	0.6955	10.7373	46.95	0.06	0.9486	0.9997	-20.9057	22.2968	-24.6627	26.0538

Simple Effect Comparisons of Group*Timepoint Least Squares Means By Timepoint												
Simple Effect Level	Group	Group	Est.	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 5	parenteral	placebo	20.8110	15.1396	67.01	1.37	0.1738	0.3563	-9.4076	51.0296	-15.0243	56.6463
Timepoint 5	oral bait	placebo	6.4535	11.9685	60.54	0.54	0.5917	0.8919	-17.4827	30.3896	-21.8759	34.7828
Timepoint 5	oral	placebo	13.4491	11.0569	50.23	1.22	0.2295	0.4499	-8.7568	35.6549	-12.7225	39.6206
Timepoint 6	parenteral	placebo	37.9392	16.3264	64.36	2.32	0.0233	0.0567	5.3269	70.5514	-0.8642	76.7426
Timepoint 6	oral bait	placebo	20.0994	12.4346	66.53	1.62	0.1107	0.2448	-4.7234	44.9222	-9.4543	49.6530
Timepoint 6	oral	placebo	7.6680	11.0355	50.23	0.69	0.4904	0.8118	-14.4948	29.8308	-18.5603	33.8963
Timepoint 7	parenteral	placebo	-33.6740	15.1396	67.01	-2.22	0.0295	0.0708	-63.8926	-3.4554	-69.6025	2.2545
Timepoint 7	oral bait	placebo	-29.3059	12.6095	66.63	-2.32	0.0232	0.0562	-54.4772	-4.1346	-59.2303	0.6184
Timepoint 7	oral	placebo	-41.4671	11.0350	50.23	-3.76	0.0004	0.0009	-63.6290	-19.3051	-67.6548	-15.2793
Timepoint 8	parenteral	placebo	-3.8045	15.1396	67.01	-0.25	0.8024	0.9872	-34.0231	26.4141	-39.7512	32.1422
Timepoint 8	oral bait	placebo	-0.3261	12.7467	65.88	-0.03	0.9797	1.0000	-25.7766	25.1244	-30.5912	29.9391
Timepoint 8	oral	placebo	7.2380	11.0350	50.23	0.66	0.5149	0.8334	-14.9239	29.3999	-18.9630	33.4390
Timepoint 9	parenteral	placebo	7.4144	15.9411	68.59	0.47	0.6433	0.9227	-24.3907	39.2194	-30.2086	45.0374
Timepoint 9	oral bait	placebo	-3.6704	13.7259	62.32	-0.27	0.7900	0.9830	-31.1052	23.7644	-36.0652	28.7243
Timepoint 9	oral	placebo	-3.0176	12.1113	47.69	-0.25	0.8043	0.9861	-27.3731	21.3378	-31.6018	25.5665

Avian PPD
(Model 3 was utilized based on lowest AICC value)

The GLIMMIX Procedure
Response Distribution Gaussian
Link Function Identity
Variance Function Default
Estimation Technique Restricted Maximum Likelihood
Degrees of Freedom Method Kenward-Roger
Fixed Effects SE Adjustment Kenward-Roger

Model 1 Information
DeltaA repeated measures data
DeltaA = group|timepoint
Random animalid(group)
Random timepoint/sub=animalid(group) ar(1) residual
Variance matrix not blocked
AICC (smaller is better) 1384.23

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	17.26	1.50	0.2503
Timepoint	8	92.66	4.96	0.0001
Group*Timepoint	24	99.85	0.86	0.6569

Model 2 Information
Predicted DeltaA by group
DeltaA = group|timepoint
No Random animalid(group)
Random timepoint/sub=animalid(group) group=group ar(1) residual
Variance matrix blocked by AnimalID(Group)
AICC (smaller is better) 1260.74

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	13.23	3.06	0.0654
Timepoint	2	31.40	5.32	0.0003
Group*Timepoint	6	60.60	2.73	0.0008

Model 3 Information

DeltaA = group|timepoint
Random animalid(group) / group=group
No residual correlation structure (no repeated measures effect)
Variance matrix diagonal
AICC (smaller is better) 1252.63

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	67.76	3.24	0.0272
Timepoint	6	60.08	5.67	<0.0001
Group*Timepoint	24	93.98	2.91	0.0001

Group*Timepoint Least Square Means

Group	Timepoint	Est	Std Error	DF	tValue	Pr > |t|	Lower	Upper
Placebo	1	0.4050	10.5140	34	0.04	0.9695	-20.9619	21.7719
Placebo	2	2.0100	10.5140	34	0.19	0.8495	-19.3569	23.3769
Placebo	3	0.5450	10.5140	34	0.05	0.9590	-20.8219	21.9119
Placebo	4	0.1610	10.5140	34	0.02	0.9879	-21.2059	21.5279
Placebo	5	0.7520	10.5140	34	0.07	0.9434	-20.6149	22.1189
Placebo	6	0.2580	10.5140	34	0.02	0.9806	-21.1089	21.6249
Placebo	7	39.0050	10.5140	34	3.71	0.0007	17.6381	60.3719
Placebo	8	1.0875	11.7550	34	0.09	0.9268	-22.8015	24.9765
Placebo	9	1.0363	11.7550	34	0.09	0.9303	-22.8527	24.9252
Parenteral	1	-232E-15	19.3846	35	-0.00	1.0000	-39.3528	39.3528
Parenteral	2	0.9600	19.3846	35	0.05	0.9608	-38.3928	40.3128
Parenteral	3	9.4050	19.3846	35	0.49	0.6306	-29.9478	48.7578
Parenteral	4	4.5160	19.3846	35	0.23	0.8171	-34.8368	43.8688
Parenteral	5	7.9280	19.3846	35	0.41	0.6850	-31.4248	47.2808
Parenteral	6	2.4850	21.6726	35	0.11	0.9094	-41.5127	46.4827
Parenteral	7	88.9870	19.3846	35	4.59	<.0001	49.6342	128.34
Parenteral	8	2.3020	19.3846	35	0.12	0.9061	-37.0508	41.6548
Parenteral	9	14.0935	19.3846	35	0.73	0.4720	-25.2593	53.4463
Oral Bait	1	1.5300	4.2226	49	0.36	0.7187	-6.9555	10.0155
Oral Bait	2	5.3250	4.2226	49	1.26	0.2133	-3.1605	13.8105
Oral Bait	3	2.3269	4.2226	49	0.55	0.5841	-6.1587	10.8124
Oral Bait	4	0.1396	4.5141	49	0.03	0.9754	-8.9318	9.2111

Group*Timpepoint Least Square Means								
Group	Timepoint	Est	Std Error	DF	tValue	Pr > |t|	Lower	Upper
Oral Bait	5	2.1750	4.5141	49	0.48	0.6321	-6.8964	11.2464
Oral Bait	6	0.2100	5.3412	49	0.04	0.9688	-10.5235	10.9435
Oral Bait	7	42.6330	5.3412	49	7.98	<.0001	31.8995	53.3665
Oral Bait	8	2.5310	5.3412	49	0.47	0.6377	-8.2025	13.2645
Oral Bait	9	2.8210	5.3412	49	0.53	0.5998	-7.9125	13.5545
Oral	1	0.1313	1.8735	24	0.07	0.9447	-3.7355	3.9980
Oral	2	0.3100	1.6757	24	0.18	0.8548	-3.1486	3.7686
Oral	3	2.6510	1.6757	24	1.58	0.1267	-0.8076	6.1096
Oral	4	0.3594	1.8735	24	0.19	0.8495	-3.5074	4.2262
Oral	5	8.1500	2.1634	24	3.77	0.0009	3.6850	12.6150
Oral	6	0.09833	2.1634	24	0.05	0.9641	-4.3667	4.5633
Oral	7	1.7433	2.1634	24	0.81	0.4282	-2.7217	6.2083
Oral	8	4.6875	2.1634	24	2.17	0.0404	0.2225	9.1525
Oral	9	1.8000	2.1634	24	0.83	0.4136	-2.6650	6.2650

Simple Effect Comparisons of Group*Timepoint Least Squares Means By Timepoint												
Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 1	parenteral	placebo	0.4050	22.0523	53.83	0.02	0.9854	1.0000	-44.6205	43.8105	-51.7173	50.9073
Timepoint 1	oral bait	placebo	1.1250	11.3302	45.04	0.10	0.9213	0.9986	-21.6946	23.9446	-25.2386	27.4886
Timepoint 1	oral	placebo	-0.2737	10.6796	36.14	-0.03	0.9797	1.0000	-21.9300	21.3825	-25.1235	24.5760
Timepoint 2	parenteral	placebo	-1.0500	22.0523	53.83	0.9622	-0.05	0.9998	-45.2655	43.1655	-52.3365	50.2365

Simple Effect Comparisons of Group*Timepoint Least Squares Means By Timepoint												
Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 2	oral bait	placebo	3.3150	11.3302	45.04	0.29	0.7712	0.9698	-19.5046	26.1346	-23.0354	29.6654
Timepoint 2	oral	placebo	-1.7000	10.6467	35.72	-0.16	0.8740	0.9944	-23.2984	19.8984	-26.4607	23.0607
Timepoint 3	parenteral	placebo	8.8600	22.0523	53.83	0.40	0.6894	0.9320	-35.3555	53.0755	-42.4265	60.1465
Timepoint 3	oral bait	placebo	1.7819	11.3302	45.04	0.16	0.8757	0.9947	-21.0378	24.6015	-24.5685	28.1322
Timepoint 3	oral	placebo	2.1060	10.6467	35.72	0.20	0.8443	0.9897	-19.4924	23.7044	-22.6547	26.8667
Timepoint 4	parenteral	placebo	4.3550	22.0523	53.83	0.20	0.8442	0.9903	-39.8605	48.5705	-47.0340	55.7440
Timepoint 4	oral bait	placebo	0.02136	11.4421	46.59	-0.00	0.9985	1.0000	-23.0451	23.0024	-26.6850	26.6423
Timepoint 4	oral	placebo	0.1984	10.6796	36.14	0.02	0.9853	1.0000	-21.4579	21.8546	-24.6885	25.0853
Timepoint 5	parenteral	placebo	7.1760	22.0523	53.83	0.33	0.7461	0.9624	-37.0395	51.3915	-44.2592	58.6112
Timepoint 5	oral bait	placebo	1.4230	11.4421	46.59	0.12	0.9016	0.9975	-21.6008	24.4468	-25.2646	28.1106
Timepoint 5	oral	placebo	7.3980	10.7342	36.85	0.69	0.4950	0.7795	-14.3547	29.1507	-17.6387	32.4347
Timepoint 6	parenteral	placebo	2.2270	24.0883	50.53	0.09	0.9267	0.9991	-46.1431	50.5971	-54.2525	58.7065
Timepoint 6	oral bait	placebo	-0.04800	11.7929	51.44	-0.00	0.9968	1.0000	-23.7183	23.6223	-27.6986	27.6026
Timepoint 6	oral	placebo	-0.1597	10.7342	36.85	-0.01	0.9882	1.0000	-21.9123	21.5930	-25.3281	25.0087
Timepoint 7	parenteral	placebo	49.9820	22.0523	53.83	2.27	0.0275	0.0597	5.7665	94.1975	-1.6442	101.61
Timepoint 7	oral bait	placebo	3.6280	11.7929	51.44	0.31	0.7596	0.9702	-20.0423	27.2983	-23.9800	31.2360
Timepoint 7	oral	placebo	-37.2617	10.7342	36.85	-3.47	0.0013	0.0020	-59.0143	-15.5090	-62.3913	-12.1320

Simple Effect Comparisons of Group*Timepoint Least Squares Means By Timepoint												
Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 8	parenteral	placebo	1.2145	22.6703	57.47	0.05	0.9575	0.9998	-44.1738	46.6028	-51.5924	54.0214
Timepoint 8	oral bait	placebo	1.4435	12.9115	48.07	0.11	0.9114	0.9982	-24.5159	27.4029	-28.6319	31.5189
Timepoint 8	oral	placebo	3.6000	11.9524	36.28	0.30	0.7650	0.9691	-20.6340	27.8340	-24.2412	31.4412
Timepoint 9	parenteral	placebo	13.0573	22.6703	57.47	0.58	0.5669	0.8470	-32.3310	58.4455	-39.7496	65.8641
Timepoint 9	oral bait	placebo	1.7847	12.9115	48.07	0.14	0.8906	0.9966	-24.1747	27.7442	-28.2906	31.8601
Timepoint 9	oral	placebo	0.7637	11.9524	36.28	0.06	0.9494	0.9997	-23.4702	24.9977	-27.0775	28.6050

ESAT6:CFP10
(Model 2 was utilized based on lowest AICC value)

The GLIMMIX Procedure
Response Distribution Gaussian
Link Function Identity
Variance Function Default
Estimation Technique Restricted Maximum Likelihood
Degrees of Freedom Method Kenward-Roger
Fixed Effects SE Adjustment Kenward-Roger

Model 1 Information
DeltaEC repeated measures data
DeltaEC = group|timepoint
Random animalid(group)
Random timepoint/sub=animalid(group) ar(1) residual
Variance matrix not blocked
AICC (smaller is better) 581.49

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	37.58	1.05	0.3838
Timepoint	2	52.87	1.36	0.2649
Group*Timepoint	6	53.49	0.35	0.9070

Model 2 Information
Predicted DeltaEC by group
DeltaEC = group|timepoint
No Random animalid(group)
Random timepoint/sub=animalid(group) group=group ar(1)
residual
Variance matrix blocked by AnimalID(Group)
AICC (smaller is better) 551.47

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	13.99	0.	0.5651
Timepoint	2	22.32	1.43	0.2605
Group*Timepoint	6	26.07	0.91	0.5016

Model 2 Information

DeltaEC = group|timepoint
Random animalid(group) / group=group
No residual correlation structure (no repeatedd measures effect)
Variance matrix diagonal
AICC (smaller is better) 983.41

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	12.55	0.38	0.7663
Timepoint	8	34.08	1.70	0.1340
Group*Timepoint	24	81.79	1.08	0.3869

Simple Effect Comparisons of Group*Timepoint
Least Squares Means By Timepoint

Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 1	parenteral	placebo	1.9430	8.8605	35.65	0.22	0.8277	0.9460	-16.0331	19.9191	-17.1097	20.9957
Timepoint 1	oral bait	placebo	1.3385	9.1644	40.46	0.15	0.8846	0.9803	-17.1769	19.8539	-18.3675	21.0445
Timepoint 1	oral	placebo	1.7147	8.8473	35.36	0.19	0.8474	0.9597	-16.2398	19.6691	-17.3096	20.7389
Timepoint 2	parenteral	placebo	2.6600	8.8605	35.65	0.30	0.7658	0.8921	-15.3161	20.6361	-16.3734	21.6934
Timepoint 2	oral bait	placebo	-2.5688	9.1644	40.46	-0.28	0.7807	0.9060	-21.0841	15.9466	-22.2548	17.1173
Timepoint 2	oral	placebo	-1.0700	8.8325	35.18	-0.12	0.9043	0.9876	-18.9976	16.8576	-20.0431	17.9031
Timepoint 3	parenteral	placebo	0.3240	8.8605	35.65	0.04	0.9710	0.9996	-17.6521	18.3001	-18.7094	19.3574
Timepoint 3	oral bait	placebo	0.6085	9.1644	40.46	0.07	0.9474	0.9977	-17.9069	19.1239	-19.0776	20.2946
Timepoint 3	oral	placebo	1.1790	8.8325	35.18	0.13	0.8946	0.9840	-16.7486	19.1066	-17.7941	20.1521
Timepoint 4	parenteral	placebo	-0.5730	8.8605	35.65	-0.06	0.9488	0.9981	-18.5491	17.4031	-19.6656	18.5196
Timepoint 4	oral bait	placebo	-1.3318	9.2131	41.21	-0.14	0.8858	0.9816	-19.9352	17.2716	-21.1842	18.5206
Timepoint 4	oral	placebo	-0.7042	8.8473	35.36	-0.08	0.9370	0.9965	-18.6586	17.2503	-19.7683	18.3599
Timepoint 5	parenteral	placebo	1.9090	8.8605	35.65	0.22	0.8306	0.9525	-16.0671	19.8851	-17.2273	21.0453

Simple Effect Comparisons of Group*Timepoint Least Squares Means By Timepoint												
Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 5	oral bait	placebo	-0.03530	9.2218	41.34	-0.00	0.9970	1.0000	-18.6545	18.5839	-19.9519	19.8813
Timepoint 5	oral	placebo	0.2505	8.8781	35.73	0.03	0.9776	0.9998	-17.7599	18.2609	-18.9238	19.4248
Timepoint 6	parenteral	placebo	0.006194	8.8902	36.07	0.00	0.9994	1.0000	-18.0227	18.0351	-19.3376	19.3500
Timepoint 6	oral bait	placebo	3.1686	9.3620	43.57	0.34	0.7366	0.8856	-15.7045	22.0417	-17.2016	23.5389
Timepoint 6	oral	placebo	0.2160	8.8898	35.87	0.02	0.9808	0.9999	-17.8156	18.2475	-19.1268	19.5588
Timepoint 7	parenteral	placebo	-27.4150	8.8605	35.65	-3.09	0.0038	0.0046	-45.3911	-9.4389	-46.6817	-8.1483
Timepoint 7	oral bait	placebo	-17.6072	9.3881	43.94	-1.88	0.0674	0.0952	-36.5283	1.3139	-38.0210	2.8066
Timepoint 7	oral	placebo	-28.1494	8.8918	35.92	-3.17	0.0031	0.0037	-46.1842	-10.1146	-47.4841	-8.8147
Timepoint 8	parenteral	placebo	-1.4058	9.9371	35.32	-0.14	0.8883	0.9831	-21.5727	18.7610	-22.8578	20.0462
Timepoint 8	oral bait	placebo	0.6065	10.4245	42.2	0.06	0.9539	0.9986	-20.4281	21.6410	-21.8976	23.1106
Timepoint 8	oral	placebo	-1.0072	9.9639	35.57	-0.10	0.9200	0.9933	-21.2235	19.2090	-22.5169	20.5025
Timepoint 9	parenteral	placebo	-0.5716	9.8826	35.32	-0.06	0.9542	0.9987	-20.6279	19.4847	-21.9117	20.7685
Timepoint 9	oral bait	placebo	1.0844	10.3728	42.22	0.10	0.9172	0.9927	-19.8454	22.0143	-21.3141	23.4830
Timepoint 9	oral	placebo	2.0544	9.9078	35.56	0.21	0.8369	0.9558	-18.0481	22.1569	-19.3401	23.4490

APPENDIX C

Models evaluated for the analysis of lymphocyte blastogenesis data obtained from responses to the following antigens: bovine PPD (SiB), avian PPD (SiA), and ESAT6:CFP10 (SiEC)

<u>BOVINE PPD</u> (Model 3 was utilized based on lowest AICC value as outlined below)*

The GLIMMIX Procedure
Response Distribution Gaussian
Link Function Identity
Variance Function Default
Estimation Technique Restricted Maximum Likelihood
Degrees of Freedom Method Kenward-Roger
Fixed Effects SE Adjustment Kenward-Roger

Model 1 Information
SiB repeated measures data
SiB = group|timepoint
Random animalid(group)
Random timepoint/sub=animalid(group) ar(1) residual
Variance matrix not blocked
AICC (smaller is better) 586.06

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	29.12	0.24	0.8647
Timepoint	2	44.07	0.24	0.24
Group*Timepoint	6	45.56	0.82	0.5622

Model 2 Information
Predicted SiB by group
SiB = group|timepoint
No Random animalid(group)
Random timepoint/sub=animalid(group) group=group ar(1) residual
Variance matrix blocked by AnimalID(Group)
AICC (smaller is better) 587.54

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	14.51	0.43	0.7320
Timepoint	2	31.55	0.26	0.7709
Group*Timepoint	6	24.07	0.70	0.6489

Model 3 Information
SiB = group|timepoint
Random animalid(group) / group=group
No residual correlation structure (no reptd meas effect)
Variance matrix diagonal
*AICC (smaller is better) 578.63

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	35.32	0.52	0.6740
Timepoint	2	55.14	0.25	0.7760
Group*Timepoint	6	41.05	0.80	0.5724

Simple Effect Comparisons of Group*Timepoint
Least Squares Means By Timepoint

Simple Effect Level	Group	Group	Est.	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 1	parenteral	placebo	-3.5212	5.8441	32.31	-0.60	0.5510	0.8983	-15.4208	8.3784	-17.9664	10.9240
Timepoint 1	oral bait	placebo	5.6581	7.5934	27.26	0.75	0.4626	0.8269	-9.9153	21.2315	-13.1110	24.4272
Timepoint 1	oral	placebo	-7.0472	7.3240	26.57	-0.96	0.3446	0.6924	-22.0863	7.9919	-25.1504	11.0560
Timepoint 2	parenteral	placebo	8.1118	5.5161	30.31	1.47	0.1517	0.3690	-3.1489	19.3724	-5.5489	21.7724
Timepoint 2	oral bait	placebo	3.1371	7.3440	24.99	0.43	0.6729	0.9611	-11.9884	18.2625	-15.0503	21.3244
Timepoint 2	oral	placebo	5.7013	6.6963	25.13	0.85	0.4026	0.7691	-8.0865	19.4890	-10.8821	22.2846
Timepoint 3	parenteral	placebo	5.7820	5.6551	31.34	1.02	0.3144	0.6603	-5.7466	17.3107	-8.2329	19.7970
Timepoint 3	oral bait	placebo	2.5586	9.0696	23.25	0.28	0.7804	0.9883	-16.1922	21.3094	-19.9185	25.0358
Timepoint 3	oral	placebo	10.7683	8.2285	23.16	1.31	0.2035	0.4693	-6.2470	27.7836	-9.6242	31.1608

<u>Avian PPD</u> (Model 3 was utilized based on lowest AICC value as outlined below)*

The GLIMMIX Procedure
Response Distribution Gaussian
Link Function Identity
Variance Function Default
Estimation Technique Restricted Maximum Likelihood
Degrees of Freedom Method Kenward-Roger
Fixed Effects SE Adjustment Kenward-Roger

Model 1 Information
SiA repeated measures data
SiA = group|timepoint
Random animalid(group)
Random timepoint/sub=animalid(group) ar(1) residual
Variance matrix not blocked
AICC (smaller is better) 610.15

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	30.79	0.55	0.6546
Timepoint	2	26.35	1.07	0.3585
Group*Timepoint	6	30.22	1.46	0.2267

Model 2 Information
Predicted SiA by group
SiA = group|timepoint
No Random animalid(group)
Random timepoint/sub=animalid(group) group=group ar(1) residual
Variance matrix blocked by AnimalID(Group)
AICC (smaller is better) 575.07

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	13.91	1.37	0.2915
Timepoint	2	19.90	1.91	0.1737
Group*Timepoint	6	24.31	0.70	0.6555

Model 3 Information
SiA = group|timepoint
Random animalid(group) / group=group
No residual correlation structure (no repeatedd measures effect)
Variance matrix diagonal
*AICC (smaller is better) 568.12

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	31.23	1.69	0.3697
Timepoint	2	28.43	1.72	0.3932
Group*Timepoint	6	36.16	0.64	0.6946

Simple Effect Comparisons of Group*Timepoint
Least Squares Means By Timepoint

Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 1	parenteral	placebo	-5.8905	14.7079	24.99	-0.40	0.6922	0.9475	-36.1822	24.4013	-41.5130	29.7321
Timepoint 1	oral bait	placebo	-2.0530	7.1155	24.92	-0.29	0.7753	0.9785	-16.7101	12.6040	-19.2869	15.1808
Timepoint 1	oral	placebo	-6.4620	6.3732	17.6	-1.01	0.3243	0.5899	-19.8732	6.9491	-21.8979	8.9738
Timepoint 2	parenteral	placebo	20.2788	14.3403	23.15	1.41	0.1706	0.3559	-9.3756	49.9332	-14.6662	55.2239
Timepoint 2	oral bait	placebo	-0.7725	6.3209	27.87	-0.12	0.9036	0.9984	-13.7230	12.1779	-16.1756	14.6305
Timepoint 2	oral	placebo	-7.0613	5.4345	18.18	-1.30	0.2101	0.4211	-18.4706	4.3480	-20.3044	6.1818
Timepoint 3	parenteral	placebo	-2.5077	14.4946	23.94	0.17	0.8641	0.9960	-32.4270	27.4117	-37.9594	32.9440
Timepoint 3	oral bait	placebo	-6.7497	7.2360	30.58	-0.93	0.3582	0.6684	-21.5160	8.0165	-24.4480	10.9485
Timepoint 3	oral	placebo	-4.6994	5.9740	19.34	-0.79	0.4410	0.7651	-17.1883	7.7895	-19.3109	9.9121

<u>ESAT6:CFP10</u> (Model 3 was utilized based on lowest AICC value as outlined below)*

The GLIMMIX Procedure
Response Distribution Gaussian
Link Function Identity
Variance Function Default
Estimation Technique Restricted Maximum Likelihood
Degrees of Freedom Method Kenward-Roger
Fixed Effects SE Adjustment Kenward-Roger

Model 1 Information
SiEC repeated measures data
SiEC = group|timepoint
Random animalid(group)
Random timepoint/sub=animalid(group) ar(1) residual
Variance matrix not blocked
AICC (smaller is better) 581.49

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	37.58	1.05	0.3838
Timepoint	2	52.87	1.36	0.2649
Group*Timepoint	6	53.49	0.35	0.9070

Model 2 Information
Predicted SiEC by group
SiEC = group|timepoint
No Random animalid(group)
Random timepoint/sub=animalid(group) group=group ar(1) residual
Variance matrix blocked by AnimalID(Group)
AICC (smaller is better) 551.47

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	13.99	0.	0.5651
Timepoint	2	22.32	1.43	0.2605
Group*Timepoint	6	26.07	0.91	0.5016

Model 3 Information

SiEC = group|timepoint
Random animalid(group) / group=group
No residual correlation structure (no repeatedd measures effect)
Variance matrix diagonal
AICC (smaller is better) 610.15

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	3	30.79	0.55	0.6546
Timepoint	2	26.35	1.07	0.3585
Group*Timepoint	6	30.22	1.46	0.2267

Simple Effect Comparisons of Group*Timepoint
Least Squares Means By Timepoint

Simple Effect Level	Group	Group	Est	Std Error	DF	tValue	Pr > |t|	Adj P	Lower	Upper	Adj Lower	Adj Upper
Timepoint 1	parenteral	placebo	3.9697	10.1909	21.27	0.39	0.7008	0.9676	-17.2072	25.1466	-21.1610	29.1004
Timepoint 1	oral bait	placebo	-3.2320	4.6322	32.74	-0.70	0.4903	0.8477	-12.6591	6.1951	-14.6550	8.1910
Timepoint 1	oral	placebo	-5.0941	3.9977	31.17	-1.27	0.2120	0.4768	-13.2457	3.0576	-14.9525	4.7643
Timepoint 2	parenteral	placebo	14.5212	10.0668	20.35	1.44	0.1644	0.3804	-6.4546	35.4970	-10.3614	39.4038
Timepoint 2	oral bait	placebo	7.8408	4.3525	31.11	1.80	0.0813	0.2072	-1.0349	16.7165	-2.9174	18.5990
Timepoint 2	oral	placebo	2.7378	3.5455	31.88	0.77	0.4457	0.8116	-4.4852	9.9608	-6.0258	11.5013
Timepoint 3	parenteral	placebo	6.3160	10.1187	20.74	0.62	0.5393	0.8910	-14.7434	27.3753	-18.7313	31.3632
Timepoint 3	oral bait	placebo	3.3275	5.2524	28.85	0.63	0.5314	0.8868	-7.4173	14.0723	-9.6740	16.3290
Timepoint 3	oral	placebo	0.5958	4.1701	31.72	0.14	0.8873	0.9984	-7.9015	9.0931	-9.7267	10.9184

APPENDIX D

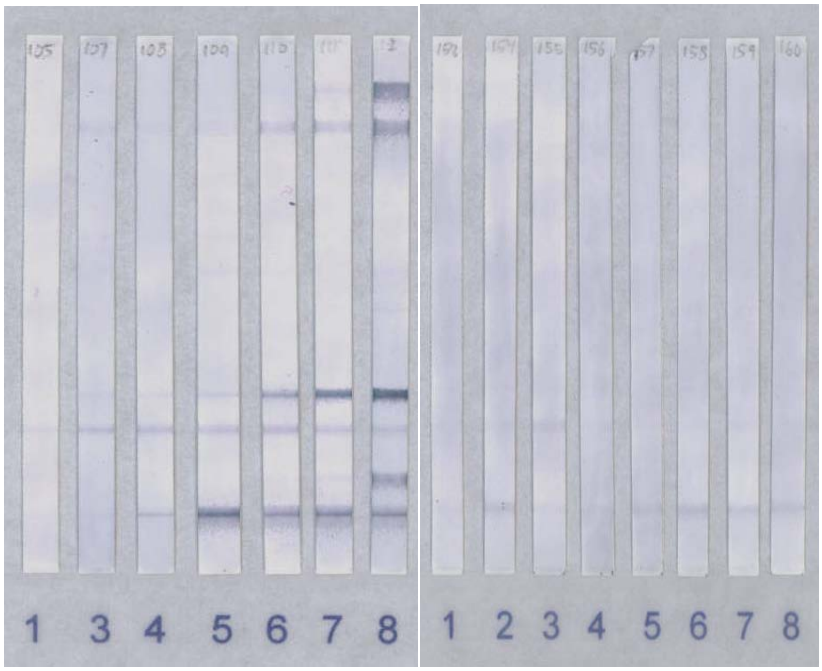
CervidTB STAT-PAK

Control Line

Test Line

APPENDIX E

MAPIA



Nonvaccinate Vaccinate