THESIS

EVALUATING THE EFFICACY OF A MYCOBACTERIUM BOVIS VACCINE IN FERAL SWINE

Submitted by

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ABSTRACT

EVALUATING THE EFFICACY OF A MYCOBACTERIUM BOVIS VACCINE IN FERAL SWINE

Bovine tuberculosis (bTB) is a globally significant zoonotic disease caused primarily by *Mycobacterium bovis* (*M. bovis*) transmission between wildlife, domestic livestock, and humans. Unfortunately in wildlife reservoirs of bTB, disease rates are increasing worldwide due to ecological dynamics and challenges in wildlife management. Despite effective, long-standing *M. bovis* eradication programs in the US, expanding wildlife reservoir habitat and importation of people, animals, and products from the Mexican dairy industry have become sources of zoonotic bTB infection. Currently, no tuberculosis vaccine is labeled for use in animals, although a vaccine could provide a new tool in preventing bTB in wildlife and domestic livestock. Bacille Calmette-Guerin (BCG), a live, attenuated *M. bovis* strain vaccine used for tuberculosis prevention in humans has been variably effective in reducing bTB development in studies on various species. We hypothesize that Texas-origin feral swine vaccinated orally with either modified-live BCG or inactivated *M. bovis* vaccine will have fewer, less severe lesions than non-vaccinated feral swine after virulent *M. bovis* challenge. In this study we test this hypothesis along with the immunologic response to vaccination and infection by measuring antibody levels in vaccinated and unvaccinated swine. Our results demonstrate that vaccination with BCG or inactivated strains of *M. bovis* do not confer protection against infectious challenge with a virulent Michigan strain of *M. bovis*.

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1. Introduction and Literature Review

1.1 *Mycobacterium bovis* – a long history

Mycobacterium bovis (*M. bovis*) is a member of the "*M. tuberculosis* complex" group of *Mycobacterium* bacteria that cause the disease tuberculosis in most mammalian species. *Mycobacterium bovis* is also an etiologic agent of bovine tuberculosis (bTB), the most significant zoonotic form of tuberculosis – primarily infecting cattle, but transmitted between wildlife, other domestic livestock, and humans (1-3). Tuberculosis has plagued the earth since the beginning of recorded human history, if not earlier, and is still in the top 10 causes of death in the world as of 2015 (4, 5). In the early to mid-1900's the United States (US) and the United Kingdom (UK) implemented test and cull eradication campaigns in hopes of ridding their cattle herds of bTB. Their efforts eventually reduced the incidence of bTB in both countries to under 1% for the national cattle population, and to completely disease-free herds and areas of the country (2-4). In the UK and US, the disease rates remained very low until the 1980's and 90's when bTB began to spread in greater numbers of animals, herds, and across species (6). The Eurasian Badger (*Meles meles*) in the UK and the white-tailed deer (*Odocoileus virginianus*) in Michigan, USA are well-established wildlife reservoirs of bTB that are infected by and transmit to domestic livestock (2). These badger and deer populations, serving as maintenance reservoirs for bTB, are an important factor in the failure to eradicate bTB in these countries over the past few decades. The UK and US examples among multiple countries, highlight the necessity to develop new, improved control and prevention efforts in developed areas of the world where wildlife

reservoirs are currently involved in reemergence of bTB as a significant source of zoonotic disease. If better methods being engineered to reduce bTB prevalence in the developed world have practical application, ideally, they could be translated for use in the developing world.

1.2 Microbiology of *Mycobacterium bovis*

Mycobacterium species *(spp.)* are a gram positive, rod-shaped bacterial species with a thick, wax and lipid-rich cell membrane which make them essentially impervious to traditional Gram staining techniques, so acid fast stains such as Ziehl-Neelson must be utilized to visualize them under a light microscope. The outer α-glucan capsular layer, along with the waxy and lipid-rich cell wall, can adapt to its host and environment, making *M. bovis* and other mycobacteria particularly resistant to host immune destruction, desiccation, acidity/low pH, and antibiotics (7). Standard histopathologic fixation and slicing of tissues includes hematoxylin-eosin (H & E) staining of the tissues in order to visualize the hallmark of *M. bovis* and other *M*. *tuberculosis* complex species infections, the tuberculous granuloma, which will be discussed in following sections. Also difficult to harvest in bacterial culture outside of the living host, these slow-growing organisms are grown over 8-12 weeks – much longer than other bacteria (8).

1.2.1 *Pathophysiology*

Transmission of pathogenic *M. bovis* bacteria occurs during direct contact with another animal by exposure to bodily fluids and excretions (blood, mucus, urine, milk, saliva, feces, etc.) or tissues – e.g. adult animals inhaling aerosolized particles from

each other, neonates drinking infected milk from their mother, or a fetus acquiring the infection transplacentally through maternal to fetal blood supply (4). It can also occur indirectly by ingestion of contaminated food or milk, skin penetration of the organism via wounds, or by coming in contact with fomites and grazing areas on which shedding animals have deposited infectious particles (2). Aerosol routes of transmission are believed to be the most common between adults of the same species, while ingestion is the most common route of transmission to dairy calves and people drinking raw milk, as well as for grazing domestic livestock and wildlife (4, 9).

Once a host is infected with *M. bovis*, the bacteria will multiply in macrophages, cells that trigger the host's immune system, to develop characteristic granulomatous structures, known as tubercles, at the site of bacterial multiplication. Depending on the species, tubercles may consist of various combinations of immune cell layers including B cells (bone marrow lymphocytes), T cells (thymic lymphocytes), neutrophils, natural killer (NK) cells, Langerhan's multinucleated giant cells, foam cells, and dendritic cells that form around an accumulation of bacteria as they invade host macrophages (4). As granulomas mature, they may form caseous, necrotic centers of debris that can further develop calcification as the bacteria proliferate and accumulate dead cells and waste products in these lesions. Tubercles typically have a thick fibrous tissue encapsulation, but less so in some species like deer, in which they grossly appear more like abscesses. The tubercles can be large - inches across in diameter - or microscopic. This granulomatous process may occur in nearly every host tissue, but are more likely in some tissues depending on the host species and disease ecology in a particular area of the world.

1.2.2 *Immunology*

Much of the immunologic mechanism behind Mycobacterial host immunity has not yet been elucidated or is contradictory, and most of our knowledge is from *M. tuberculosis* studies with humans and mice. We do know that innate and acquired immune defenses are both involved in fighting against *M. bovis* infections, and will vary to a significant extent in each host, depending on virulence of an *M. bovis* strain, the host species, and individual host genetic variation. The innate immune system is a non-specific defense unit of the host's immunity and involves cells such as neutrophils, macrophages, T cells, as well as inflammation-mediating chemicals known as cytokines (9). Alternatively, the acquired/adaptive immune system learns to develop defenses against pathogens after being exposed to them in a vaccine or from an infection, so that a more rapid, efficient response to pathogen invasion can be produced upon subsequent infection. This adaptive process is led by antigen-presenting cells (APC), T cells, B cells, cytokines such as interferon gamma (IFNγ) and interleukins (IL), and antibodies among other immune system components (10).

Most of the cells and molecules responsible for the formation of tuberculous granulomas and the necrosis or caseation at their interior, are the result of innate immune responses to *M. bovis* infection initiated by macrophages that both harbor and help destroy this pathogen (11). Delayed-type hypersensitivity reactions and chronic inflammation are involved in granuloma development, recruiting immune cells to surround infected macrophages and wall off the site with fibrous tissue. Isolating infection from the rest of the body and aiding macrophages in destroying *M. bovis*, these granulomas may successfully shield the rest of the immune system from

developing a fulminant infection and acquired immunity. Mycobacteria have their own protective mechanisms to evade the immune system by manipulating the complement system and macrophage dynamics. For example, after phagocytosis of *M. bovis* by macrophages, the bacteria can bind complement receptor 3 and mannose receptors to down-regulate the immune response (3). This prevents release of cytotoxic reactive oxygen molecules and blocks microbicidal phagosomal activity within macrophages and activation of adaptive immunity, leading to intracellular sequestration and avoidance of immune system recognition of mycobacteria.

If mycobacteria are successful in escaping the innate immune system defenses of macrophages and the tuberculous granuloma, the bacteria will multiply and spread to other parts of the body, triggering adaptive immune system responses while developing fulminant infection. A component of acquired immunity involves the cells mentioned above, and relies on the host immune system to recognize and adapt to antigenic stimulation, resulting in antibody production. Antigens are chemical structures recognized by the immune system as foreign, presented on the surface of APC's, stimulating T cells with many defensive roles and B cells to construct antibodies that recognize, bind, and help systematically destroy an organism, in this case mycobacteria. The level of antibody formation following mycobacterial infections is somewhat dependent on species and immune competency. Because cattle mount a significant cell-mediated innate immune response, successfully walling off *M. bovis* in granulomatous foci, antibody production is limited until advanced progression of disease (11). In cattle, high antibody levels are believed to be an indication of poor immune defense and inability to isolate bacteria in tubercles. Mycobacteria of multiple species

and strains have similar antigenic structures, allowing the antibodies of a host to bind a variety of mycobacteria, despite only being elicited by a single species. This is one mechanism by which vaccines that are produced with *M. bovis* may be used to defend against tuberculosis caused by other mycobacterial species (e.g. *M. tuberculosis*), but often lead to an incomplete protective immunity against infection and can interfere with testing to differentiate between infected and vaccinated animals.

Cattle or other bovine species are the primary hosts for *M. bovis* and tend to have better species-specific immunity against this bacteria. In comparision to most mammals, cattle have more of the unconventional γδ T cells that make them more resistant to *M bovis* infection (11). Cattle and other ruminants also have a higher concentration of the cytokines Interleukin-4 (IL-4) and IFN-γ that are released in to the bloodstream in response to mycobacterial infections, making these immunologic markers for detecting infection or response to vaccination in these species. Vaccines designed to prepare the adaptive immune system for *M. bovis* infection and diagnostic tests to detect immunologic response to vaccination have been goals of ongoing research with *M. bovis*.

Initial studies in European wild boar allowed for the discovery of genes expressing complement component 3 (C3), IFN-γ, IL-4, “Regulated on Activation Normal T Expressed and Secreted Cytokine” (RANTES or CCL5), and methylmalonyl CoA mutase (MUT) levels are upregulated in vaccinated animals and serve a protective and inflammatory function against *M. bovis* infection (12, 13). The level of C3 and MPB83/NADPAD antibodies produced against *M. bovis* in vaccinated animals was also found to correlate with lack of disease severity measured by lesion and culture scoring

(13). Ongoing identification of molecular markers (genes, cytokines, proteins, etc) of *M. bovis* infection and vaccine-mediated immunity in feral swine and other species is key to further development of diagnostic and preventative technologies for species-specific monitoring and control of tuberculosis (14-17).

1.2.3 *Clinical manifestation*

Bovine tuberculosis is typically chronic, and slowly progressive in onset, although acute forms can develop. The disease can progress more rapidly in patients with immunosuppression, including those with comorbidities, those that are older with waning immune system function as well as a longer and higher risk of exposure over time, and those with high or chronic levels of stress – which allow for fulminant infection or reactivation of a latent infection in an asymptomatic carrier (1). Unfortunately, outward appearances make it difficult to identify disease or transmission risk, as not all infected hosts are shedding the organism that can transmit disease, and not all shedding animals have obvious clinical signs of disease.

Clinical signs or symptoms of tuberculosis vary widely, but are often associated with lymphadenopathy or pulmonary pathology. The lungs and lymph nodes of the head, neck and thorax are the common sites of lesions in hosts infected by inhalation (9). Other lymph nodes, organs, gastrointestinal tract, nervous system, genitourinary, and hematogenous (bloodstream) sites of infection are less common locations seen across the many host species. Lymphadenopathy may lead to fistula tracts draining caseous material from superficial lymph nodes to the skin surface. Other signs may include ptyalism respiratory distress, dysphagia, airway turbulence, edema and pain.

Pneumonia and other respiratory or cardiac dysfunction related to lung damage such as pleuritis, cough, hemoptysis, dyspnea, and pericarditis can be seen. Abscesses, organ failure, diarrhea or constipation, disorientation and lack of mental appropriateness, and severe weight loss are other signs of fulminant *M. bovis* infection (4).

In field studies on wild boar performed in Spain and Italy, *M. bovis* infection was predominantly found in the mandibular, bronchial, mediastinal, and mesenteric lymph nodes as well as in the lungs of naturally infected animals hunted in the wild (18, 19). All infected animals had mandibular lymph node lesions and one third (30%) of those infected only had mandibular lymph node lesions (18). This was in contrast to field studies performed on feral pigs in Australia, where a greater percentage (62%) of animals had only mandibular lymph node lesions, no pulmonary involvement, and less disseminated (systemic) disease manifestation (20). This difference in disease severity is important to note due to epidemiologic implications that will be discussed in the following section. No draining fistulae at the skin surface originating from the lymph nodes were found in pigs; these lesions are suspected to be a source of transmission from some other species (2). Recognition of the mandibular lymph nodes as the major site of infection, and histologic identification of granulomatous lesions breaking out into excretory ducts of the mandibular salivary glands, suggests that oral secretions containing *M. bovis* are one likely source of transmission from pigs. In the Spanish study conducted by Martín et. al.,2006, 17% of the wild boar that were euthanized for diagnosis of tuberculosis that cultured positive for tuberculosis (22 out of 127) showed no macroscopic lesions, and only half of these pigs (11 out of 22) had detectable

microscopic lesions, demonstrating the importance of performing tests beyond gross post-mortem examination to definitively diagnose tuberculosis (18).

1.3 Epidemiology

1.3.1 *The Global Picture*

According to the World Health Organization, the annual incidence (new cases) of tuberculosis in humans is increasing every year, and in 2015, 10.4 million people were newly infected and 1.8 million died from the disease (5). Approximately 35% of people with human immunodeficiency virus infection die because they develop tuberculosis, emphasizing the danger of contracting this disease in immune-compromised populations (1, 5). Zoonotic transmission risk of *M. bovis* is higher in developing countries where livestock and wildlife management, sanitation protocols, and food safety regulation are not easily achieved (1, 21). The morbidity and mortality rates associated with bTB will vary significantly depending on the species and environment in which the disease is found. Nevertheless, bTB can be a very costly disease for the farmers and governments responsible for a region's cattle industry. Approximately 50 million cattle were infected with *M. bovis* globally as of the late 1990's, with a US $3-4 billion annual cost to agriculture at that time (2). On a domestic scale, eradication efforts put forward to successfully create a bTB-free designation in the state of Minnesota alone after an outbreak in 2005 cost federal and state agricultural organizations over US $85 million dollars (2).

1.3.2 *Human-Wildlife-Livestock Interface*

In some developed countries like the US, New Zealand, Spain, Ireland, and the UK, there have been outbreaks and increasing incidence of bTB despite long-standing test and cull programs for domestic cattle (2, 3, 22, 23). In regions with established "maintenance" wildlife reservoirs (animals that readily transmit the pathogen to and from other susceptible host species) of bTB such as white-tailed deer (*Odocoileus virginianus*) in Michigan, European badger (*Meles meles*) in Ireland and the UK, and wild boar (*Sus scrofa*) in Europe, the incidence of tuberculosis is increasing due to these wildlife reservoir populations outgrowing their habitats and due to the complexities in their management (24-26). New Zealand is one country with an established wildlife reservoir of bTB, the brushtail possum (*Trichosurus vulpecula*), that has successfully reduced disease incidence by intensive population control of this invasive non-native species, as it simultaneously promotes conservation of forest ecosystems (27). The likelihood of spillover to wildlife and spillback to domestic livestock can be encouraged by a number of factors including transport of wildlife to new areas, feed supplementation of wildlife that crowds animals into one area, protected wildlife species legislation leading to uncontrolled population growth, and failed attempts to keep wildlife off of land that domestic livestock inhabit (2).

Typically, zoonotic and reverse-zoonotic spread of bTB is via direct contact with cattle or consumption of contaminated raw dairy or meat products, but can also be the result of direct contact with captive wildlife (in zoos, rehabilitation centers, sanctuaries) and infected game carcasses (4, 28, 29). Transmission between animals and people also depends on disease ecology – population density, severity and length of disease

as it affects shedding capacity, geographical influences on the range and overlap of multiple-species habitats, and climate in regards to survival of host and pathogen in the environment (2, 18). *M. bovis* is particularly good at surviving in cool, moist climates with limited sunlight, and was found to persist up to 88 days in soil, 58 days in water and hay, and 43 days on corn in a study done in Michigan to investigate the risk of indirect transmission between cattle and white-tailed deer (30). Studies in Spain on wild boar populations have identified artificial water and feeding sites, scarce water sources in drought season, containment in fenced hunting and wildlife refuges, and translocations as factors encouraging aggregation and close interaction that increases *M. bovis* transmission risk within and across species (26).

"Dead-end" hosts, as opposed to maintenance reservoir hosts, may be designated in species that tend to be infected, but typically develop mild to non-existent clinical signs or pathologic lesions, so that transmission does not readily occur from them to other hosts. Determining whether a particular species is considered a maintenance reservoir versus dead-end host will vary based on the disease ecology, socioeconomic environment, and epidemiology – e.g. disease prevalence and incidence – in a particular area of the world (2). For example, feral pigs in the previously mentioned Australian study were considered a dead-end host of bTB, because the vast majority of cases had lesions limited to a localized area of the body – the mandibular lymph nodes (20). In studies with European wild boars, disease manifestation was disseminated throughout the body, more severely affecting this species, so they are considered a maintenance reservoir in this location (18). DNA sequencing, restriction fragment length polymorphism (RFLP) analysis, and spoligotyping (DNA fingerprinting

techniques) have confirmed that the genotypes of *M. bovis* strains in European wild boar and North American white-tailed deer matched those from cattle within their respective regions. These molecular epidemiologic tools by which wildlife hosts of bTB can be identified as maintenance reservoirs are used to trace and implicate transmission events (21, 31, 32).

Despite previously effective *M. bovis* eradication strategies in domestic cattle herds and federally regulated pasteurization of dairy products in the US, bTB persistence in wildlife reservoirs and constant importation of cattle and dairy products from Mexico are expanding sources of zoonotic bTB infection in the US (22, 29, 33-35). Along with the northern US (Michigan) having to improve bTB control efforts with the region's white-tailed deer bTB reservoir, there has also been a notable increase in bTB incidence in Southern California. The incidence of bTB in cattle has persisted in both Michigan and California, not dropping low enough to designate a tuberculosis-free status (36). In pockets of the US, where the increasingly popular practice of consuming unpasteurized, fresh cow milk and cheese is occurring, people are contracting bTB from contaminated raw dairy products. A global study on zoonotic tuberculosis found particularly high percentages of bTB incidence in Latin American communities of San Diego, New York, and from Mexico compared to the proportion of human tuberculosis due to *M. bovis* around the world (4). In San Diego county, California from 1994-2005, the incidence of *M. tuberculosis* was falling while the incidence of *M. bovis* was increasing, with 45% of childhood and 6% of adult tuberculosis cases caused by *M. bovis* (37). Also concerning is that the likelihood of death from zoonotic *M. bovis* in this setting is twice the rate of death caused by *M. tuberculosis*, and 2.6-8.3 times higher in

HIV patients in various parts of the US (4). The exact cause of this higher mortality rate is not fully understood, but it may be attributed to limited health care access for this ethnic population, inappropriate treatment with *M. tuberculosis* targeted drugs and timeframes, and perhaps the difficulty of treating extra-pulmonary forms of tuberculosis due to ingestion rather than inhalation of *M. bovis* (29).

1.3.3 *Wild and Feral Swine*

More than 15 countries have discovered bTB in wild or feral swine species (22, 29, 34, 38-40). A few broad surveillance studies on feral swine in the Southern Texas border region (41), other US mainland states, and Hawaii (35, 42, 43) found a lack of *M. bovis*, despite some of these animals' proximity to known outbreaks of bTB in cattle and cervid populations. It is important to determine whether feral swine in the US are currently negative for *M bovis*, as they pose a potential risk for becoming a reservoir, and epidemiologic surveillance should be ongoing if outbreaks continue to occur in cattle and human populations. The map of the US mainland and Hawai'i (Figure 1) illustrates the range of expansion in the feral swine population as it has grown from 1982 to 2015 (44).

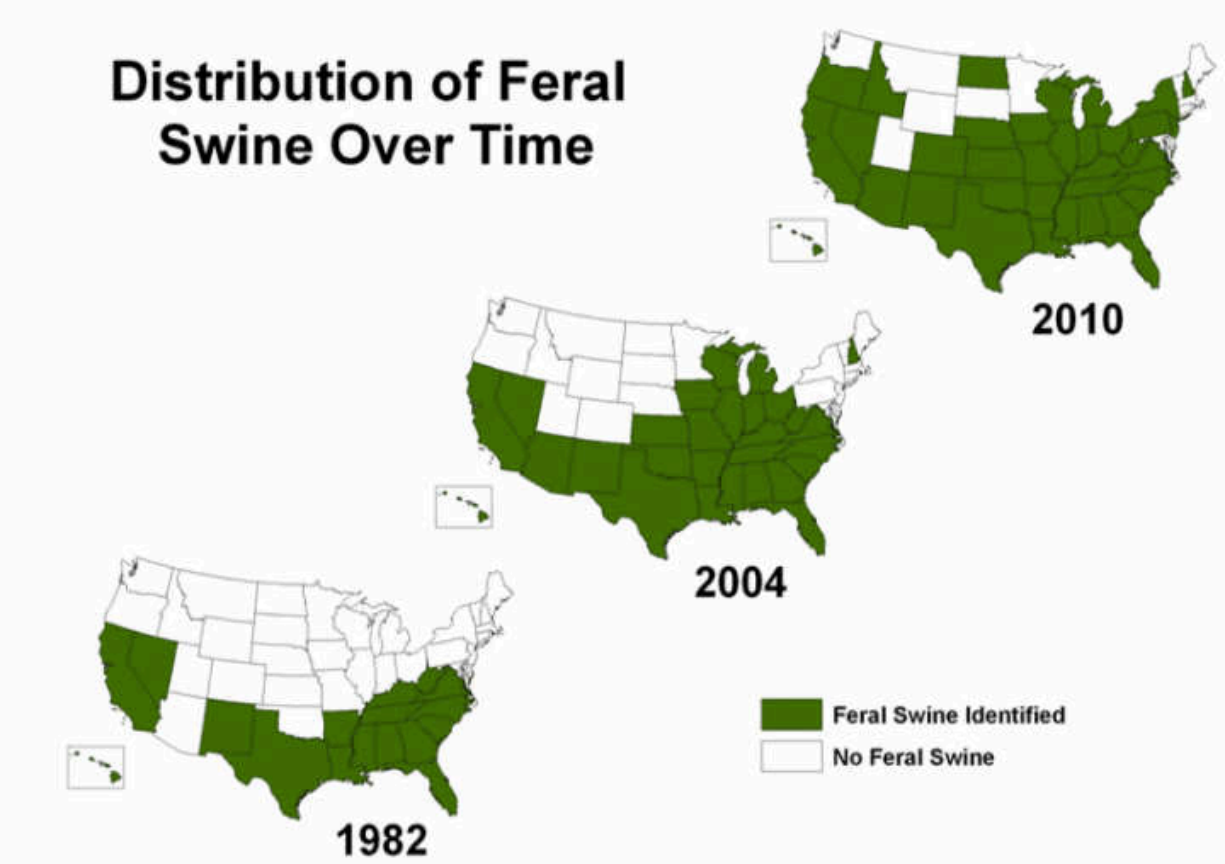

Figure 1: USDA map of range of expansion in the feral swine population from 1982 to 2010 (44).

As these swine populations expand across states and into northern Mexico where bTB infection exists in cattle, white-tailed deer, and humans, the risk of feral swine becoming maintenance reservoirs is increasing (35, 36, 45). Ongoing epidemiologic and ecologic surveillance of infectious disease, behavior, migration, and interspecies interactions in feral swine in these areas of North America will be essential (35). Updated studies should be performed on Molokai island where bTB last had a significant prevalence of approximately 20% (38), but was largely mitigated by culling infected cattle, swine, and deer. A more recent survey of feral swine found 5 out of 482 animals testing positive for *M. bovis* (1%) on Molokai, with transmission being prevented by keeping cattle away from areas inhabited by feral swine (35). Because swine are culturally protected and

prized as hunting bounty on Molokai, population growth as well as exposure of people to infected game carcasses could pose a transmission risk. Feral swine population dynamics, intensive livestock grazing, the practice of wild game feeding, baiting, and hunting in broadening ranges of the US form a combined threat of multi-species *M. bovis* transmission as it has been demonstrated in European wild boar and North American white-tailed deer (22, 46).

1.4 Diagnosis, Treatment, Prevention

1.4.1 *Diagnosis*

Culture is the gold standard for diagnosis of *M. bovis*, but the most practical methods for testing large populations of people and animals are less time consuming and more cost effective (28). *Mycobacterium bovis* is harvested using one of the following culture media: modified Löwenstein-Jensen (with pyruvate and without glycerol), Stonebrink's, tuberculosis bovine blood agar, or modified Middlebrook 7H11 agar. Culture samples are typically collected from post-mortem tissues in animals, but is more often an ante-mortem diagnostic tool when used in humans.

Intradermal testing is the most readily performed test to diagnose tuberculosis in developed countries. It can be done using tuberculin purified protein derivative (PPD), an *M. bovis* antigen that is injected into the dermal layer of skin and examined after 72 hours for a delayed type hypersensitivity (DTH) reaction caused by recognition of the acquired cellular immune system to the antigen after pre-exposure or infection with *Mycobacterium spp.* (skin swelling and induration/reddening at site of injection). This type of skin test is used routinely in dairy cattle herds and in the human population to

detect infection or exposure to *M. bovis* or *M. tuberculosis* with a sensitivity of about 70-75% (11). It is used in countries that do not have endemic tuberculosis, because a positive result would occur in too many patients being tested in parts of the world where vaccination or disease are common, as it does not differentiate vaccination from exposure/infection. There can also be a cross-reaction between different mycobacterial species, complicating this test result, so confirmatory testing may need to be performed. For example, human patients are most often infected with *M. tuberculosis* and dairy cattle may be infected with *M. avium paratuberculosis* (MAP), although they both have the potential to react to the *M. bovis* antigen whether they are actually infected with *M. bovis* or not (4). A comparative cervical intradermal tuberculin skin test with antigen from MAP is one test that can differentiate an *M bovis* infection from a non-specific DTH reaction to mycobacteria in animals. Additionally, a test that must be read 72 hours later is not a feasible way to monitor wildlife that are difficult to capture and should not be subjected to repeated stressful handling in such a short time (28). Other confirmatory tests recognizing cell-mediated immune responses to disease are lymphocyte proliferation and IFN-γ whole blood assays (4). The cytokine IFN-γ, as discussed in the immunology section, is upregulated during mycobacterial infection, so levels can be monitored in some ruminant, human, and non-human primate species that the test has been validated for (11, 28). The IFN-γ release and Interleukin-17A assays, as well as a skin test using *M. tuberculosis* complex rather than *M. bovis* PPD antigens are some useful diagnostic tests to differentiate infected from vaccinated animals (DIVA tests), but more of these types of DIVA tests are needed (47-52).

Enzyme linked immunosorbant assays (ELISA) and similar tests have been developed to detect specific anti-*M. bovis* antibody detection in serum. Serologic tests tend to be logistically better for application in the field, as the antibodies are stable during sample transport, and the costs are relatively low compared to other tests with similarly high specificity (28). As discussed in the above immunology section, animals may have varying levels of antibody response to bTB and *M. bovis* vaccination based on species, age, and stage of disease. Serological assays such as the lateral-flow immunochromatographic test, the species-specific multi-antigen print immunoassay (MAPIA), and the dual-path platform assay (DPP) have been developed to detect a variety of antibodies. Antibody detection is especially ideal for wildlife *M. bovis* testing, as some of them are rapid, “animal-side” tests or can be run on large numbers of animals at once, but are currently only commercially available for use in elephants, some deer and camelid species, badgers, and primates (28). In one study with wild boar, the DPP assay had the highest specificity (89.6%) and the bovine purified protein derivative (bPPD) ELISA was the test with the highest sensitivity (100%) depending on the cutoff value used (28). A PPD based ELISA has the potential for cross-reactivity, as it does in the tuberculin skin test form, so this can lower specificity due to the presence of environmental mycobacteria in some animals. Additionally, testing an animal once for antibody levels has limited utility for interpreting disease prevalence on its own, as it can indicate previous exposure as well as concurrent infection.

Polymerase chain reaction (PCR) or other genotyping technologies for DNA fingerprinting of mycobacterial strains (Restriction Fragment Length Polymorphism or spoligotyping) are more precise although lengthy techniques, as some are used to

identify *Mycobacterium spp.* after culture isolation or post-mortem examination has been performed (31, 32, 53). These methods can be used to test populations of subjects in any epidemiological climate, although with more significant costs associated with laboratory equipment and supplies.

Ultimately, the goal is to develop a diagnostic that combines a DIVA test with high specificity for *M. bovis*, availability at a reasonable cost, high efficiency to test with one sampling event, compatibility with field use, and production of timely results when simultaneously testing large numbers of multiple species (28). In most developing countries, advanced confirmatory testing methods are not available, so differentiation between infections with *M. bovis* and other *Mycobacteria spp.* are not made (4). This limits making the distinction between zoonotic and human-to-human transmitted tuberculosis, therefore limiting the ability to adequately track, monitor, prevent and treat infections as well.

1.4.2 *Treatment*

The conventional treatment for tuberculosis is a 6-month course of isoniazid, rifampin, ethambutol, and pyrazinamide antibiotics in people. Although there is an effective medical cure for human patients with uncomplicated *M. tuberculosis* infections, extrapulmonary tuberculosis, especially caused by *M. bovis,* is notoriously difficult to treat (29). *Mycobacterium bovis* has an innate resistance to pyrazinamide antibiotics, and an extended course of antibiotics should be used to effectively treat *M. bovis* infections. Unfortunately, most physicians around the world do not differentiate between *M. tuberculosis* and *M. bovis* before initiating treatment in people, and research has

shown that *M. bovis* causes a higher rate of mortality than *M. tuberculosis* (29). Multi-drug resistant and rifampin resistant strains of tuberculosis also complicate therapy, and were found in 580,000 combined new cases in 2015 (4). Treating animals with multi-drug therapy is not typically practiced, as it does not inhibit spread of infection, complete cure is difficult to achieve, it tends to be cost prohibitive, and it may not be allowed in food-producing species.

1.4.3 *Prevention and Control*

Since the early 1900's, most of the developed world has kept the incidence of bTB in domestic livestock extremely low with monitoring and control programs (33, 54, 55). Government and industry organizations in the US have carried out effective bTB eradication efforts in domestic livestock using tuberculin skin testing and removal protocols, as well as carcass inspection and condemnation at slaughter (56). Complete eradication will not be possible if wildlife reservoirs continue to maintain the disease due to domestic livestock transmission (spillover) and wildlife transmission back to the domestic species (spillback) (2). While culling wildlife reservoirs may appear to be a feasible solution to the threat of bTB at the wildlife-livestock-human interface, it may go against protections for certain wildlife species – based on whether the species is native or non-native, invasive or not, socially valued, etc. – and may not be effective given the particular environment in which these control efforts are made (3, 6, 29, 57). As described above, multiple approaches to enhanced disease surveillance, wildlife management, and control in all species involved have been explored over the last few decades (26, 35, 42, 58-61), and goals have been to minimize disruption of natural

habitats, avoid stressful handling of animals, and optimize efficacy of biotechnology in a restrictive field setting. No tuberculosis vaccine is available for widespread distribution in animals, but an effective and practical, ideally species-specific, vaccine would provide a key addition to preventing the spread of disease and reducing financial burden of bTB in wildlife, humans, and domestic livestock (2, 55, 62).

1.5 *Mycobacterium bovis* Vaccination

1.5.1 *Bacille Calmette-Guérin (BCG) Modified-live M. bovis Vaccine*

BCG is a live, attenuated strain of *M. bovis* that has been used to vaccinate people against tuberculosis worldwide since the 1920's. It was developed by the two French scientists it was named after, and is used regularly to vaccinate babies and young children in populations with endemic tuberculosis. Although BCG has been the standard for tuberculosis vaccination for nearly a century, and is the only vaccine licensed for use in people, there are notable disadvantages to its use in human patients. BCG only induces the most consistent and effective protection in immunologically naïve human vaccinates (3). Efficacy is measured by a more consistent reduction in the severity of clinical disease, thereby reducing the shedding and transmission of virulent tuberculosis-causing organisms into the environment as well (3, 55). Ongoing research has determined factors interfering with vaccine success in people, most notably in developing countries, such as exposure to environmental mycobacteria, chronic parasitic infestation, co-morbidities with immunosuppressing infections (e.g. HIV), malnutrition, and dosage of tuberculosis pathogen exposure (55). These factors certainly translate to vaccination failure in animals, as BCG has shown variable efficacy

in animal studies as well – geographic location, breed or species of animal, age of the animal at vaccination, and the disease ecology can all play a role (3, 63, 64). Although veterinary research trials being conducted around the world have shown BCG to impart some protection against experimental or natural challenge with *M. bovis* in animal reservoirs such as badgers, white-tailed deer, possums, cattle, buffalo, and wild boar, it is not always reliable at preventing significant disease, and limited testing has been performed in the field (11, 12, 24, 64-72). Additional disadvantages to using the vaccine in animals is that, as a modified-live vaccine, BCG is unstable for extreme temperature fluctuations in an outdoor setting and poses an exposure hazard to people and non-target species when administered in non-endemic areas (73). BCG must be used cautiously, if at all, in locations where exposure to the vaccine can cause a disease-positive test result – i.e. countries like the US where skin testing is the primary method for diagnosis in people and cattle (3). Although feral swine that have been vaccinated with BCG did not carry traces of the *M. bovis* vaccine strain in their tissues on necropsy, and did not cause unvaccinated animals inhabiting the same environment to react to TB skin testing, another study on white-tailed deer did demonstrate persistence of BCG in tissues, but not in co-habiting animals (39, 54, 65, 95). This would theoretically allow us to administer BCG safely in a controlled setting, but may be impractical for dispersal to large populations of wildlife that have the potential to be consumed by people. If the vaccine were released into the environment itself from spillage or in a dispensed form, it could cause unwanted exposure and positive tuberculin skin tests in both domestic cattle and people (68).

This is one reason why ongoing improvements to diagnostic tests that differentiate infection from vaccination (DIVA tests) will also make development of a universally adaptable vaccine strategy easier as well (3, 54, 55, 62, 74).

1.5.2 *Alternatives to BCG vaccines*

Development of a more reliably efficacious, practical, stable, and less biohazardous vaccine than BCG is needed for use in vaccinating wildlife and domestic animals (3, 4, 14, 23, 24, 26). In the last few decades, vaccine trials in animals have demonstrated improved efficacy of vaccination when using BCG in combination with a booster of DNA, protein, or viral vector subunit vaccine (11, 75-77), but they are not efficacious when used on their own. Oral baits for delivery of vaccines have been developed for non-invasive, mass distribution to wildlife populations, and some successful palatability and efficacy results in feral swine/wild boar studies in Spain and the US have been demonstrated (39, 59, 73, 78-81). Heat-inactivated vaccines have been tested as well, as they are more stable under temperature fluctuations than attenuated vaccines, and do not pose a risk of tuberculin skin or IFN-γ release assay testing reactions in domestic cattle that ingest them in oral baits (13, 73, 82).

The first research on *M. bovis* vaccination in wild boar was performed in Spain, showing that oral bait administration of either BCG or an inactivated (heat killed) *M. bovis* vaccine resulted in significant decreases in bTB disease severity after experimental infection when compared to unvaccinated control groups (12, 13, 73). This was the first attempt to vaccinate against tuberculosis in any wildlife species using a temperature-stable, killed-inactivated *Mycobacterium spp.* vaccine (73).

2. Evaluating the Efficacy of *Mycobacterium bovis* Vaccines in Feral Swine

2.1 Introduction

The threat of bovine tuberculosis (bTB) transmission in the US is rising as a result of greater interaction between wildlife, livestock and humans, lack of consistently safe and effective control measures in wildlife, importation of cattle and dairy products from Mexico, and wildlife population expansion (36, 55). Eradication efforts in developed countries, including the US, helped reduce the prevalence of *M. bovis* in domestic livestock to very low levels, but these populations are continually being threatened with outbreaks as the wildlife reservoirs of bTB are maintaining the disease with spillover and spillback phenomena (2, 83, 84). As wild boar serve as established wildlife reservoirs of bTB in several European countries, feral swine have the potential to become reservoirs in North America under similar pressures from disease ecology, and development of a reliable vaccination scheme would help mitigate an outbreak involving this species (22, 35).

While the modified-live *M. bovis* bacille Calmette-Guerin (BCG) is the only vaccine labeled for use in humans, it has not been able to consistently stop transmission of disease in enough people on its own to control tuberculosis in most of the world. Likewise, it confers variable protection against *M. bovis* infection in human and animal species (3, 55, 64). An ideal bTB vaccine template for use in wildlife species would be stable in the field, minimize stressful animal handling, and be safe for any person or animal exposed to it during distribution. Testing of inactivated *M. bovis* strains and oral bait delivery systems have had some promising results in initial wild boar

studies in Spain (12, 73). Demonstrating the palatability of oral baits, and efficacy of BCG and inactivated *M. bovis* vaccines, Spanish wild boar trials have shown less bTB disease severity in vaccinates when compared to unvaccinated controls (12, 59, 78, 79). Wild boar vaccinated with an initial and booster BCG vaccine and then challenged with virulent *M. bovis* revealed a 75.8% and 66.9% reduction in lesion and culture scores, respectively, when compared to control animals (24). Another Spanish study using parenteral and orally-administered inactivated *M. bovis,* as well as oral modified-live BCG vaccines, demonstrated a reduction in average lesion scores by 43.3%, 43.3% and 52.2%, and a reduction in average thoracic culture scores by 66.7%, 33.3%, and 66.7%, respectively, in comparison to non-vaccinated controls (73).

In this study, we compared the efficacies of orally administered heat-inactivated *M. bovis* strains obtained from Spain and Michigan, USA to each other and to modified-live BCG vaccines administered before experimental infection with virulent *M. bovis* in Texas-origin feral swine. Our study was in collaboration with researchers from previous Spanish trials at the Instituto de Investigación en Recursos Cinegéticos (IREC) Universidad de Castilla-La Mancha in Ciudad Real, Spain and Neiker Tecnalia in Bilbao, Spain. We were not able to demonstrate that any of the three *M. bovis* vaccines were protective in the face of pathogenic *M. bovis* challenge. This research also showed no significant difference between IgG antibody levels against bPPD antigen, macroscopic or microscopic (histopathology) lesion scores, or culture between vaccinated and control pigs.

2.2 Materials and Methods

2.2.1 *Subjects*

Twenty nine piglets were produced from a tuberculosis-free captive population of Texas-origin feral swine housed at the United States Department of Agriculture (USDA)/Colorado State University (CSU) Wildlife Research Facility (WRF) in Fort Collins, Colorado, USA. The piglets were born in early spring 2015 and were housed with access to fresh water and pelleted pig feed in outdoor pens at the WRF and later indoors at the Animal Disease Laboratory (ADL), a biosecurity level 3 facility on the CSU Foothills campus. All experiments were approved by the CSU Institutional Animal Care and Use Committee (IACUC) under protocol #14-5367A.

2.2.2 *Inactivated Michigan/Spanish strain M. bovis vaccine preparation*

The Spanish and Michigan *M. bovis* strain killed-inactivated vaccines were prepared by Neiker Tecnalia in Bilbao, Spain as previously described (73). The Spanish *M. bovis* strain (#1403, Neiker) was obtained from a naturally infected wild boar in Spain. The Michigan *M. bovis* strain (14-03411) was obtained from a cow in a bTB outbreak in 2014 in Michigan, USA. The isolates were grown in Middlebrook 7H9 enriched broth containing Oleic acid-Albumin-Dextrose-Catalase (OADC Enrichment; Difco) for 2-3 weeks. After harvesting via centrifugation at 2500 x g for 20 minutes, cells were washed twice in phosphate buffered saline (PBS). Bacterial pellets were resuspended in PBS and a fine needle syringe was used for declumping. The optical density of suspension turbidity was adjusted to 5 McFarland units. The antigenic dose was measured by determining the colony forming units (CFU) per unit volume in the

production batch, with ten-fold serial dilutions prepared and plated onto agar-solidified 7H9 with OADC in quadruplicate. The inocula were inactivated in an airtight bottle in a water bath at 83-85°C for 45 minutes. Four 0.5mL samples of the inactivated batch were inoculated in BACTEC Mycobacterial Growth Indicator tubes (Becton Dickinson) and on separate OADC agar solidified 7H9 plates (100μL each) and monitored for 60 days to confirm negative growth and viability of the bacterial isolates. The oral vaccine was shipped from the manufacturer at a concentration of $1x10^6$ CFU killed *M. bovis* per 0.2mL and was diluted at 3mL per 12mL of 7H9 to provide $1x10^6$ CFU killed *M. bovis* in 1mL of diluted fluid volume.

2.2.3 *Modified-live BCG strain M. bovis vaccine preparation*

Frozen BCG stock was received courtesy of T. Thacker at the USDA/ARS National Animal Disease Center, Ames, Iowa, USA. The BCG stock was frozen at a concentration of 7.9 x 109 cfu/mL and thawed and diluted to 2.6 x 106 cfu/mL in phosphate buffered saline (PBS) as a working solution.

2.2.4 *Vaccination*

Swine subjects were randomly divided into four treatment groups: 1) eight animals were orally administered 1mL of $1x10^6$ CFU heat-killed *M. bovis* Michigan strain [Michigan group], 2) seven animals were orally administered 1mL of $1x10^6$ CFU heat-killed *M. bovis* Spanish strain [Spanish group], 3) seven animals were orally administered 1mL of $1x10^6$ CFU modified-live BCG [BCG group], and 4) seven animals were orally administered 1mL water as controls [control group]. All four groups were

administered vaccine or water via syringe attached to a urinary catheter aimed at the oropharyngeal mucosa, introduced into the mouth through an oral speculum. Animals were manually restrained in a Panepinto pig sling (Figure 2) for these procedures (85). The two groups receiving killed *M. bovis* (Spanish or Michigan) were vaccinated in August 2015 (prime/initial) and September 2015 (1 month booster). The two groups receiving BCG and placebo vaccine were administered only one dose in August 2015. Each group was housed completely isolated from one another throughout the entire experiment.

Figure 2: Pig subject restrained in the Panepinto sling
For vaccination via oral speculum and catheter.

2.2.5 *Challenge with Pathogenic Mycobacterium bovis*

Two months after initial vaccination (October 2015), all animals were anesthetized and transported in a standard stock trailer a distance of 0.5 miles from the WRF to the ADL. Either a combination of intramuscular butorphanol-medetomidine-

midazolam (0.3 mg/kg; 0.06 mg/kg; 0.3 mg/kg respectively) or telazol-medetomidine (3 mg/kg; 0.05 mg/kg respectively) was used for anesthesia in each subject administered.

Stocks of Michigan deer *M. bovis* strain TB14-03411 were received from the National Veterinary Services Laboratory and inoculated onto agar plates. Those cultures were incubated at 37C for 17 days, at which time nice lawns had developed. Plates were flooded with phosphate buffered saline (PBS) and bacteria were harvested with a swab. Those suspensions were sonified to break up clumps and counted using a Petroff-Hausser chamber. The resulting titer was 2.3×10^9 bacteria/5 ml, and that suspension was diluted 1:2300 in PBS to yield an inoculum with a concentration of 10^6/5 ml.

After an acclimation period of 42 days in the ADL, 4 months post-initial vaccination, all animals were anesthetized with the telazol-medetomidine as previously described (86) via darts (Pneudart Inc., Williamsport, PA, USA) projected from a CO2-powered dart pistol (DanWild LLC, Austin, TX, USA). The subjects were then challenged with 1×10^6 *M. bovis* bacteria as described above, following a previously developed protocol and verified adequate dose for experimental infection via oropharyngeal route (12).

2.2.6 *Enzyme Linked Immunosorbant Assay (ELISA) Serology*

Blood samples of approximately 5mL were collected by phlebotomy using a 1.5 inch, 18 gauge needle and 10mL syringe from the anterior vena cava of all swine either while in Panepinto sling restraint at the WRF or under anesthesia in the ADL. Samples were obtained from pigs pre-vaccination (time 1), four weeks post-vaccination with killed

vaccines (time 2), sixteen weeks post-vaccination/time of pathogenic *M. bovis* challenge (time 3), five weeks post-challenge (time 4), and at time of euthanasia and necropsy/fifteen weeks post-challenge (time 5). The serum was analyzed for immunoglobulin G (IgG) antibodies against bovine purified protein derivative (bPPD) *M. bovis* antigen using ELISA. Serum antibody levels were determined using a bovine purified protein derivative (bPPD) ELISA protocol developed by researchers at the Instituto de Investigación en Recursos Cinegéticos (Institute for Game and Wildlife Research - IREC) Sanidad y Biotecnoligía (Health and Biotechnology - SaBio) group at the Universidad de Castilla-La Mancha (University of Castilla-La Mancha) in Ciudad Real, Spain. The bPPD ELISA was designed to detect protoplasmic antigen antibodies associated with *M. bovis* infection. The protocol was optimized for performance at the CSU Animal Population Health Institute Laboratory using an ELx 405 autoplate washer (Bio-Tek Instruments, Inc.) as described previously (87) with some alterations (see Figure 4 for schematic of technique): after coating the plates with 50 μL of cervical bPPD tuberculin (lot # 31-CER 1401, June 2016, NVL – Ames, IA) at a concentration of 5 μL PPD/mL of bicarbonate buffer (Pierce Biotechnology, Inc., Rockford, IL) for 48 hours at 4˚C, the wells were washed 6 times with phosphate buffered saline (PBS) (AMRESCO MidSci, Solon, Ohio) solution containing 0.05% Tween 20 – (BioRad Labs, Hercules, California) (PBST) and blocked for 1 hour at room temperature (RT) by adding 100 μl of 5% skim milk (BioRad Labs) in PBST to each well. After aspirating the blocking solutions out of each well, a 1:40 dilution of sera in PBS were added to plates (50 μl/well) diluted down the plate into 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560 concentrations. For the positive control, dilutions of 1:200, 1:400, 1:800, 1:1600,

1:3200, and 1:6400 were tested, and for the negative control a 1:40 dilution was used. Plates were incubated for 1 hour at RT. Samples were tested in duplicate on each plate, while positive and negative controls were tested in singles on every testing plate. Positive and negative control serum was obtained from confirmed *M. bovis* culture-positive and culture-negative wild boar from Spain (kindly provided by C. Gortazar and T. Anderson). After washing 6 times with PBST, recombinant protein G-peroxidase conjugate (1mg/mL, catalog number 31449 ThermoFisher Scientific, Waltham, MA USA) milk blocking solution was added to each well at a concentration of 0.5 μL protein G/mL as a conjugate (50 μL per well) and incubated at RT for 80 minutes in the dark before washing again 6 times with PBST. Color production was induced using 50 μL/well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Promega Corp., Madison, WI, USA) for 20 minutes in darkness. The reaction was stopped with 50 μL/well of 3N sulfuric acid, and optical density (OD) was measured in a Spectra MAX plus 384 microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) at 450nm absorbance. Serum sample results were represented by the average OD (at a 1:40 dilution) for each sample and compared at each time frame (Time 1-Time 5). Antibody levels were also represented using an ELISA percentage (E%) and calculated as previously described (13, 60, 73, 87) using the formula:

Sample E = (mean sample OD/2 X mean negative control OD) X 100

If a sample E% was greater than 1, it was considered positive.

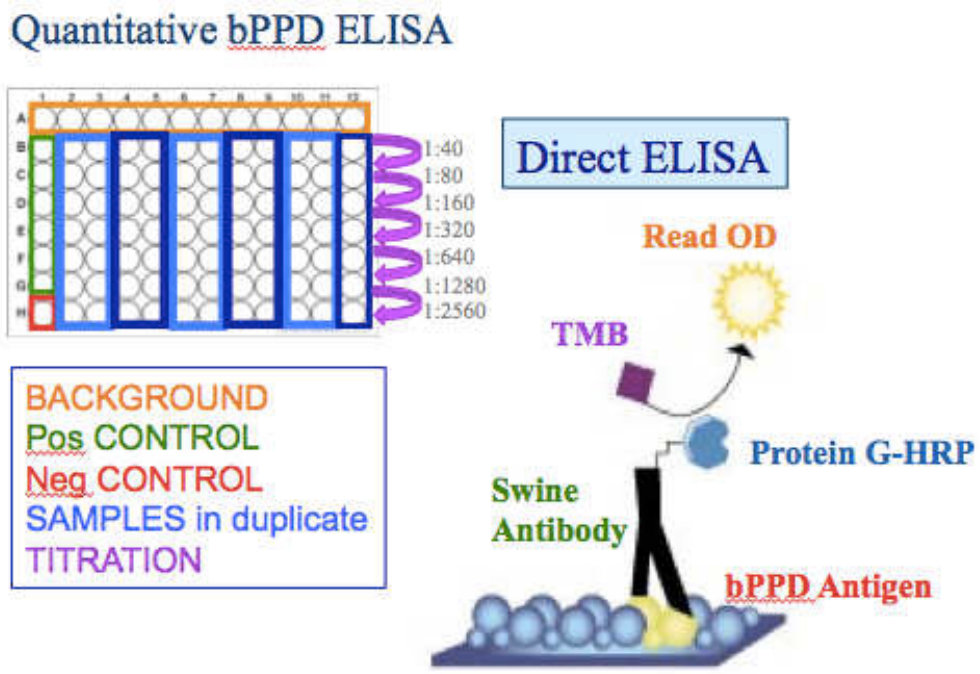

Figure 3: Enzyme linked immunosorbant assay (ELISA) diagram illustrating the titration technique used on each individual plate and the reagent steps use in the protocol developed by SaBio-IREC (Ciudad Real, Spain).

2.2.7 *Euthanasia and Necropsy with Lesion Scoring*

In March 2016, 15 weeks post-challenge with pathogenic *M. bovis*, all groups were anesthetized with the telazol-medetomidine protocol described above, humanely euthanized via captive bolt gun, and necropsied at the ADL immediately after time of death. Prior to euthanasia, none of the animals in any group displayed overt signs of clinical disease. Assessment of gross pathology was noted on post mortem examination. Tissues targeted for examination included: lung lobes (diaphragmatic, cranial, cardiac, accessory), visceral organs (liver, spleen, kidney, ileocecal intestinal junction), and lymph nodes (mandibular, parotid, retropharyngeal, mediastinal, tracheobronchial, bronchial, superficial cervical, mesenteric, ileocecal, tonsils, and hepatic) – see score worksheet in Appendix 1. Macroscopic lesion scoring was

performed according to a previously published rubric (73) and was based on size and number of lesions. Lung lobe scoring ranged from 0-5: 0 = no visible lesion, 1 = few <1cm caseous foci ("A" lesions) scattered throughout lobe, 2 = numerous or clustered "A" lesions with some coalescence of foci, 3 = "A" lesions densely clustered throughout the lobe, 4 = at least one lesion >1cm ("B" lesion), 5 = two or more "B" lesions. Visceral organ scoring ranged from 0-2: 0 = no visible lesion, 1 = 1-2mm foci scattered throughout the organ, 2 = 5-10mm diameter clusters of 1-2mm foci or a single "B" lesion. Lymph node scoring ranged from 0-4: 0 = no visible lesion, 1 = 1-2 small "A" lesions, 2 = several "A" lesions, 3 = several foci with at least one "B" lesion, 4 = diffusely distributed lesions. Calcification and necrosis were also noted for each target tissue examined as an indication of more advanced lesion pathology.

2.2.8 *Histopathology and Bacterial Culture*

Histopathologic examination was performed on all target lymph nodes and tissues listed above and in Appendix 1 by fixing cut tissues in 10% neutral-buffered formaldehyde, embedding them in paraffin, and slicing them at approximately 4-5μm thickness before staining with hematoxylin-eosin stain. Some slides were also selected to be fixed with acid fast stain as well to help detect the presence of acid-fast bacilli. Slide fixation was performed at USDA, Animal and Plant Health Inspection Service (APHIS), National Veterinary Services Laboratory (NVSL) in Ames, Iowa. Slides were read on a standard light microscope to detect the presence of microscopic lesions (with guidance kindly provided by Jack Rhyan, USDA/APHIS National Wildlife Research Center, Fort Collins, CO). The presence or absence of microgranulomas (early lesions),

characteristic tuberculous granulomas and mature lesions with calcified, necrotic centers were all noted in each target tissue. Any detection of acid fast bacilli was also noted.

Mycobacterial cultures were performed on the same target tissues that were collected in Whirl Pak® (Nasco, Fort Atkinson, Wisconsin, USA) bags and stored at -70 ^{o}C until being sent to the USDA APHIS NVSL in Ames, Iowa. Mycobacterial cultures were performed as previously described (88). Briefly, tissues were trimmed, homogenized in a phenol red broth and decontaminated with 4% NaOH for 10 minutes. Once samples were neutralized with 6% HCl, they were centrifuged at 4,600 x g and the pellet was inoculated into both BACTEC MGIT media and 7H11 Middlebrooks with hemolyzed blood, calf serum, pyruvate and malachite green as additives. Media were incubated according to manufacturer's recommendations, and signal positive tubes or bottles were examined for the presence of acid fast bacteria. If the media signaled positive prior to 42 days and no acid fast organisms were detected, they were incubated at 37C for the full 42 days before being restained. All suspicious colonies were identified as *M. bovis* by PCR (Dykema, 2016).

2.3 Statistical Analysis

R: A language and environment for statistical computing (Vienna, Austria) was used for analyses online. Data across all four groups and all variables showed skewed distribution when plotted as histograms. Taking that into consideration, without normality, Spearman non-parametric correlation and linear regression were both used to analyze the relationship of lesion scores with histopathology, culture, and serum

antibody levels between all pigs. Kruskall-Wallis non-parametric tests were used to compare treatment groups by these individual variables (lesion score, histopathology, culture, antibody levels), to compare average antibody levels at each time point (1-5), and to test for confounding by gender bias.

2.4 Results

2.4.1 *Necropsy: Gross pathology*

On necropsy, the severity of gross pathology was assessed by macroscopic lesion scoring of target tissues listed in the methods sections and displayed on the necropsy worksheet (Appendix 1). Any exceptions to those listed in the rubric were also noted at the time of post mortem examination and during tissue preparation of histology slides. These exceptions included areas of tissue pathology that could not be grossly confirmed as a tuberculosis-related lesion or may have been in a tissue that was not considered a target organ. Superficial cervical lymph nodes with areas of characteristic granuloma formation were seen in 7 out of 8 pigs from the Michigan group. Suspect lymphoid hyperplasia of mesenteric, hepatic, ileocecal, parotid lymph node or tonsilar tissues were seen in 3 Michigan, 3 Spanish, 3 BCG, and 1 control group pig. Abscesses of the tonsils were seen in one Michigan pig. Central calcification or necrosis was noted in the mandibular or hepatic lymph nodes of 2 control, 2 BCG, and 1 Michigan pig, and was noted in the liver and spleen of 1 control and 1 Michigan pig.

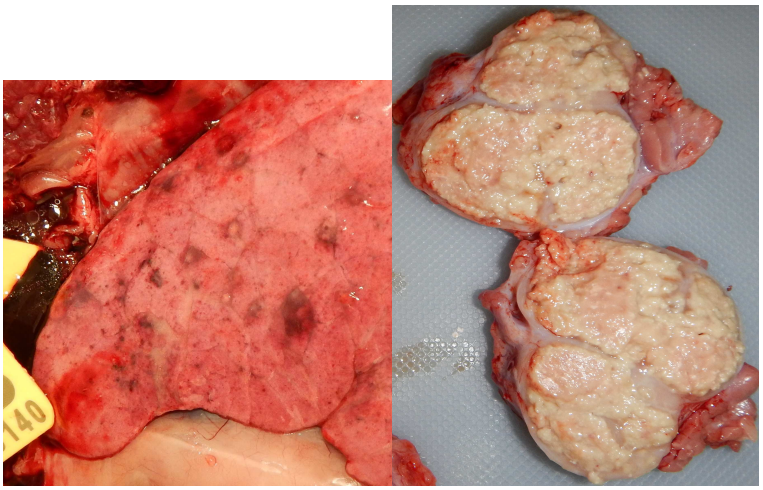

Figure 4 **Figure 5**

Figure 4: macroscopic granulomatous tubercules at parenchymal surface of the lung lobe.
Figure 5: cross section of a mandibular lymph node with lesions measuring 8x10cm across.

2.4.2 *Necropsy: Lesion scoring*

Total lesion scores from each pig were tallied and graphed as a group then compared to the other treatment groups – see Tables 1-4 and Figure 7. The mean treatment group scores from lowest to highest were: 6.1 (Spanish), 6.7 (Control), 8.1 (BCG), and 15.25 (Michigan). The median scores from lowest to highest were: 5 (Control and BCG), 6 (Spanish), and 11.5 (Michigan). Total group scores from lowest to highest were: 43 (Spanish, n=7), 47 (Control, n=7), 57 (BCG, n=7), and 122 (Michigan, n=8). Every animal, regardless of group, had macroscopic lymph node lesions. The BCG vaccination group had the lowest total lymph node lesion scores, although this group had the animal with the most severe lung lesions. The Control and Spanish *M. bovis* vaccination groups had no lung lesions. The Spanish *M. bovis* vaccination group was the only group that had no lesions on major organs (liver, spleen, kidney). The Michigan *M. bovis* vaccination group had the animals with the most severe organ and lymph node lesions as well as the animal with the highest lesion scores overall (Pig ID: 38 with a score of 37). Lesions scores ranged from 2 (BCG ID: 4, Spanish ID: 20) to 37.

Table 1: Gross lesion scores in the Control group with individual pig totals, system (lung/organ/lymph node) totals, and overall group total, mean, and median.

GROUP	Control							
PIG ID	37	17	2	3	19	26	30	Total
LUNGS	0	0	0	0	0	0	0	0
ORGANS	0	0	1	2	0	1	0	4
LYMPH N	10	4	12	5	4	4	4	43
TOTAL	10	4	13	7	4	5	4	47
Control group total = 47, Mean = 6.7, Median = 5								

Table 2: Gross lesion scores in the Michigan *M bovis* strain vaccine group with individual pig totals, system (lung/organ/lymph node) totals, and overall group total, mean, and median.

GROUP	Michigan								
PIG ID	9	27	38	28	8	4	22	29	Total
LUNGS	0	4	8	0	0	0	0	4	16
ORGANS	2	2	4	1	0	3	3	3	18
LNN.	10	14	25	10	8	3	6	12	85
TOTAL	12	20	37	11	8	6	9	19	122
Michigan group total = 122, Mean = 15.25, Median = 11.5									

Table 3: Gross lesion scores in the Spanish *M bovis* strain vaccine group with individual pig totals, system (lung/organ/lymph node) totals, and overall group total, mean, and median.

GROUP	Spanish							
PIG ID	39	10	18	21	1	20	7	Total
LUNGS	0	0	0	0	0	0	0	0
ORGANS	0	0	0	0	0	0	0	0
LYMPH N	6	6	5	3	7	2	14	43
TOTAL	6	6	5	3	7	2	14	43
Spanish group total = 43, Mean = 6.1, Median = 6								

Table 4: Gross lesion scores in the BCG *M bovis* strain vaccine group with individual pig totals, system (lung/organ/lymph node) totals, and overall group total, mean, and median.

GROUP	BCG							
PIG ID	6	11	5	23	25	12	24	Total
LUNGS	0	0	0	0	15	0	0	15
ORGANS	0	0	1	0	0	1	0	2
LYMPH N	4	6	4	8	12	4	2	40
TOTAL	4	6	5	8	27	5	2	57
BCG group total = 57, Mean = 8.1, Median = 5								

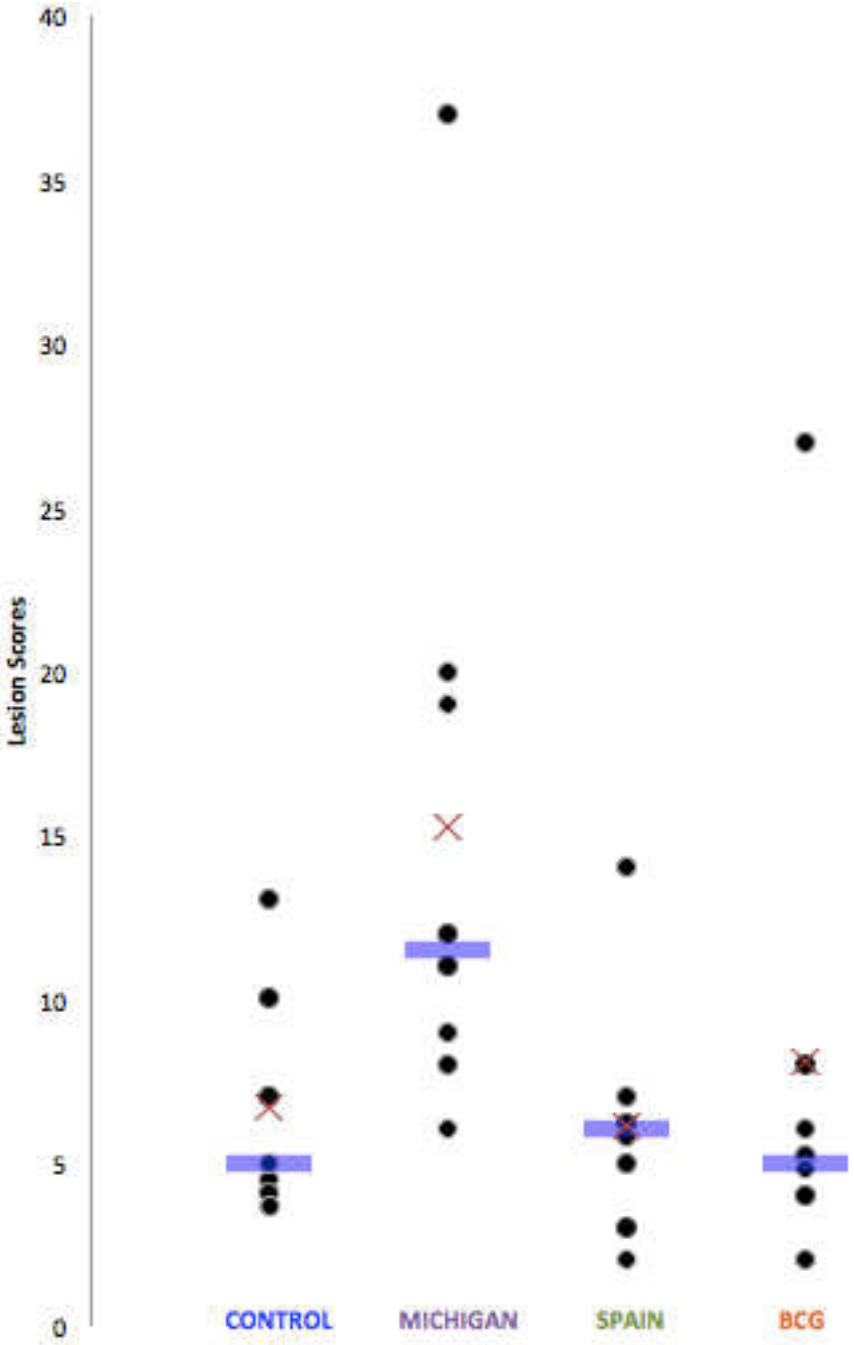

Figure 6: Gross lesion scores (totals from each individual animal) compared across all 4 groups. The red X's signify means and blue bars signify medians for each group.

2.4.3 *Histopathology*

Microscopic lesions were identified as present or not, and differentiation between immature and mature lesions with calcified/mineralized centers was made in each tissue. The presence of acid fast bacilli on special stained slides was noted in very few slides, and typically only 1 organism was identified on the entire slide (results not reported). Table 5 shows the results of histopathologic analysis on each pig. All pigs, regardless of treatment group, developed lymph node lesions, and only 2 (1 control and 1 BCG pig) did not have calcified necrosis of these lesions. 7 out of 8 Michigan pigs and 1 BCG pig were the only animals with major organ lesions on histopathology. Lung lesions were scattered among all treatment groups. The Michigan treatment group had animals with the most lung, major organ, and lymph node lesion locations. The average number of locations with lesions per group from smallest to largest were 3.6 (Control and Spanish), 4.6 (BCG), and 7 (Michigan).

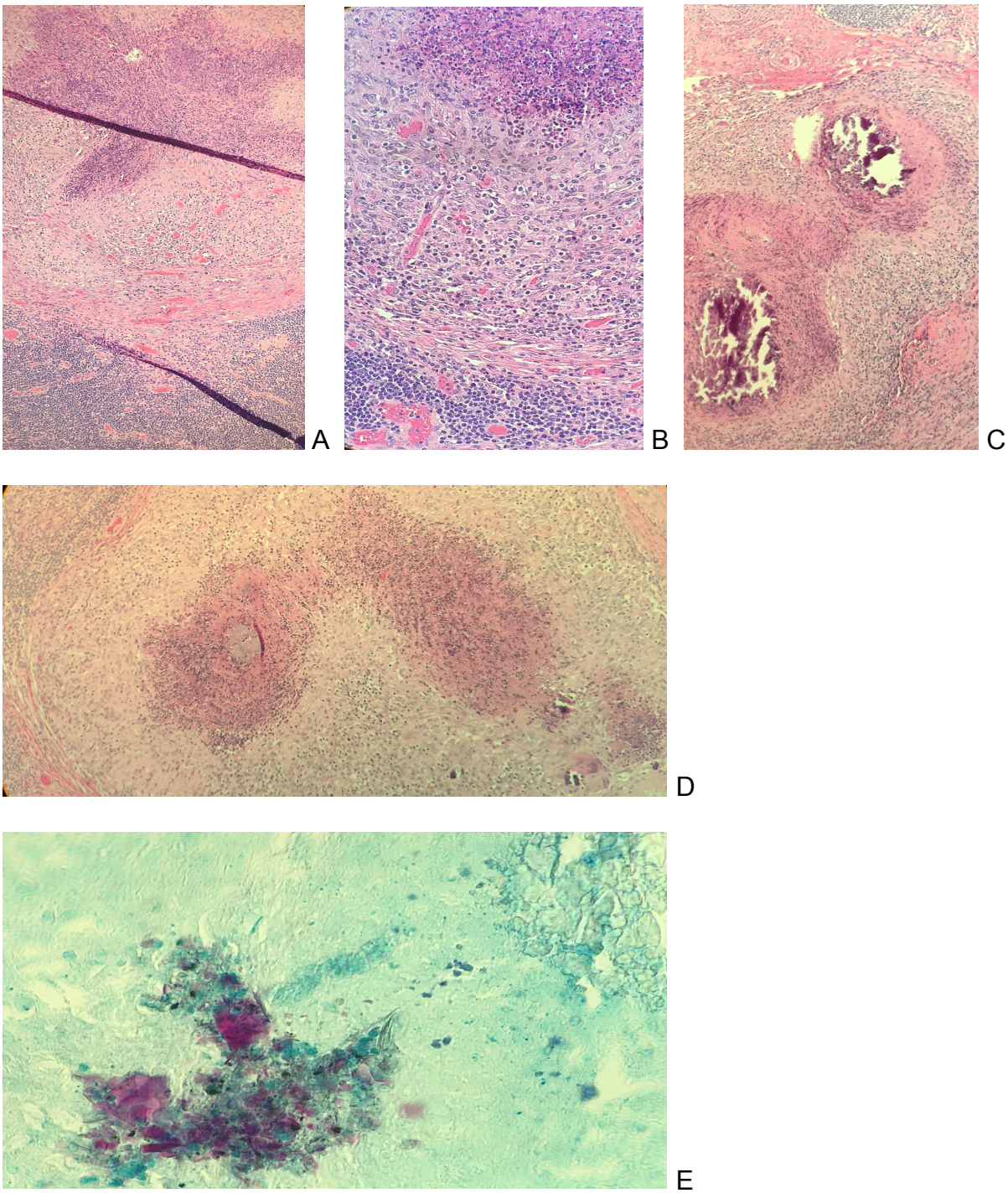

Figure 7: Histopathologic lesions with characteristic tuberculosis granulomas using hematoxylin-eosin stain (A-D) and acid fast stain (E) on light microscopy at 10x (A, C, D) and 40x (B, E) magnification. Central areas of mineralized, caseous necrosis can be seen (C, E).

Table 5: Number of tissues with bTB consistent granuloma lesions on histopathologic exam of H&E stained sections from target lungs, organs, and lymph nodes. Tallies from each pig with early or calcified lesions were totaled for each treatment group, and the group mean of pathologic tissues was calculated.

Group	Pig ID	Early lesion locations			Calcified locations			Total	Mean
		Lungs	Organs	Lym N	Lungs	Organs	Lym N		
Control	37			1			2	3	
	17			4			1	5	
	2	2		4				6	
	3			1			1	2	
	19			1			2	3	
	26			2			1	3	
	30			1			2	3	
Group	Total	2		14			9	25	3.6
Michigan	9		1	1			4	6	
	27	1	3	3			3	10	
	38	2	1	1		1	4	9	
	28			1			3	4	
	8	1	1	1			5	8	
	4		1	2			1	4	
	22	1	1	4			1	7	
	29	1	1	2			4	8	
Group	Total	6	9	15		1	25	56	7
Spain	39	1		2			1	4	
	10			2			3	5	
	18			1			1	2	
	21			1			1	2	
	1			4			1	5	
	20			2			1	3	
	7	2					2	4	
Group	Total	3		12			10	25	3.6
BCG	6			1			2	3	
	11			1			1	2	
	5	2		2			2	6	
	23			2			1	3	
	25	1		5	2	1		9	
	12	1		3			1	5	
	24			3			1	4	
Group	Total	4		17	2	1	8	32	4.6

2.4.4 *Culture Scores*

On necropsy, tissues scored for lesions were also sampled for culture (Table 5, Figure 8). Every animal had mandibular lymph nodes that cultured positive for *M. bovis*. The next most common site for positive culture was the palatine tonsil (in 23 out of 29 animals) followed by the tracheobronchial and bronchial lymph nodes (16 and 15 animals, respectively). The Michigan *M. bovis* strain vaccination group had the highest

combined number of culture positive samples (61) and three of the four pigs with the highest number of culture positive sites. Supervicial cervical lymph nodes only cultured positive in the Michigan group, of which 7 out of 8 pigs cultured positive, consistent with gross lesion data. The group with the lowest number of combined culture positive samples was the control group (32) followed by BCG (35) and the Spanish *M. bovis* strain (42) vaccine groups. A score was assigned to each pig as the number of sampled tissues culturing positive for *M. bovis*.

Table 6: the results of culture samples for each animal in all 4 groups. B = *M. bovis* positive culture result in the specified tissue. 3 in the cranial lung row for Pig ID: 8 signifies that 3 of 4 lung tissues were culture positive. The miscellaneous row designates a positive, unidentified lymph node culture from Pig ID: 8, 9.

Pig ID:	37	17	2	3	19	26	30	9	27	38	28	8	4	22	29	39	10	18	21	1	20	7	6	11	5	23	25	12	24
Cranial lung			B				B		B	B	B	3			B	B	B						B			B	B		
Cardiac lung	B		B				B		B	B					B	B							B				B		
Diaph. lung			B				B		B	B						B	B	B					B		B	B	B	B	
Access. lung			B				B		B	B					B	B	B												
Liver										B																			
Spleen										B																			
Kidney																													
Ileocecal Jxn																													
Mandib lnn.	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Parotid lnn	B				B	B						B					B	B		B	B	B			B		B		B
Retroph lnn					B	B			B	B	B	B					B			B					B	B			B
Mediast lnn										B																	B		
TrachB lnn.		B	B			B			B	B	B	B	B	B	B		B				B	B				B	B	B	
Bronchial lnn	B	B	B			B			B	B	B				B	B	B	B		B		B				B	B		
S Cerv lnn.								B	B	B	B	B		B	B														
Mesent lnn.																	B					B			B				
Ileocecal lnn																						B							
Tonsil	B	B	B	B	B	B	B	B	B	B	B			B	B	B	B	B	B	B	B		B			B	B		B
Hepatic lnn	B		B			B	B			B	B	B			B	B				B		B	B						
Misc.								B				B																	
Total	6	4	9	2	4	7	7	4	10	14	8	10	2	4	9	8	10	5	2	6	4	7	6	1	5	7	9	3	4
Group total	Control = 32 Average = 4.6							Michigan = 61 Average = 7.6								Spanish = 42 Average = 6							BCG = 35 Average = 5						

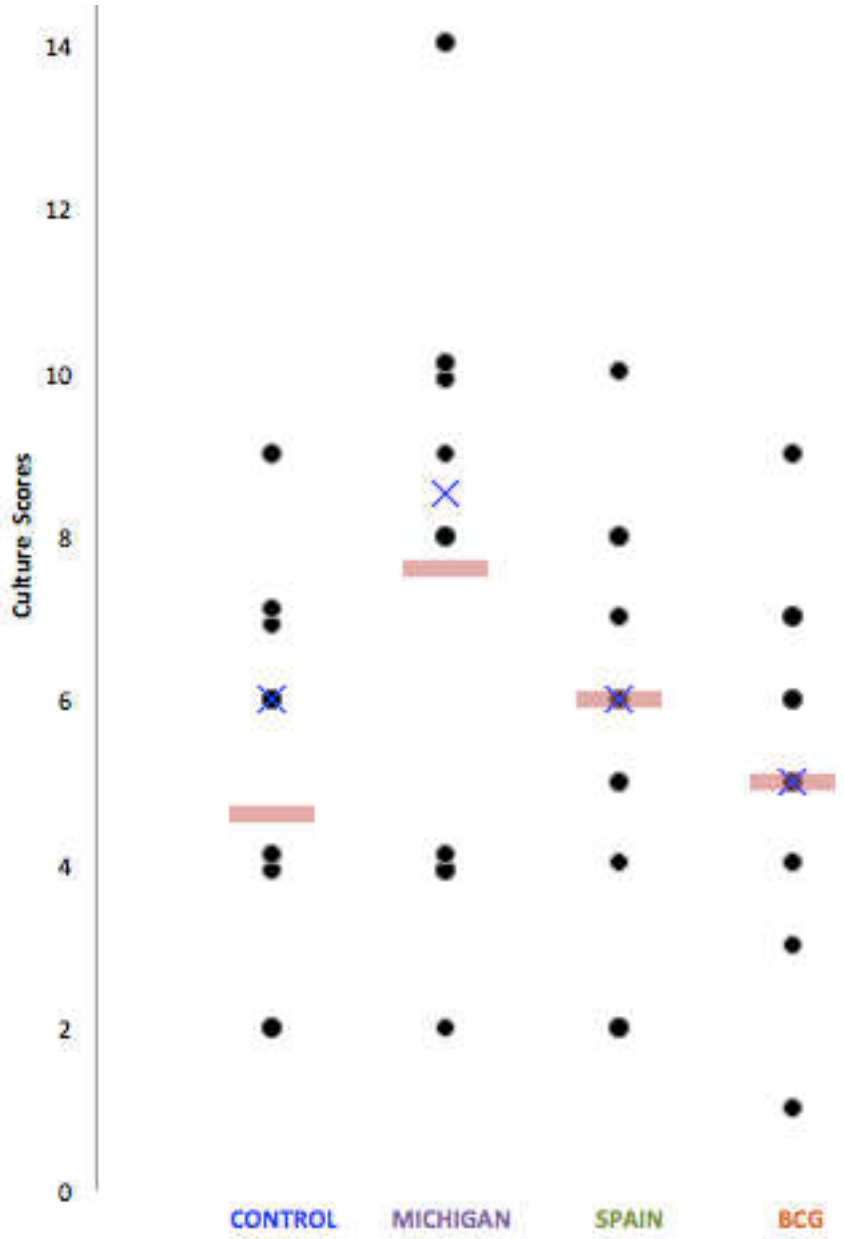

Figure 8: Culture scores (number of *M. bovis* positive tissue cultures per animal) compared across all 4 groups. The blue X's signify means and red bars signify medians for each group.

2.4.5 *Enzyme Linked Immunosorbant Assay – ELISA*

The sensitivity of our ELISA test was 25 out of 29 (86.2%) that had a positive antibody level/E% results out of the pigs that were culture confirmed positive. ELISAs conducted on serum from time points 1, 2, and 3 revealed negative E% readings (<100), indicating a lack of anti-bPPD antibody production in any of the treatment

groups prior to (Time 1), four weeks-post (Time 2), and sixteen weeks-post (Time 3) vaccination. The first detectable antibodies were produced in all four groups at Time 4, four weeks post-challenge with the pathogenic field strain of *M. bovis*. Antibodies levels persisted in animals until Time 5, fifteen weeks post-challenge. Antibodies were quantified by average E% and optical density at a ELISA 1:40 plate dilution from each animal sample run in duplicate. Figure 10 compares the average E% at time points 1-5 between each group.

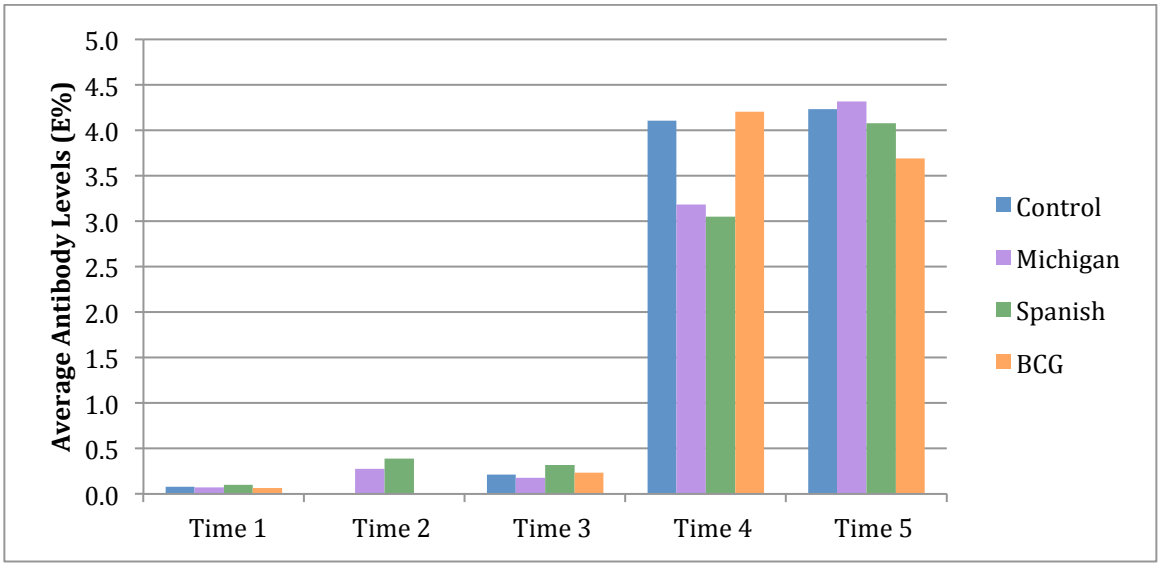

Figure 9: Antibody levels against mycobacterial antigens from serum samples in each vaccination group were determined from bPPD ELISA. The average E% from each vaccination group at time points 1-5 were compared. E% values below 1 are negative, and values above 1 are positive.

2.4.6 *Statistical analyses*

Spearman non-parametric rank-based correlation tests revealed a moderate relationship between lesion scores and histopathology (0.609), cultures (0.575), and an insignificant relationship between lesions and antibody levels/E% (with outliers:0.204, without outliers/negatives: 0.259).

Kruskall Wallis rank sum tests demonstrated a statistically significant difference between the Michigan group and the rest of the groups when comparing lesion scores

(p=0.0122) and histopathology scores (p=0.0030). All groups were not significantly different from each other when comparing culture (x^2 = 2.4278, p=0.49) and antibody levels (E%/OD, x^2 = 0.3702, p=0.94). Boxplots of the data comparing groups with each variable (lesions, histopathology, culture, antibody levels) are displayed in Appendix 2. Confounding due to gender bias was not found when comparing female and male results for lesions (x2 = 0.0019, p = 0.4824) or antibody levels (x2 = 0.56, p=0.228). Boxplots comparing data between female and male pigs are displayed in Appendix 2.

Regression analysis scatterplots are displayed for visualization (Appendix 3) which indicated the same moderate relationship between lesions and histopathology (R^2 = 0.5426, p<0.05), lesions and culture (R^2 = 0.4764, p<0.05), as well as no significant relationship between lesions and antibody levels, with or without outliers included (OD: R^2 = 0.0089, p>0.05; E% without outliers: R^2 = 0.0269, p>0.05).

2.5 Discussion

This vaccine trial is the first investigation on inactivated *M. bovis* vaccination as a sustainable approach to bTB control in North American feral swine. Our study is also the first to examine BCG vaccinated North American feral swine after experimental challenge with pathogenic *M. bovis*. Comparisons between lesion scores, microbial cultures, histopathology, and anti-bPPD antibody levels in vaccinated and non-vaccinated animals after *Mycobacterium bovis* challenge were made. We planned to deem vaccination successful if the numbers of animals with lesions in the control group was at or exceeded 90% and the number of animals in the vaccine groups with lesions was at or below 30%. Instead, 100% of pigs in the trial developed lesions. It has been

shown that the number of lesions and number of tissue sites containing lesions compatible with bTB have a linear, positive correlation with severity of disease and level of shedding capacity in calves (90). We suspect swine develop a similar association between disease severity and transmission potential by pathogen shedding. Vaccine efficacy imparting protection against lesion development, measured by a statistically significant reduction in lesion scores and a reduction in the number of animals with lesions, would serve as a tool for control of bTB by lowering transmission risk.

Immunologic research has shown us that cell-mediated response to *M. bovis* infection tends to occur earlier in disease progression, while humoral antibody responses are seen during further development of fulminant disease or exposure in cattle, swine, and other species (25, 28, 91). This may limit antibody detection in animals that are orally vaccinated with an inactivated dose of *M. bovis*, as previous vaccine trials have demonstrated that significant antibody levels were reached only after parenteral administration of inactivated vaccine or challenge with virulent *M. bovis* (13, 73). This is the same trend we saw in our results, as none of our orally vaccinated pigs had antibody production after vaccination, but did produce antibodies after pathogenic *M. bovis* challenge. Results of previous *M. bovis* vaccine and experimental infection trials revealed that tuberculosis lesion and culture scores were both negatively correlated with antibody levels against NADP-dependent alcohol dehydrogenase C (NADPAD) and MPB83 *M. bovis* antigens while positively correlated with antibody levels against purified bovine tuberculin protein derivative (bPPD) in wild boar – indicating a protective or pathologic association with humoral response depending on

the specific antibody produced (13, 73). Our study showed no correlation, negative or positive, between lesions and antibody responses.

We expected the vaccines produced with inactivated Michigan strain *M. bovis* or Spanish strain *M. bovis*, as well as modified-live BCG would impart some level of protection (at least a 60% reduction in lesions) in feral swine from disease caused by virulent *M. bovis* challenge with $1x10^6$ CFU. Unfortunately, pigs in the Spanish and Control groups developed similar, less severe disease than those in BCG and Michigan vaccination groups based on gross lesion scoring and histopathology. Pigs in the BCG and control groups also had the fewest tissues culturing positive, while pigs in the Michigan group developed the most severe tissue pathology in all lesion and culture assessments. We also anticipated that lesion scores and microbial cultures would be positively correlated to one another, and that was confirmed based on our statistical analyses. Knowing that antibody levels increase with the progression of clinical disease in swine species, we expected to see lesion severity positively correlated with antibody production, but this was not the trend seen in our data. This may be because lesion severity may indicate immune system ability to wall-off the organism in granulomatous structures, while antibody levels can indicate a systemic infection that has spread beyond isolated tissues or simply an exposure to the pathogen in the absence of fulminant infection. We hypothesized that the most significant reduction in clinical severity of bTB using an orally administered inactivated regional strain (i.e. Michigan) of *M. bovis* in our subjects, as it could impart the most specific immune defense against experimental infection with the same, genetically identical strain. Instead, we saw the most severe disease develop in the Michigan *M. bovis* strain vaccination group, and had

statistically significant gross lesion and histopathology scores that were much worse than the other groups. Without fully understanding the complex immunologic mechanism behind *M. bovis* infection and vaccination, especially in North American feral swine species, we can only speculate why the bovine strain of Michigan *M. bovis* used for vaccination was incapable of inducing an adequate immunologic defense against the virulence of the same strain in our swine subjects.

The reason for failure of all three vaccines we tested is unknown, but we have considered a few theories. A study on wild boar in Spain revealed that co-infection with Porcine Circovirus 2 or pseudorabies disease virus, age, and *Metastrongylus spp.* lung nematode infestation was positively correlated with more severe bTB disease lesions (63). Our research subjects tested negative to pseudorabies and were on a deworming regimen, but we may consider concurrent parasitic infection or pathogenic immune system interference as contributors to *M. bovis* vaccination failure, although we do not consider this a highly likely cause. Undetected environmental mycobacterial exposure may have contributed to lack of protective immunity as has been demonstrated previously (92). Additionally, our pigs were approximately 6 months of age at the time of vaccination, and may have been too mature to develop an appropriate immunogenic response to the vaccines before experimental infection occurred. Successful *M. bovis* vaccination in previous studies was performed in pigs at 3-4 months of age (12, 13, 24, 73), and other vaccine studies in people and animals have determined that age can interfere with protective response to vaccination (3, 69, 92).

Other considerations we have made in regards to vaccination failure involve booster vaccination and route of administration. One study demonstrated that BCG

given twice (prime and booster) increased the protective capabilities of this vaccine in wild boar (24), while two vaccines of BCG have failed to protect from bTB development in African buffalo and were worse with two compared to one vaccine in calves (64, 69). The two vaccination trials using oral baits to deliver inactivated vaccine to wild boar were delivered in one dose (73) or with a booster dose 52 days after the initial vaccine (13), and did impart protection in piglets after challenge with virulent *M. bovis* innoculation. Our trial instead administered a booster of inactivated vaccine to the subjects 30 days after the first, no booster to the BCG vaccinates, and vaccines were delivered via syringe to the oropharynx rather than in bait form.

Another difference in our trial from previous studies in Spanish wild boar was the dose of inactivated *M. bovis* vaccine given and of the pathogenic *M. bovis* strain challenge. Two doses of $1x10^7$ CFU (13) or one dose of $6x10^6$ CFU (73) inactivated *M. bovis* strain vaccine were delivered via oral baits to Spanish wild boar, while our trial administered two doses of $1x10^6$ CFU inactivated *M. bovis* strains (Michigan or Spanish) to the oropharynx. Two doses of $1x10^6$ CFU (24) and one dose of $1x10^5$ CFU (12) BCG vaccine were administered in baits to Spanish boar piglets, while our trial administered one dose of $1x10^6$ CFU BCG strain vaccine to the oropharynx. Five mLs of $1x10^5$ CFU (13, 24) and five mLs of $1x10^6$ CFU (73) suspended pathogenic *M. bovis* was administered at challenge. Our study challenged with $1x10^6$ CFU pathogenic Michigan field strain *M. bovis*. Although the CFU of our BCG vaccine was the same, the inactivated vaccine CFU was smaller than in the vaccine study by Ballesteros et. al. 2009 (12). A low dose of BCG *M. bovis* vaccine has been shown to lack protective effects that are imparted by larger, verified doses of vaccine (93). The equivalent

pathogenic challenge dose we gave should have been equally likely to cause disease or be protected against after vaccination. Our use of a completely different virulent strain of *M. bovis* (Michigan vs. Spanish) that came from an entirely different species (bovine vs. porcine origin), may have contributed to a difference in pathogenicity and failure of the vaccine to protect against it compared to the European research.

Different breeds, or even individuals, of the same species have the potential to mount different immunities after vaccination and to be more or less susceptible to infection with a pathogen. A study performed across South Central Spain sampled multiple populations of wild boar, and lack of genetic variation (homozygosity) was linked to increased likelihood of infection and disease progression in the Iberian wild boar population (94). If across one region of Spain, native wild boar populations show contrasting genetic influences on immunity, we speculate that there must be differences in genetic makeup between Spanish boar and feral swine of North America. Feral swine in North America are a non-native population of hybrids carrying multiple breeds of domesticated swine genetics. This genetic variability may explain a difference in immunity after *M. bovis* vaccination and the ability to develop infection with virulent *M. bovis* challenge.

3. Concluding Remarks and Future Directions

The ultimate goal of our vaccine trial and studies like it was to demonstrate the protective benefits and reduced clinical severity of bTB after vaccinating with an inactivated *M. bovis* vaccine adapted for oral delivery to North American feral swine. The application of such preventative measures to decrease disease burden and environmental *M. bovis* shedding would theoretically reduce the shedding and therefore the transmission rate of disease. Determining whether shedding capacity is correlated with level of disease severity in vaccinated and unvaccinated animals would also be helpful for assessing the risk of *M. bovis* transmission from North American feral swine.

Follow-up research will continue to investigate the efficacy of orally administered inactivated *M. bovis* vaccine in feral swine originating from Texas as well as swine originating from Molokai. These follow-up experiments will be needed to investigate vaccine efficacy and immune parameters in North American feral swine, and study focus should be on specifications for vaccine delivery (route, booster time-frame, dose, vaccine strain, etc.) that can lead to practical application in a field setting. Additionally, groups that are administered inactivated vaccine and controls will be intermingled to account for the possible bias caused by keeping each treatment in individual rooms. The BCG vaccinates will have to be kept separate so that potential shedding of the vaccine does not expose the other animals in the study.

While we used bPPD ELISA technology to assess disease severity as it correlates with other quantifiable diagnostics (culture and lesion scores), a more species-specific assay differentiating vaccine-associated and infection-associated

antibodies (DIVA tests) in feral swine would be a more precise way to identify immune responses. Likewise, identifying the virulence and inoculation dose at which fulminant infection occurs from virulent strains, from Michigan or elsewhere, of *M. bovis* would be useful. Molecular markers such as IFN-γ, C3, IL-β, MUT, anti-MPB83 antibodies, etc. may be another way to qualitatively evaluate the innate and adaptive immune system defenses being stimulated by a particular vaccine in a particular strain of pig.

Finally, updated tuberculosis epidemiology, and animal ecology surveys are needed in areas of the US where feral swine populations are migrating into locations with reemerging or persistent *M. bovis* incidence in domestic livestock and humans.

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Appendices

Appendix 1

Killed *M. bovis* Feral Swine Study 2016

Date:_____

Animal ID:_____ Age: Sex: M/F

Method of euthanasia_____ Blood obtained_____

Lesion Scoring:

Lung Lobe	0= No Lesions	1= a few <1cm foci	2= numerous scattered <1cm foci	3= densely clustered <1cm foci	4= one >1cm lesion	5= multiple >1cm lesions	Calcified Or Necrotic ?
Diaphragm.	0	1	2	3	4	5	C / N
Cranial	0	1	2	3	4	5	C / N
Cardiac	0	1	2	3	4	5	C / N
Accessory	0	1	2	3	4	5	C / N

Visceral Organs	0= No lesions	1= 1-2mm scattered foci	2= clusters 1-2mm foci OR foci >1cm	Calcified or Necrotic?
Liver	0	1	2	C / N
Spleen	0	1	2	C / N
Kidney	0	1	2	C / N
Ileocecal Jxn	0	1	2	C / N

Lymph Nodes	0= No lesions	1=1-2 foci <1cm	2= many <1cm	3= >1cm lesion	4= diffuse foci	Calcified or Necrotic?
Mandib (2)	0	1	2	3	4	C / N
Parotid (2)	0	1	2	3	4	C / N
Retrophar (2)	0	1	2	3	4	C / N
Mediastinal	0	1	2	3	4	C / N
Tracheobron	0	1	2	3	4	C / N
Bronchial	0	1	2	3	4	C / N
Super. Cerv.	0	1	2	3	4	C / N
Mesenteric	0	1	2	3	4	C / N
Ileocecal	0	1	2	3	4	C / N
Tonsil	0	1	2	3	4	C / N
Hepatic	0	1	2	3	4	C / N

Appendix 2
Boxplots

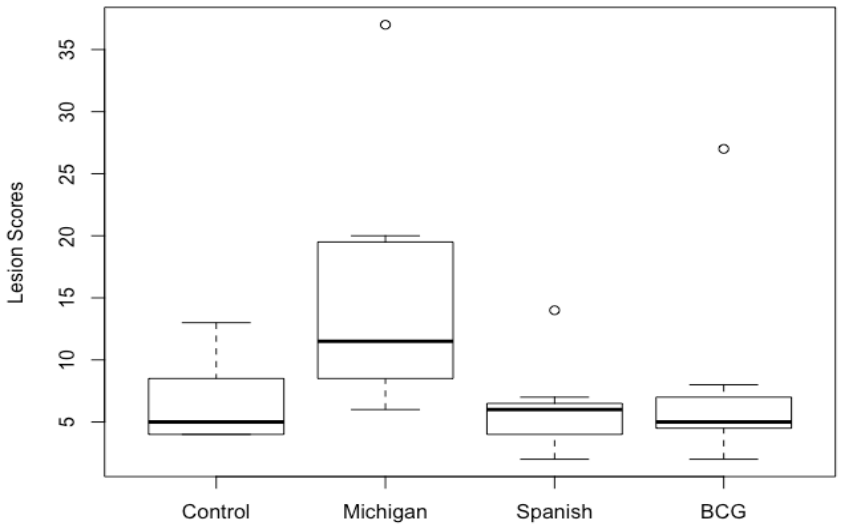

Figure 10: Comparison of Control, Michigan, Spanish, BCG group lesion score data. Kruskall Wallis rank sum test: Michigan was statistically different ($p < 0.05$).

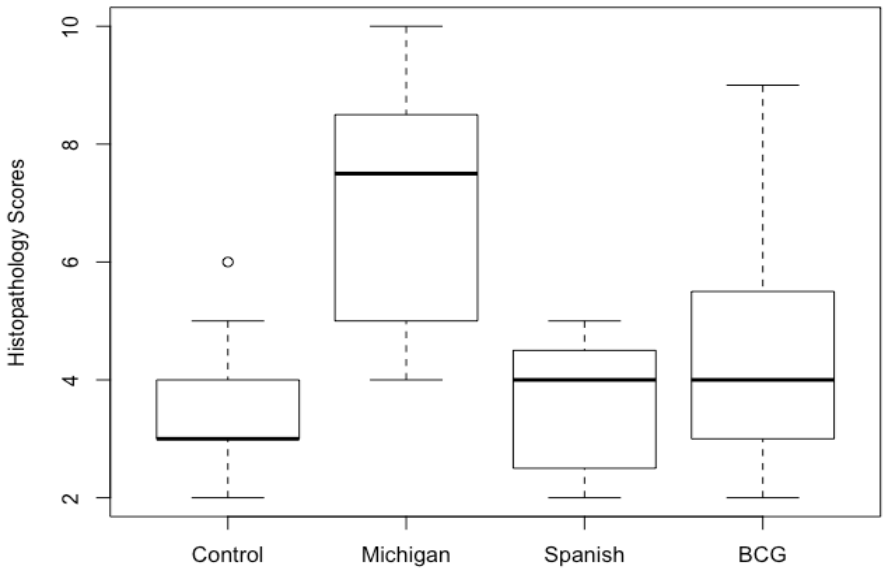

Figure 11: Comparison of Control, Michigan, Spanish, BCG group histopathology data. Kruskall Wallis rank sum test: Michigan was statistically different ($p < 0.05$).

Appendix 2 (continued)

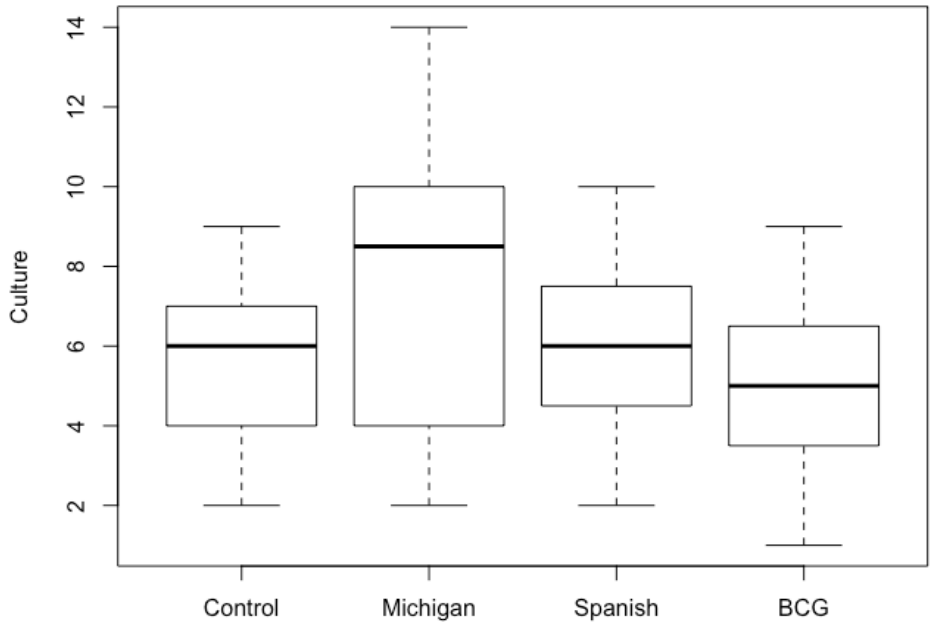

Figure 12: Comparison of Control, Michigan, Spanish, BCG group culture results.
Kruskall Wallis rank sum tests: all groups were not statistically different (x^2 = 2.4278, p=0.49).

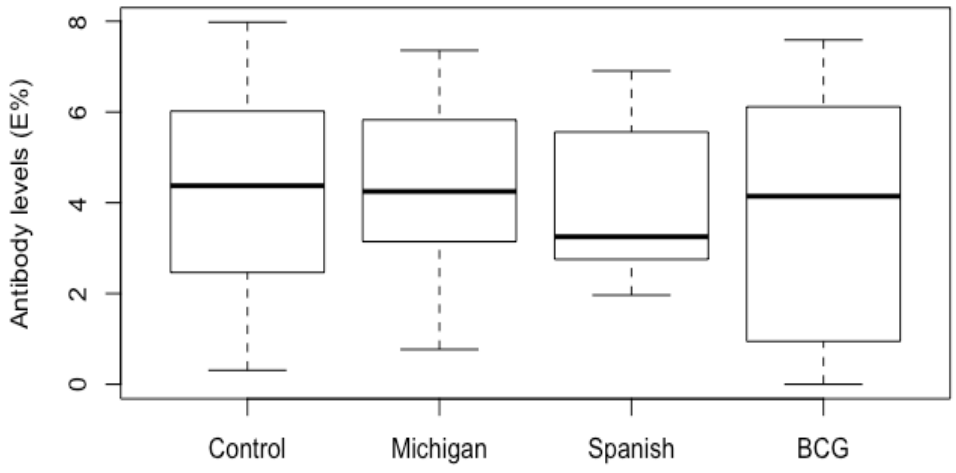

Figure 13: Comparison of Control, Michigan, Spanish, BCG group antibody levels.
Kruskall Wallis rank sum tests: all groups were not statistically different (x^2 = 0.3702, p=0.94).

Appendix 2 (continued)

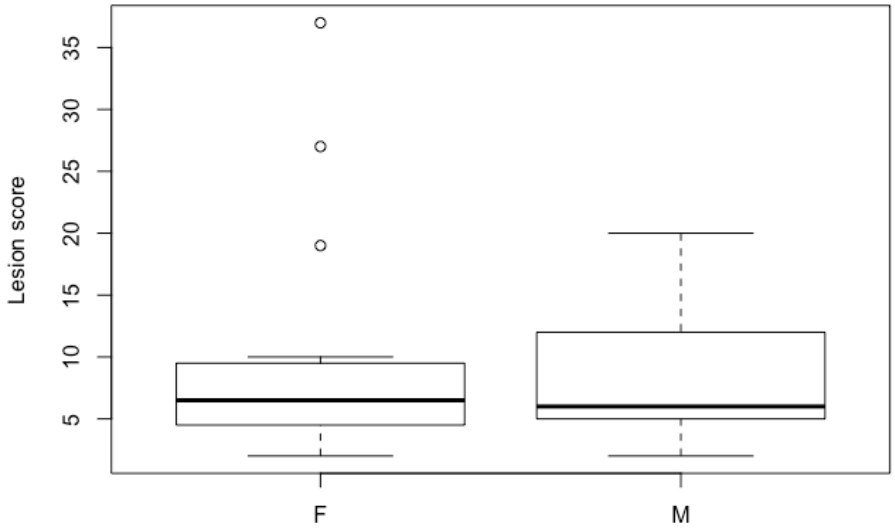

Figure 14: Comparison of female and male lesion score data.
Kruskall Wallis rank sum test: groups were not statistically different (x^2 = 0.0019, p = 0.4824).

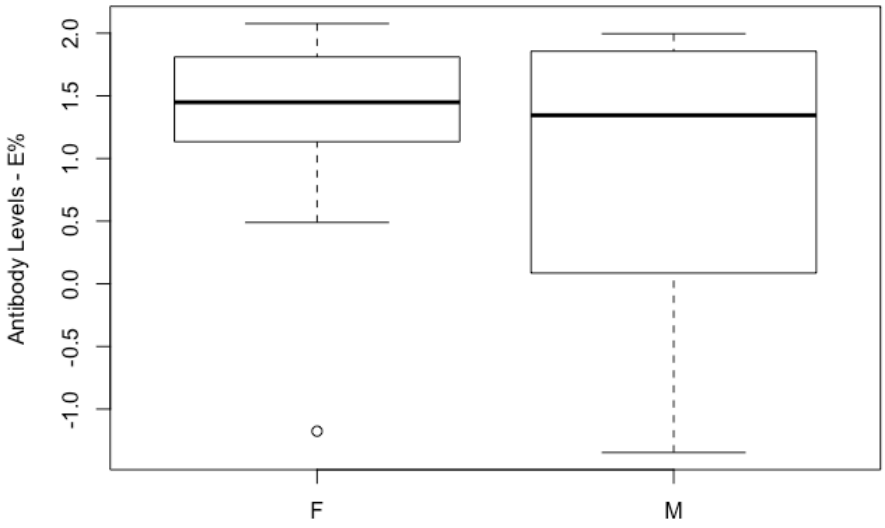

Figure 15: Comparison of female and male antibody levels (E%)
Kruskall Wallis rank sum test: groups were not statistically different (x^2 = 0.56, p=0.228).

Appendix 3
Regression plots

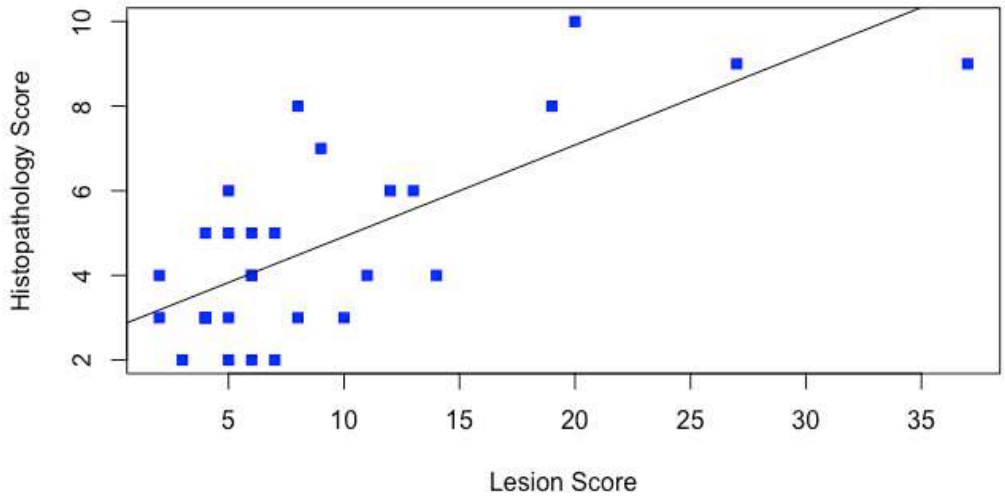

Figure 16: Linear regression relationship between histopathology and lesion scores from all pigs in the study. Analysis of lesions versus histopathology (R^2 = 0.5426, p <0.05).

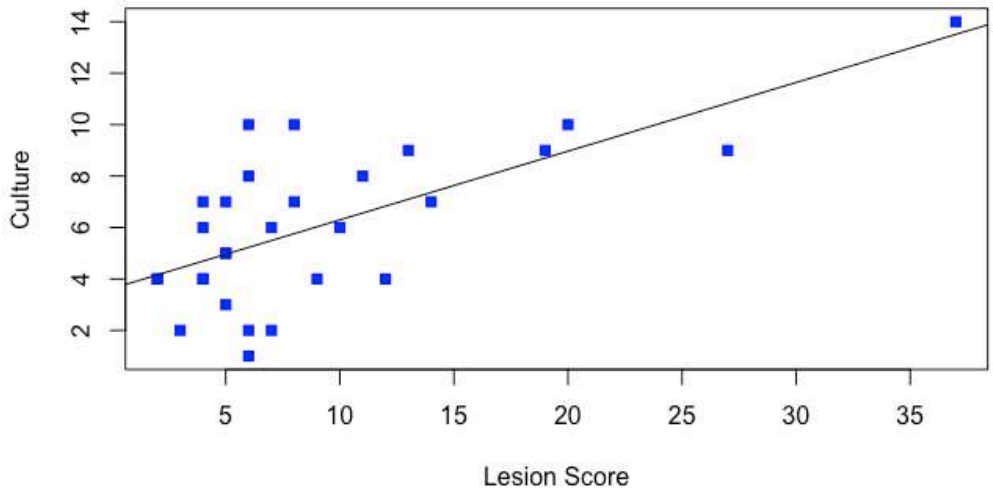

Figure 17: Linear regression relationship between culture and lesion scores from all pigs in the study. Analysis of lesions versus culture (R^2 = 0.4764, p <0.05).

Appendix 3 (continued)

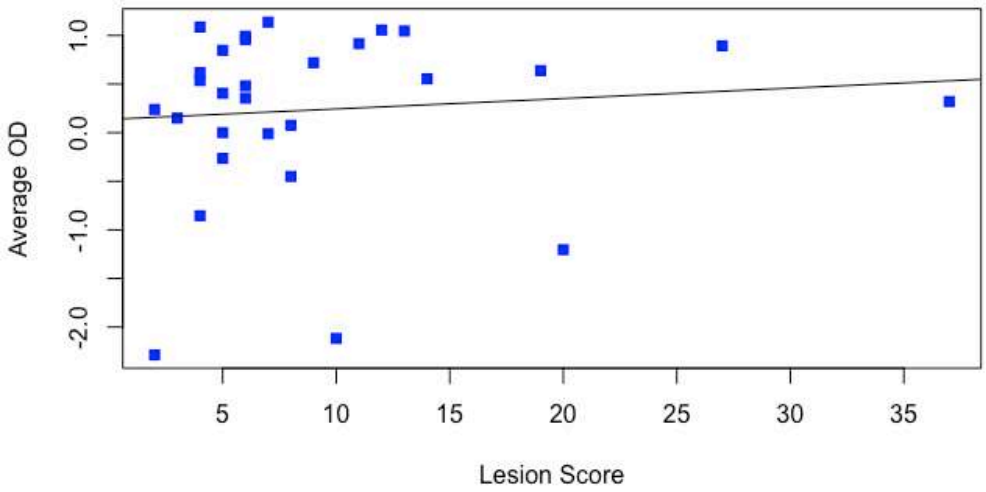

Figure 18: Linear regression relationship between average optical density (OD) and lesion scores from all pigs in the study. Analysis of lesions versus average OD with outliers (R^2 = 0.008932, p = 0.6258).

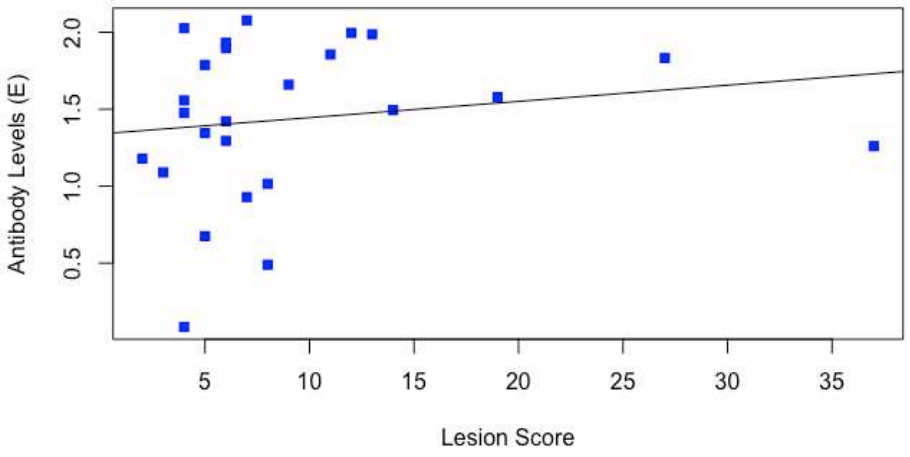

Figure 19: Linear regression relationship between E% and lesion scores from all pigs in the study. Analysis of lesions versus Antibody Levels/E% without outliers (R^2 = 0.02686, p = 0.434).