Mycobacterium bovis in U.S. feral swine – history, diagnostics, and comparative experimental infection

by

Dana C. Hill

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology (Cellular and Molecular Pathology)

Program of Study Committee:
Douglas Jones, Co-major Professor
Tyler Thacker, Co-major Professor
Aaron Lehmkuhl
Michael Yaeger
Olufemi Fasina

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2023

TABLE OF CONTENTS

NOMENCLATURE

AFB	Acid fast bacilli
AG	Arabinogalactan
AO	Auramine-acridine orange
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
BSL	Biosafety level
bTB	Bovine tuberculosis
C3b	Complement component 3b
CCT	Comparative cervical skin test
CFP	Culture filtrate protein
CFT	Caudal fold test
CFU	Colony forming units
ConA	Concanavalin A
CR	Complement receptor
DC	Dendritic cell
DC-SIGN	Dendritic cell intercellular-adhesion molecule-3 grabbing non-integrin
DPI/dpi	Days post infection
DPP	Dual path platform
ECH	ESAT-6/CFP-10 fusion peptide
ELISA	Enzyme-linked immunosorbent assay
ESAT	Early secretory antigenic target

FFPE	Formalin-fixed paraffin-embedded
γδ T-cell	gamma-delta T-cell
H&E	Hematoxylin and eosin
IFN-γ/IFN-g	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IS	Insertion sequence
LAM	Lipoarabinomannan
LIMS	Laboratory information management system
LM	Lipomannan
Man-LAM	Mannose-capped oligosaccharides of LAM
MAZ	Modified-accredited zone
MDR	Multi-drug resistant
MGC	Multinucleated giant cell
MMP9	Matrix metalloproteinase 9
MOTT	Mycobacteria other than tuberculosis
MPB	Mycobacterial protein
MTC	*Mycobacterium tuberculosis* complex
Na2B4O7	Sodium borate
NCAH	National Centers for Animal Health
NF	New fuchsin
NO	Nitric oxide
NTM	Nontuberculous mycobacteria

NVSL	National Veterinary Services Laboratories
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimycocerosate
PE	Proline-glutamate
PF	Preventive fraction
PIM	Phosphatidylinositol mannoside
PPE	Proline-proline-glutamate
PPD	Purified protein derivative
PPDa	Purified protein derivative, *Mycobacterium avium*
PPDb	Purified protein derivative, *Mycobacterium bovis*
PWM	Pokeweed mitogen
qPCR	Real-time PCR
RD	Region of difference
RFLP	Restriction fragment length polymorphism
RLU	Relative light unit
SNP	Single nucleotide polymorphism
T1	Test line 1 on DPP, corresponding to MPB83
T2	Test line 2 on DPP, corresponding to ECH
TB	Tuberculosis
USDA	United State Department of Agriculture
VNTR	Variable number tandem repeats

WGS	whole genome sequencing
WTD	White-tailed deer

ACKNOWLEDGMENTS

I would like to thank my committee members, Doug Jones, Tyler Thacker, Aaron Lehmkuhl, Mike Yaeger, and Olufemi Fasina for their guidance and support throughout the course of this research.

In addition, I would like to extend my gratitude to the many individuals at the National Centers for Animal Health and other branches of USDA who helped to make this project possible. More specifically, I would like to thank the pathology sections of NVSL and NADC for their assistance with sample processing and histopathology; the labs of Mitch Palmer and Crystal Loving for always having open doors and nurturing a collaborative research environment; Nick Lewis for assisting with data collection; the Animal Resource Unit veterinarians and caretakers for their daily care and support of the animals used for this research; Tim Smyser, Courtney Bowden, Anna Mangan, and Rachael Giglio at the National Wildlife Research Center for ongoing collaborations in swine genomics; Nehemias Ulloa and Amy Lock for their statistical expertise; and my resident mates and colleagues at Iowa State University for their guidance. Lastly, a special thank you to my friends, family, and husband for their unwavering support, love, and encouragement.

ABSTRACT

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, has a long history on the Hawaiian island of Moloka'i where disease spillover from infected cattle into feral swine was identified in the 1980s, cattle were temporarily removed from the island, and disease spillback from feral swine into cattle was subsequently confirmed in the early 1990s. This ignited a 10-year-long bTB surveillance study of Moloka'i wildlife, including feral swine, axis deer, and mongoose from 1999-2008. The last bTB positive cow on Moloka'i was identified in 1997 followed by 26 consecutive years of negative annual testing. That is, up until 2021 when a recent epizootic bTB outbreak on Moloka'i confirmed that several cattle are infected with a strain of *M. bovis* with single-nucleotide polymorphism (SNP) pattern homologous to 2008 isolates taken from feral swine. The debate remains whether Moloka'i feral swine fit the criteria of a spillover host, an amplifier host, or a wildlife maintenance host for bTB. Determination of bTB host status is complex, requiring extensive epidemiological data from naturally infected populations and is crucial in bTB-disease mitigation and eradication efforts. We aimed to identify surveillance and diagnostic challenges of bTB and infer bTB-host status of genetically distinct populations of U.S. feral swine. Diagnostic test performance for bTB was retroactively compared between a wildlife surveillance study from Moloka'i feral swine (1999-2008) and routine U.S. cattle surveillance (1999-2010). Additionally, experimental infection of U.S. feral swine from distinct genetic backgrounds provides insights into bTB host status potential by highlighting differing pathophysiology and immunology.

Tissues from Moloka'i feral swine and cattle originating from the United States of America were submitted to the National Veterinary Services Laboratories (NVSL) in Ames, IA for bTB testing. Mycobacterial culture (current gold standard) from abattoir cattle samples

identified more bTB-positive cases than formalin-fixed paraffin-embedded (FFPE) polymerase chain reaction (PCR), while for field sample collections from Moloka'i feral swine the opposite was observed. A total of 63,764 cattle samples were received by NVSL compared with 452 feral swine submissions. bTB was confirmed by culture and/or PCR in 741 cases for cattle (1.16% positive) and 15 cases for feral swine (3.32% positive). Results and disparities between histopathology, PCR, and culture were investigated. Culture identified 39 additional bTB cases from cattle that were missed by PCR while, for feral swine, PCR identified more bTB cases (n = 13) than culture (n = 8). Histopathology identified more lesions as mycobacteriosis compatible than were verified by culture or FFPE PCR for both cattle and feral swine samples but proved to be a valuable and rapid initial screening test in the bTB diagnostic decision-making tree. A multimodal approach to bTB diagnostics, utilizing histopathology, culture, and PCR in parallel, was beneficial in identifying and correcting discrepant results for both cattle and feral swine submissions.

Eurasian wild boar descendants from Texas and Polynesian swine from Hawaii were experimentally infected with *M. bovis* to compare disease susceptibility, pathology, and immunology. A low dose of *M. bovis* produced tuberculous lesions within 5/6 (83%) Moloka'i and 3/4 (75%) Texas pigs, with distinct lesion profiles and host immunity. Longitudinal sampling of peripheral blood throughout infection indicates highly variable humoral and cell-mediated immune responses as evidenced by antibody production on dual path platform (DPP) and interferon-gamma (IFN-γ) production from peripheral blood mononuclear cells (PBMCs) restimulated with various mycobacterial antigens. Moloka'i-origin swine mounted cell mediated and humoral immune responses to bTB infection on while infected Texas-origin pigs had undetectable antibody responses against MPB83 and ECH.

These results suggest unique immune responses and lesion profiles in genetically distinct U.S. feral swine experimentally infected with *M. bovis*. Feral swine are susceptible to disease caused by *M. bovis* and diagnostic test performance varies with species and method of collection. The historical information and inherent differences observed between the two groups of pigs used for this study support the hypothesis that Moloka'i feral swine are acting as a maintenance host for bTB and contribute to disease spillback events into domestic livestock.

CHAPTER 1. GENERAL INTRODUCTION

Mycobacteria are a diverse group of Gram-positive, acid-fast, aerobic or micro-aerophilic bacteria belonging to the phylum *Actinomycetota*, class *Actinomycetes*, order *Mycobacteriales*, family *Mycobacteriaceae*. The *Mycobacterium* genus comprises over 200 species with the majority being free-living environmental organisms found ubiquitously in soil and water sources (Brosch et al., 2000; Parte, 2020). The mycobacterial envelope consists of a plasma membrane, cell wall, and capsule. The peptidoglycan layer present in the cell wall of Gram-positive bacteria is separated from the plasma membrane by a thin periplasmic space and shielded by a capsule. The cell wall is spanned by phosphatidylinositol mannosides (PIMs) with glycosylated lipomannans (LM) and lipoarabinomannan (LAM) extending from the outer leaflet of the plasma membrane to the capsular surface (Brennan & Nikaido, 1995; Dulberger et al., 2020).

A large component of the asymmetrical cell wall is the polysaccharide arabinogalactan (AG) that is unique to mycobacteria and esterified to high molecular weight long chain fatty acids (mycolic acids) (Brennan & Nikaido, 1995). The waxy outer membrane is composed of mycolic acids that create a barrier notoriously impermeable to various hydrophilic and lipophilic solutes, including dyes and detergents. For this reason, mycobacteria require acid-fast staining techniques such as Ziehl-Neelsen and New Fuchsin for microscopic visualization (Sheehan & Hrapchak, 1973). Several surface-associated or secreted proteins, free lipids (phthiocerol dimycocerosate, PDIMs), and glycosylated carbohydrates (mannan/mannose) are scattered throughout the outer cell membrane and capsular surface, providing a dynamic framework for stages of growth and survival among different mycobacterial species (Torrelles & Schlesinger, 2010). One of the most abundant macromolecules present at the cell surface are the mannose-

capped oligosaccharides of LAM (Man-LAM), which are unique to slow-growing, pathogenic mycobacteria.

Differentially expressed cell wall components including complex lipids and surface-associated or secreted proteins play an important role in bacterial homeostasis and replication as well as participate in host-cell interactions. Specifically, proline-glutamate (PE) and proline-proline-glutamate (PPE) are two important genes unique to the *Mycobacteria* genus encoding for an estimated 200 proteins, many of which localize to the cell wall (Brennan et al., 2001; Cole & Barrell, 1998; Flores & Espitia, 2003). The PE and PPE N-terminal domains are followed by protein coding sequences and GC-rich C-terminal repeats (PE-PGRS and PPE-MPTR) which vary greatly in length and sequence. PE and PPE gene regions are common targets for recombination, insertions, and mutations, resulting in dynamic cell wall profiles and creating molecular signatures for bacterial strains (Hermans et al., 1992; Poulet & Cole, 1995; Van Soolingen et al., 1993).

Active secretion of both endogenous and exogenous bacterial components assist mycobacteria in survival and contribute to antimicrobial resistance. The most well-characterized example is the ESX type VII secretion system, encoded by *esx* gene regions. ESX is regulated by the PhoPR two-component system involving *phoP* and *EspR* (Malone et al., 2018; Simeone et al., 2015). The *esx* gene cluster region carries the PE/PPE gene region although the two are not directly related. It is suggested that PE/PPE was inserted into the *esx* gene of a common mycobacterial ancestor then underwent numerous duplications along with *esx* copying events, resulting in multiple *esx* gene cluster regions (ESX-1 through ESX-5) and highly variable PE/PPE gene regions (Gey van Pittius et al., 2001; Gey van Pittius et al., 2006). These small but significant genomic variations translate into phenotypic and functional differences in cell surface

interactions, growth/survival characteristics, and antigenicity between mycobacterial species and strains *in vivo* and *in vitro* (Bansal et al., 2010; Karboul et al., 2008; Li et al., 2005).

Mycobacteria are classified as pathogenic, opportunistic, and environmental mycobacteria based on phylogenetic attributes as well as their ability to cause disease in humans and animals. Environmental mycobacteria are saprophytic microbes with rapid or slow growth characteristics and often referred to as mycobacteria other than tuberculosis (MOTT), or nontuberculous mycobacteria (NTM). The genome length of NTM is small (5-7 mb) with high GC content (60-70%) compared with other bacteria (Fedrizzi et al., 2017; Rahman et al., 2014). Opportunistic or atypical mycobacteria account for approximately 25 out of over 180 NTM species that are documented to cause disease in immunocompromised humans and animals (van Ingen, 2013; Wolinsky, 1979). Pathogenic mycobacteria are set apart as slow-growing, intracellular pathogens typically adapted to a primary host species. This group of human- and animal-adapted virulent species are collectively referred to as the *Mycobacterium tuberculosis* complex (MTC). Belonging to the MTC are 11 species of mycobacteria (and their primary hosts) including *M. tuberculosis* (humans), *M. africanum* (humans), *M. canettii* (humans), *M. microti* (voles), *M. pinnipedii* (seals and sea lions), *M. orygis* (oryx), *M. suricattae* (meerkat) *M. mungi* (banded mongoose), *M. bovis* (cattle), *M. caprae* (goats and sheep), Dassie bacillus (rock hyrax) and chimpanzee bacillus (Alexander et al., 2010; Aranaz et al., 2003; Collins & Grange, 1983; Coscolla et al., 2013; Cousins et al., 2003; Grange & Collins, 1987); Koch (1890); (Niemann et al., 2002; Parsons et al., 2008; Parsons et al., 2013; Smith, 1898; van Ingen et al., 2012; Van Soolingen et al., 1997).

Relative to environmental mycobacteria, MTC genomes are small, ranging from 4.4 million bp (*M. tuberculosis*) to 4.3 million bp (*M. bovis*) (Brosch et al., 2000; Cole & Barrell,

1998; Cole et al., 1998; Garnier et al., 2003). This would suggest that loss of genetic material led to an evolutionary shift from free-living saprophytes to intracellular pathogens dependent upon host factors for survival. Interestingly, when comparing genomes within MTC, there is 99.9% homology at the nucleotide level yet significant differences in host range and disease outcomes (Brosch et al., 2002; Sreevatsan, Pan, Stockbauer, et al., 1997). Regions of difference (RD) are sections of the mycobacterial genome that differ between species due to deletions of specific genomic segments (Mahairas et al., 1996). The product(s) of various RDs have the potential to affect mycobacterial species' survivability and pathogenicity. Numerous RDs (RD1-RD16) were originally identified as missing from the genome of *M. bovis* strain bacille Calmette-Guérin (BCG) in comparison with that of *M. tuberculosis.*

BCG ~~is sometimes included in the MTC grouping as it~~ was developed by a human physician and veterinarian team, Léon Charles Albert Calmette and Jean-Marie Camille Guérin, respectively, in 1920 from a live *M. bovis* strain (Calmette et al., 1926). The original bacillus underwent serial passages on potato wedges and bovine bile liquid culture media supplemented with glycerin and serially passaged over 200 times until, ultimately, the attenuated vaccinate strain was produced. The original BCG strain, BCG Pasteur, has been subjected to nearly a century of cultivation and serial passage, changing its genome drastically, but characteristically lacks the RD1 locus, which is conserved among all virulent MTC members (Brosch et al., 2000; Gordon et al., 1999; Lange et al., 2022; Mahairas et al., 1996). Deletion of RD1 contributes to the attenuation of BCG presumably through decreased production of effector proteins, cell wall components, and/or virulence factors (Abdallah et al., 2007; Lewis et al., 2003).

Two proteins found in RD1, early secretory antigenic target of 6kDa (ESAT-6) and culture filtrate protein of 10kDa (CFP-10), have been identified as highly immunogenic antigens

specific to all virulent MTC species and few atypical mycobacteria (Berthet et al., 1998; Pollock & Andersen, 1997a, 1997b; Sørensen et al., 1995). These proteins are encoded by the ESX-1 gene cluster region on the RD1 locus and are dimerized and secreted by a type VII secretion system to play important roles in intracellular survival and as antigenic targets for memory T cells (Andersen et al., 1995; Van Pinxteren et al., 2000). In addition to RD1, BCG contains a mutation which significantly reduces the expression of two other mycobacterial proteins, MPB83 and MPB70, a surface-associated lipoprotein and secreted antigen, respectively (Charlet et al., 2005; Harboe et al., 1996; Harboe et al., 1998; Wiker, 2009; Wiker et al., 1998). The exact functions of MPB83 and MPB70 remain unknown but both are serodominant antigens normally constitutively expressed at high levels in cattle infected with *M. bovis* (Veyrier et al., 2008). The subtle differences in bacterial surface markers and virulence factors encoded by RD1 and other genomic deletions manifest as differences in disease severity and host range.

MTC members, which all retain RD1 and the associated virulence factors, cause an array of disease syndromes with the most classic being tuberculosis (TB) in humans, which involves development of tuberculoid granulomas most commonly throughout the lungs and associated lymph nodes. TB has inflicted disease on humans and animals while largely evading scientific efforts to produce efficacious vaccines and mitigate disease spread. It is estimated that 25% of the global human population is infected with TB and, in 2021, TB was responsible for 1.6 million deaths making it the second most deadly infectious disease (WHO, 2022). Human TB cases are predominantly caused by *M. tuberculosis*, and less commonly, other human-adapted or animal-adapted MTC species. Transmission is primarily aerogenous for *M. tuberculosis* and many other MTC members, facilitated by direct and indirect contact. The infectious dose of *M.*

tuberculosis is remarkably low, with as few as 10 bacilli capable of infecting humans (WHO, 2022).

Symptoms of active TB in humans are non-specific and may include general malaise, cough, hemoptysis, fever, night sweats, and/or weight loss with intermittent shedding of infectious bacilli (WHO, 2022). Pulmonary lesions may or may not be evident on diagnostic imaging depending on the route of transmission, time course of infection, and severity of disease. Latency is a chronic, subclinical infection characterized by a positive reaction on the intradermal tuberculin skin test, no evidence of pulmonary granulomas, and a lack of bacterial shedding (Young et al., 2008; Young et al., 2009). Clinical disease or loss of latency and progression to active TB is most often reported in immunocompromised patients with comorbidities such as human immunodeficiency virus, diabetes, or immune-mediated diseases from iatrogenic treatment using immunomodulatory therapies (Eldholm & Balloux, 2016; Guerra-Assunção et al., 2015; Middelkoop et al., 2009; Morris et al., 2011; WHO, 2022).

Active or latent TB in humans is treated through long-term multimodal antimicrobial therapy with isoniazid, rifampicin, and pyrazinamide being the first-line antimicrobials (WHO, 2022). The emergence of muti-drug resistant (MDR) TB or zoonotic infection by *M. bovis*, which is naturally resistant to pyrazinamide and more likely to cause extrapulmonary disease, require modification of the classic treatment regimen to achieve clearance (Shafer et al., 1995; Small et al., 1993; Sreevatsan, Pan, Zhang, et al., 1997). Speciation of mycobacterial infections in humans is not routinely performed in a clinical setting as it requires specialized genetic fingerprinting/sequencing or biochemical analysis. Multiple reports suggest that this contributes to underestimation of zoonotic TB cases caused by *M. bovis* and risks compromising successful

treatment outcome for those patients (Allix-Béguec et al., 2010; Cosivi et al., 1995; Grange, 2001; Grange & Yates, 1994; Saukkonen et al., 2006).

Interestingly, exposure to *M. tuberculosis* does not invariably cause infection or result in disease. Active infection with clinical disease is observed in only a proportion of exposed individuals and the majority either immediately clear the bacteria or become latently infected with a 5-10% risk of disease reactivating later in life (WHO, 2022). The reason why 90% of exposed individuals clear TB infection is poorly understood but likely a culmination of multiple factors attributable to the host, bacterial load, and interactions between immune cells and the pathogen. Effective innate immune responses are implicated as a major contributing factor as certain exposed individuals do not show evidence of cell-mediated immunity even with repeated exposures to TB (Barry 3rd et al., 2009; WHO, 2022; Young et al., 2009).

Apparent variations in bacterial virulence for both *M. tuberculosis* and *M. bovis* have been reported in natural and experimental cases of human and animal tuberculosis, often correlating to specific phylogenetic clades and geographic lineages (Alvarez et al., 2009; Bigi et al., 2019; Tsolaki et al., 2004). Given the high level of genomic sequence homology for MTC members, it is suggested that the observed differences in disease prevalence geographically and severity within infected individuals may be explained by host genetics, environment, and/or differential gene expression of bacterial cell wall components and other virulence factors influencing immune outcomes (Castillo-Velázquez et al., 2013; Coscolla & Gagneux, 2010; Flores & Espitia, 2003; Reed et al., 2004).

As the name suggests, *M. bovis* is the primary cause of bovine tuberculosis (bTB) with cattle being the primary maintenance host. The economic impact of bTB resulting from trade restrictions, epidemiological investigations, regulatory testing, compensation to producers, as

well as long-term socioeconomic effects, are substantial (Caminiti et al., 2016; Krebs, 1997; Pérez-Morote et al., 2020). Natural infections of bTB are documented in over 40 animal species including domestic livestock and free-ranging wildlife (Corner, 2006; A. R. Spickler, 2019). Importantly, bTB is a zoonotic disease with human cases being especially prevalent at the human-animal interface involving livestock producers, veterinarians, zookeepers, and abattoir workers (Cosivi et al., 1995; A. R. Spickler, 2019). Contaminated meat and dairy products are also potential sources of bTB in humans. Historically, consumption of raw unpasteurized milk accounted for approximately 30% of primary gastrointestinal TB in children (McFadyean, 1900). Over a century of research has increased our understanding about bTB pathophysiology and transmission, contributing to improvement of diagnostics and control measures. Through the implementation of pasteurization, test and cull programs of affected cattle herds, and regular abattoir surveillance, the number of bTB cases in both cattle and humans in the U.S. has drastically decreased (Grange, 2001; Hardie & Watson, 1992; Michel et al., 2010; O'Reilly & Daborn, 1995; Pérez-Lago et al., 2014). However, it is suggested that an accurate depiction of bTB cases in humans around the globe is significantly underestimated as speciation of mycobacteria in human TB cases is not routinely performed (El-Sayed et al., 2016; Middelkoop et al., 2009). Whole genome sequencing (WGS) has become more readily available and practical for laboratory use, allowing for molecular epidemiology to be conducted during outbreaks (Van Soolingen, 2001).

In many ways, bTB parallels disease in humans caused by *M. tuberculosis,* however, there are notable differences in the host range, pathophysiology, and epidemiology (De la Rua-Domenech et al., 2006). Cattle are the primary maintenance host species for bTB but *M. bovis* has the widest host range of all MTC members, capable of causing disease in virtually any

mammal (Corner, 2006; R. S. Miller & S. J. Sweeney, 2013). In the late 1800s, Theobold Smith was the first to suggest that *M. bovis* was a distinct disease agent from *M. tuberculosis* based on culture growth characteristics, bacterial morphology, and different virulence observed in bioassays (Smith, 1898). Both are slow-growing but *M. tuberculosis*, comparatively, displayed more rapid growth on culture media and was observed to be slender, curved bacilli approximately 2-3X the length of *M. bovis*, which typically measures 5-7µm long and 0.2-0.5um wide. Within their respective primary hosts, *M. tuberculosis* is more likely to result in latent infection in humans while *M. bovis* tends to cause more severe and acute disease in cattle (Alvarez et al., 2009). Human cases of bTB are also shown to have a higher incidence of extrapulmonary disease which complicates detection based on conventional diagnostic methods for TB, which are usually focused on respiratory infection (Allix-Béguec et al., 2010). Both TB and bTB are complex diseases shaped by intricate host-pathogen interactions and environmental influences. Understanding the pathophysiology in susceptible hosts is crucial to our understanding of disease and implementing effective control strategies.

M. bovis and *M. tuberculosis* are most commonly transmitted via inhalation and reach the terminal bronchioles where they interact with host pattern recognition receptors and cell surface integrins on alveolar macrophages (Fenton et al., 2004; Kleinnijenhuis et al., 2011). Two C-type lectin receptors, the mannose receptor on alveolar macrophages and dendritic cell intercellular-adhesion molecule-3 grabbing non-integrin (DC-SIGN) on dendritic cells (DCs) are important in initial recognition of LAM and Man-LAM on the cell wall of pathogenic mycobacteria, ultimately inducing phagocytosis and cytokine production (Hoebe et al., 2005; Rojas et al., 2000; Torrelles & Schlesinger, 2010). Toll-like receptors TLR2 and TLR4 on alveolar macrophages become engaged through interactions with Man-LAM and phosphatidylinositol mannosides

(PIM), causing release of pro-inflammatory cytokines (IL-1β, IL-12, IL-6, IFN-γ, TNF-α) and chemokines (CXCL8, MCP-1, RANTES) via MyD88-dependent signaling pathway (Ballesteros et al., 2009; Bowdish et al., 2009; Gilleron et al., 2003; Magee et al., 2014). Complement component 3b (C3b) and complement receptors CR1, CR3, and CR4 contribute to mycobacterial opsonization and complement-mediated phagocytosis into phagocytes (Le Cabec et al., 2002; Schlesinger et al., 1990).

Following internalization, successful bactericidal events result in mycobacterial killing in the vast majority of infections. Host receptor interactions with TNF-α and IFN-γ, other endogenous signaling molecules (i.e., 1,25-dihydroxyvitamin D3), and mycobacterial cell wall components (i.e., PE-PGRS, LAM) stimulate phagolysosome fusion and production of inducible nitric oxide synthase (iNOS) (Chan et al., 2001; Esquivel-Solís et al., 2013; García-Jiménez et al., 2012; Rockett et al., 1998). This leads to phagolysosomal maturation and production of reactive oxygen and nitrogen species, including nitric oxide (NO), contributing to early bacterial clearance (Balaji et al., 2007; Bansal et al., 2010; Basu et al., 2007).

Mycobacteria have evolved mechanisms to survive in the host by influencing the process of granuloma formation and taking advantage of its unforgiving environment (Volkman et al., 2004). Host cells infected with MTC succumb to immunomodulation and bacterial persistence and may undergo cellular death. Pathogenic MTC modulate tyrosine kinase cell signaling pathways and second messengers causing attenuation of Th1 pro-inflammatory cytokine secretion and inhibit phagolysosome fusion/acidification which promotes bacterial growth and survival (Koul et al., 2004; Sturgill-Koszycki et al., 1994). ESAT-6 and CFP-10 heterodimer secretion by the ESX-1 type VII secretion system ruptures the phagosome and releases mycobacteria into the cytoplasm (Augenstreich et al., 2017; Simeone et al., 2012). Phagosomal

escape is beneficial for intracellular bacterial survival but, in turn, also activates cell-mediated immunity (Jones et al., 2010; Pollock & Andersen, 1997b). Antigen presenting cells (APCs) parasitized by mycobacteria secrete Th1 cytokines while ESAT-6 and CFP-10 induces matrix metalloproteinase 9 (MMP9) from surrounding epithelial cells, ultimately recruiting monocytes to the granuloma and propagating infection (Maggioli et al., 2016; Volkman et al., 2004; Volkman et al., 2010; Vordermeier et al., 2002). Downregulation of MHC I on infected host monocytes, mediated by ESAT-6, further contributes to the internal struggle between host and pathogen by abrogating the adaptive immune response (Sreejit et al., 2014). The result is formation of an organized, complex structure of activated and naïve immune cells with a center of caseous necrosis – the granuloma.

The general function of a granuloma is to wall off an infection or foreign material that immune cells are unable to clear through normal lysosomal, autophagic, and exocytic mechanisms. Essentially, a failure of innate immune clearance stimulates cell-mediated immunity and granuloma formation through complex interactions between specialized immune cells. Macrophages, multinucleated giant cells (MGCs), and DCs are all monocytic cells capable of supporting mycobacterial growth and are crucial producers of cytokines and chemokines throughout bTB lesion development (Palmer et al., 2016). Innate cellular homing mechanisms allow DCs to migrate to regional lymph nodes and activate lymphocytes, which, in turn, migrate to the site of infection. However, migration of DCs from the site of infection to regional lymph nodes may contribute to disease spread (Humphreys et al., 2006).

Ultimately, cytokine expression shapes the host immune response and determines the outcome of bTB lesion development in susceptible animals. Throughout chronic TB infection, cytokine expression is dynamic but Th1 cytokines are essential for preventing unchecked

bacterial replication and dissemination (Cooper, 2009). Infected macrophages produce IL-12 and TNF-α to stimulate IFN-γ release by CD4 T-helper cells. In turn, IFN-γ further activates internal macrophage bactericidal mechanisms for efficient mycobacterial killing (Clay et al., 2008). The containment of bacilli within the granuloma is largely due to polarization of macrophages to an M1 phenotype, leading to conversion of L-arginine to NO which exerts bactericidal effects on mycobacteria (Yang et al., 2016).

The essential role TNF-α and IFN-γ have in the formation and maintenance of granulomas and control of disease caused by pathogenic mycobacteria is highlighted by knockout studies and case reports of patients receiving anti-TNF or -IFN therapy. Inhibition of TNF-α or IFN-γ expression or downstream signaling often results in disease progression and/or death (Maggioli et al., 2016; Maggioli et al., 2015; Pollock, McNair, et al., 2001; Thacker et al., 2007). TNF-α deficiency causes loss of latency through granuloma breakdown, bacterial replication, and dissemination. The granuloma helps to control TB progression by serving as both a physical barrier, whereby bacterial replication is impeded by the anaerobic environment of the necrotic core, and through cytokine-dependent bactericidal mechanisms which prevent cellular turnover and bacterial dissemination (Clay et al., 2008; Ramakrishnan, 2012).

Mycobacterial growth and dissemination are promoted in certain instances where a bias toward Th2 cytokine responses drives humoral immunity (Castillo-Velázquez et al., 2013; Lucena et al., 2017; Ritacco et al., 1991; Welsh et al., 2005). Interleukins-4, -5, and -10 activate Th2 host immunity resulting in antibody production and M2 macrophage differentiation. Macrophages in this environment transcribe arginase which converts L-arginine to proline/polyamine and urea. Instead of promoting cytotoxicity and phagocytosis through reactive

nitrogen species, M2 macrophages are anti-inflammatory and stimulate angiogenesis, in favor of mycobacterial persistence.

The recruitment of uninfected macrophages and activation of T helper and cytotoxic T cells contributes to granuloma formation, maturation. CD4 T helper lymphocytes provide IFN-γ for macrophage activation. CD8 T cells serve dual protective and damaging roles by mediating cytotoxicity of infected macrophages and MGCs but release bacteria into the caseonecrotic center (Liebana et al., 2000). Surrounding the necrotic core of the prototypical mycobacterial granuloma are uniquely differentiated epithelioid macrophages and Langhans-type MGCs. These cells may be present in any granulomatous immune response and display decreased (for epithelioid) to absent (MGCs and Langhans-type MGCs) bacterial internalization capability compared with other macrophages, however, they maintain antigen presentation capability (Lay et al., 2007; Mustafa et al., 2008). Macrophages and MGCs lack CD1 expression, therefore, can only present protein antigens and are limited in class I MHC by mycobacterial evasion tactics (Schaible et al., 2003). DCs in *M. tuberculosis* infection are essential for recognition of mycobacteria's antigenic glycolipids (i.e., LAM) through CD1, allowing for cross-presentation to class I MHC on group I CD1-restricted CD8 T cells (Moody et al., 2000). CD8 T cells produce IFN-γ and induce apoptosis of infected cells, further contributing to antigen presentation by delivering apoptotic blebs containing mycobacterial antigens to uninfected APCs. MGCs are the most common cell type to show intracytoplasmic acid-fast bacilli, carrying a higher antigen load.

The majority of lymphocytes in Cattle, swine, and other animal species have the gamma-delta T-cell (γδ T-cell) receptor immunophenotype. In bTB, γδ T-cells are thought to play a role both in innate defenses and activation of adaptive immunity (Guzman et al., 2014; Guzman et al.,

2012; McGill et al., 2014). Following bTB infection, γδ T-cells respond by participating in cytokine production, granuloma formation, and mycobacterial killing. A surface scavenger receptor, WC1, recognizes mycobacterial antigens, resulting in T cell recruitment and subsequent production of IFN-γ and CCL2 within tubercles during chronic disease (Pollock, McNair, et al., 2001; Rusk et al., 2017). The role of γδ T-cells in innate defenses and adaptive disease control to bTB in livestock are apparent, yet the significance of their contribution is unclear (Kennedy et al., 2002).

Apoptosis is an orderly cell death whereby membrane-bound cytoplasmic blebs of dying cells are released from the dying cell only to be engulfed by inactivated phagocytes. In contrast, necrosis is a disorderly process where cellular contents are released into the extracellular space, stimulating an inflammatory reaction. Although apoptosis may appear preferable to avoid bacterial spread, resident macrophages involved in clearing cellular debris may become infected by phagocytosing apoptotic vesicles containing MTC (Davis & Ramakrishnan, 2009). Virulent mycobacteria are shown to induce apoptosis through ESAT-6 production and signaling (Ramakrishnan, 2012). Conversely, MTC also inhibit cellular apoptotic pathways by skewing immune signaling (Balcewicz-Sablinska et al., 1998; Queval et al., 2016). Down-regulation of TNF-α and/or its receptor by mycobacteria induces host cell necrosis and interferes with IFN-γ signaling (Chen et al., 2008; Clay et al., 2008; Tobin et al., 2012; Tobin et al., 2010). Both apoptotic and necrotic cell death are proposed to occur from mycobacterial infection and potentially contribute to perpetuating disease (Augenstreich et al., 2017; Basu et al., 2007; Forrellad et al., 2013; Keane et al., 2000). The interplay between host defensive mechanisms and MTC immunomodulatory effects fluctuate throughout the course of disease and determines the rate of bacterial growth, dissemination, and lesion severity.

Histologically, a classic tuberculoid granuloma consists of a necrotic or mineralized center surrounded by organized cellular layers of epithelioid macrophages and MGCs, an outer rim of lymphocytes, and, usually, a fibrous connective tissue capsule. MTC bacteria are usually present in low numbers (paucibacillary) compared with lesions from NTM. Lesions may be assigned a histologic grade (I-IV) according to the chronicity, determined by the level of cellular organization, appearance of the fibrous capsule, extent of central necrosis, and the presence/absence of dystrophic mineralization (Palmer et al., 2007). An array of I – IV granulomas are visualized in most bTB-infected tissues, with small satellite granulomas forming at the edge of much more mature lesions, suggesting processes involved in early lesion development and maturation occur throughout chronic infection. It has been proposed that TGF-β expression is higher in stage III and IV granulomas owing to collagen production of the fibrous capsule, however, encapsulation is an inconsistent finding, and some studies showed higher TGF-β expression within stage I and II granulomas (Aranday-Cortes et al., 2013; Palmer et al., 2007). Mycobacteria are typically concentrated either within the necrotic center of the granuloma or within surrounding macrophages and MGCs.

Cell-mediated immunity is an important part of controlling tuberculosis and the basis of current diagnostic assays. The delayed-type hypersensitivity elicited by memory T cells in tuberculosis assists in disease control, but neither infection nor vaccination achieve protective, lifelong, sterilizing immunity (Pollock, McNair, et al., 2001). Antemortem bTB diagnostics offer convenient screening tests but are limited in their sensitivity and specificity. The tuberculin skin test in cattle consists of intradermal injection of purified protein derivative (PPD) from heat-killed mycobacteria in the caudal fold of the tailhead and relies on cell-mediated immunity mounted in exposed animals to produce measurable inflammation at the site of injection within

72 hours (Schiller et al., 2010; Waddington, 2004). This on-farm test requires animal handling by an accredited veterinarian on two separate occasions, to inject PPD on day 1, followed by test result interpretation 48-72 hours later. Variations in immune response, PPD composition/preparation, test administration and interpretation, BCG vaccine status, and/or coinfection with NTM or atypical mycobacteria all confound accuracy of the tuberculin skin test. Cross reactivity with NTM especially from *Mycobacterium avium* ssp. *paratuberculosis* (the cause of Johne's disease) commonly results in false positive results (Aranaz et al., 2004; Biet et al., 2005; De la Rua-Domenech et al., 2006; Gcebe et al., 2016; Schiller et al., 2010). Test interpretation may also be variable among veterinarians, leading to bTB-infected animals going unnoticed as well as reactors lacking postmortem lesions (Doherty & Cassidy, 2002; Pollock, Buddle, et al., 2001). In cattle, the sensitivity of the caudal fold test (CFT) ranges from 68% to 96.8% while the specificity achieves 96-98.8%. The comparative cervical skin test (CCT) uses two different PPDs, one derived from *M. bovis* (PPDb) and one from *M. avium* (PPDa) to help to differentiate between animals with Johne's vs. bTB (Schiller et al., 2010). However, there remains overlapping protein antigens between PPD derived from NTM and MTC species contributing to lower specificity in tests utilizing these tuberculins (De la Rua-Domenech et al., 2006; Gcebe et al., 2016). The CCT is available as a parallel or follow-up test to help increase tuberculin skin test specificity but achieves an estimated 55.1-93.5% and 88.8-100% sensitivity and specificity, respectively (Monaghan et al., 1994; Schiller et al., 2010). To increase test specificity of tuberculins, using a cocktail of antigens or those conserved predominantly among MTC have growing appeal. ESAT-6 and CFP-10 show promising roles in the future development of MTC vaccination and/or more specific antigenic targets for diagnostic use (Maggioli et al., 2016; Volkman et al., 2004; Vordermeier et al., 2002). ESAT-6 is cited as a

more specific and robust T cell antigen than PPD and has been exploited for diagnostic purposes in MTC infection Pollock 1997b (Pollock & Andersen, 1997a; Vordermeier et al., 2001). Other drawbacks to traditional skin testing are repeated handling of animals, availability of PPD, and requiring experienced personnel to administer then read test results 72 hours apart, making it impractical in certain situations, especially for non-domesticated animal species.

The IFN-γ release assay is another ancillary antemortem diagnostic test available that relies upon the cell-mediated immune response in bTB-infected animals and shares similarities with the QuantiFERON gold test utilized in humans (Schiller et al., 2010). Briefly, an enzyme-linked immunosorbent assay (ELISA) is used to quantify IFN-γ production from ex vivo stimulated PBMCs, predominantly T lymphocytes, in whole blood samples (Rothel et al., 1990; Schiller et al., 2010). PPD is the most common stimulant used to induce IFN-γ production but PBMCs may be stimulated with other mycobacterial antigens (i.e., ESAT-6, CFP-10). A non-stimulatory reagent (i.e., PBS) as well as a non-specific mitogen (i.e. pokeweed mitogen, PWM) are generally included as a negative and positive control, respectively. The observed optical density of IFN-γ in response to PPDa is measured via ELISA plate reader, subtracted from that of PPDb, and compared to PWM to determine a positive result in cattle. The IFN-γ release assay has an average sensitivity and specificity of 87.6% and 96.6%, respectively, and is more rapid than the CFT and CCT which is beneficial but also a limiting factor as whole blood must be analyzed within 24 hours from the time of collection. Constraints associated with a higher cost and specificity concerns with tuberculins are drawbacks to the IFN-γ release assay (Pollock et al., 2005; Schiller et al., 2010; Schiller et al., 2009).

Serology is less commonly used for domestic cattle, as antibody production is inconsistent, but has proven useful as an initial screening test in a handful of wildlife and zoo

animal species. Specifically, the dual path platform (DPP) is a commercialized lateral-flow based assay (Chembio©) with two test lines containing MTC-specific antigens, MPB83 and ESAT-6/CFP-10 (ECH), coated to colloidal gold particles on a nitrocellulose membrane. Sample is loaded passing through each test line and a control line allowing antibody to bind and creating a visible band. In the United States, visible bands are read by a spectrophotometer and reported in relative light units (RLU). The DPP is validated for white tailed deer and elephants using a threshold value of 50 and 3.0 RLU, respectively, has been established to determine a positive result (Greenwald et al., 2009; Lyashchenko et al., 2013). In these two species where DPP is validated, the sensitivity ranges from 65.1-100% while specificity ranges from 97.8-100%. Other wildlife maintenance hosts of bTB have also been assessed on DPP yielding sensitivity and specificity of 55% and 98% in badgers (Ashford et al., 2020) and 89.6% and 90.4% in the Eurasian wild boar (Boadella et al., 2011).

Since its development in 1921, BCG remains the only licensed vaccine for TB in humans. While BCG decreases disease severity and minimizes mortality associated with meningeal and disseminated TB in infants, it does not prevent infection by *M. tuberculosis* or other MTC species. BCG efficacy in adults has additional limitations as it does not prevent pulmonary disease or progression from latency to active infection, allowing TB transmission to perpetuate (WHO, 2022). BCG vaccination has never been shown to revert to a virulent state, however, when administered in the absence of TB infection it can produce lymphadenitis and small, mild TB-like lesions which can increase in severity particularly with immunosuppression (Norouzi et al., 2012). The efficacy and widespread use of BCG is debatable. BCG is regularly administered to healthy neonatal humans in endemic regions but not in countries with a relatively low prevalence of TB, such as the United States (WHO, 2022). Protection is provided by primarily

memory T cells but the duration of efficacy and ability to differentiate vaccinated from infected animals complicates diagnostics and control efforts (Ballesteros et al., 2009; Maggioli et al., 2016; Maggioli et al., 2015).

Although reported on nearly every civilized continent aside from Australia, the prevalence of bTB varies greatly worldwide. Many proposed factors contribute to geographic differences in bTB, including producer management practices, socioeconomic status, implementation of surveillance and eradication programs, host-pathogen interactions, and the presence/absence of wildlife maintenance hosts (Ameni et al., 2007; McInerney et al., 1995; Queirós et al., 2018; Vicente et al., 2013; Vicente et al., 2006; Vicente et al., 2007). Animal overcrowding in the production system causes stress and exacerbates transmission of communicable disease especially among aerogenous agents like *M. bovis* (Phillips et al., 2003; Serraino et al., 1999). The development and execution of bTB control programs helps mitigate disease spread, however, efforts for complete eradication become exceedingly impossible following establishment of wildlife maintenance hosts (Aranaz et al., 2004; Bany & Freier, 2000; Beltrán-Beck et al., 2012; R. Miller & S. Sweeney, 2013; R. S. Miller & S. J. Sweeney, 2013).

Despite the characterization of human- and animal-adapted strains and the general acceptance of a primary reservoir host for each, all MTC members are disease-causing agents in more than one species. *M. tuberculosis* shows tropism for humans with evidence of natural infection in elephants, non-human primates, dogs, cats, and isolated reports in other wildlife and zoo animals (Ghodbane & Drancourt, 2013). *M. orygis* and *M. caprae* have been isolated from primates, humans, and various ungulates aside from oryx and goats, their respective primary hosts (Aranaz et al., 2003; Aranaz et al., 2004; van Ingen et al., 2012). The MTC member

exhibiting the most extensive host range, however, is *M. bovis*, capable of infecting nearly any terrestrial mammal and certain species of birds (Corner, 2006; A. R. Spickler, 2019).

With such a variety of potential hosts, identifying those which play an integral part in perpetual maintenance of bTB through documented inter- and intra-species transmission (maintenance host) is essential. Certain animals act as sentinel species for bTB as they participate in disease spillover events from maintenance hosts. Sentinel species may be spillover hosts or amplifier hosts for bTB but do not maintain bTB within their population in the absence of other maintenance hosts. They may be more prone to developing acute disease and disseminated lesions, which may or may not involve the lungs (Coleman et al., 1999; Corner et al., 1981; Corner, 2006). Animals which succumb more quickly do not shed bacilli for as long and extrapulmonary disease is less likely to aerosolize infectious bacilli, however, many factors are involved and depend largely on the host species involved. Numerous wildlife bTB maintenance hosts have been documented across the globe. These include red deer/elk in Europe, white-tailed deer (WTD) in Michigan, elk and wood bison in Canada, the Eurasian wild boar in the Mediterranean, brushtail possums in New Zealand, European badger in the United Kingdom and Republic of Ireland, and the African buffalo (Corner, 2006; A. R. Spickler, 2019).

Worldwide, the Eurasian/European wild boar (*Sus scrofa scrofa*) has been proposed as a sentinel species for bTB in New Zealand, a spillover host in the U.K., and a maintenance host in Spain (Victoria Naranjo et al., 2008; Nugent, 2011; Palmer & Waters, 2006; Richomme et al., 2010; Wakelin & Churchman, 1991). Conversely, domestic pigs (*Sus scrofa domesticus*) are generally considered a spillover host for bTB. The Eurasian wild boar was originally introduced to North America by Spanish explorers and settlers in the 1500s and have since outbred with backyard domestic breeds of pigs, resulting in a hybrid species with a mixed genetic background

at present day (Goedbloed et al., 2013; Smyser et al., 2020; USDA, 2023a; Wood & Barrett, 1979). In contrast, feral swine on Hawaii were originally introduced by the Polynesians and the majority (70% from one study) retain the Pacific clade lineage, similar to the pot-bellied pig (Linderholm et al., 2016). A smaller proportion (30%) of Hawaiian feral swine showed European clade origins from the later introduction of the Eurasian wild boar by Captain Cook. Only two pigs from Moloka'i were included in this study but one carried the Pacific clade allele while the other carried the European allele.

Feral swine on the Hawaiian island of Moloka'i were first found to be infected with *M. bovis* in 1980 with an estimated 19.67% prevalence (ranging from 11-35% at 95% CI), dropping to 3.22% (>0-17% at 95% CI) in 1983 (Essey et al., 1981; Essey et al., 1983). The drop in prevalence was attributed to actions taken to control bTB, including depopulation of the affected cattle herd on the eastern end of the island and open hunting declared for feral swine surrounding affected cattle ranches and within the bordering state forest. It was surmised that bTB was first introduced to cattle on Moloka'i in the 1940s and spread until 1985 when the decision was made to depopulate all cattle on the entire island, totaling approximately 9,400 head (Meyer, 2000). No cattle were on the island for a full year following depopulation and only steers were allowed the 2nd year, who were immediately sent to slaughter upon maturing. Slowly, after another 2 years, breeding animals with two negative tuberculin skin tests were introduced and annual regulatory testing was implemented.

Moloka'i cattle remained bTB-free up until 1997 when a cow from a herd of 450 cattle on the eastern end of the island tested positive (Meyer, 2000). The affected herd was depopulated and 4,922 cattle from surrounding ranches were tested but no reactors were detected. Additionally, a hunter-assisted wildlife surveillance study targeting axis deer, feral swine, feral

goats, and mongoose was conducted from 1998 until early 2008. Earlier surveillance efforts between 1961-1985 detected a handful of positive axis deer (n=5) and mongoose (single *M. bovis* colony from a pooled sampled of 3 mongoose). However, out of approximately 350 axis deer, over 70 feral goats, 70 mongoose, and 450 feral swine, only feral swine showed evidence of bTB from the wildlife survey (Meyer, 2000).

In the U.S., and around the globe, feral swine are regarded as an invasive pest species responsible for causing significant damage to crops, fragile ecosystems, and threatened/endangered species (Bevins et al., 2014). Feral swine in the continental U.S. harbor multiple diseases of agricultural and public health significance, such as brucellosis (caused by *Brucella suis* and other *Brucella* sp.) and pseudorabies (caused by suid herpesvirus-1) (Miller et al., 2013; Pedersen et al., 2017; VerCauteren et al., 2019). They are susceptible to foreign animal diseases such as African swine fever, classical swine fever, and foot and mouth disease virus, which carry devastating consequences for food production systems (USDA, 2023a). The reproductive cycle of feral swine is more prolific than any other wild ungulate and their natural behaviors and opportunistic, omnivorous diet drive them to congregate at shared watering holes and wildlife baiting stations, which overlap with domestic livestock and perpetuate disease transmission (Taylor et al., 1998; Wyckoff et al., 2009). Since 1982, the population of U.S. feral swine has increased exponentially to an estimated 6 million at present day and their range has expanded from primarily affecting 11 states in the southern U.S. to 35 states including central and northern U.S. (Bevins et al., 2014; Wood & Barrett, 1979). Furthermore, along the southern Canadian border, expanding populations of feral swine of Eurasian wild boar descent have escaped captivity and wreaked havoc on farmlands and forested habitats for decades (Aschim &

Brook, 2019). The expanding range and population densities of feral swine across the continental U.S. create a high-risk scenario for infectious disease spillover.

Given recent disease occurrence on Moloka'i and a continued threat of bTB disease spillover into feral swine in the continental U.S. from infected cattle or wildlife, we sought to infer bTB host status of feral swine in North America by looking retroactively at surveillance samples and modeling bTB disease in the species of interest using experimental infection. Chapter 2 will discuss the historical data available for documented cases of bTB in Moloka'i feral swine from wildlife surveillance samples to compare diagnostic test performance with cattle bTB submissions and investigate discrepant results. The potential role of Molokai'i pigs as a maintenance host for bTB is further investigated in Chapter 3 through comparative pathology of experimental bTB infection in two genetically distinct populations of feral swine. This information will inform epidemiologists and researchers of the important factors contributing to disease outcomes in feral swine and will hopefully impress consideration of suids as susceptible species to bTB with the potential to amplify or maintain disease in specific situations which target host immunity or perpetuate disease transmission.

References

Abdallah, A. M., Gey van Pittius, N. C., DiGiuseppe Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J., & Bitter, W. (2007). Type VII secretion—mycobacteria show the way. Nature Reviews Microbiology, 5(11), 883-891.

Alexander, K. A., Laver, P. N., Michel, A. L., Williams, M., van Helden, P. D., Warren, R. M., & van Pittius, N. C. G. (2010). Novel *Mycobacterium tuberculosis* complex pathogen, M. mungi. Emerging infectious diseases, 16(8), 1296.

Allix-Béguec, C., Fauville-Dufaux, M., Stoffels, K., Ommeslag, D., Walravens, K., Saegerman, C., & Supply, P. (2010). Importance of identifying *Mycobacterium bovis* as a causative agent of human tuberculosis. European Respiratory Journal, 35(3), 692-694.

Alvarez, A. H., Estrada-Chávez, C., & Flores-Valdez, M. A. (2009). Molecular findings and approaches spotlighting *Mycobacterium bovis* persistence in cattle. Veterinary Research, 40(3).

Ameni, G., Aseffa, A., Engers, H., Young, D., Gordon, S., Hewinson, G., & Vordermeier, M. (2007). High prevalence and increased severity of pathology of bovine tuberculosis in Holsteins compared to zebu breeds under field cattle husbandry in central Ethiopia. Clinical and Vaccine Immunology, 14(10), 1356-1361.

Andersen, P., Andersen, A. B., Sørensen, A., & Nagai, S. (1995). Recall of long-lived immunity to *Mycobacterium tuberculosis* infection in mice. Journal of immunology (Baltimore, Md.: 1950), 154(7), 3359-3372.

Aranaz, A., Cousins, D., Mateos, A., & Domínguez, L. (2003). Elevation of *Mycobacterium tuberculosis* subsp. caprae Aranaz et al. 1999 to species rank as Mycobacterium caprae comb. nov., sp. nov. International journal of systematic and evolutionary microbiology, 53(6), 1785-1789.

Aranaz, A., De Juan, L., Montero, N., Sánchez, C., Galka, M., Delso, C., Alvarez, J., Romero, B., Bezos, J., & Vela, A. I. (2004). Bovine tuberculosis (*Mycobacterium bovis*) in wildlife in Spain. Journal of Clinical Microbiology, 42(6), 2602-2608.

Aranday-Cortes, E., Bull, N., Villarreal-Ramos, B., Gough, J., Hicks, D., Ortiz-Peláez, A., Vordermeier, H., & Salguero, F. (2013). Upregulation of IL-17 A, CXCL 9 and CXCL 10 in Early-Stage Granulomas Induced by M ycobacterium bovis in Cattle. Transboundary and Emerging Diseases, 60(6), 525-537.

Aschim, R. A., & Brook, R. K. (2019). Evaluating cost-effective methods for rapid and repeatable national scale detection and mapping of invasive species spread. Scientific Reports, 9(1), 7254.

Ashford, R. T., Anderson, P., Waring, L., Davé, D., Smith, F., Delahay, R. J., Gormley, E., Chambers, M. A., Sawyer, J., & Lesellier, S. (2020). Evaluation of the Dual Path Platform (DPP) VetTB assay for the detection of *Mycobacterium bovis* infection in badgers. Preventive Veterinary Medicine, 180, 105005.

Augenstreich, J., Arbues, A., Simeone, R., Haanappel, E., Wegener, A., Sayes, F., Le Chevalier, F., Chalut, C., Malaga, W., & Guilhot, C. (2017). ESX-1 and phthiocerol dimycocerosates of *Mycobacterium tuberculosis* act in concert to cause phagosomal rupture and host cell apoptosis. Cellular microbiology, 19(7), e12726.

Balaji, K. N., Goyal, G., Narayana, Y., Srinivas, M., Chaturvedi, R., & Mohammad, S. (2007). Apoptosis triggered by Rv1818c, a PE family gene from *Mycobacterium tuberculosis* is regulated by mitochondrial intermediates in T cells. Microbes and Infection, 9(3), 271-281.

Balcewicz-Sablinska, M. K., Keane, J., Kornfeld, H., & Remold, H. G. (1998). Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-α. The Journal of Immunology, 161(5), 2636-2641.

Ballesteros, C., Garrido, J. M., Vicente, J., Romero, B., Galindo, R. C., Minguijón, E., Villar, M., Martín-Hernando, M. P., Sevilla, I., & Juste, R. (2009). First data on Eurasian wild boar response to oral immunization with BCG and challenge with a *Mycobacterium bovis* field strain. Vaccine, 27(48), 6662-6668.

Bansal, K., Elluru, S. R., Narayana, Y., Chaturvedi, R., Patil, S. A., Kaveri, S. V., Bayry, J., & Balaji, K. N. (2010). PE_PGRS antigens of *Mycobacterium tuberculosis* induce maturation and activation of human dendritic cells. The Journal of Immunology, 184(7), 3495-3504.

Bany, S., & Freier, J. (2000). Use of GIS to evaluate livestock-wildlife interactions relative to tuberculosis spread on Molokai Island, Hawaii.

Barry 3rd, C. E., Boshoff, H. I., Dartois, V., Dick, T., Ehrt, S., Flynn, J., Schnappinger, D., Wilkinson, R. J., & Young, D. (2009). The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nature Reviews Microbiology, 7(12), 845-855.

Basu, S., Pathak, S. K., Banerjee, A., Pathak, S., Bhattacharyya, A., Yang, Z., Talarico, S., Kundu, M., & Basu, J. (2007). Execution of macrophage apoptosis by PE_PGRS33 of *Mycobacterium tuberculosis* is mediated by Toll-like receptor 2-dependent release of tumor necrosis factor-α. Journal of Biological Chemistry, 282(2), 1039-1050.

Beltrán-Beck, B., Ballesteros, C., Vicente, J., De la Fuente, J., & Gortázar, C. (2012). Progress in oral vaccination against tuberculosis in its main wildlife reservoir in Iberia, the Eurasian wild boar. Veterinary Medicine International, 2012.

Berthet, F.-X., Rasmussen, P. B., Rosenkrands, I., Andersen, P., & Gicquel, B. (1998). A *Mycobacterium tuberculosis* operon encoding ESAT= 6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology, 144(11), 3195-3203.

Bevins, S. N., Pedersen, K., Lutman, M. W., Gidlewski, T., & Deliberto, T. J. (2014). Consequences associated with the recent range expansion of nonnative feral swine. BioScience, 64(4), 291-299.

Biet, F., Boschiroli, M., Thorel, M., & Guilloteau, L. L. (2005). Zoonotic aspects of *Mycobacterium bovis* and Mycobacterium avium-intracellulare complex (MAC). Veterinary Research, 36(3), 411-436.

Bigi, M., Vazquez, C. L., Castelão, A. B. C., Garcia, E. A., Cataldi, A. A., Jackson, M., McNeil, M., Soria, M., Zumárraga, M. J., & Cabruja, M. (2019). Analysing nonsynonymous mutations between two *Mycobacterium bovis* strains with contrasting pathogenic profiles. Veterinary Microbiology, 239, 108482.

Boadella, M., Lyashchenko, K., Greenwald, R., Esfandiari, J., Jaroso, R., Carta, T., Garrido, J. M., Vicente, J., de la Fuente, J., & Gortázar, C. (2011). Serologic tests for detecting antibodies against *Mycobacterium bovis* and Mycobacterium avium subspecies paratuberculosis in Eurasian wild boar (Sus scrofa scrofa). Journal of Veterinary Diagnostic Investigation, 23(1), 77-83.

Bowdish, D. M., Sakamoto, K., Kim, M.-J., Kroos, M., Mukhopadhyay, S., Leifer, C. A., Tryggvason, K., Gordon, S., & Russell, D. G. (2009). MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. PLoS pathogens, 5(6), e1000474.

Brennan, M. J., Delogu, G., Chen, Y., Bardarov, S., Kriakov, J., Alavi, M., & Jacobs Jr, W. R. (2001). Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infection and Immunity, 69(12), 7326-7333.

Brennan, P. J., & Nikaido, H. (1995). The envelope of mycobacteria. Annual review of biochemistry, 64(1), 29-63.

Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., & Kremer, K. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceedings of the National Academy of Sciences, 99(6), 3684-3689.

Brosch, R., Gordon, S. V., Pym, A., Eiglmeier, K., Garnier, T., & Cole, S. T. (2000). Comparative genomics of the mycobacteria. International journal of medical microbiology, 290(2), 143-152.

Calmette, A., Guerin, C., Negre, L., & Boquet, A. (1926). Immunization of the Newly Born against Tuberculosis. Ann. Inst. Pasteur, 40, 89-133.

Caminiti, A., Pelone, F., LaTorre, G., De Giusti, M., Saulle, R., Mannocci, A., Sala, M., Della Marta, U., & Scaramozzino, P. (2016). Control and eradication of tuberculosis in cattle: a systematic review of economic evidence. Veterinary Record, 179(3), 70-75.

Castillo-Velázquez, U., Gomez-Flores, R., Tamez-Guerra, R., Tamez-Guerra, P., & Rodríguez-Padilla, C. (2013). Differential responses of macrophages from bovines naturally resistant or susceptible to *Mycobacterium bovis* after classical and alternative activation. Veterinary Immunology and Immunopathology, 154(1-2), 8-16.

Chan, E. D., Morris, K. R., Belisle, J. T., Hill, P., Remigio, L. K., Brennan, P. J., & Riches, D. W. (2001). Induction of inducible nitric oxide synthase-NO· by lipoarabinomannan of *Mycobacterium tuberculosis* is mediated by MEK1-ERK, MKK7-JNK, and NF-κB signaling pathways. Infection and Immunity, 69(4), 2001-2010.

Charlet, D., Mostowy, S., Alexander, D., Sit, L., Wiker, H. G., & Behr, M. A. (2005). Reduced expression of antigenic proteins MPB70 and MPB83 in *Mycobacterium bovis* BCG strains due to a start codon mutation in sigK. Molecular microbiology, 56(5), 1302-1313.

Chen, M., Divangahi, M., Gan, H., Shin, D. S., Hong, S., Lee, D. M., Serhan, C. N., Behar, S. M., & Remold, H. G. (2008). Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. The Journal of experimental medicine, 205(12), 2791-2801.

Clay, H., Volkman, H. E., & Ramakrishnan, L. (2008). Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. Immunity, 29(2), 283-294.

Cole, S., & Barrell, B. (1998). Analysis of the genome of *Mycobacterium tuberculosis* H37Rv. Genetics and Tuberculosis: Novartis Foundation Symposium 217,

Cole, S., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S., Eiglmeier, K., Gas, S., & Barry Iii, C. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature, 396(6707), 190-190.

Coleman, J., Cooke, M., Jackson, R., & Webster, R. (1999). Temporal patterns in bovine tuberculosis in a brushtail possum population contiguous with infected cattle in the Ahaura Valley, Westland. New Zealand Veterinary Journal, 47(4), 119-124.

Collins, C., & Grange, J. (1983). The bovine tubercle bacillus. Journal of Applied Microbiology, 55(1), 13-29.

Cooper, A. M. (2009). Cell-mediated immune responses in tuberculosis. Annual review of immunology, 27, 393-422.

Corner, L., Barrett, R., Lepper, A., Lewis, V., & Pearson, C. (1981). A survey of mycobacteriosis of feral pigs in the Northern Territory. Australian Veterinary Journal, 57(12), 537-542.

Corner, L. A. (2006). The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Veterinary Microbiology, 112(2-4), 303-312.

Coscolla, M., & Gagneux, S. (2010). Does M. tuberculosis genomic diversity explain disease diversity? Drug Discovery Today: Disease Mechanisms, 7(1), e43-e59.

Coscolla, M., Lewin, A., Metzger, S., Maetz-Rennsing, K., Calvignac-Spencer, S., Nitsche, A., Dabrowski, P. W., Radonic, A., Niemann, S., & Parkhill, J. (2013). Novel *Mycobacterium tuberculosis* complex isolate from a wild chimpanzee.

Cosivi, O., Meslin, F., Daborn, C., & Grange, J. (1995). Epidemiology of *Mycobacterium bovis* infection in animals and humans, with particular reference to Africa. Revue scientifique et technique (International Office of Epizootics), 14(3), 733-746.

Cousins, D. V., Bastida, R., Cataldi, A., Quse, V., Redrobe, S., Dow, S., Duignan, P., Murray, A., Dupont, C., & Ahmed, N. (2003). Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: Mycobacterium pinnipedii sp. nov. International journal of systematic and evolutionary microbiology, 53(5), 1305-1314.

Davis, J. M., & Ramakrishnan, L. (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell, 136(1), 37-49.

De la Rua-Domenech, R., Goodchild, A., Vordermeier, H., Hewinson, R., Christiansen, K., & Clifton-Hadley, R. (2006). Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Research in veterinary science, 81(2), 190-210.

Doherty, M., & Cassidy, J. (2002). New perspectives on bovine tuberculosis. The Veterinary Journal, 2(163), 109-110.

Dulberger, C. L., Rubin, E. J., & Boutte, C. C. (2020). The mycobacterial cell envelope—a moving target. Nature Reviews Microbiology, 18(1), 47-59.

El-Sayed, A., El-Shannat, S., Kamel, M., Castañeda-Vazquez, M., & Castañeda-Vazquez, H. (2016). Molecular epidemiology of *Mycobacterium bovis* in humans and cattle. Zoonoses and Public Health, 63(4), 251-264.

Eldholm, V., & Balloux, F. (2016). Antimicrobial resistance in *Mycobacterium tuberculosis*: the odd one out. Trends in microbiology, 24(8), 637-648.

Esquivel-Solís, H., Vallecillo, A. J., Benítez-Guzmán, A., Adams, L. G., Lopez-Vidal, Y., & Gutierrez-Pabello, J. A. (2013). Nitric oxide not apoptosis mediates differential killing of *Mycobacterium bovis* in bovine macrophages. PLoS One, 8(5), e63464.

Essey, M., Payne, R., Himes, E., & Luchsinger, D. (1981). Bovine tuberculosis surveys of axis deer and feral swine on the Hawaiian island of Molokai. Proceedings of the United States Animal Health Association,

Essey, M., Stallknecht, D., Himes, E., & Harris, S. (1983). Follow-up survey of feral swine for *Mycobacterium bovis* infection on the Hawaiian island of Molokai. Proceedings of the United States Animal Health Association,

Fedrizzi, T., Meehan, C. J., Grottola, A., Giacobazzi, E., Fregni Serpini, G., Tagliazucchi, S., Fabio, A., Bettua, C., Bertorelli, R., & De Sanctis, V. (2017). Genomic characterization of nontuberculous mycobacteria. Scientific Reports, 7(1), 1-14.

Fenton, M. J., Riley, L. W., & Schlesinger, L. S. (2004). Receptor-mediated recognition of *Mycobacterium tuberculosis* by host cells. Tuberculosis and the Tubercle Bacillus, 403-426.

Flores, J., & Espitia, C. (2003). Differential expression of PE and PE_PGRS genes in *Mycobacterium tuberculosis* strains. Gene, 318, 75-81.

Forrellad, M. A., Klepp, L. I., Gioffré, A., Sabio y Garcia, J., Morbidoni, H. R., Santangelo, M. D. L. P., Cataldi, A. A., & Bigi, F. (2013). Virulence factors of the *Mycobacterium tuberculosis* complex. Virulence, 4(1), 3-66.

García-Jiménez, W., Fernández-Llario, P., Gómez, L., Benítez-Medina, J., García-Sánchez, A., Martínez, R., Risco, D., Gough, J., Ortiz-Peláez, A., & Smith, N. (2012). Histological and immunohistochemical characterisation of *Mycobacterium bovis* induced granulomas in naturally infected fallow deer (Dama dama). Veterinary Immunology and Immunopathology, 149(1-2), 66-75.

Garnier, T., Eiglmeier, K., Camus, J.-C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., & Monsempe, C. (2003). The complete genome sequence of *Mycobacterium bovis*. Proceedings of the National Academy of Sciences, 100(13), 7877-7882.

Gcebe, N., Michel, A., Gey van Pittius, N. C., & Rutten, V. (2016). Comparative genomics and proteomic analysis of four non-tuberculous Mycobacterium species and *Mycobacterium tuberculosis* complex: occurrence of shared immunogenic proteins. Frontiers in microbiology, 7, 795.

Gey van Pittius, N. C., Gamieldien, J., Hide, W., Brown, G. D., Siezen, R. J., & Beyers, A. D. (2001). The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+ C Gram-positive bacteria. Genome biology, 2(10), 1-18.

Gey van Pittius, N. C., Sampson, S. L., Lee, H., Kim, Y., Van Helden, P. D., & Warren, R. M. (2006). Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions. BMC evolutionary biology, 6, 1-31.

Ghodbane, R., & Drancourt, M. (2013). Non-human sources of *Mycobacterium tuberculosis*. Tuberculosis, 93(6), 589-595.

Gilleron, M., Quesniaux, V. F., & Puzo, G. (2003). Acylation state of the phosphatidylinositol hexamannosides from *Mycobacterium bovis* bacillus Calmette Guerin and *Mycobacterium tuberculosis* H37Rv and its implication in Toll-like receptor response. Journal of Biological Chemistry, 278(32), 29880-29889.

Goedbloed, D., Megens, H., Van Hooft, P., Herrero-Medrano, J., Lutz, W., Alexandri, P., Crooijmans, R., Groenen, M., Van Wieren, S., & Ydenberg, R. (2013). Genome-wide single nucleotide polymorphism analysis reveals recent genetic introgression from domestic pigs into Northwest European wild boar populations. Molecular Ecology, 22(3), 856-866.

Gordon, S. V., Heym, B., Parkhill, J., Barrell, B., & Cole, S. T. (1999). New insertion sequences and a novel repeated sequence in the genome of *Mycobacterium tuberculosis* H37Rv. Microbiology, 145(4), 881-892.

Grange, J. (2001). *Mycobacterium bovis* infection in human beings. Tuberculosis, 81(1-2), 71-77.

Grange, J., & Collins, C. (1987). Bovine tubercle bacilli and disease in animals and man. Epidemiology & Infection, 99(2), 221-234.

Grange, J. M., & Yates, M. D. (1994). Zoonotic aspects of *Mycobacterium bovis* infection. Veterinary Microbiology, 40(1-2), 137-151.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Ball, R., Dumonceaux, G., Schmitt, D., & Moller, T. (2009). Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clinical and Vaccine Immunology, 16(5), 605-612.

Guerra-Assunção, J. A., Houben, R. M., Crampin, A. C., Mzembe, T., Mallard, K., Coll, F., Khan, P., Banda, L., Chiwaya, A., & Pereira, R. P. (2015). Recurrence due to relapse or reinfection with *Mycobacterium tuberculosis*: a whole-genome sequencing approach in a large, population-based cohort with a high HIV infection prevalence and active follow-up. The Journal of infectious diseases, 211(7), 1154-1163.

Guzman, E., Hope, J., Taylor, G., Smith, A. L., Cubillos-Zapata, C., & Charleston, B. (2014). Bovine γδ T cells are a major regulatory T cell subset. The Journal of Immunology, 193(1), 208-222.

Guzman, E., Price, S., Poulsom, H., & Hope, J. (2012). Bovine γδ T cells: cells with multiple functions and important roles in immunity. Veterinary Immunology and Immunopathology, 148(1-2), 161-167.

Harboe, M., Oettinger, T., Wiker, H. G., Rosenkrands, I., & Andersen, P. (1996). Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infection and Immunity, 64(1), 16-22.

Harboe, M., Wiker, H. G., Ulvund, G., Lund-Pedersen, B., Andersen, Å. B., Hewinson, R. G., & Nagai, S. (1998). MPB70 and MPB83 as indicators of protein localization in mycobacterial cells. Infection and Immunity, 66(1), 289-296.

Hardie, R., & Watson, J. (1992). *Mycobacterium bovis* in England and Wales: past, present and future. Epidemiology and infection, 109(1), 23.

Hermans, P., van Soolingen, D., & Van Embden, J. (1992). Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of Mycobacterium kansasii and Mycobacterium gordonae. Journal of Bacteriology, 174(12), 4157-4165.

Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., & Zähringer, U. (2005). CD36 is a sensor of diacylglycerides. Nature, 433(7025), 523-527.

Humphreys, I. R., Stewart, G. R., Turner, D. J., Patel, J., Karamanou, D., Snelgrove, R. J., & Young, D. B. (2006). A role for dendritic cells in the dissemination of mycobacterial infection. Microbes and Infection, 8(5), 1339-1346.

Jones, G. J., Gordon, S. V., Hewinson, R. G., & Vordermeier, H. M. (2010). Screening of predicted secreted antigens from *Mycobacterium bovis* reveals the immunodominance of the ESAT-6 protein family. Infection and Immunity, 78(3), 1326-1332.

Karboul, A., Mazza, A., Gey van Pittius, N. C., Ho, J. L., Brousseau, R., & Mardassi, H. (2008). Frequent homologous recombination events in *Mycobacterium tuberculosis* PE/PPE multigene families: potential role in antigenic variability. Journal of Bacteriology, 190(23), 7838-7846.

Keane, J., Remold, H. G., & Kornfeld, H. (2000). Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. The Journal of Immunology, 164(4), 2016-2020.

Kennedy, H. E., Welsh, M. D., Bryson, D. G., Cassidy, J. P., Forster, F. I., Howard, C. J., Collins, R. A., & Pollock, J. M. (2002). Modulation of immune responses to *Mycobacterium bovis* in cattle depleted of WC1+ γδ T cells. Infection and Immunity, 70(3), 1488-1500.

Kleinnijenhuis, J., Oosting, M., Joosten, L. A., Netea, M. G., & Van Crevel, R. (2011). Innate immune recognition of *Mycobacterium tuberculosis*. Clinical and Developmental Immunology, 2011.

Koch, R. (1890). I. Weitere Mittheilungen über ein Heilmittel gegen Tuberculose. DMW-Deutsche Medizinische Wochenschrift, 16(46), 1029-1032.

Koul, A., Herget, T., Klebl, B., & Ullrich, A. (2004). Interplay between mycobacteria and host signalling pathways. Nature Reviews Microbiology, 2(3), 189-202.

Krebs, J. (1997). Bovine tuberculosis in cattle and badgers.

Lange, C., Aaby, P., Behr, M. A., Donald, P. R., Kaufmann, S. H., Netea, M. G., & Mandalakas, A. M. (2022). 100 years of *Mycobacterium bovis* bacille Calmette-Guérin. The Lancet Infectious Diseases, 22(1), e2-e12.

Lay, G., Poquet, Y., Salek-Peyron, P., Puissegur, M. P., Botanch, C., Bon, H., Levillain, F., Duteyrat, J. L., Emile, J. F., & Altare, F. (2007). Langhans giant cells from M. tuberculosis-induced human granulomas cannot mediate mycobacterial uptake. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 211(1), 76-85.

Le Cabec, V., Carréno, S., Moisand, A., Bordier, C., & Maridonneau-Parini, I. (2002). Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively. The Journal of Immunology, 169(4), 2003-2009.

Lewis, K. N., Liao, R., Guinn, K. M., Hickey, M. J., Smith, S., Behr, M. A., & Sherman, D. R. (2003). Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. The Journal of infectious diseases, 187(1), 117-123.

Li, Y., Miltner, E., Wu, M., Petrofsky, M., & Bermudez, L. E. (2005). A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cellular microbiology, 7(4), 539-548.

Liebana, E., Aranaz, A., Aldwell, F., McNair, J., Neill, S., Smyth, A., & Pollock, J. (2000). Cellular interactions in bovine tuberculosis: release of active mycobacteria from infected macrophages by antigen-stimulated T cells. Immunology, 99(1), 23-29.

Linderholm, A., Spencer, D., Battista, V., Frantz, L., Barnett, R., Fleischer, R. C., James, H. F., Duffy, D., Sparks, J. P., & Clements, D. R. (2016). A novel MC1R allele for black coat colour reveals the Polynesian ancestry and hybridization patterns of Hawaiian feral pigs. Royal Society Open Science, 3(9), 160304.

Lucena, A. N., Cuartero, L. G., Mulcahy, G., & Zintl, A. (2017). The immunoregulatory effects of co-infection with Fasciola hepatica: From bovine tuberculosis to Johne's disease. The Veterinary Journal, 222, 9-16.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., O'Brien, D. J., Schmitt, S. M., Palmer, M. V., & Waters, W. R. (2013). Rapid detection of serum antibody by dual-path platform VetTB assay in white-tailed deer infected with *Mycobacterium bovis*. Clinical and Vaccine Immunology, 20(6), 907-911.

Magee, D. A., Conlon, K. M., Nalpas, N. C., Browne, J. A., Pirson, C., Healy, C., McLoughlin, K. E., Chen, J., Vordermeier, H. M., & Gormley, E. (2014). Innate cytokine profiling of bovine alveolar macrophages reveals commonalities and divergence in the response to *Mycobacterium bovis* and *Mycobacterium tuberculosis* infection. Tuberculosis, 94(4), 441-450.

Maggioli, M. F., Palmer, M. V., Thacker, T. C., Vordermeier, H. M., McGill, J. L., Whelan, A. O., Larsen, M. H., Jacobs Jr, W. R., & Waters, W. R. (2016). Increased TNF-α/IFN-γ/IL-2 and decreased TNF-α/IFN-γ production by central memory T cells are associated with protective responses against bovine tuberculosis following BCG vaccination. Frontiers in Immunology, 7, 421.

Maggioli, M. F., Palmer, M. V., Thacker, T. C., Vordermeier, H. M., & Waters, W. R. (2015). Characterization of effector and memory T cell subsets in the immune response to bovine tuberculosis in cattle. PLoS One, 10(4), e0122571.

Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C., & Stover, C. K. (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent M. bovis. Journal of Bacteriology, 178(5), 1274-1282.

Malone, K. M., Rue-Albrecht, K., Magee, D. A., Conlon, K., Schubert, O. T., Nalpas, N. C., Browne, J. A., Smyth, A., Gormley, E., & Aebersold, R. (2018). Comparative'omics analyses differentiate *Mycobacterium tuberculosis* and *Mycobacterium bovis* and reveal distinct macrophage responses to infection with the human and bovine tubercle bacilli. Microbial genomics, 4(3).

McFadyean, J. (1900). Tubercle bacilli in cow's milk as a possible source of tuberculous disease in man. Public Health, 13, 814-825.

McGill, J. L., Sacco, R. E., Baldwin, C. L., Telfer, J. C., Palmer, M. V., & Waters, W. R. (2014). The role of gamma delta T cells in immunity to *Mycobacterium bovis* infection in cattle. Veterinary Immunology and Immunopathology, 159(3-4), 133-143.

McInerney, J., Small, K., & Caley, P. (1995). Prevalence of *Mycobacterium bovis* infection in feral pigs in the Northern Territory. Australian Veterinary Journal, 72(12), 448-451.

Meyer, R. (2000). Results of an Epidemiologic Investigation for*Mycobacterium bovis* in selected wildlife populations on the island of Molokai, Hawaii. USDA.

Michel, A. L., Müller, B., & Van Helden, P. D. (2010). *Mycobacterium bovis* at the animal–human interface: A problem, or not? Veterinary Microbiology, 140(3-4), 371-381.

Middelkoop, K., Bekker, L.-G., Mathema, B., Shashkina, E., Kurepina, N., Whitelaw, A., Fallows, D., Morrow, C., Kreiswirth, B., & Kaplan, G. (2009). Molecular epidemiology of *Mycobacterium tuberculosis* in a South African community with high HIV prevalence. The Journal of infectious diseases, 200(8), 1207-1211.

Miller, R., & Sweeney, S. (2013). REVIEW ARTICLE *Mycobacterium bovis* (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations.

Miller, R. S., Farnsworth, M. L., & Malmberg, J. L. (2013). Diseases at the livestock–wildlife interface: status, challenges, and opportunities in the United States. Preventive Veterinary Medicine, 110(2), 119-132.

Miller, R. S., & Sweeney, S. J. (2013). *Mycobacterium bovis* (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations. Epidemiology & Infection, 141(7), 1357-1370.

Monaghan, M., Doherty, M., Collins, J., Kazda, J., & Quinn, P. (1994). The tuberculin test. Veterinary Microbiology, 40(1-2), 111-124.

Moody, D. B., Ulrichs, T., Mühlecker, W., Young, D. C., Gurcha, S. S., Grant, E., Rosat, J.-P., Brenner, M. B., Costello, C. E., & Besra, G. S. (2000). CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. Nature, 404(6780), 884-888.

Morris, A., Crothers, K., Beck, J. M., & Huang, L. (2011). An official ATS workshop report: Emerging issues and current controversies in HIV-associated pulmonary diseases. Proceedings of the American Thoracic Society, 8(1), 17-26.

Mustafa, T., Wiker, H. G., Mørkve, O., & Sviland, L. (2008). Differential expression of mycobacterial antigen MPT64, apoptosis and inflammatory markers in multinucleated giant cells and epithelioid cells in granulomas caused by *Mycobacterium tuberculosis*. Virchows Archiv, 452, 449-456.

Niemann, S., Richter, E., & Rüsch-Gerdes, S. (2002). Biochemical and genetic evidence for the transfer of *Mycobacterium tuberculosis* subsp. caprae Aranaz et al. 1999 to the species *Mycobacterium bovis* Karlson and Lessel 1970 (approved lists 1980) as *Mycobacterium bovis* subsp. caprae comb. nov. International journal of systematic and evolutionary microbiology, 52(2), 433-436.

Norouzi, S., Aghamohammadi, A., Mamishi, S., Rosenzweig, S. D., & Rezaei, N. (2012). Bacillus Calmette-Guérin (BCG) complications associated with primary immunodeficiency diseases. Journal of Infection, 64(6), 543-554.

O'Reilly, L. M., & Daborn, C. (1995). The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. Tubercle and Lung Disease, 76, 1-46.

Palmer, M., Waters, W., & Thacker, T. (2007). Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with *Mycobacterium bovis*. Veterinary Pathology, 44(6), 863-874.

Palmer, M. V., Thacker, T. C., & Waters, W. R. (2016). Multinucleated giant cell cytokine expression in pulmonary granulomas of cattle experimentally infected with *Mycobacterium bovis*. Veterinary Immunology and Immunopathology, 180, 34-39.

Parsons, S., Smith, S. G., Martins, Q., Horsnell, W. G., Gous, T. A., Streicher, E. M., Warren, R. M., van Helden, P. D., & van Pittius, N. C. G. (2008). Pulmonary infection due to the dassie bacillus (*Mycobacterium tuberculosis* complex sp.) in a free-living dassie (rock hyrax—Procavia capensis) from South Africa. Tuberculosis, 88(1), 80-83.

Parsons, S. D., Drewe, J. A., van Pittius, N. C. G., Warren, R. M., & Van Helden, P. D. (2013). Novel cause of tuberculosis in meerkats, South Africa. Emerging infectious diseases, 19(12), 2004.

Parte, A. C., Sardà Carbasse, J., Meier-Kolthoff, J.P., Reimer, L.C. and Göker, M. (2020). List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ https://doi.org/10.1099/ijsem.0.004332

Pedersen, K., Miller, R. S., Anderson, T. D., Pabilonia, K. L., Lewis, J. R., Mihalco, R. L., Gortázar, C., & Gidlewski, T. (2017). Limited antibody evidence of exposure to *Mycobacterium bovis* in feral swine (Sus scrofa) in the USA. Journal of Wildlife Diseases, 53(1), 30-36.

Pérez-Lago, L., Navarro, Y., & García-de-Viedma, D. (2014). Current knowledge and pending challenges in zoonosis caused by *Mycobacterium bovis*: a review. Research in veterinary science, 97, S94-S100.

Pérez-Morote, R., Pontones-Rosa, C., Gortázar-Schmidt, C., & Muñoz-Cardona, Á. I. (2020). Quantifying the economic impact of bovine tuberculosis on livestock farms in south-western Spain. Animals, 10(12), 2433.

Phillips, C., Foster, C., Morris, P., & Teverson, R. (2003). The transmission of *Mycobacterium bovis* infection to cattle. Research in veterinary science, 74(1), 1-15.

Pollock, J., Buddle, B., & Andersen, P. (2001). Towards more accurate diagnosis of bovine tuberculosis using defined antigens. Tuberculosis, 81(1-2), 65-69.

Pollock, J., McNair, J., Welsh, M., Girvin, R., Kennedy, H., Mackie, D., & Neill, S. (2001). Immune responses in bovine tuberculosis. Tuberculosis, 81(1-2), 103-107.

Pollock, J., Welsh, M., & McNair, J. (2005). Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. Veterinary Immunology and Immunopathology, 108(1-2), 37-43.

Pollock, J. M., & Andersen, P. (1997a). The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. Journal of Infectious Diseases, 175(5), 1251-1254.

Pollock, J. M., & Andersen, P. (1997b). Predominant recognition of the ESAT-6 protein in the first phase of interferon with *Mycobacterium bovis* in cattle. Infection and Immunity, 65(7), 2587-2592.

Poulet, S., & Cole, S. T. (1995). Repeated DNA sequences in mycobacteria. Archives of microbiology, 163, 79-86.

Queirós, J., Alves, P. C., Vicente, J., Gortázar, C., & de la Fuente, J. (2018). Genome-wide associations identify novel candidate loci associated with genetic susceptibility to tuberculosis in wild boar. Scientific Reports, 8(1), 1-12.

Queval, C. J., Song, O.-R., Deboosère, N., Delorme, V., Debrie, A.-S., Iantomasi, R., Veyron-Churlet, R., Jouny, S., Redhage, K., & Deloison, G. (2016). STAT3 represses nitric oxide synthesis in human macrophages upon *Mycobacterium tuberculosis* infection. Scientific Reports, 6(1), 29297.

Rahman, S. A., Singh, Y., Kohli, S., Ahmad, J., Ehtesham, N. Z., Tyagi, A. K., & Hasnain, S. E. (2014). Comparative analyses of nonpathogenic, opportunistic, and totally pathogenic mycobacteria reveal genomic and biochemical variabilities and highlight the survival attributes of *Mycobacterium tuberculosis*. MBio, 5(6), e02020-02014.

Ramakrishnan, L. (2012). Revisiting the role of the granuloma in tuberculosis. Nature Reviews Immunology, 12(5), 352-366.

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., Kaplan, G., & Barry III, C. E. (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. Nature, 431(7004), 84-87.

Ritacco, V., Lopez, B., De Kantor, I., Barrera, L., Errico, F., & Nader, A. (1991). Reciprocal cellular and humoral immune responses in bovine tuberculosis. Research in veterinary science, 50(3), 365-367.

Rockett, K. A., Brookes, R., Udalova, I., Vidal, V., Hill, A. V., & Kwiatkowski, D. (1998). 1, 25-Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. Infection and Immunity, 66(11), 5314-5321.

Rojas, M., García, L. F., Nigou, J., Puzo, G., & Olivier, M. (2000). Mannosylated lipoarabinomannan antagonizes *Mycobacterium tuberculosis*-induced macrophage apoptosis by altering Ca+ 2-dependent cell signaling. The Journal of infectious diseases, 182(1), 240-251.

Rothel, J., Jones, S., Corner, L., Cox, J., & Wood, P. (1990). A sandwich enzyme immunoassay for bovine interferon-γ and its use for the detection of tuberculosis in cattle. Australian Veterinary Journal, 67(4), 134-137.

Rusk, R. A., Palmer, M. V., Waters, W. R., & McGill, J. L. (2017). Measuring bovine γδ T cell function at the site of *Mycobacterium bovis* infection. Veterinary Immunology and Immunopathology, 193, 38-49.

Saukkonen, J. J., Cohn, D. L., Jasmer, R. M., Schenker, S., Jereb, J. A., Nolan, C. M., Peloquin, C. A., Gordin, F. M., Nunes, D., & Strader, D. B. (2006). An official ATS statement: hepatotoxicity of antituberculosis therapy. American journal of respiratory and critical care medicine, 174(8), 935-952.

Schaible, U. E., Winau, F., Sieling, P. A., Fischer, K., Collins, H. L., Hagens, K., Modlin, R. L., Brinkmann, V., & Kaufmann, S. H. (2003). Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nature medicine, 9(8), 1039-1046.

Schiller, I., Oesch, B., Vordermeier, H., Palmer, M., Harris, B., Orloski, K., Buddle, B., Thacker, T., Lyashchenko, K., & Waters, W. (2010). Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transboundary and Emerging Diseases, 57(4), 205-220.

Schiller, I., Waters, W. R., Vordermeier, H. M., Nonnecke, B., Welsh, M., Keck, N., Whelan, A., Sigafoose, T., Stamm, C., & Palmer, M. (2009). Optimization of a whole-blood gamma interferon assay for detection of *Mycobacterium bovis*-infected cattle. Clinical and Vaccine Immunology, 16(8), 1196-1202.

Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R., & Horwitz, M. A. (1990). Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. Journal of immunology (Baltimore, Md.: 1950), 144(7), 2771-2780.

Serraino, A., Marchetti, G., Sanguinetti, V., Rossi, M. C., Zanoni, R. G., Catozzi, L., Bandera, A., Dini, W., Mignone, W., & Franzetti, F. (1999). Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques. Journal of Clinical Microbiology, 37(9), 2766-2771.

Shafer, R., Singh, S., Larkin, C., & Small, P. (1995). Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in an immunocompetent patient. Tubercle and Lung Disease, 76(6), 575-577.

Simeone, R., Bobard, A., Lippmann, J., Bitter, W., Majlessi, L., Brosch, R., & Enninga, J. (2012). Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. PLoS pathogens, 8(2), e1002507.

Simeone, R., Bottai, D., Frigui, W., Majlessi, L., & Brosch, R. (2015). ESX/type VII secretion systems of mycobacteria: Insights into evolution, pathogenicity and protection. Tuberculosis, 95, S150-S154.

Small, P. M., Shafer, R. W., Hopewell, P. C., Singh, S. P., Murphy, M. J., Desmond, E., Sierra, M. F., & Schoolnik, G. K. (1993). Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. New England Journal of Medicine, 328(16), 1137-1144.

Smith, T. (1898). A comparative study of bovine tubercle bacilli and of human bacilli from sputum. The Journal of experimental medicine, 3(4-5), 451.

Smyser, T. J., Tabak, M. A., Slootmaker, C., Robeson, M. S., Miller, R. S., Bosse, M., Megens, H. J., Groenen, M. A., Paiva, S. R., & de Faria, D. A. (2020). Mixed ancestry from wild and domestic lineages contributes to the rapid expansion of invasive feral swine. Molecular Ecology, 29(6), 1103-1119.

Sørensen, A. L., Nagai, S., Houen, G., Andersen, P., & Andersen, A. B. (1995). Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infection and Immunity, 63(5), 1710-1717.

Spickler, A. R. (2019). Zoonotic Tuberculosis in Mammals, including Bovine and Caprine Tuberculosis. Center for Food Security and Public Health. Retrieved 01/11/2023 from https://www.cfsph.iastate.edu/diseaseinfo/disease/?disease=bovine-tuberculosis&lang=en

Sreejit, G., Ahmed, A., Parveen, N., Jha, V., Valluri, V. L., Ghosh, S., & Mukhopadhyay, S. (2014). The ESAT-6 protein of *Mycobacterium tuberculosis* interacts with beta-2-microglobulin (β2M) affecting antigen presentation function of macrophage. PLoS pathogens, 10(10), e1004446.

Sreevatsan, S., Pan, X., Stockbauer, K. E., Connell, N. D., Kreiswirth, B. N., Whittam, T. S., & Musser, J. M. (1997). Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proceedings of the National Academy of Sciences, 94(18), 9869-9874.

Sreevatsan, S., Pan, X., Zhang, Y., Kreiswirth, B. N., & Musser, J. M. (1997). Mutations associated with pyrazinamide resistance in pncA of *Mycobacterium tuberculosis* complex organisms. Antimicrobial agents and chemotherapy, 41(3), 636-640.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J., & Russell, D. G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science, 263(5147), 678-681.

Taylor, R. B., Hellgren, E. C., Gabor, T. M., & Ilse, L. M. (1998). Reproduction of feral pigs in southern Texas. Journal of Mammalogy, 79(4), 1325-1331.

Thacker, T. C., Palmer, M. V., & Waters, W. R. (2007). Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle. Veterinary Immunology and Immunopathology, 119(3-4), 204-213.

Tobin, D. M., Roca, F. J., Oh, S. F., McFarland, R., Vickery, T. W., Ray, J. P., Ko, D. C., Zou, Y., Bang, N. D., & Chau, T. T. (2012). Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. Cell, 148(3), 434-446.

Tobin, D. M., Vary, J. C., Ray, J. P., Walsh, G. S., Dunstan, S. J., Bang, N. D., Hagge, D. A., Khadge, S., King, M.-C., & Hawn, T. R. (2010). The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. Cell, 140(5), 717-730.

Torrelles, J. B., & Schlesinger, L. S. (2010). Diversity in *Mycobacterium tuberculosis* mannosylated cell wall determinants impacts adaptation to the host. Tuberculosis, 90(2), 84-93.

Tsolaki, A. G., Hirsh, A. E., DeRiemer, K., Enciso, J. A., Wong, M. Z., Hannan, M., de la Salmoniere, Y.-O. L. G., Aman, K., Kato-Maeda, M., & Small, P. M. (2004). Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. Proceedings of the National Academy of Sciences, 101(14), 4865-4870.

USDA. (2023, March 09, 2023). History of Feral Swine in the Americas. USDA Wildlife Services. https://www.aphis.usda.gov/aphis/ourfocus/wildlifedamage/operational-activities/feral-swine/sa-fs-history

van Ingen, J. (2013). Diagnosis of nontuberculous mycobacterial infections. Seminars in respiratory and critical care medicine,

van Ingen, J., Rahim, Z., Mulder, A., Boeree, M. J., Simeone, R., Brosch, R., & Van Soolingen, D. (2012). Characterization of Mycobacterium orygis as M. tuberculosis complex subspecies. Emerging infectious diseases, 18(4), 653.

Van Pinxteren, L. A., Ravn, P., Agger, E. M., Pollock, J., & Andersen, P. (2000). Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. Clinical Diagnostic Laboratory Immunology, 7(2), 155-160.

Van Soolingen, D. (2001). Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. Journal of internal medicine, 249(1), 1-26.

Van Soolingen, D., De Haas, P., Hermans, P., Groenen, P., & Van Embden, J. (1993). Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology, 31(8), 1987-1995.

Van Soolingen, D., Hoogenboezem, T., De Haas, P. E., Hermans, P. W., Koedam, M. A., Teppema, K. S., Brennan, P. J., Besra, G. S., Portaels, F., & Top, J. (1997). A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. International journal of systematic bacteriology, 47(4), 1236-1245.

VerCauteren, K. C., Beasley, J. C., Ditchkoff, S. S., Mayer, J. J., Roloff, G. J., & Strickland, B. K. (2019). Invasive wild pigs in North America: ecology, impacts, and management. CRC Press.

Veyrier, F., Saïd-Salim, B., & Behr, M. A. (2008). Evolution of the mycobacterial SigK regulon. Journal of Bacteriology, 190(6), 1891-1899.

Vicente, J., Barasona, J. A., Acevedo, P., Ruiz-Fons, J., Boadella, M., Diez-Delgado, I., Beltran-Beck, B., González-Barrio, D., Queirós, J., & Montoro, V. (2013). Temporal trend of tuberculosis in wild ungulates from Mediterranean S pain. Transboundary and Emerging Diseases, 60, 92-103.

Vicente, J., Höfle, U., Garrido, J., Fernández-De-Mera, I. G., Juste, R., Barral, M., & Gortazar, C. (2006). Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. Veterinary Research, 37(1), 107-119.

Vicente, J., Hofle, U., Garrido, J. M., Fernandez-de-Mera, I. G., Acevedo, P., Juste, R., Barral, M., & Gortazar, C. (2007). Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain.

Volkman, H. E., Clay, H., Beery, D., Chang, J. C. W., Sherman, D. R., & Ramakrishnan, L. (2004). Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS biology, 2(11), e367.

Volkman, H. E., Pozos, T. C., Zheng, J., Davis, J. M., Rawls, J. F., & Ramakrishnan, L. (2010). Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science, 327(5964), 466-469.

Vordermeier, H., Whelan, A., Cockle, P., Farrant, L., Palmer, N., & Hewinson, R. (2001). Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clinical Diagnostic Laboratory Immunology, 8(3), 571-578.

Vordermeier, H. M., Chambers, M. A., Cockle, P. J., Whelan, A. O., Simmons, J., & Hewinson, R. G. (2002). Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. Infection and Immunity, 70(6), 3026-3032.

Waddington, K. (2004). To stamp out "so terrible a malady": Bovine tuberculosis and tuberculin testing in Britain, 1890–1939. Medical history, 48(1), 29-48.

Welsh, M. D., Cunningham, R. T., Corbett, D. M., Girvin, R. M., McNair, J., Skuce, R. A., Bryson, D. G., & Pollock, J. M. (2005). Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology, 114(1), 101-111.

WHO. (2022, 10/27/2022). Tuberculosis. World Health Organization. Retrieved 1/3/2023 from https://www.who.int/news-room/fact-sheets/detail/tuberculosis

Wiker, H. (2009). MPB70 and MPB83–major antigens of *Mycobacterium bovis*. Scandinavian Journal of Immunology, 69(6), 492-499.

Wiker, H. G., Lyashchenko, K. P., Aksoy, A. M., Lightbody, K. A., Pollock, J. M., Komissarenko, S. V., Bobrovnik, S. O., Kolesnikova, I. N., Mykhalsky, L. O., & Gennaro, M. L. (1998). Immunochemical characterization of the MPB70/80 and MPB83 proteins of *Mycobacterium bovis*. Infection and Immunity, 66(4), 1445-1452.

Wolinsky, E. (1979). Nontuberculous mycobacteria and associated diseases. American Review of Respiratory Disease, 119(1), 107-159.

Wood, G. W., & Barrett, R. H. (1979). Status of wild pigs in the United States. Wildlife Society Bulletin, 237-246.

Wyckoff, A. C., Henke, S. E., Campbell, T. A., Hewitt, D. G., & VerCauteren, K. C. (2009). Feral swine contact with domestic swine: a serologic survey and assessment of potential for disease transmission. Journal of Wildlife Diseases, 45(2), 422-429.

Yang, K., Wu, Y., Xie, H., Li, M., Ming, S., Li, L., Li, M., Wu, M., Gong, S., & Huang, X. (2016). Macrophage-mediated inflammatory response decreases mycobacterial survival in mouse MSCs by augmenting NO production. Scientific Reports, 6(1), 1-11.

Young, D., Stark, J., & Kirschner, D. (2008). Systems biology of persistent infection: tuberculosis as a case study. Nature Reviews Microbiology, 6(7), 520-528.

Young, D. B., Gideon, H. P., & Wilkinson, R. J. (2009). Eliminating latent tuberculosis. Trends in microbiology, 17(5), 183-188.

CHAPTER 2. TEN-YEAR SURVEILLANCE AND DIAGNOSTIC TEST PERFORMANCE FOR BOVINE TUBERCULOSIS IN U.S. CATTLE AND FERAL SWINE

Dana Hill*, Tyler Thacker*, Aaron Lehmkuhl*, Suelee Robbe-Austerman*

*United States of America Department of Agriculture, Veterinary Services, National Veterinary Services Laboratories, Ames, IA

Modified from a manuscript to be submitted to *Journal of Veterinary Diagnostic Investigation*[1]

See supplementary document, "FS bTB Figures and Tables"[2]

Abstract

Bovine tuberculosis (bTB) is the disease caused by *Mycobacterium bovis. M. bovis* is one of multiple pathogenic mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTC) group. Cattle are the primary maintenance host for bTB but *M. bovis* has a wide host range and has become established in multiple wildlife maintenance hosts. Feral swine on the Hawaiian island of Moloka'i have maintained bTB in their population since the 1980s with historical and recent disease spillback into cattle and other domestic livestock. The primary method of bTB detection in cattle in the United States is abattoir surveillance followed by test and cull. Surveillance of bTB in wildlife relies heavily on postmortem submissions from hunter harvest or targeted efforts of wildlife regulatory officials. Postmortem bTB diagnostics have drastically evolved over the last 20-30 years following implementation of molecular diagnostic techniques such as polymerase chain reaction (PCR) and whole genome sequencing (WGS). This study reviewed 63,764 cattle samples and 452 Hawaiian feral swine surveillance samples submitted to the United States Department of Agriculture (USDA) National Veterinary Services

[1]This chapter is currently a work in progress. It may differ in significant ways from the published version.
[2]Figures and tables for Chapter 2 are included in supplementary document entitled, "FS bTB Figures and Tables"

Laboratories (NVSL) in Ames, IA from 1999 through 2010. Factors affecting bTB diagnostic test accuracy were investigated for both cattle and Moloka'i feral swine surveillance samples. Performance of histopathology (n = 63,765 and n = 428 for cattle and feral swine, respectively), PCR on formalin fixed paraffin embedded (FFPE) tissues (n = 657, 19), and culture (n = 1,838, 450), according to NVSL diagnostic flow charts, were compared. Submissions increased annually after the bovine tuberculosis eradication program was reinvigorated by an emergency declaration in the fall of 2000, reaching approximately 7000-8000 cultured samples each year throughout 2005-2008 before dropping back to 3000-4000 in 2009 and 2010. While agreement between histology and culture was remarkably high, a review of the 20 discrepant cases from cattle revealed that pathologist error reading the initial slide (11 cases) and cross contamination in the culture laboratory (4 cases) were the most common errors. Additionally, FFPE PCR proved to be an important ancillary diagnostic for the identification of bTB in field-collected samples from Moloka'i feral swine. Actively investigating discrepant results from parallel testing and genotyping appear to have been important process improvement mechanisms for the laboratory. These data suggest that parallel testing a significant portion of granuloma submissions and genotyping all positive cases can be beneficial by identifying error and improving overall test accuracy.

Introduction

Mycobacterium bovis, the causal agent of bovine tuberculosis (bTB), is an important zoonotic pathogen that impacts public health and agricultural trade. Consequently, many countries have developed bTB control or eradication programs. Since the United States instituted the State - Federal Cooperative Bovine Tuberculosis Eradication program in 1917, the herd prevalence of bTB has declined from 5% to less than 0.002% (Portacci et al., 2011). This sharp

decline in prevalence impacted the positive/negative predictive value of antemortem test results, and in order to maintain an efficient national program, program officials made periodic adjustments to testing paradigms. By the early 1960s the herd prevalence in the US had been reduced to below 0.5% nationally, and officials suspended routine area antemortem skin testing in bTB accredited free zones; relying instead on slaughter surveillance as the primary method to detect new cases.

Throughout the 1970s-1990s a greater percentage of states became accredited bTB-free and little antemortem skin testing was being conducted. In the late 1990s, state and federal bTB program officials became concerned with the robustness of the slaughter surveillance program and a review was conducted by USDA (Naugle et al., 2014). During 1999, only 1062 granulomas were submitted despite over 30 million cattle slaughtered; it was readily apparent an insufficient number of granuloma-like lesions were being submitted for bTB testing to adequately monitor the national herd. On October 23, 2000, the Secretary of Agriculture issued an emergency declaration to accelerate the eradication of bTB from the United States (Naugle et al., 2014). The slaughter surveillance program was reinvigorated by instituting training programs for abattoir inspectors and veterinarians, formalizing the granuloma submission standard of one granuloma per 2000 head of adult (> 2 years of age) cattle inspected into regulation, and providing awards to Food Safety Inspection Service inspectors and veterinarians for identifying cases of bTB (Portacci et al., 2011).

The emergency declaration also enhanced funding to the NVSL which allowed the histology and mycobacterial culture laboratories to add staff and equipment as submission rates increased. An extensive genotyping project was underway at NVSL during the early 2000s, providing valuable molecular epidemiological data for the increasing number of suspect bTB

cases during these years (Orloski et al., 2018). Consequently, throughout these 10 years, a significant amount of parallel testing with histology, PCR, culture, and genotyping was done.

In areas with established wildlife maintenance hosts, control of bTB is especially challenging with complete eradication being nearly impossible. Management practices, environmental influences, and socioeconomic factors affecting surveillance efforts and wildlife population densities all contribute to increased or decreased contact at the wildlife-livestock interface (Taylor et al., 1998; Wyckoff et al., 2009). Interactions resulting in direct or indirect contact through shared water or feeding stations have the potential to result in bTB disease spillover from cattle into wildlife, or spillback from infected wildlife into livestock (Nugent, 2011). In the U.S., bTB-infected white-tailed deer (WTD) in the modified accredited zone (MAZ) of Michigan and feral swine on the Hawaiian island of Moloka'i pose an ongoing threat to domestic livestock and public health (USDA, 2023b). Members of the Suidae family are generally regarded as spillover hosts for bTB, requiring cattle or another maintenance host for continual bTB transmission. However, in certain situations they may act as maintenance hosts or amplifier hosts – the latter being a spillover host for a disease with the capability of temporarily fulfilling a maintenance host role by perpetuating inter- and intra-species disease transmission in certain environmental situations and/or population structures (Coleman & Cooke, 2001; Nugent, 2011). The Eurasian wild boar in Spain has been widely recognized as a maintenance host for bTB (Aranaz et al., 2004; Vicente et al., 2006). Feral swine on Moloka'i appear to be maintaining a low-level of disease since bTB-infected cattle were introduced in the 1940s and bTB-infected feral swine were first identified in the 1980s (Essey et al., 1981; Essey et al., 1983). Historical wildlife surveillance studies conducted on Moloka'i between 1961-1985 initially showed a drastic decrease in bTB disease prevalence in feral swine after cattle were

removed from the island completely. Following a confirmed positive domestic cow in 1997 from the Eastern side of the island, a second surveillance study became underway between the years of 1999-early 2008. The results of this study are compiled within this article alongside cattle submissions from the years of 1999-2010.

We sought to evaluate bTB postmortem diagnostic test performance for the primary host, domestic cattle, in addition to feral swine from Moloka'i between the years of 1999-2010. Our objectives were to describe the impact of the policies to improve submissions, describe the laboratory performance of histology, FFPE PCR, and mycobacterial culture, and to identify the most common causes of discrepant test results on granulomas identified during regular bTB cattle surveillance from January 1, 1999, through December 31, 2010 and feral swine wildlife surveillance samples collected between 1999-2008.

Methods

Study population

Cattle and feral swine surveillance samples between 1999 and 2010 submitted to the NVSL were included in the study. Data were retroactively compiled from the laboratory information management system (LIMS) to include cattle abattoir submissions (6-35) from across the U.S. and feral swine wildlife surveillance samples originating from Moloka'i. This excluded cattle from known infected herds or antemortem test-positive cattle. Animals originating from outside of the U.S. were also excluded from the study. Federal inspectors and veterinarians at abattoirs identified suspicious lesions predominantly in lymph nodes and lungs consistent with bTB granulomas. Excised lesions were split, submitting half in 10% buffered formalin and the other half in a saturated solution of sodium borate ($Na_2B_4O_7$). Samples were shipped to NVSL for overnight delivery. Feral swine samples were collected by wildlife officials

and hunter-harvest split as described above and sent to NVSL, typically within a few days of collection.

Case selection and definition

All samples were accessioned and tested according to a diagnostic decision-making tree that varied slightly for abattoir cattle (Figure 1) and feral swine (Figure 8). Briefly, cattle submissions were initially screened by histopathology and those with suspect microscopic lesions (see methods for histology) or of unknown etiology were routed for mycobacterial culture. In contrast, all but two feral swine submissions were cultured. FFPE PCR (methods under FFPE PCR) was performed on a subset of suspect histology cases for both species depending on the year submitted (first implemented in 2000), the discretion of the pathologist reading the case, and at the request of the submitting USDA area veterinarian in charge.

The NVSL LIMS that had been implemented in the fall of 1999 was replaced with a more comprehensive system during summer of 2009. The original LIMS generated the official reports and maintained manually entered summary results of laboratory tests. Including the electronic record, detailed laboratory paperwork was available for all cases with positive culture results, but not necessarily for all negative results. Histology blocks and the original slides for many cases were available for review, if needed. Since all discrepant histopathology cases were reviewed as soon as a positive culture result was obtained, only four cases in the early years of the study did not have enough documentation and required microscopic review by the authors. However, discrepant culture results were not necessarily rigorously questioned prior to the full implementation of genotyping in 2005. Because the isolates associated with this study were being pulled for a WGS project, those results along with the original laboratory records, histopathology slides, and FFPE PCR results were used to review discrepant culture cases (Orloski et al., 2018). Additionally, FFPE PCR did not become routinely implemented until

2000, therefore, earlier cases without PCR results where tissue blocks were still available were tested in present day. A confirmed case of bTB in cattle or feral swine for this study was based on a histopathology result of mycobacteriosis compatible and MTC/*M. bovis* was isolated by culture or FFPE PCR positive by insertion sequence 6110 (IS6110) primers.

Histology

Formalin fixed tissues were processed and embedded into paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Slides were examined by veterinary pathologists and either a final (negative) histologic diagnosis was determined, or if necessary, additional special stains were performed for the identification of intra-lesional bacteria. Generally, Gram stain, Auramine-Acridine Orange (AO), and a modified Ziehl-Neelson, New Fuchsin (NF) were applied to suspicious lesions to determine the presence/absence of acid-fast bacilli (AFB) or other bacteria. Cases which contained granulomas and AFB with morphology consistent with *M. bovis* on microscopy were diagnosed as "mycobacteriosis compatible" and further tested by mycobacterial culture. All mycobacteriosis compatible cases were reviewed by a second pathologist prior to reporting the diagnosis. All cases with granulomas in which a specific etiologic agent was not identified on histopathology were also cultured. Cases were not submitted for culture based on the histopathologic diagnosis when neoplastic lesions or inflammatory lesions with identifiable etiologic agents were observed. A subset of histo cases contained AFB but a non-compatible lesion which were designated as "histo other" for mycobacteria other than tuberculosis (MOTT). Diagnoses designated as "histo other" include: "granuloma – atypical mycobacteria" (AFB with an atypical lesion or bacterial morphology), "paratuberculosis" (Johne's disease), "mycobacterial lymphadenitis" (primarily Johne's cases but also atypical mycobacteria), and "Johne's vaccine reaction" (moderate to abundant AFB within lipid vacuoles usually affecting prescapular or cervical lymph node).

PCR on formalin-fixed paraffin embedded tissues (FFPE PCR)

Mycobacteriosis compatible cases were further tested by conventional PCR from FFPE tissues using primers for *Mycobacterium tuberculosis* complex (MTC) and *Mycobacterium avium* species. A result was considered positive when IS6110 primers, used to identify MTC bacteria, produced a 123 base pair amplicon, which was observed after electrophoresis and ethidium bromide staining of the PCR product (Miller et al., 1997; Palmer et al., 2006). An "other "result on PCR resulted from 16S rRNA amplification for *M. avium* and/or IS900 for *M. avium* ssp. *paratuberculosis*.

Mycobacterial culture

Samples in sodium borate were held at 4ºC until histopathology was completed to determine which samples advanced for culture. Mycobacterial cultures were performed using a NaOH decontamination method as described previously with a combination of liquid and solid media (Robbe-Austerman et al., 2013). However, during the 10 years of data in this study, culture methods were updated. Of most significance, in late 2003, the MGIT culture system (Becton Dickinson and Company, Sparks, MD, USA) was incorporated into the laboratory. Samples that were diagnosed as mycobacteriosis compatible were inoculated into both the MGIT and BACTEC 460 systems (Becton Dickinson and Company, Sparks, MD) as well as solid media; in all other cases a single liquid media system (BACTEC 460 or MGIT) and 4 tubes of solid media were used.

Results

Figure 1 details the sample flow chart for cattle bTB submissions and Figure 2 summarizes the number of bTB-confirmed cases by year based on histology, PCR and culture results. A total of 63,764 cattle samples were submitted between 1999 through 2010 compared with 452 feral swine samples from Moloka'i during 1999-2008 (Figure 8). Of the 63,764

histology samples from cattle, 863 (1.3%) were determined to be “Mycobacteriosis compatible,” 105 (0.2%) “other mycobacteria,” and 62,796 (98%) were given a diagnosis of “histo negative.” There was a total of 1,840 cases with culture results for cases with a histo diagnosis of compatible (n=819), other mycobacteria (n = 92), or negative (n = 929). PCR results were available for 657 histo cases including compatible (n = 590) and other mycobacteria (n = 67).

There was a total of 741 confirmed positive bTB cattle cases by either culture or FFPE PCR. *M. bovis*/MTC was cultured from 685 of 819 histo compatible cases (84%) and 708 of 1840 total cultured cases (38%). FFPE PCR was performed on 590 out of 863 compatible cases and was positive for IS6110 in 447 cases (76%). Only 611 cattle cases had parallel testing of histopathology, culture, and PCR and 470 of these cases were confirmed and considered for diagnostic test comparison (Table 1). Of these, 453 (96%) and 431 (92%) samples were positive on culture and PCR, respectively. Culture identified 39 additional cases of bTB of histo compatible lesions with other (n = 1) and negative (n = 38) PCR results, while PCR detected *M. bovis* nucleic acid in 17 cases where MOTT were isolated (n = 4) or no isolation was made (n = 13). PCR was not performed on 266 of 819 histo compatible cases that were cultured, of which, MTC or *M. bovis* was isolated in 232 cases. Similarly, culture was not performed on 44 histo compatible lesions, presumably because only fixed tissues were received by NVSL. PCR completed on 37 of these samples yielded an additional 16 bTB-positive cases.

There were 62,797 negative histopathology results for cattle, of which 61,868 samples did not have any additional testing. A total of 928 samples with negative histology results were cultured – either due to high suspicion or at the request of the area veterinarian in charge – yielding 23 samples with *M. bovis* or MTC isolated. Cases which were initially missed on histology but later identified as positive on culture did not have PCR performed. After cultures

identified *M. bovis* in these cases, fixed tissues and tissue blocks were re-evaluated and mycobacteriosis compatible lesions were confirmed in 12 available cattle cases. The most common histological diagnoses for cases not cultured were actinobacillosis, actinomycosis, carcinoma, and coccidiomycosis.

A "histo other" result was recorded for 105 histology cases in which a granuloma contained AFB with morphology inconsistent with *M. bovis* (Figure 3). These included 55 paratuberculosis/Johne's, 23 granuloma – atypical mycobacteria, 13 Johne's vaccine reaction, and 10 mycobacterial lymphadenitis diagnoses. Of the 55 paratuberculosis/Johne's histo diagnoses, 49 culture and 25 PCR results were available. *Mycobacterium avium* ssp. *paratuberculosis* was confirmed in 25 (51%) and 23 (92%) cases by culture and PCR respectively, with 14 cases in agreement. *M. avium*/*Mycobacterium avium* complex (MAC) were confirmed in 17 cases, 4 of which were PCR positive for both 16S rRNA and IS900. One case reported as Johne's on histo was PCR positive for IS900 but *M. smegmatis* was isolated on culture. A microscopic diagnosis of mycobacterial lymphadenitis coincided with 3 *M. avium* ssp. *paratuberculosis* (all IS900 PCR positive), 2 MAC isolations (one IS900 and one 16s rRNA + IS900 PCR positive), and 5 culture results of "no isolation made" (2 IS900 PCR positive and 3 PCR negative). "Granuloma - atypical mycobacteria" diagnoses consisted of *M. fortuitum* (n=1), *M. kansasii* (n=2), MAC (n=2), and 13 with no isolation made (1 IS900, 1 16s rRNA + IS900, 2 16s rRNA PCR positives, and 9 PCR negative). Johne's vaccine reaction lesions most commonly yielded no isolation made (n = 8; 3 *M. avium* PCR positive, 1 16s rRNA + IS900 positive, 3 PCR negative) with *M. avium, M. fortuitum,* and a non-speciated MOTT being isolated from three other cases. Culture results were unavailable for two cases of vaccine reactions, one of which was PCR positive for *M. avium* and the other was PCR negative. The remaining four "other"

histopathology cases cultured MAC (n=3, one PCR positive for both 16s rRNA and IS900) and MOTT (n =1).

All isolates of mycobacteria from histo negative or other diagnoses, excluding histo compatibles and isolates of *M. bovis* and MTC, are shown in Figure 4. A total of 798 isolates were made including 31 different species and six mycobacteria complex groups. The "other" category includes non-speciated MOTT (n = 115) and 1 isolate each of *M. acapulcensis, M. chitae, M. nonchromogenicum, M. confluentis, M. engbackii, M. gastri, M. gordonae, M. lentiflavum, M. monacense, M. moriokaense,* and *M. phlei*. *Mycobacterium avium* complex was the most abundant followed by *M. fortuitum* and *M. smegmatis*. These results are consistent with previous reports on atypical mycobacterial infections in domestic animals (Thacker et al., 2013).

Histologic diagnoses made from cultured cattle submissions between 1999-2010 were most commonly mycobacteriosis compatible (45%, Figure 5). "No significant findings" were recorded for 9% of cattle samples routed for culture. Recognizing that due to sectioning, histologic diagnoses don't always reflect the lesion produced by the mycobacteria species isolated, other relatively common diagnoses include pyogranuloma (17%), abscess (6%), lymphadenitis (5%), and lymphoid hyperplasia (4%).

Histopathology correctly identified mycobacteriosis compatible bTB lesions in 83% of cattle cases where *M. bovis* or MTC were confirmed by culture (Figure 6). No isolation was made in 76 histo compatible cases, reconfirming that halving tissues may contribute to discrepant results. FFPE PCR results were available for 54 of these samples and IS6110 was amplified from 13 tissues, highlighting the usefulness of having an additional confirmatory diagnostic for fixed tissues. The second most common isolate cultured from compatible lesions in cattle were MAC members (n = 27) where speciation could not be achieved or was not attempted.

For feral swine, 452 samples were submitted from the Moloka'i wildlife survey between 1999 and 2008 when the study concluded. The percent positive of bTB-confirmed cases increased toward the study terminus, with 11.6% and 20% positive in 2006 and 2007, respectively (Figure 7). In contrast to the flow chart used for cattle, all histology results were cultured including non-compatible lesions (Figure 8). Histopathology results were available for 396 cases with the remainder recorded as "not done" or "no diagnosis", the latter usually due to severe autolysis obscuring an accurate microscopic interpretation.

The total number confirmed bTB cases for feral swine by either FFPE PCR or culture was 16 (3.5%). Twenty-four histo compatible cases were identified and 19 of these had PCR performed. There were 13 PCR positive samples for IS6110, 2 samples positive for *M. avium*, and 4 samples negative on PCR. Sixteen of the 19 PCR samples had corresponding culture results. *M. bovis* (n = 6) and MTC (n = 2) were isolated in 8/16 cases. Culture identified an additional 3 bTB cases in feral swine where PCR was not performed. *M. bovis* isolates made up 9% of all culture samples that recovered mycobacteria. MAC members were the most common isolate (44%), similar to cattle (Figure 9).

Discussion

The policies to improve the submission rates implemented in early 2000s successfully increased submissions from abattoir cattle, peaking in 2005-2008 before decreasing. Furthermore, by 2010 the 40 largest adult cattle abattoirs, responsible for killing 95% or more of adult cattle in the US, were meeting the submission standard (Tsao et al., 2014). Along with this increase came a subsequent decrease in granulomas that were histologically diagnosed as mycobacteriosis compatible, from 3.6% in 2001 down to 0.5% in 2010. While implementation of submission standard may explain some of this decrease by lowering the "quality" of granulomas submitted, bTB granulomas can mimic the gross appearance of other granulomatous diseases

such as actinomycosis, actinobacillosis, and coccidiomycosis, as well as neoplastic diseases such as metastatic squamous cell carcinoma. Despite fluctuations in the number of submissions and positive bTB cases in cattle, infected herds detected through slaughter surveillance and associated investigations remained fairly constant, ranging from no herds (2004) to 10 herds (2010), for a total of 35 herds detected as a result of the slaughter surveillance program. Slaughter surveillance detected 35/85 (41%) of all affected herds identified during these 10 years (Portacci et al., 2011; Tsao et al., 2014).

Histology was the most critical component to the slaughter surveillance program as disposition of the carcass was, in part, determined by the histology and results could generally be reported within 24 hours of receipt, causing minimal interruption to commerce. Seven-hundred-forty-one confirmed cases in cattle were identified from the 63,754 submissions for an overall percent positive of 1.16%, compared with 3.32% for feral swine. In this study, histopathology initially misclassified 12 of the 453 confirmed *M. bovis* cases. Interestingly, five of those cases were misclassified by one pathologist on a single day. The other seven cases were missed sporadically throughout the study years. The most common misdiagnosis was pyogranuloma (n = 7), followed by granuloma of unknown etiology (n = 3), then abscess (n = 2). Eleven of the 12 misdiagnoses (false negatives) were due to pathologist error, as intralesional acid-fast bacilli were later identified on the original slides. The other misdiagnosis was confirmed after the isolation of *M. bovis* by retesting additional formalin fixed tissue that originally had not been prepared for microscopic examination. In paucibacillary cases, it is common to only find 1 or 2 bacilli on a slide with multiple lesions from various tissues. Therefore, for cases of high suspicion serial sections may be necessary to locate AFB.

PCR on FFPE tissues has been an important addition to the slaughter surveillance program. When suspect cases are first identified through slaughter surveillance, rapid FFPE PCR results are critical for prioritizing the investigation and for confirming the infection status of a herd when the first animals are removed from the herd after whole herd testing. Of all histo compatible cases subjected to molecular testing (n = 657), FFPE PCR detected IS6110 in 68%. FFPE PCR failed to detect 39 of the 453 culture positive samples (8.6% false negatives). Performance of FFPE PCR directly corresponded to the number of bacteria visible by histology and pathologists could usually predict which samples would be FFPE PCR negative. Due to NVSL's policy of only using FFPE PCR on mycobacteriosis compatible histo samples, there was complete agreement between histo and PCR positives.

Complete diagnostic test agreement for histopathology, culture, and PCR was reached in 414/553 (75%) cattle cases and 8/17 (47%) feral swine cases where results of all three tests were available. Mycobacterial culture detected all but 17 FFPE PCR-confirmed cases in cattle, a false negative rate of 4%. All but 4 of these cases occurred prior to 2004, before the practice of setting up 2 liquid media cultures on mycobacteriosis compatible samples began. It is important to note that over these 10 years there were 82 mycobacteriosis compatible cases that were negative by both FFPE PCR and culture (122 cases when considering a negative result on either culture or PCR). These cases either had atypical mycobacteria detected (n = 27 FFPE PCR and n = 42 MOTT from culture), had mycobacteria that were not cultivable (i.e., due to contamination or low bacterial load), or were potentially missed by both assays. The latter is somewhat unlikely, as 20 of these cases were still investigated epidemiologically and no affected herds were identified.

Over these 10 years, there were 4 false positive *M. bovis* culture results. Two of these cases were due to cross-contamination with an ATCC strain of *Mycobacterium africanum* that had been manipulated in the biosafety cabinet prior to processing cases. Once this was determined, the results were redacted within a few weeks of the initial report. The other 2 false positive cases were caused by either cross-contamination with another positive field case cultured on the same day or occurred during isolate identification. They were discovered 10-14 years later, when these isolates were pulled from the archive for WGS and they matched isolates that were not epidemiologically related. A review of the case records showed they were handled in the laboratory at the same time as the matching cases. Most, 410/414 (99%) of the confirmed cases reported in this study and all 4 of the false positive cases, were subjected to WGS. The 4 false positive culture cases were the only slaughter surveillance samples where additional extensive histopathology testing from submitted tissue could not retroactively identify a mycobacteriosis compatible lesion once the conflict between the culture and histopathology results had been identified. This further confirms the importance of investigating conflicting test results, and only when sampling error (evaluated by tissue matching - genotyping the cattle tissue in the formalin and borate) and cross contamination (evaluated by genotyping, preferably WGS of the isolate recovered) are ruled as unlikely should discrepant results be released. It is important to note that truly discrepant results are not unusual in animals from infected herds, or in high prevalence populations, due to animals that are in the early stages of disease and lack grossly visible lesions. The U.S. slaughter surveillance system however, does not contain antemortem bTB test positive animals or animals from infected herds. Samples are submitted only because grossly visible lesions were identified during the inspection process.

Genotyping is an important tool for laboratory quality monitoring, as well as for epidemiological tracing. In the human literature, a review of 14 large studies (≥ 100 patients) found a median of 3.1 % falsely diagnosed cases of *M. tuberculosis*, with roughly 0.8%, due to laboratory cross contamination (Burman & Reves, 2000). The NVSL started genotyping in 2003 and was genotyping all diagnostic cases by 2005. While cross contamination continued to be a rare occurrence after 2004, we believe we identified all the potential false positive cross contamination cases prior to the release of results due to genotyping, careful evaluation of cases batched during that testing period, and working with the pathologist to review histopathology.

While *M. bovis* was recovered from 678 (78.5 %) of the mycobacteriosis compatible lesions in cattle, atypical mycobacteria were detected by FFPE PCR or isolated from 63 (7.3 %) and a causal organism was not detected in 75 (8.7 %) of the compatible lesions. Mycobacterium avium complex (MAC) organisms were the most common (n = 27) atypical mycobacteria detected in compatible lesions. From all non-compatible cases with other histological diagnoses, there were 798 samples (1.3%) where atypical mycobacteria were recovered (Figure 4). These cases were included in a previous study describing these *Mycobacterium* spp. in greater detail (Thacker et al., 2013).

For feral swine, bTB was confirmed in 16/452 cases (3.5% positive overall). Direct comparisons regarding diagnostic test performance for cattle vs. feral swine cannot be made considering differences in the bTB diagnostic flow charts for each species. However, it is interesting that for feral swine submissions, FFPE PCR detected more bTB cases than culture, while the reverse is true for cattle. The nature of sample collection for these subsets was drastically different, with cattle samples being collected in an indoor, controlled environment by professionally trained individuals and feral swine samples collected by hunter harvest or wildlife

officials in rigorous terrain from a tropical environment. Estimates of overall bTB prevalence in U.S. cattle and Moloka'i feral swine are suspected to be higher for feral swine which may also contribute to diagnostic test performance (Essey et al., 1981; Essey et al., 1983). The presence of coinfections by rapid-growing, atypical mycobacteria confound culture by out-competing MTC. Minor differences in *M. bovis* strains can also affect bacterial growth rate and efficiency. The Moloka'i strain of *M. bovis* is cultivated at particularly slow growth rates compared with other strains circulating throughout the continental U.S. (unpublished, internal observation).

As with any comprehensive retrospective diagnostic study that spans years, some inherent biases do occur in these data. Only 60% of the cases were cultured and those were determined by the histological diagnosis; consequently, the sensitivity of histology could have been overestimated. Furthermore, these samples were tested under a quality management system and errors in testing were reviewed, documented and if needed, processes altered, which likely improved laboratory performance over time. Mycobacterial cultures and FFPE PCR were not conducted blindly and mycobacteriosis compatible samples were cultured with additional media; consequently, the performance of both culture and FFPE PCR are likely overestimated. A non-gold standard/Bayesian analysis would allow for determination of each individual diagnostic test, including culture, but is obscured by diagnostic biases built into the NVSL decision making tree for bTB. Furthermore, technology greatly impacted the mycobacterial culture laboratory, and over these 10 years the laboratory incorporated restriction fragment length polymorphism (RFLP), spoligotyping, variable number tandem repeats (VNTR) to characterize *M. bovis*, and Sanger sequencing for atypical mycobacterial identification. These new technologies likely improved specificity of culture within the time period of the study.

Conclusion

This study examined the diagnostic performance of histology, FFPE PCR and culture to detect bTB in surveillance cases from abattoir cattle and Moloka'i feral swine over a 10-year period in a low prevalence country. It appears that the efforts resulting in the invigoration of the State - Federal Cooperative Bovine Tuberculosis Eradication program had significant success. By 2010, all 40 of the largest adult cattle abattoirs were meeting the submission standard, and the detection rate of bTB continued to decline, despite increases in submissions. Parallel testing of histology and culture, and the investigation of discrepant results, identified laboratory error and improved test performance. Genotyping is not only important for epidemiological tracing, but also important to detect cross contamination events in the culture laboratory.

Acknowledgments

The authors would like to thank the veterinarians and inspectors at the United States Department of Agriculture's Food Safety and Inspection Service and the many pathologists, microbiologists, and laboratory technicians who performed the tests over these 10 years.

References

Aranaz, A., De Juan, L., Montero, N., Sánchez, C., Galka, M., Delso, C., Alvarez, J., Romero, B., Bezos, J., & Vela, A. I. (2004). Bovine tuberculosis (*Mycobacterium bovis*) in wildlife in Spain. Journal of Clinical Microbiology, 42(6), 2602-2608.

Burman, W. J., & Reves, R. R. (2000). Review of false-positive cultures for *Mycobacterium tuberculosis* and recommendations for avoiding unnecessary treatment. Clinical infectious diseases, 31(6), 1390-1395.

Coleman, J., & Cooke, M. (2001). *Mycobacterium bovis* infection in wildlife in New Zealand. Tuberculosis, 81(3), 191-202.

Essey, M., Payne, R., Himes, E., & Luchsinger, D. (1981). Bovine tuberculosis surveys of axis deer and feral swine on the Hawaiian island of Molokai. Proceedings of the United States Animal Health Association,

Essey, M., Stallknecht, D., Himes, E., & Harris, S. (1983). Follow-up survey of feral swine for *Mycobacterium bovis* infection on the Hawaiian island of Molokai. Proceedings of the United States Animal Health Association,

Miller, J., Jenny, A., Rhyan, J., Saari, D., & Suarez, D. (1997). Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. Journal of Veterinary Diagnostic Investigation, 9(3), 244-249.

Naugle, A. L., Schoenbaum, M., Hench, C. W., Henderson, O. L., & Shere, J. (2014). Bovine tuberculosis eradication in the United States: a century of progress. Zoonotic tuberculosis: *Mycobacterium bovis* and other pathogenic mycobacteria, 235-251.

Nugent, G. (2011). Maintenance, spillover and spillback transmission of bovine tuberculosis in multi-host wildlife complexes: a New Zealand case study. Veterinary Microbiology, 151(1-2), 34-42.

Orloski, K., Robbe-Austerman, S., Stuber, T., Hench, B., & Schoenbaum, M. (2018). Whole genome sequencing of *Mycobacterium bovis* isolated from livestock in the United States, 1989–2018. Frontiers in Veterinary Science, 5, 253.

Palmer, M. V., Waters, W. R., Thacker, T. C., Stoffregen, W. C., & Thomsen, B. V. (2006). Experimentally induced infection of reindeer (Rangifer tarandus) with *Mycobacterium bovis*. Journal of Veterinary Diagnostic Investigation, 18(1), 52-60.

Portacci, K., Lombard, J., Abrahamsen, L., Bush, E., Fossler, C., Harris, R., Johnson, K., Miller, R. S., Mitchell, D., & Pritchard, R. (2011). Assessment of Pathways for the Introduction and Spread of *Mycobacterium bovis* in the United States.

Robbe-Austerman, S., Bravo, D. M., & Harris, B. (2013). Comparison of the MGIT 960, BACTEC 460 TB and solid media for isolation of *Mycobacterium bovis* in United States veterinary specimens. BMC Veterinary Research, 9, 1-6.

Taylor, R. B., Hellgren, E. C., Gabor, T. M., & Ilse, L. M. (1998). Reproduction of feral pigs in southern Texas. Journal of Mammalogy, 79(4), 1325-1331.

Thacker, T. C., Robbe-Austerman, S., Harris, B., Palmer, M. V., & Waters, W. R. (2013). Isolation of mycobacteria from clinical samples collected in the United States from 2004 to 2011. BMC Veterinary Research, 9, 1-10.

Tsao, K., Robbe-Austerman, S., Miller, R. S., Portacci, K., Grear, D. A., & Webb, C. (2014). Sources of bovine tuberculosis in the United States. Infection, Genetics and Evolution, 28, 137-143.

USDA. (2023, 03/07/2023). TB Brucellosis Summary Reports and Affected Herd Maps. Retrieved 01/11/2023 from https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/cattle-disease-information/tuberculosis-brucellosis-monthly-report/tb-bruc-reports

Vicente, J., Höfle, U., Garrido, J., Fernández-De-Mera, I. G., Juste, R., Barral, M., & Gortazar, C. (2006). Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. Veterinary Research, 37(1), 107-119.

Wyckoff, A. C., Henke, S. E., Campbell, T. A., Hewitt, D. G., & VerCauteren, K. C. (2009). Feral swine contact with domestic swine: a serologic survey and assessment of potential for disease transmission. Journal of Wildlife Diseases, 45(2), 422-429.

CHAPTER 3. EVALUATING *MYCOBACTERIUM BOVIS* IN TWO DISTINCT POPULATIONS OF U.S. FERAL SWINE

Dana Hill*, Tyler Thacker*, Aaron Lehmkuhl*, Suelee Robbe-Austerman*

*United States of America Department of Agriculture, Veterinary Services, National Veterinary Services Laboratories, Ames, IA

Modified from a manuscript to be submitted to *Journal of Veterinary Diagnostic Investigation*[1]

See supplementary document, "FS bTB Figures and Tables"[2]

Abstract

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, has a long history on the Hawaiian island of Moloka'i where disease spillover from infected cattle into feral swine was identified in the 1980s and disease spillback from feral swine into cattle was confirmed in the early 1990s following temporary removal of cattle from the island. Despite evidence of historic (1997) and recent (2021) disease spillback from feral swine into cattle, the debate remains whether Moloka'i feral swine fit the criteria of a spillover host, an amplifier host, or a maintenance host for bTB. To infer host status of European wild boar descendants from Texas and Polynesian swine from Hawaii, disease susceptibility, pathology, and immunology were compared between these genetically distinct groups experimentally infected with *M. bovis.* Experimental infection of Moloka'i- and Texas-origin, captive-bred feral swine infected with a low-dose of *M. bovis* showed distinct lesion profiles. Longitudinal sampling of peripheral blood throughout infection indicates highly variable humoral and cell-mediated immune responses as

[1] This chapter is currently a work in progress. It may differ in significant ways from the published version.
[2]Figures and tables for Chapter 2 are included in supplementary document entitled, "FS bTB Figures and Tables"

evidenced by antibody production on dual path platform (DPP) and interferon-gamma IFN-γ production from peripheral blood mononuclear cells (PBMCs) stimulated with various mycobacterial antigens. Specifically, 5/6 (83%) and 3/6 (50%) bTB-infected Hawaiian pigs were positive on DPP for MPB83 and ECH, respectively, at one or more timepoints. In contrast, 2/4 (50%) of infected Texas pigs were positive for only MPB83 during infection. Purified protein derivative from *M. bovis* (PPDb) was identified as an immunodominant stimulant recognized by PBMCs in *M. bovis*-infected feral swine, regardless of genetic background. Inherent differences in immune responses of these groups may contribute to bTB disease development, ultimately shaping host suitability of U.S. feral swine populations. Additional multi-omics and epidemiological investigations of host and pathogen characteristics and interactions affecting disease susceptibility will help to further elucidate bTB pathophysiology and disease outcome in feral swine.

Introduction

Bovine tuberculosis (bTB) is the disease caused by *Mycobacterium bovis,* one of several Gram-positive, acid-fast, aerobic or microaerophilic bacilli belonging to the *Mycobacterium tuberculosis* complex (MTC) (Brosch et al., 2000). It is an intracellular, predominantly respiratory, pathogen capable of evading host defenses and surviving within host macrophages, dendritic cells (DCs), and multinucleated giant cells (MGCs) throughout chronic infection. Tuberculoid granulomas are caseonecrotic and often mineralized lesions that form due to chronic immune stimulation and may be found in single or multiple tissues within infected individuals. Most commonly, granulomas caused by *M. bovis* are found within the lungs and associated lymph nodes of the head and thorax (e.g. submandibular, retropharyngeal, parotid, mediastinal, and tracheobronchial). In some cases, bTB may become disseminated resulting in lesions within

abdominal viscera, the udder, pleural and parietal surfaces, and extrathoracic lymph nodes (Palmer et al., 2007).

Numerous clinicopathologic outcomes are possible following infection, the most common being chronic, slowly progressive disease with or without clinical symptoms and bacterial shedding, making detection and control a challenge. In the U.S., a bTB-free nation, abattoir surveillance is the primary avenue by which cattle are screened. Grossly visible granulomas are collected by abattoir workers and/or veterinary inspectors and sent to the National Veterinary Services Laboratories (NVSL) in Ames, IA for confirmatory testing. Bacterial culture is the gold standard diagnostic test for bTB but is time consuming (6-8 weeks for mycobacterial growth), requires specialized biosafety level-3 (BSL-3) laboratory conditions, and typically is only conducted on tissues collected postmortem. Histopathology is also a postmortem diagnostic test and provides the most rapid results but is considered an ancillary diagnostic with low specificity. A histologic diagnosis of "mycobacteriosis – compatible" is assigned to granulomas with specific features consistent with bTB: central necrosis, mineralization, Langhans-type MGCs, satellite granulomas, paucibacillary, and appropriate bacterial morphology for *M. bovis* (short, typically straight, 1-2um, acid-fast bacilli within central necrosis or cytoplasm of histiocytes or MGCs). Polymerase chain reaction (PCR) from formalin-fixed paraffin-embedded (FFPE) tissues is validated for use at NVSL as a secondary confirmatory diagnostic (Miller et al., 1997). A real-time PCR (qPCR) is routinely used as a screening test for fresh tissue samples being subjected to mycobacterial culture. Primers for FFPE PCR amplify an insertion sequence (IS6110/IS6110T) conserved among MTC species but not unique to *M. bovis* (Miller et al., 2002; Thacker et al., 2011). Speciation of *M. bovis* and

molecular epidemiology for bTB cases may be confirmed by whole genome sequencing (WGS) from culture isolates (Orloski et al., 2018).

Antemortem diagnostics most routinely used for cattle include the caudal fold test (CFT), comparative cervical skin test (CCT), and the IFN-γ release assay. The CFT involves intradermal injection of purified protein derivative (PPD) originating from *M. bovis* (PPDb) at the tailhead whereas CCT includes a second injection with PPD from *Mycobacterium avium* (PPDa) to help differentiate animals with atypical mycobacterial infection, such as Johne's disease caused by *M. avium* ssp. *paratuberculosis* (Monaghan et al., 1994; Waddington, 2004). Exposed animals generally develop a measurable inflammatory reaction at the injection site within 48-72 hours of injection resulting from delayed-type hypersensitivity reaction induced by mycobacterial infection as well as vaccination from *M. bovis* strain bacille Calmette-Guérin (BCG). The IFN-γ release assay quantifies IFN-γ production from whole blood samples through *ex vivo* stimulation of mononuclear cells by mycobacterial antigens, most commonly involving PPDa, PPDb, in addition to positive (pokeweed mitogen, PWM) and negative controls (Rothel et al., 1990; Schiller et al., 2010). These diagnostics are generally used as herd screening tests prior to animal movement or in test and cull operations within exposed herds.

The commercialized DPP test kit by ChemoBio® is a serologic test validated for use in white-tailed deer (WTD) and elephants (Greenwald et al., 2009; Lyashchenko et al., 2013). This lateral flow-based assay screens for IgG produced against two mycobacterial antigens on separate test lines (T1 and T2); T1 corresponding to mycobacterial protein 83 (MPB83) and T2 a recombinant protein (ECH), composed of early secretory antigenic target of 6kDa (ESAT-6) and culture filtrate protein of 10kDa (CFP-10) (Maggioli et al., 2016; Volkman et al., 2004; Vordermeier et al., 2001).

Control of bTB in the U.S. relies on abattoir surveillance and removal of test positive animals from exposed herds. Repeated disease spillover events from infected cattle into wildlife risks establishment of novel maintenance hosts and disease spillback into naïve cattle, making eradication virtually impossible (Aranaz et al., 2004; R. Miller & S. Sweeney, 2013). A few wildlife species accepted as bTB maintenance hosts include WTD in the modified accredited zone (MAZ) in Michigan, the European badger in the U.K., the brushtail possum in New Zealand, and the Eurasian wild boar in Spain (Corner, 2006; A. Spickler, 2019). The geographic and host species barriers of *M. bovis* are not clearly understood, however, livestock and wildlife management practices, environmental, and host factors (e.g., genetics, immune response) are all thought to play a role in disease transmission and maintenance within a population (Brosch et al., 2002; Garnier et al., 2003; Phillips et al., 2002).

Undoubtedly, cattle are the primary reservoir for *M. bovis*, showing pulmonary and lymph node involvement and transmitting bTB via aerosolized respiratory secretions (Cousins, 2001). Humans are generally considered a spillover host as human-human transmission of bTB is relatively uncommon, although it does occur in immunocompromised and HIV+ individuals where disease may be acute, severe, and fatal (Rangaka et al., 2007). People are exposed through the respiratory route most notably in livestock ranchers, veterinarians, zoo personnel, and slaughterhouse workers, and, prior to pasteurization, through consumption of contaminated milk (Cosivi et al., 1995; McFadyean, 1900; A. Spickler, 2019). Humans infected with bTB may show extensive pulmonary disease and have served as a source of infection in cattle through bTB spillback (Grange & Collins, 1987; Lombard et al., 2021; Portacci et al., 2011; Tsao et al., 2014).

The distinction of reservoir vs. spillover host becomes much more complicated when factoring in numerous susceptible species inadvertently exposed to bTB-infected cattle. The U.S.

holds bTB-free status among the national cattle herd, allowing for exportation of meat and dairy products without trade restrictions. Occasionally, illegal or natural movement of bTB-infected cattle across the Mexico-U.S. border or exposure to wildlife with endemic bTB results in identification of infected cattle during routine testing, posing a threat to agriculture and public health (Portacci et al., 2011; Tsao et al., 2014). Environmental and molecular epidemiological analyses of the MAZ have revealed cattle as the origin of infection in WTD with subsequent disease spillback into cattle (Corner, 2006). Infected deer show tuberculoid lesions with a range of severity and distribution, affecting the lungs, parietal pleura, individual or multiple lymph nodes within the head or chest, and/or abdominal viscera (Thacker et al., 2006). In contrast, lesions in bTB-infected cattle are often restricted to the lungs, thoracic lymph nodes (e.g., mediastinal, tracheobronchial), and the medial retropharyngeal lymph nodes (Palmer et al., 2007).

Detailed epidemiological studies of other bTB-infected wildlife species, such as badgers in the United Kingdom, brushtail possums in New Zealand, and the European wild boar in Spain, suggest a bTB maintenance host role (Coleman & Cooke, 2001; Coleman et al., 1999; Phillips et al., 2003). These wildlife species have proven their ability to perpetuate bTB transmission within their populations and back into cattle, posing a significant challenge to eradication efforts (Corner, 2006). Although wild and domestic swine are generally thought of as spillover hosts for bTB, the European wild boar in Spain is an exception (Aranaz et al., 2004; De Mendoza et al., 2006; V Naranjo et al., 2008; Parra et al., 2003; Vicente et al., 2006). In this region, bTB prevalence ranges from 44% upwards to 100% in certain populations of wild boar.

Feral swine on the Hawaiian island of Moloka'i were first found to be infected with *M. bovis* in the 1980s with an estimated prevalence of 19.67% (ranging from 11-35% at 95% CI),

dropping to 3.22% (>0-17% at 95% CI) in 1983 (Essey et al., 1981; Essey et al., 1983). The drop in prevalence was attributed to actions taken to control bTB, including depopulation of the affected cattle herd on the eastern end of the island and open hunting declared for feral swine surrounding affected cattle ranches and within the bordering state forest. It was surmised that bTB was first introduced to cattle on Moloka'i in the 1940s and remained until 1985 when the decision was made to depopulate all cattle on the entire island, totaling approximately 9,400 head (Meyer, 2000). No cattle were on the island for a full year following depopulation and only steers were allowed the 2nd year, who were immediately sent to slaughter upon maturing. Slowly, after another 2 years, breeding animals with two negative tuberculin skin tests were introduced and annual regulatory testing of cattle on the eastern (affected) part of the island was implemented.

Moloka'i cattle remained bTB-free up until 1997 when a cow from a herd of 450 cattle on the eastern end of the island tested positive (Meyer, 2000). The affected herd was depopulated and 4,922 cattle from surrounding ranches were tested but no reactors were detected. Additionally, a hunter-assisted wildlife surveillance study targeting axis deer, feral swine, feral goats, and mongoose was conducted from 1998 until early 2008. Earlier surveillance efforts between 1961-1985 detected a handful of positive axis deer (n=5) and mongoose (single *M. bovis* colony from a pooled sampled of 3 mongoose). However, out of approximately 350 axis deer, over 70 feral goats, 70 mongoose, and 450 feral swine, only feral swine showed evidence of bTB from the wildlife survey initiated in 1998 (Meyer, 2000).

Subsequently, cattle remained bTB-negative on annual regulatory testing up until 2021 when a cow originating from the eastern part of Moloka'i reacted on the caudal fold test and was later confirmed bTB-positive by NVSL. WGS of the *M. bovis* isolate from the infected cow

determined it originated from the endemic strain present within feral swine (NVSL unpublished findings). Historical epidemiological investigations from Moloka'i combined with the molecular evidence from the most recent isolates recovered from cattle and other domestic livestock, including domestic swine, all suggest that feral swine may be acting as a maintenance host for bTB within this geographical niche.

Feral swine occupying the lower 48 U.S. states have not been investigated in a similar capacity since naturally occurring bTB is largely absent from the continental U.S. (Campbell et al., 2011; Pedersen et al., 2017). Feral swine are susceptible to tuberculosis caused by *M. bovis* as well as *M. tuberculosis* and swine satisfy many high-risk criteria for bTB transmission and amplification (Bevins et al., 2014; Nol et al., 2016; Pedersen et al., 2017). Populations of feral swine in the continental U.S. have continually risen since 1982, reaching an estimated 6 million today (USDA, 2023a). The range of feral swine overlaps with bTB-infected Mexican cattle and is rapidly expanding (Campbell et al., 2013; Delgado-Acevedo et al., 2007; Delgado-Acevedo et al., 2010; Wyckoff et al., 2009). Recent sightings of feral swine in Michigan within the bTB-infected WTD zone are also concerning for disease spillover (USDA, 2023a). Baiting of wild game, legally or illegally, results in wildlife aggregation and increased contact between feral swine, deer, along with numerous other bTB-susceptible species (Brown & Cooper, 2006; Brown et al., 2018).

While free-range populations of feral swine are generally considered heterogenous with hybrid genetics, illegal movement of animals outside their normal range and into hunting estates contributes to species isolation, inbreeding, and population bottlenecks. Texas feral swine share a common ancestor with an established bTB-reservoir host in Spain, the European/Eurasian wild boar (McCann et al., 2018; Serraino et al., 1999). At present, Texas feral swine are a genetic

mixture of Eurasian wild boar outbred to domestic swine (Bailey et al., 2013; Smyser et al., 2020). In contrast, feral swine on Hawaii were originally introduced by the Polynesians and the majority (70% from one study) retain the Pacific clade lineage, similar to the pot-bellied pig (Linderholm et al., 2016). In this study, a smaller proportion (30%) of Hawaiian feral swine showed European clade origins from the later introduction of the Eurasian wild boar by Captain Cook. Only two pigs from Moloka'i were included in this study and one carried the Pacific clade allele while the other carried the European allele.

The debate remains whether Moloka'i feral swine fit the criteria of a spillover host, an amplifier host (who exacerbates disease maintenance and spread under specific population and environmental dynamics), or a wildlife maintenance host for bTB (similar to WTD deer in Michigan and the Eurasian wild boar in Spain). Determination of bTB host status is complex, requiring extensive epidemiological data from naturally infected populations and is crucial in bTB-disease mitigation and eradication efforts. Investigation of bTB in U.S. feral swine offers a unique opportunity to compare and contrast host and pathogen interactions in a potential maintenance host on Moloka'i alongside feral swine of slightly distinct genetic background with limited exposure potential to different strains of *M. bovis*.

Given the Eurasian wild boar's established role as a wildlife maintenance host for bTB in Spain, our aims were to compare disease severity using an experimental disease model to infer the potential bTB-host status of U.S. feral swine with mixed genetic backgrounds. We hypothesized that since Moloka'i feral swine have historically maintained bTB within their geographical niche that this group would show more extensive lesions/bacterial loads supporting disease transmission. Feral swine originating from Moloka'i and Texas were infected with *M. bovis* and evaluated for lesion profiles, cell-mediated and humoral immunity, and genetic factors

contributing to bTB infection. Our results demonstrate distinct lesion distribution patterns and immunological responses throughout the course of infection in Moloka'i- compared with Texas-origin swine. Pathophysiology of bTB in these groups of pigs was assessed, used to improve bTB diagnostics for feral swine, and inform researchers and epidemiologists about host and pathogen factors contributing to disease development and maintenance in a non-bovid host species.

Materials and methods

Breeding and Farrowing

All procedures and animals enrolled for this study were approved by the institutional animal care and use committee at the National Centers for Animal Health (NCAH) in Ames, IA according to American Association of Laboratory Animal Science and American Veterinary Medical Association guidelines for the ethical and humane treatment of animals used for research. Breeding, farrowing, and rearing of animals occurred at the NCAH campus. Two captive breeding colonies of feral swine originating from either Texas or Moloka'i were maintained and bred yielding twelve piglets farrowed indoors within a BSL-2 barn, including seven Moloka'i and five Texas origin. Piglets were ear tagged and separated into two treatment groups according to genetic background (Moloka'i or Texas) and two pigs, one Moloka'i and one Texas, were randomly assigned as negative controls. Treatment group pigs were relocated to BSL-3 biocontainment into separate rooms according to genetic background and acclimated for a minimum of 2 weeks while negative controls remained in BSL-2 confinement.

Inoculum Preparation and Infection

A low dose of 10^4 colony forming units (CFU) of *M. bovis* was cultured and quantified as previously described (Larsen et al., 2007; Waters et al., 2010). Briefly, *M. bovis* strain 95-1315 (isolated from WTD in Michigan in 1995) (Schmitt et al., 1997) was grown on Middlebrook 7H9

media (BD, Oxford, UK) enriched volume/volume with 10% Middlebrook acid-albumin-dextrose-catalase (BD) and 0.05% Tween 80 (Sigma-Aldrich) and supplemented with 4.16 g/L sodium pyruvate (Sigma-Aldrich Co., Poole, UK). Serial dilutions on modified Middlebrook 7H11 agar were performed to achieve 10^4 CFU per milliliter (CFU/ml) (Gallagher & Horwill, 1977). Two milliliters of phosphate buffered saline (PBS) was mixed with 1ml of inoculum immediately prior to administration to the treated (infected) pigs and 3 mls of PBS was used for mock-infected negative controls. At 8-9 months old pigs were sedated, placed in dorsal recumbency, and an oral speculum was used to deliver the infectious dose via pharyngeal lavage. Pigs were gently rolled between dorsal and right/left lateral recumbency for 30 seconds and then released and monitored from a distance until fully recovered from sedation.

Longitudinal Sampling

Pooled oral fluids from hanging cotton rope and feces were collected 7 days prior to infection, at 1-day post-infection (dpi) and weekly thereafter through 182 dpi from infected and negative control pig rooms. Cotton ropes were hung at shoulder height prior to feeding and remained hanging in each pig room for approximately 20 minutes. Mycobacterial culture (Larsen et al., 2007; Waters et al., 2010) and real time PCR (qPCR) targeting IS6110 (Thacker et al., 2011) were performed on pooled feces and oral fluids. Blood was collected at 7 days prior to infection, 1 day post infection, and then every 14 days to analyze humoral and cell mediated immune responses.

Dual Path Platform

Feral swine serum samples collected at -7 dpi, 1 dpi, and at 14-day intervals until 182 dpi were tested for antibody responses to mycobacterial antigens using the commercialized ChemBio® VetTB DPP (ChemBio Diagnostic Systems, Medford, NY) kit. Briefly, in addition to an internal control, two nitrocellulose test strips (T1 and T2) with immobilized mycobacterial

antigens (MPB83 and ECH, respectively) were exposed to serum and test buffer containing colloid gold particles. The presence of IgG in the sample to either antigen results in *in vitro* immune complex formation and production of a visible band detectable by an optical reader device reporting in relative light units (RLU). Any samples with positive reflectance on the initial run was retested to confirm the presence/absence of immune complex formation and ultimately determine a positive or negative result. Being an unvalidated test for feral swine, any visible band in T1 or T2 (RLU value > 0) was considered positive (Greenwald et al., 2009; Lyashchenko et al., 2013).

PBMC Isolation and Restimulation

Whole blood from each pig was collected at -7 dpi, 1 dpi, and at 14-day intervals until 182 dpi into two CPT sodium citrate tubes and one serum separator tube (BD Vacutainer®). Blood tubes were kept at room temp during sampling (1-1.5 hours) then centrifuged at 1800 x g, 25ºC for 25 minutes (Beckman Coulter benchtop bucket centrifuge). PBMCs were isolated by carefully removing from the buffy coat then transferred to a 15ml conical tube, wash three times in PBS, filtered through a 40um cell strainer, and resuspended in 3-4 mls of complete RMPI 1640 media (cRMPI, ThermoFisher) containing 8% fetal bovine serum (ThermoFisher), 25mM HEPES (ThermoFisher), 1% M5550 EAA (Sigma-Aldrich), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich), and 2mM L-glutamine (ThermoFisher). Cells were counted on the Muse cell counter (Luminex Corporation), resuspended in cRMPI media to achieve a final concentration of 10 million cells/ml, and plated into culture treated, U-bottom 96-well plates at 500,000 cells/well. *Ex vivo* restimulation of PBMCs was performed in duplicate using PPDa at 4.3 ug/ml, PPDb at 10.6 ug/ml, and MPB70 and MPB83, and ECH, at 0.5 ug/ml, in addition to a cRMPI negative control (media only) and PWM positive control at 5ug/ml. PPDa, PPDb and PWM were obtained as part of the PPD pack from ID Vet (Innovative Diagnostics, Grabel,

France). MPB70, MPB83, and ECH originated from Lionex Diagnostics and Therapeutics (Braunschweig, Germany). Cells were incubated at 39ºC in 5% CO2 for 72 hours then pelleted and supernatants collected and stored at -80ºC.

Interferon-gamma ELISA

Supernatants from PBMC restimulation were thawed and IFN-γ quantitated using the Porcine DuoSet IFN-γ solid phase sandwich enzyme-linked immunosorbent assay (ELISA, biotechne, R&D Systems) according to manufacturer instructions at a 1:2 sample dilution. Plates were washed using an automatic microplate washer (BioTek 405) and reflectance values read by ELISA plate reader (Synergy HT, Gen 5 V. 3.08) at 450nm and 540 nm. Results were exported and reflectance values corrected for background removal and wavelength correction. The final concentration of IFN-γ (pg/ml) was calculated following interpolation and transformation of corrected OD values using GraphPad Prism and adjusted sample dilution factor of 1:2. Duplicate sample values were averaged and negative control media concentrations were subtracted from stimulant sample values.

Necropsy and Tissue Processing

Animals were maintained within biocontainment for approximately 6.7 months (196-203 days) and then humanely euthanatized and postmortem examinations were performed. A complete set of study tissues (Table 2) were collected from each animal in addition to any grossly visible lesions. Tissues were sectioned at 0.5-1cm in the biocontainment necropsy suite where grossly visible lesions were documented, halved for culture from fresh-frozen tissue or sectioned into cassettes and placed into 10% neutral buffered formalin on an agitator for 24-hour fixation. One section of each lymph node, two sections from each lung lobe, and two sections of liver were collected for fixation or fresh-frozen prior culture. After 24 hours, fixed tissues were transferred to 70% ethanol and processed for routine histopathology.

Histopathology

Slides were stained with hematoxylin and eosin (H&E) and examined for the presence of microscopic lesions. Tissues with marked histiocytic infiltration, epithelioid macrophages, multinucleated giant cells, the presence of necrosis, granuloma formation, or other inflammation were documented and special stains were applied, including Gram stain, New Fuchsin acid fast (NF), and auramine-acridine orange (AO) (Sheehan & Hrapchak, 1973). Lesions were examined for the presence/absence of acid-fast bacilli (AFB) by light microscopy (NF) and fluorescent microscopy (AO). Histologic lesions were scored from I-IV according to previously published criteria for cattle and wild boar (Martín-Hernando et al., 2007; Palmer et al., 2007). Lesion scores were defined as: I, increased infiltrates and disorganized aggregates of epithelioid macrophages, occasional multinucleated giant cells, with low number of neutrophils and lymphocytes; II, small to medium organized clusters of epithelioid macrophages, multinucleated giant cells, +/- few neutrophils, a partial to complete fibrous connective tissue capsule, necrotic core absent; III, organized granulomas with a necrotic core, epithelioid macrophages, multinucleated giant cells, a peripheral rim of lymphocytes, +/- fibrous connective tissue capsule; IV, organized granulomas as described for "score III" with the presence of dystrophic mineralization. Tissues were assigned a score for the most advanced lesion present within the section even though more advanced lesions were often surrounded by smaller, less organized, satellite granulomas.

Pilot study

A pilot study was conducted using four captive-bred, Texas-origin feral swine piglets 5.5 months old for assay optimization and to analyze a mixed, low-dose infection containing 50% of *M. bovis* strain originating from Moloka'i (feral swine isolate) and 50% of a Mexican cattle strain. Animals were maintained in BSL-3 biocontainment for approximately 120 days post-

infection with pooled feces and oral fluids and blood/serum collected at 0, 32, and 120 dpi. Pooled fecal and oral fluid samples were analyzed by mycobacterial culture and qPCR and serum was submitted for DPP. PBMCs were separated from whole blood collected in CPT tubes as described above and cells were cultured for 72 hours with cRMPI media (negative control), concanavalin A (ConA, positive control), avian and bovine tuberculin (PPDa 2500 and PPDb 3000, Prionics, BOVIGAM), ECH (generously provided by the Palmer laboratory). The porcine DuoSet ELISA (R&D) was used to quantify IFN-γ concentration (pg/ml) from duplicate samples of *ex vivo* stimulated PBMCs.

Results

Antibody production and mycobacterial shedding

Antibody production was most consistent among infected Moloka'i pigs from 28 dpi through the study terminus against both MBP83 and ECH (Table 3). Texas feral swine showed limited antibody production against only MBP83 at intermittent timepoints (28, 42, 56, and 140 dpi) throughout the study. Two Moloka'i pigs, one negative control and one treated prior to infection, showed false positive results on DPP primarily on the T2 channel. DPP results for individual pigs are shown in Figure 10 with different treatment groups overlaid in the first 2 columns for negative controls (MPB83 in light green, ECH in dark green) and 4 columns for *M. bovis*-infected Texas pigs (MPB83 in orange, ECH in red) and dpi repeating along the x-axis. The average RLU correlating to test line 1 (MPB83) and 2 (ECH) is shown for each pig from -7 through 182 dpi (Figure 10). Antibody production against MPB83 and ECH was evident among 5/6 (83%) of infected Moloka'i pigs at much higher RLU readings relative to two Texas-origin pigs (50%) with positive results for only MPB83. The false positive results for ECH observed within the negative control Moloka'i pig reached readings over 200 RLU, presumably from cross-reactivity to NTM.

Mycobacterial detection via qPCR from pooled feces and oral fluids was sporadic and only detected within the Texas group (Table 3). *M. bovis* was not isolated from any pooled feces or oral fluid samples through 189 dpi. At 1 dpi, MTC IS6110 was detected by qPCR from the pooled feces collected from the Texas group indicating that inoculum had been swallowed and passed from at least one feral pig, but no mycobacteria were grown on culture. PCR was positive from oral fluid samples originating from the Texas group at 84 and 119 dpi. All samples of pooled feces and oral fluids were negative on qPCR for the negative controls and Moloka'i pigs at all time points. Mycobacterial culture was negative for all groups at all time points except for AFB isolated from pooled oral fluids collected from the negative control room at 14, 112, and 161 dpi, yielding a nontuberculous mycobacteria not MTC. Occasional contamination of oral fluid samples noted at 56 dpi for all groups in addition to 70 and 112 dpi for Texas pigs, 105 dpi for Moloka'i pigs, and 133 dpi for negative controls.

IFN-γ production

Ex vivo restimulated PBMCs from all pigs produced IFN-γ in response to PWM (positive control) at all timepoints (Figure 11). PWM successfully induced IFN-γ from PBMCs of all pigs at all timepoints indicating that PBMCs were viable and the assay worked as expected. Moloka'i pigs, regardless of infection status, showed a much more robust interferon response relative to that of Texas pigs.

Texas-origin pigs consistently displayed lower levels of IFN-γ production than Moloka'i-origin pigs when stimulated by PWM and mycobacterial antigens. *M. bovis*-infected pigs showed the most robust IFN-γ production in response to PPDb (Figure 12), with 5/6 (83%) Moloka'i and 3/4 (75%) Texas responding. IFN-γ induced from PPDb was observed as early as 14 to 28 dpi and continued throughout 182 dpi, peaking around 70-98 dpi. PPDa induced moderate to marked IFN-γ production from PBMCs isolated from 4/6 (66.7%) Moloka'i pigs and 1/4 Texas pigs with

mild IFN-γ produced by PBMCs from 2 additional Texas-origin animals (75% total, Figure 13). Out of 6 infected Moloka'i pigs, 5 (83%) showed moderate IFN-γ production from PBMCs stimulated with ECH while all (100%) of infected Texas pigs had minimal or no IFN-γ production in response to ECH restimulation (Figure 14). Relative to PPDb, PPDa, and ECH, cell mediated immunity directed against MPB70 and MPB83 were negligible in all animals at all timepoints (Figure 15 and 16, respectively). A lower level of IFN-γ (<100,000 pg/ml) was measured from cells isolated from 4/6 (66.7%) Moloka'i pigs restimulated with MPB83. Moloka'i pig #5 (FS #6) and Texas pig #3 (FS #10) did not show evidence of antibody production or cell-mediated immunity to any mycobacterial antigens at any timepoint despite being inoculated with *M. bovis*.

Necropsy and histopathology

Findings of postmortem examination including gross and microscopic lesion distribution, histologic lesion scoring, and presence/absence of acid-fast bacteria are summarized in Table 2. Gross lesions were visible in the head and thoracic lymph nodes in at least one pig from each infected group. Five out of 6 Moloka'i pigs showed gross lesions within the hepatic lymph node and one had a diffuse interstitial pneumonia without grossly discernable pulmonary granulomas. In addition to head and thoracic lymph node lesions, one Texas pig had a single, 4mm diameter pulmonary granuloma in the right caudal lung lobe and numerous, 2mm diameter, miliary caseonecrotic foci within a cervical lymph node located near the thoracic inlet. Considerably more granulomas were identified microscopically, with 57 and 35 lesions present in various tissue sections for infected Moloka'i (n = 6) and Texas (n = 4) groups, respectively. In contrast, negative control pigs showed a total of 5 microscopic lesions which were granulomatous or caseous in nature affecting the palatine tonsils (both control pigs), ileocecal lymphoid tissue (Moloka'i control), and right middle lung lobe (Moloka'i control). Within the tonsils were crypt

abscesses associated with abundant Gram + coccoid bacteria and long branching filamentous bacilli (Texas control) and multifocal microabscesses with occasional multinucleated giant cells (Moloka'i control). The Moloka'i control also had a focal microgranuloma within a Peyer's patch of the ileum and an aggregate of epithelioid macrophages and MGCs in the alveolar septum of the lung engulfing a small amount of black granular material (suspect anthracosis). One infected Moloka'i pig had a single, small (0.4 cm diameter) pyogranuloma located along the ventral surface of the caudal mandible associated with abundant Gram + coccoid bacteria and Splendore-Hoeppli material. All lesions which were histiocytic/granulomatous or caseonecrotic in nature observed within negative controls or associated with Gram positive/negative bacteria (lacking acid-fast bacilli) were presumed a normal, low level of a background lesions.

Special stains (Gram, NF, and AO) were applied to 55 and 27 lesioned tissue sections from infected Moloka'i and Texas pigs, respectively, with 32 (58%) and 8 (30%) containing histologic evidence of AFB. *M. bovis*-infected Moloka'i pigs showed AFB within the mandibular, retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric lymph nodes, palatine tonsils, and in all lung lobes for at least one animal. Texas-origin infected animals had AFB present within mandibular, mediastinal, tracheobronchial, and sternal lymph nodes, as well as right cranial and right caudal lung lobes (Table 2).

Microscopic lesions from all treatment groups and negative controls were assigned a number according to the histologic scoring system (I: histiocytic inflammation +/- multinucleated giant cells; II: microgranulomas; III: small to medium granulomas with central necrosis; IV: mineralized granulomas) for the most advanced lesion present within the tissue section (Figure 17). Out of 57 microscopic granulomas for the Moloka'i pigs, 38 (67%) were

scored IV and 7 (12%) a III, totaling 45/57 (79%) lesions graded III or IV. In contrast, 9/35 (26%) lesions achieved a score of III (1/35, 3%) or IV (8/35, 23%) for the Texas pigs.

Given identical exposures to bTB, the preventive fraction (PF) represents the reduction in risk that having Texas genetic background provides for having a gross or microscopic bTB lesion at that particular site. The analysis was ran with Texas pigs representing the control (or, having a Texas background helped to prevent disease) whereby a positive PF value closest to 1 means being a Texas-origin pig conferred a decreased chance of having a lesion/being diseased at that particular site. In this analysis, the assumption was made that being a Moloka'i pig was considered an "intervention" which potentially causes harm or an increased risk for bTB, and a negative PF indicated Texas pigs were more likely to be lesioned/affected. A PF of 0 indicated there was no difference in the occurrence of disease for either the Texas or Moloka'i groups. Out of 15 comparisons made between Moloka'i and Texas bTB lesions, 10 PFs were positive and only 3 negative, demonstrating that being a Texas pig reduced the risk for disease occurring at these sites at a 95% confidence interval (Table 4). This was statistically significant for the hepatic lymph node (PF 1, 95% CI -0.94, 1).

The lesion distribution and severity varied greatly between individual animals, with one animal each from both Moloka'i and Texas groups showing more severe, disseminated disease especially throughout the lungs. Five out of six inoculated Moloka'i pigs (5/5 with tuberculous disease) produced IFN-γ (>50,000 pg/mL) in response ECH versus none of the Texas pigs (Figure 14). Additionally, both Moloka'i and Texas bTB-infected groups each had one animal (FS #5 and #10, respectively) without evidence of gross or microscopic lesions, corresponding to the two animals lacking humoral and cell-mediated immune responses.

Pilot Study

Three out of the four bTB-infected Texas-origin feral swine produced antibodies against MPB83 evidenced by DPP at 32 and 120 dpi. Reflectance values were over 200 for these three pigs at both timepoints (Figure 18). All four pilot study pigs developed gross lesions in the mandibular lymph nodes and microscopic lesions in the tracheobronchial lymph nodes (data not shown). Three out of four (75%) had suspect bTB pulmonary granulomas while the other showed extrapulmonary disease within the mesenteric lymph node. Stimulated PBMCs from Texas pilot study pigs produced IFN-γ in response to ECH for 2/4 (50%) of pigs by 32 dpi and 100% of pigs by 120 dpi (Figure 19).

Discussion

U.S. feral swine were investigated as potential maintenance hosts for bTB through investigation of historical epidemiologic and diagnostic data and comparing genetically distinct populations of pigs in low-dose experimental infection with *M. bovis*. Antemortem longitudinal data and postmortem findings were documented and compared between Texas and Moloka'i captive-bred feral swine. Measurements of cell-mediated and humoral immune responses were recorded prior to infection and every 14 days through 182 dpi (approximately 6 months). Pooled oral fluid and fecal samples were analyzed by PCR and mycobacterial culture weekly for transmission potential. Differences in disease progression and immunity were compared between groups of pigs.

Serological responses in bTB-infected feral swine were inconsistent and noticeably different between Moloka'i and Texas feral swine. Five out of six (83%) infected Moloka'i pigs responded to at least one test line on DPP by 28-56 dpi. The only inoculated Moloka'i pig to not respond on DPP also lacked evidence of gross lesions and cell mediated immunity typical of bTB. For all non-validated species tested on DPP, including *Sus Scrofa*, any RLU >0 is

considered positive while a threshold RLU value is determined during the validation process for species more routinely tested on DPP, such as white tailed-deer and elephants (Greenwald et al., 2009; Lyashchenko et al., 2013). Cross reactivity on DPP from NTM exposure wad documented in these studies and may help explain false positive results observed in this study.

Pooled feces from the infected Texas group tested positive by qPCR at 1dpi, presumably from swallowed inoculum. Oral fluids collected from bTB-infected Texas pigs at 84 and 119 dpi were also positive by qPCR which may indicate intermittent bacterial shedding from the oropharynx or false positives considering mycobacterial culture was negative at all time points. AFB were isolated from oral fluid samples taken from the negative control room was determined to be NTM other than MTC. No gross lesions were present in mock-infected controls and no other atypical mycobacteria were cultured from tuberculous granulomas in any infected pigs, therefore, is presumed an environmental contaminant. Inadvertent exposure to environmental mycobacteria through feed/water sources cannot be completely eliminated even though study animals were farrowed, reared, and studied within BSL-2 and BSL-3 spaces only.

The discordance in IFN-γ production observed between the two genetic backgrounds in response to a non-specific mitogen (PWM) and mycobacterial antigens suggests immune responses causing either over-production by Moloka'i pigs or underproduction by Texas pigs. This could be due to inherent differences in lymphocyte subsets or downstream signaling pathways involved in cell mediated immunity. The cell mediated immune response to bTB infection in cattle can be measured by IFN-γ release assay which usually determines a positive result by subtracting the concentration of IFN-γ induced by PPDa from that of PPDb (Schiller et al., 2010; Schiller et al., 2009). Exposure to NTM in the environment and/or reactivity of cell wall constituents to various antigens present in PPD preparations can result in cross-reactivity on

mycobacterial diagnostic assays (Aranaz et al., 2004; Biet et al., 2005; De la Rua-Domenech et al., 2006; Gcebe et al., 2016; Schiller et al., 2010). For this reason, evaluating immunodominant antigens in different bTB-infected species and determining suitable diagnostic assays is essential for establishing screening tests to improve bTB wildlife surveillance.

A few disparities were observed between bTB immune responses for Texas-origin pigs from this study and those used for the pilot study. IFN-γ responses on PBMC restimulation were minimal for Texas pigs in the current study (Figure 14) yet fairly robust for pilot study pigs in response to ESAT-6 (Figure 19). Additionally, 3/4 (75%) Texas pigs from the pilot study mounted humoral immune responses against MPB83 on DPP by 32 dpi (Figure 18), compared with 2/4 (50%) bTB-infected Texas pigs from the current study, albeit, the latter showing much lower RLU values (Figure 10).

All Texas pigs from the current and pilot study originated from the same dam and sire, were inoculated with the same dose of *M. bovis* via pharyngeal lavage, and the same PBMC isolation and mycobacterial restimulation protocol was used. Differences between the two studies which may have impacted these observations include: different strains of *M. bovis* (50:50 mixture of the Moloka'i:Mexican cattle strain for pilot study vs. Michigan 95-1315 WTD strain for comparison study), the age of inoculation (6 months for pilot study, 9-10 months for current study), study length (4 months for pilot vs. 6 months for comparative study), and certain mycobacterial stimulants utilized for PBMC stimulation (pilot study did not include MPB83, MPB70, and ConA rather than PWM was used for a positive control).

The lesion severity and distribution was distinct between the Moloka'i and Texas feral swine. Out of 6 Moloka'i and 4 Texas pigs, 15 and 7 gross lesions were evident upon postmortem examination, respectively. Considerably more granulomas were identified

microscopically, with a total of 57 Moloka'i and 35 Texas histologic lesions present. The presence of grossly visible tuberculoid granulomas within the hepatic lymph nodes of 5/6 (83%) infected Moloka'i pigs was an unexpected finding. A preventive fraction calculated with Texas pigs representing the control determined this gross lesion to be statistically significant with a preventive fraction of 1 (Table 4). When grossly visible, the presence of a granuloma was a good indicator of bTB infected animals and correlated with the presence of AFB on histology in 94% of cases, with only one grade IV hepatic lymph node from an infected Moloka'i pig which did not show AFB on the initial tissue section.

For feral swine, screening for bTB solely based on presence/absence of gross lesions risks missing infected animals. Three out of 6 (50%) of infected Moloka'i pigs had only one gross lesion present (one each within a mandibular lymph node, the tracheobronchial, and the hepatic lymph node). The histologic lesions observed in the negative control pigs are not uncommon or unexpected and provide a baseline level of granulomatous lesions for pigs of similar genetic background and environmental exposures. Two animals, one from each genetic background, appear to have evaded development of bTB disease following infection and 6 months of cohabitation with other infected pen mates potentially shedding *M. bovis*.

Genome-wide association studies particularly benefit TB research given the complexities of the host-pathogen interactions involved. Identifying genes associated with beneficial immune traits or heritable resistance to disease provide insight into pathophysiology and avenues to overcome persistent disease threats, including bTB (Acevedo-Whitehouse et al., 2005; Amos & Acevedo-Whitehouse, 2009; Azad et al., 2012; Bothamley et al., 1989; V Naranjo et al., 2008; Schaible et al., 2003). Inbreeding causes runs of homozygosity within the genome and limits allelic diversity which can impact immune function and contribute to susceptibility/resistance to

various diseases. Increased susceptibility to infection and disseminated disease has been observed within inbred populations of cattle and feral swine infected with *M. bovis*, owing to inherent immune defects and altered pathophysiology (Azad et al., 2012; Stein, 2011). The captive breeding stock of Texas origin have been inbred for 3-4 generations compared with 1-2 generations for the Moloka'i breeding group. Ultimately, with some inbreeding in the pedigree, the individual effects (as expressed as heterozygosity) come down to the stochastic draw from 18 autosomal chromosomes inherited from the dam and the sire. For future directions, the genetic and transcriptional differences between these populations of pigs and how those difference manifest as individual immune responses and bTB disease susceptibility will be further explored using samples collected from the current study and ancestral breeding stock.

References

Acevedo-Whitehouse, K., Vicente, J., Gortazar, C., Höfle, U., Fernandez-de-Mera, I. G., & Amos, W. (2005). Genetic resistance to bovine tuberculosis in the Iberian wild boar. Molecular Ecology, 14(10), 3209-3217.

Amos, W., & Acevedo-Whitehouse, K. (2009). A new test for genotype–fitness associations reveals a single microsatellite allele that strongly predicts the nature of tuberculosis infections in wild boar. Molecular Ecology Resources, 9(4), 1102-1111.

Aranaz, A., De Juan, L., Montero, N., Sánchez, C., Galka, M., Delso, C., Alvarez, J., Romero, B., Bezos, J., & Vela, A. I. (2004). Bovine tuberculosis (*Mycobacterium bovis*) in wildlife in Spain. Journal of Clinical Microbiology, 42(6), 2602-2608.

Azad, A. K., Sadee, W., & Schlesinger, L. S. (2012). Innate immune gene polymorphisms in tuberculosis. Infection and Immunity, 80(10), 3343-3359.

Bailey, S. S., Crawshaw, T. R., Smith, N. H., & Palgrave, C. J. (2013). *Mycobacterium bovis* infection in domestic pigs in Great Britain. The Veterinary Journal, 198(2), 391-397.

Bevins, S. N., Pedersen, K., Lutman, M. W., Gidlewski, T., & Deliberto, T. J. (2014). Consequences associated with the recent range expansion of nonnative feral swine. BioScience, 64(4), 291-299.

Biet, F., Boschiroli, M., Thorel, M., & Guilloteau, L. L. (2005). Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). Veterinary Research, 36(3), 411-436.

Bothamley, G. H., Beck, J. S., Schreuder, G. M. T., D'Amaro, J., de Vries, R. R., Kardjito, T., & Ivanyi, J. (1989). Association of tuberculosis and M. tuberculosis-specific antibody levels with HLA. Journal of Infectious Diseases, 159(3), 549-555.

Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., & Kremer, K. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceedings of the National Academy of Sciences, 99(6), 3684-3689.

Brosch, R., Gordon, S. V., Pym, A., Eiglmeier, K., Garnier, T., & Cole, S. T. (2000). Comparative genomics of the mycobacteria. International journal of medical microbiology, 290(2), 143-152.

Brown, R. D., & Cooper, S. M. (2006). In my opinion: the nutritional, ecological, and ethical arguments against baiting and feeding white-tailed deer. Wildlife Society Bulletin, 34(2), 519-524.

Brown, V. R., Bowen, R. A., & Bosco-Lauth, A. M. (2018). Zoonotic pathogens from feral swine that pose a significant threat to public health. Transboundary and Emerging Diseases, 65(3), 649-659.

Campbell, T. A., Long, D. B., Bazan, L. R., Thomsen, B. V., Robbe-Austerman, S., Davey, R. B., Soliz, L. A., Swafford, S. R., & VerCauteren, K. C. (2011). Absence of *Mycobacterium bovis* in feral swine (Sus scrofa) from the southern Texas border region. Journal of Wildlife Diseases, 47(4), 974-978.

Campbell, T. A., Long, D. B., & Shriner, S. A. (2013). Wildlife contact rates at artificial feeding sites in Texas. Environmental management, 51(6), 1187-1193.

Coleman, J., & Cooke, M. (2001). *Mycobacterium bovis* infection in wildlife in New Zealand. Tuberculosis, 81(3), 191-202.

Coleman, J., Cooke, M., Jackson, R., & Webster, R. (1999). Temporal patterns in bovine tuberculosis in a brushtail possum population contiguous with infected cattle in the Ahaura Valley, Westland. New Zealand Veterinary Journal, 47(4), 119-124.

Corner, L. A. (2006). The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Veterinary Microbiology, 112(2-4), 303-312.

Cosivi, O., Meslin, F., Daborn, C., & Grange, J. (1995). Epidemiology of *Mycobacterium bovis* infection in animals and humans, with particular reference to Africa. Revue scientifique et technique (International Office of Epizootics), 14(3), 733-746.

De la Rua-Domenech, R., Goodchild, A., Vordermeier, H., Hewinson, R., Christiansen, K., & Clifton-Hadley, R. (2006). Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Research in veterinary science, 81(2), 190-210.

De Mendoza, J. H., Parra, A., Tato, A., Alonso, J., Rey, J., Peña, J., García-Sánchez, A., Larrasa, J., Teixidó, J., & Manzano, G. (2006). Bovine tuberculosis in wild boar (Sus scrofa), red deer (Cervus elaphus) and cattle (Bos taurus) in a Mediterranean ecosystem (1992–2004). Preventive Veterinary Medicine, 74(2-3), 239-247.

Delgado-Acevedo, J., DeYoung, R. W., & Campbell, T. A. (2007). Landscape genetics of feral swine and implications for management.

Delgado-Acevedo, J., Zamorano, A., DeYoung, R. W., Campbell, T. A., Hewitt, D. G., & Long, D. B. (2010). Promiscuous mating in feral pigs (Sus scrofa) from Texas, USA. Wildlife Research, 37(7), 539-546.

Essey, M., Payne, R., Himes, E., & Luchsinger, D. (1981). Bovine tuberculosis surveys of axis deer and feral swine on the Hawaiian island of Molokai. Proceedings of the United States Animal Health Association,

Essey, M., Stallknecht, D., Himes, E., & Harris, S. (1983). Follow-up survey of feral swine for *Mycobacterium bovis* infection on the Hawaiian island of Molokai. Proceedings of the United States Animal Health Association,

Gallagher, J., & Horwill, D. (1977). A selective oleic acid albumin agar medium for the cultivation of *Mycobacterium bovis*. Epidemiology & Infection, 79(1), 155-160.

Garnier, T., Eiglmeier, K., Camus, J.-C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., & Monsempe, C. (2003). The complete genome sequence of *Mycobacterium bovis*. Proceedings of the National Academy of Sciences, 100(13), 7877-7882.

Gcebe, N., Michel, A., Gey van Pittius, N. C., & Rutten, V. (2016). Comparative genomics and proteomic analysis of four non-tuberculous Mycobacterium species and *Mycobacterium tuberculosis* complex: occurrence of shared immunogenic proteins. Frontiers in microbiology, 7, 795.

Grange, J., & Collins, C. (1987). Bovine tubercle bacilli and disease in animals and man. Epidemiology & Infection, 99(2), 221-234.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Ball, R., Dumonceaux, G., Schmitt, D., & Moller, T. (2009). Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clinical and Vaccine Immunology, 16(5), 605-612.

Larsen, M. H., Biermann, K., & Jacobs Jr, W. R. (2007). Laboratory maintenance of *Mycobacterium tuberculosis*. Current protocols in microbiology, 6(1), 10A. 11.11-10A. 11.18.

Linderholm, A., Spencer, D., Battista, V., Frantz, L., Barnett, R., Fleischer, R. C., James, H. F., Duffy, D., Sparks, J. P., & Clements, D. R. (2016). A novel MC1R allele for black coat colour reveals the Polynesian ancestry and hybridization patterns of Hawaiian feral pigs. Royal Society Open Science, 3(9), 160304.

Lombard, J. E., Patton, E. A., Gibbons-Burgener, S. N., Klos, R. F., Tans-Kersten, J. L., Carlson, B. W., Keller, S. J., Pritschet, D. J., Rollo, S., & Dutcher, T. V. (2021). Human-to-Cattle *Mycobacterium tuberculosis* Complex Transmission in the United States. Frontiers in Veterinary Science, 8, 691192.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., O'Brien, D. J., Schmitt, S. M., Palmer, M. V., & Waters, W. R. (2013). Rapid detection of serum antibody by dual-path platform VetTB assay in white-tailed deer infected with *Mycobacterium bovis*. Clinical and Vaccine Immunology, 20(6), 907-911.

Maggioli, M. F., Palmer, M. V., Thacker, T. C., Vordermeier, H. M., McGill, J. L., Whelan, A. O., Larsen, M. H., Jacobs Jr, W. R., & Waters, W. R. (2016). Increased TNF-α/IFN-γ/IL-2 and decreased TNF-α/IFN-γ production by central memory T cells are associated with protective responses against bovine tuberculosis following BCG vaccination. Frontiers in Immunology, 7, 421.

Martín-Hernando, M. P., Höfle, U., Vicente, J., Ruiz-Fons, F., Vidal, D., Barral, M., Garrido, J. M., de la Fuente, J., & Gortazar, C. (2007). Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. Tuberculosis, 87(4), 360-367.

McCann, B. E., Smyser, T. J., Schmit, B. S., Newman, R. A., Piaggio, A. J., Malek, M. J., Swafford, S. R., Sweitzer, R. A., & Simmons, R. B. (2018). Molecular population structure for feral swine in the United States. The Journal of Wildlife Management, 82(4), 821-832.

McFadyean, J. (1900). Tubercle bacilli in cow's milk as a possible source of tuberculous disease in man. Public Health, 13, 814-825.

Meyer, R. (2000). Results of an Epidemiologic Investigation for*Mycobacterium bovis* in selected wildlife populations on the island of Molokai, Hawaii. USDA.

Miller, J., Jenny, A., Rhyan, J., Saari, D., & Suarez, D. (1997). Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. Journal of Veterinary Diagnostic Investigation, 9(3), 244-249.

Miller, J. M., Jenny, A. L., & Payeur, J. B. (2002). Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and Mycobacterium avium organisms in formalin-fixed tissues from culture-negative ruminants. Veterinary Microbiology, 87(1), 15-23.

Miller, R., & Sweeney, S. (2013). REVIEW ARTICLE *Mycobacterium bovis* (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations.

Monaghan, M., Doherty, M., Collins, J., Kazda, J., & Quinn, P. (1994). The tuberculin test. Veterinary Microbiology, 40(1-2), 111-124.

Naranjo, V., Acevedo-Whitehouse, K., Vicente, J., Gortázar, C., & De La Fuente, J. (2008). Influence of methylmalonyl-CoA mutase alleles on resistance to bovine tuberculosis in the European wild boar (Sus scrofa). Animal genetics, 39(3), 316-320.

Nol, P., Robbe-Austerman, S., Rhyan, J. C., McCollum, M. P., Triantis, J. M., Beltrán-Beck, B., & Salman, M. D. (2016). Determining the persistence of *Mycobacterium bovis* bacille Calmette–Guerin Danish in select tissues of orally vaccinated feral swine (Sus scrofa ssp.). Research in veterinary science, 104, 50-52.

Orloski, K., Robbe-Austerman, S., Stuber, T., Hench, B., & Schoenbaum, M. (2018). Whole genome sequencing of *Mycobacterium bovis* isolated from livestock in the United States, 1989–2018. Frontiers in Veterinary Science, 5, 253.

Palmer, M., Waters, W., & Thacker, T. (2007). Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with *Mycobacterium bovis*. Veterinary Pathology, 44(6), 863-874.

Parra, A., Fernández-Llario, P., Tato, A., Larrasa, J., Garcıa, A., Alonso, J., De Mendoza, M. H., & De Mendoza, J. H. (2003). Epidemiology of *Mycobacterium bovis* infections of pigs and wild boars using a molecular approach. Veterinary Microbiology, 97(1-2), 123-133.

Pedersen, K., Miller, R. S., Anderson, T. D., Pabilonia, K. L., Lewis, J. R., Mihalco, R. L., Gortázar, C., & Gidlewski, T. (2017). Limited antibody evidence of exposure to *Mycobacterium bovis* in feral swine (Sus scrofa) in the USA. Journal of Wildlife Diseases, 53(1), 30-36.

Phillips, C., Foster, C., Morris, P., & Teverson, R. (2003). The transmission of *Mycobacterium bovis* infection to cattle. Research in veterinary science, 74(1), 1-15.

Phillips, C. J., Foster, C. R., Morris, P. A., & Teverson, R. (2002). Genetic and management factors that influence the susceptibility of cattle to *Mycobacterium bovis* infection. Animal Health Research Reviews, 3(1), 3-13.

Portacci, K., Lombard, J., Abrahamsen, L., Bush, E., Fossler, C., Harris, R., Johnson, K., Miller, R. S., Mitchell, D., & Pritchard, R. (2011). Assessment of Pathways for the Introduction and Spread of *Mycobacterium bovis* in the United States.

Rangaka, M. X., Diwakar, L., Seldon, R., van Cutsem, G., Meintjes, G. A., Morroni, C., Mouton, P., Shey, M. S., Maartens, G., & Wilkinson, K. A. (2007). Clinical, immunological, and epidemiological importance of antituberculosis T cell responses in HIV-infected Africans. Clinical infectious diseases, 44(12), 1639-1646.

Rothel, J., Jones, S., Corner, L., Cox, J., & Wood, P. (1990). A sandwich enzyme immunoassay for bovine interferon-γ and its use for the detection of tuberculosis in cattle. Australian Veterinary Journal, 67(4), 134-137.

Schaible, U. E., Winau, F., Sieling, P. A., Fischer, K., Collins, H. L., Hagens, K., Modlin, R. L., Brinkmann, V., & Kaufmann, S. H. (2003). Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nature medicine, 9(8), 1039-1046.

Schiller, I., Oesch, B., Vordermeier, H., Palmer, M., Harris, B., Orloski, K., Buddle, B., Thacker, T., Lyashchenko, K., & Waters, W. (2010). Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transboundary and Emerging Diseases, 57(4), 205-220.

Schiller, I., Waters, W. R., Vordermeier, H. M., Nonnecke, B., Welsh, M., Keck, N., Whelan, A., Sigafoose, T., Stamm, C., & Palmer, M. (2009). Optimization of a whole-blood gamma interferon assay for detection of *Mycobacterium bovis*-infected cattle. Clinical and Vaccine Immunology, 16(8), 1196-1202.

Schmitt, S. M., Fitzgerald, S. D., Cooley, T. M., Bruning-Fann, C. S., Sullivan, L., Berry, D., Carlson, T., Minnis, R. B., Payeur, J. B., & Sikarskie, J. (1997). Bovine tuberculosis in free-ranging white-tailed deer from Michigan. Journal of Wildlife Diseases, 33(4), 749-758.

Serraino, A., Marchetti, G., Sanguinetti, V., Rossi, M. C., Zanoni, R. G., Catozzi, L., Bandera, A., Dini, W., Mignone, W., & Franzetti, F. (1999). Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques. Journal of Clinical Microbiology, 37(9), 2766-2771.

Sheehan, D. C., & Hrapchak, B. B. (1973). Theory and practice of histotechnology.

Smyser, T. J., Tabak, M. A., Slootmaker, C., Robeson, M. S., Miller, R. S., Bosse, M., Megens, H. J., Groenen, M. A., Paiva, S. R., & de Faria, D. A. (2020). Mixed ancestry from wild and domestic lineages contributes to the rapid expansion of invasive feral swine. Molecular Ecology, 29(6), 1103-1119.

Spickler, A. (2019). Zoonotic Tuberculosis in Mammals, including Bovine and Caprine Tuberculosis. Retrieved January 1, 2022, from https://www.cfsph.iastate.edu/Factsheets/pdfs/bovine_tuberculosis.pdf

Stein, C. M. (2011). Genetic epidemiology of tuberculosis susceptibility: impact of study design. PLoS pathogens, 7(1), e1001189.

Thacker, T. C., Harris, B., Palmer, M. V., & Waters, W. R. (2011). Improved specificity for detection of *Mycobacterium bovis* in fresh tissues using IS6110 real-time PCR. BMC Veterinary Research, 7(1), 1-5.

Thacker, T. C., Palmer, M. V., & Waters, W. R. (2006). Correlation of cytokine gene expression with pathology in white-tailed deer (Odocoileus virginianus) infected with *Mycobacterium bovis*. Clinical and Vaccine Immunology, 13(6), 640-647.

Tsao, K., Robbe-Austerman, S., Miller, R. S., Portacci, K., Grear, D. A., & Webb, C. (2014). Sources of bovine tuberculosis in the United States. Infection, Genetics and Evolution, 28, 137-143.

USDA. (March 09, 2023). History of Feral Swine in the Americas. USDA Wildlife Services. https://www.aphis.usda.gov/aphis/ourfocus/wildlifedamage/operational-activities/feral-swine/sa-fs-history

Vicente, J., Höfle, U., Garrido, J., Fernández-De-Mera, I. G., Juste, R., Barral, M., & Gortazar, C. (2006). Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. Veterinary Research, 37(1), 107-119.

Volkman, H. E., Clay, H., Beery, D., Chang, J. C. W., Sherman, D. R., & Ramakrishnan, L. (2004). Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS biology, 2(11), e367.

Vordermeier, H., Whelan, A., Cockle, P., Farrant, L., Palmer, N., & Hewinson, R. (2001). Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clinical Diagnostic Laboratory Immunology, 8(3), 571-578.

Waddington, K. (2004). To stamp out "so terrible a malady": Bovine tuberculosis and tuberculin testing in Britain, 1890–1939. Medical history, 48(1), 29-48.

Waters, W., Whelan, A., Lyashchenko, K., Greenwald, R., Palmer, M., Harris, B., Hewinson, R., & Vordermeier, H. (2010). Immune responses in cattle inoculated with *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium kansasii*. Clinical and Vaccine Immunology, 17(2), 247-252.

Wyckoff, A. C., Henke, S. E., Campbell, T. A., Hewitt, D. G., & VerCauteren, K. C. (2009). Feral swine contact with domestic swine: a serologic survey and assessment of potential for disease transmission. Journal of Wildlife Diseases, 45(2), 422-429.

CHAPTER 4. GENERAL CONCLUSION

Parallel testing of fixed and fresh tissues using ancillary (i.e. histopathology) and confirmatory (PCR on FFPE tissues and culture) diagnostic tests for bTB allowed for correct identification of positive cases in cattle and feral swine and aided in the investigation of discrepant results. Culture and FFPE PCR were useful in both cattle and feral swine submissions though, FFPE detected more positive cases in field-collected feral swine samples. Causes of false negatives include missing the lesion in the tissues used for histopathology or culture, having few or absent bacilli within the lesion tested, or, occasionally, variations in the *M. bovis* genome affecting target regions for molecular diagnostics. Serial sectioning tissues being submitted for diagnostics, rather than halving, might be a more sensitive method for finding lesions and ensuring the presence of bacilli. Since tissues are generally halved before being placed in either formalin or sodium borate and prior to their arrival at NVSL, sectioning would have to be performed on fresh tissues by the submitter. This may prove more challenging for submitters and subtle lesions may go unnoticed. A recent clinical isolate of *M. bovis* from a cow was consistently positive on qPCR from fresh tissue but negative on FFPE PCR from crude and purified extracts (NVSL unpublished findings). WGS of the culture isolate revealed a deletion affecting IS6110, causing FFPE PCR primers to fail but not those used for qPCR, IS1081 (Collins & Stephens, 1991; Selim et al., 2014).

Experimental infection of Moloka'i and Texas feral swine reconfirmed the susceptibility of both to bTB yet revealed noteworthy differences in the resulting pathology and immunology. Cell-mediated and humoral immunity was mounted primarily by the Moloka'i pigs with tuberculous granulomas but not in exposed animals lacking observable lesions. Antibodies were produced against MPB83 and/or ECH as early as 28 dpi in 3-5 Moloka'i pigs, in contrast with

MPB83 response only in 1-2 Texas pigs at 4 timepoints. Cross-reactivity between PPDa and PPDb was evidenced by IFN-γ production by stimulated PBMCs from both infected groups. False positive results for the Moloka'i negative control on DPP was recorded a single time for MPB83 antibody production and at 4 timepoints for ECH. This may imply that a higher threshold for RLU needs to be investigated through validation studies for determination of a positive result on DPP for bTB in U.S. feral swine. Exposure to environmental mycobacteria cannot be completely excluded as an explanation as both antigens are produced by a handful of NTM that could be present within food and/or water sources, even within animal biocontainment. However, results of mycobacterial culture from infected study animals did not yield any culture isolates other than *M. bovis*, making unintentional exposure less likely. Pooled oral fluid samples collected from the negative control room at two time points cultured an AFB most consistent with NTM. No grossly apparent lesions that would suggest mycobacteriosis were discovered. Multiple tonsillar microabscesses/microgranulomas were present in 7 out of 12 study animals overall and suspected to be a normal background finding.

Initial pathogen recognition and subsequent priming of Th1 adaptive immunity within the host contribute to mycobacterial clearance and disease control. By using an experimental infection and controlling for certain pathogen and environmental factors, our results suggest that host genetics influence the immune responses and cytokine signaling pathways involved in bTB disease susceptibility for feral swine. Collaboration with the USDA National Wildlife Research Center feral swine genomics group is underway to pursue a multi-omics approach and identify additional host and pathogen factors contributing to host status for bTB. RNA sequencing on viable tissue collected adjacent to tuberculoid granulomas would provide valuable insight into differential gene expression between the two groups of pigs at the local level. Specifically,

differences impacting immune cell signaling, which was observed systemically through peripheral sampling for serology and restimulation of PBMCs, may also be appreciable within the lesion.

A total of 7 Moloka'i and 5 Texas pigs were used for the current experimental infection study and these animals originated from an already inbred captive population of "feral" swine. At least two and three generations of inbreeding are estimated for the Moloka'i and Texas group lineages, respectively. Genomic data on the subset of study pigs will reveal the rates of heterozygosity and characterization of runs of homozygosity. This additional genomic information, when compared with their respective larger populations, will inform about frequency and timing of inbreeding within Texas and Hawaiian pig lineages. Population bottlenecks and loss of allelic diversity are documented circumstances influencing TB disease susceptibility in humans, particularly involving the human leukocyte antigen (HLA) and susceptibility to pulmonary TB (Oliveira-Cortez et al., 2016). This effect could be pronounced for Moloka'i feral swine given restricted animal movements on and off the island and a small overall geographic area limiting range expansion.

A metered host immune response, mounted according to antigenic load and long-term capability of the interferon response, ultimately determines clearance or persistence of *M. bovis*. The maintenance or amplification of disease within a particular population depends on complex interplay involving all aspects of the host, pathogen, and environment and is difficult to predict. Epidemiological evidence confirming successful intra- and inter-species transmission is documented in maintenance hosts for bTB.

Undeniably, members of the Suidae family are susceptible to bTB and capable of transmitting disease within their population in certain circumstances. After the bTB-positive cow

was confirmed in 1997 and the following surveillance study identified feral swine as a potential source of disease, the cattle on Moloka'i remained bTB-negative on annual regulatory testing up until 2021 when a cow originating from the eastern part of Moloka'i reacted on the caudal fold test and was later confirmed bTB-positive by NVSL. WGS of the *M. bovis* isolate from the infected cow determined it originated from the endemic strain present within feral swine, nearly matching the archived genotype from 2008 (unpublished findings). Historical epidemiological investigations from Moloka'i combined with the molecular evidence from the most recent cattle isolate all suggest that feral swine may be acting as a maintenance host for bTB within this unique geographical niche.

Feral swine should not be disregarded as a host for bTB particularly when ranges overlap with domestic cattle or another maintenance host species. Hunting feral swine for sport has become increasingly popular and bounty programs commissioned by regulatory wildlife officials in areas of the U.S. with high population densities of this invasive and poorly-regarded pest species have been attempted to control disease spread. Unfortunately, purposeful and illegal relocations of feral swine and processing of meat for personal consumption resulted, which exacerbated regulatory officials' concerns (Bevins et al., 2014). Careful evaluation of tissues at slaughter for gross lesions has limitations in field applications with an uncontrolled environment. Cases of early disease with only microscopic lesions or atypical lesion location/distribution (i.e. extrapulmonary) may also result in missing bTB granulomas, even for highly trained abattoir workers, veterinary inspectors, and pathologists.

The historical information and inherent differences observed between the two groups of pigs used for this study support the hypothesis that Moloka'i feral swine are acting as a maintenance host for bTB and contributing to disease spillback events into livestock. Similar

investigations of naturally-infected feral swine and implementation of a “multi-omics” approach will better characterize naturally-occurring bTB for complete analysis of host and pathogen factors (Comas & Gagneux, 2009).

References

Bevins, S. N., Pedersen, K., Lutman, M. W., Gidlewski, T., & Deliberto, T. J. (2014). Consequences associated with the recent range expansion of nonnative feral swine. BioScience, 64(4), 291-299.

Collins, D. M., & Stephens, D. M. (1991). Identification of an insertion sequence, IS1081, in *Mycobacterium bovis*. FEMS microbiology letters, 83(1), 11-15.

Comas, I., & Gagneux, S. (2009). The past and future of tuberculosis research. PLoS pathogens, 5(10), e1000600.

Oliveira-Cortez, A., Melo, A., Chaves, V., Condino-Neto, A., & Camargos, P. (2016). Do HLA class II genes protect against pulmonary tuberculosis? A systematic review and meta-analysis. European Journal of Clinical Microbiology & Infectious Diseases, 35(10), 1567-1580.

Selim, A., El-Haig, M., & Gaede, W. (2014). Duplex real-time PCR assay targeting insertion elements IS1081 and IS6110 for detection of *Mycobacterium bovis* in lymph nodes of cattle. Biotechnology in Animal Husbandry, 30(1), 45-59.