DISSERTATION

EVALUATION OF FERAL SWINE AS POTENTIAL RESERVOIRS, SENTINELS, AND VECTORS OF EMERGING AND RE-EMERGING ZOONOTIC PATHOGENS IN THE UNITED STATES

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2023

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ABSTRACT

EVALUATION OF FERAL SWINE AS POTENTIAL RESERVOIRS, SENTINELS, AND VECTORS OF EMERGING AND RE-EMERGING ZOONOTIC PATHOGENS IN THE UNITED STATES

Feral swine are an extremely adaptable and prolific invasive species present in many regions of the United States. A generalist diet, high reproductive capacity, and opportunistic nature make them a significant threat to native flora and fauna, and their destructive foraging behaviors have been attributed to substantial crop loss and property damage throughout their range. Feral swine have also been demonstrated as vectors and reservoirs for many diseases, some of which are transmissible to humans. Despite the efforts of government agencies to impede range expansion of feral swine and monitor populations for disease, human-mediated movements as well as continued natural dispersal in some regions have made management difficult. Subsequently, this invasive species continues to threaten human and animal health throughout its current range.

Reported herein are a series of laboratory and field-based investigations that seek to address the potential role(s) of feral swine in the epidemiology of a few zoonotic pathogens that are emerging or re-emerging in the United States. As pigs interact closely with soils through their natural rooting and wallowing behaviors, we were primarily interested in soil-dwelling zoonoses, and identified three of particular interest. The two bacterial species *Bacillus anthracis* and *Burkholderia pseudomallei*, as well as the fungus *Coccidioides*, are the causative agents of anthrax, melioidosis, and coccidioidomycosis respectively, and each is understudied as they

relate to feral swine. While each of these organisms is unique in their biology and the relative threat(s) they've posed to humans and animals throughout their respective histories, they are similar in that much of their ecology remains undescribed. The often-complex ecological relationships exhibited by pathogenic organisms are vital to their continued survival and evolution and can play an important role in their dissemination to human populations. The presence of high-density populations of feral swine in many regions of the United States, as well as their ability to occupy a range of habitats, may be playing a significant role in the persistence and dissemination of these organisms. Furthermore, the interactions of feral swine with contaminated substrates within their environment, as well as high levels of interspecies interactions throughout their current range could make them a likely source of additional emerging or novel pathogens. After a summary of the literature concerning feral swine in the United States, followed by that for the causative agents of anthrax, coccidioidomycosis, melioidosis, and a few select emerging disease threats, each respective chapter within this work details investigations largely by pathogen.

Within Chapter 2, we present the results of a field serosurvey in feral swine residing across regions of known anthrax endemicity in Texas, and retrospectively document bacterial exposure via enzyme-linked immunosorbent assay (ELISA). This study was performed principally to evaluate the biosentinel utility of feral swine for anthrax contaminated environments, and the ELISA utilized was developed and optimized for detecting antibodies of swine origin to *B. anthracis* protective antigen. We additionally report on a laboratory-based experimental infection study where which a group of juvenile feral swine were either intranasally or subcutaneously exposed to varying amounts of *B. anthracis* strain Sterne 34F2 spores, and seroconversion as well as bacterial shedding through the nasal passages documented over time.

Through both the field-based serosurvey, as well as the experimental inoculation study, we report that feral swine serology may be used as a management strategy to indirectly identify regions contaminated with anthrax bacteria. Moreover, we report that some feral swine intranasally exposed to high amounts of *B. anthracis* Sterne contained detectable and viable spores within their nasal passages up to 56 days past their initial exposure event as demonstrated by bacterial culture. The presence of viable *B. anthracis* within the nasal mucosa well after an exposure event suggests that feral swine may be capable of spreading infectious anthrax bacteria as mechanical vectors.

Chapter 3 of this work details an experimental infection study similar to that reported in the previous chapter, however, instead intranasally exposes a group of juvenile feral swine to *Coccidioides posadasii* strain Silveira spores. This study was performed to evaluate the pathogenesis and immune response of feral swine to one of the causative agents of coccidioidomycosis. Fungal culture of tissues collected from inoculated individuals at necropsy was performed to assess whether animals might act as reservoirs for the fungus in addition to describing the characteristics of infection. Feral swine utilized for this study did not display overt clinical signs of disease, and despite being inoculated with a large dose of fungal spores, also did not seroconvert based on the results of porcine agar gel immunodiffusion assays. Fungal culture of lung and mediastinal lymph node tissues of a subset of pigs revealed the presence of viable *C. posadasii*, however, examination of additional sections of the same tissues did not reveal the presence of the granulomatous lesions often associated with coccidioidomycosis. Most individuals had significant comorbidities as illustrated via histology, most notably *Metastrongylus* nematodes in the lungs. Despite an absence of lesions and organisms histologically, isolation of the fungus from tissues by culture indicated active infection and imply

that feral swine are mildly susceptible to acute *Coccidioides* infection. These results suggest that in some instances, feral swine may aid in fungal dispersal on the landscape due to the presence of spherules in tissues post-mortem.

Chapter 4, concerning melioidosis, reports on laboratory-based experiments evaluating an indirect ELISA for measuring antibodies to *B. pseudomallei* in domestic and wild swine. Described previously to test human sera, this ELISA utilized whole-cell lysate derived from *B. pseudomallei* strain Bp82 and was optimized and preliminarily validated by testing sera from domestic goats and domestic swine experimentally inoculated with virulent and avirulent strains of *B. pseudomallei*, respectively. Further evaluation of assay specificity was performed by testing sera against similar antigen preparations of the closely related bacterial species *Pseudomonas aeruginosa* strain PAO1. Examination of sera from laboratory animals against Bp82 and PAO1 whole-cell lysate antigens revealed that most animals seroconverted and displayed *B. pseudomallei* antibodies, and that little cross-reactivity existed between antigens. After preliminary assay validation, serum from wild pig populations originating from Arizona, California, and Puerto Rico were assessed using both antigens to document *B. pseudomallei* exposure in wild pigs residing in regions where bacterial absence or endemicity can be confidently inferred at this time. In stark contrast to the serology displayed by laboratory animals, analysis of field sera collected from these regions demonstrated high levels of cross-reactivity between Bp82 and PAO1 antigens, suggesting the assay is not suitable for use in wild pig populations.

Lastly, Chapter 5 describes a general survey of feral swine removed from the Aransas National Wildlife Refuge in Texas for each of the organisms investigated in the proceeding

chapters, as well as additional bacterial and viral pathogens; added pathogens that were included within this survey included *Francisella tularensis*, and SARS-CoV-2, the causative agents of tularemia and COVID-19, respectively. A suite of samples, including external parasites (e.g., ticks), nasal swabs, blood, and a series of tissues were collected from each feral swine in the field and analyzed by bacterial and fungal culture, serology, and viral assays to determine if any pigs were actively infected or previously exposed to a range of pathogens. General and selective culture of tissue samples did not reveal active infection with *B. anthracis*, *Coccidioides spp*., *B. pseudomallei*, or other pathogenic bacteria, however, serology illustrated that a subset of pigs were previously exposed to *B. anthracis*. Further examination of tissues histologically illustrated a high degree of parasitic infection in the majority of pigs, particularly with *Metastrongylus* nematodes. Finally, three ixodid tick species, all adults, were collected off of the majority of feral swine sampled, none of which appeared to be carrying infectious virus based on the results of general cytopathic effects assays. Results generated for this study further confirm that feral swine are hosts for a range of ixodid ticks present in Texas and include species that are known to pose a risk to the health of livestock, wildlife, and humans. Moreover, serological results indicating previous exposure to anthrax-causing bacteria agree with past ecological modeling studies that have suggested environmental suitability for *B. anthracis* in the region of Aransas National Wildlife Refuge.

Taken together, these investigations demonstrate that feral swine in the United States very likely are contributing to the ecology of anthrax and coccidioidomycosis by being mechanical vectors and possible reservoirs of *B. anthracis* and *Coccidioides*, respectively. Serological data demonstrated from field and laboratory studies additionally support the use of feral swine as biosentinels within landscapes undergoing invasive species management and that may be

contaminated with *B. anthracis*. While the serological data generated through the *B. pseudomallei* investigations reported here currently do not support use of a crude whole-cell lysate-based ELISA to evaluate samples collected from the field, we hypothesize that feral swine may be reservoirs and/or vectors of melioidosis-causing bacteria. Future investigations should continue to examine additional antigens for serologic testing, as laboratory-infected swine demonstrated a measurable antibody response after intranasal exposure with Bp82. Additional follow-up studies should also be conducted to further describe the relative role(s) that feral swine may play in the ecology and epidemiology of *B. anthracis* and *Coccidioides*, particularly if regions with high incidence of animal or human disease correspond with high densities and activity of feral swine. Future research should continue to screen feral swine as well as the ectoparasites that feed upon them for emerging or re-emerging pathogens, especially in areas with high biodiversity or species of conservation concern, as well as in areas with high levels of human activity.

ACKNOWLEDGEMENTS

The research presented herein would not have been possible without the support and guidance from numerous people. I would first like to thank Dr. Richard Bowen and Dr. Angela Bosco-Lauth for giving me the opportunity to work in their laboratory. You both took me in as a student with very little experience or background in the realm of zoonotic disease research, gave me a myriad of opportunities to learn and grow under your supervision, and also trusted me to design experiments and explore some of my own research interests. I'm incredibly lucky to have been mentored by such giants in the field of infectious disease, and to have experienced such a supportive space for learning and growth.

I would next like to thank each of my committee members, Dr. Bradley Borlee, Dr. Sushan Han, and Dr. Vienna Brown, for their continued support and sound scientific advice whenever I've needed it, as well as their unique contributions to the work presented here. Dr. Borlee, thank you for your patience with me as I continue to try to understand bacteria and all their complexity, and for being a guide for many of my laboratory methods. Dr. Han, thank you for taking the time to provide your histological expertise, particularly for reading the (many) slides from our laboratory animals, as well as your continued support despite a major job change; it's been a pleasure to continue working with you. I would lastly like to give a special thank you to Dr. Brown, who is largely responsible for me taking the leap to go to graduate school in the first place and is the reason why any of these projects had funding. Vienna, thank you for helping to create this extraordinary opportunity for me; I am forever indebted to you.

I would also like to thank everyone in the Bowen/Bosco-Lauth lab, past and present, for their assistance, advice, and support during my graduate career. Not only have I gained a great

network of professional colleagues by working with each of you, but also a second family. The guidance, knowledge, passion for science and scientific debate, and endless supply of humor each of you give both professionally and personally every day make coming to work a true gift.

I would next like to thank all the collaborations that made this work possible. First, to Michael Bodenchuk, and everyone with USDA-APHIS-Wildlife Services and the Feral Swine Damage Management Program at the National Wildlife Research Center. None of this research would have been possible without the funding provided through the Feral Swine Program, nor the collaboration of Michael Bodenchuk and countless agency personnel in Texas who helped us to acquire laboratory animals, and the numerous field samples necessary for this work. Thank you, Courtney Pierce with the National Wildlife Research Center, for your assistance with all the statistical modeling performed for our anthrax field study, and for your patience with me, a very non-quantitative individual. Thank you, Dr. Michael Norris, at the University of Florida, for your guidance with ELISA troubleshooting, and anthrax expertise. I would like to thank all the faculty and staff at the University of Arizona Valley Fever Center for Excellence, for their support and guidance regarding all things *Coccidioides* and diagnostic troubleshooting. Lastly, thank you Dr. Helle Bielefeldt-Ohmann at the University of Queensland, for your enthusiastic support and assistance in reading and interpreting the histology slides for the tissues we collected in the field.

I would finally like to thank all my friends and family. To my parents, thank you for always supporting my goals, no matter how big or small. To my Aunt Laurie and Uncle Jeff, who from an early age helped nurture a love for the outdoors and animal health, thank you for giving me much needed support; without you I likely would not have made it through my undergraduate career, let alone graduate school. And, finally, my dear Kathryn, thank you for talking me down

the many mountains I've made from molehills, and for your boundless love and support; I truly could not have done any of this without you.

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CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

INTRODUTION: FERAL SWINE IN THE UNITED STATES

Feral Swine: Taxonomy and Definitions

Feral swine (*Sus scrofa*) are a harmful and destructive invasive species in the United States (U.S.). True wild pigs in the family Suidae (class Mammalia; order Artiodactyla) are not indigenous to North America[1,2], and instead were historically found only in the Palearctic, Oriental, and Ethiopian realms based on fossil evidence[3]. Their range expansion into the Nearctic realm is believed to be solely due to human-mediated movements, which include both intentional and accidental introduction events[1,3].

Since their primary introduction into North America, wild pigs have and continue to exhibit a wide range of morphological and physical diversity, which can be explained by the varying taxonomy of their ancestors[1]. Genetic evidence has suggested that the Eurasian wild boar (*S. scrofa*) is the wild ancestor of both ancient and modern breeds of domestic swine throughout the world[4-10], and members of either lineage, domestic or wild, are considered conspecifics[1]. Taxonomy of higher resolution for North American feral swine, however, is complicated by additional introductions of subspecies of *S. scrofa*, of which there are 15 currently recognized to have originated in the Old World[1,3,4]. Additionally, multiple introductions and releases of domesticated pig breeds onto the same landscapes have allowed for repeated hybridization of the two lineages[11]. The hybridization between wild boar lineages with various domestic swine breeds have in some cases caused conflicts regarding species definition and subsequent management by state wildlife agencies[1]. Notwithstanding the genetic and taxonomic complexity of invasive

swine in North America, and particularly, the U.S., the majority of systematic zoologists refer to Eurasian wild boar, domestic swine, and hybridizations of the two as *S. scrofa*[1]. Furthermore, as currently most populations of feral swine present throughout the U.S. have varying ancestral ties to Eurasian wild boar and their subspecies, as well as domesticated swine breeds, use of any particular subspecies designation is considered taxonomically invalid; therefore, all pigs, domestic or wild, are most accurately classified as *S. scrofa*[1,3,12].

Within this work, the term "feral swine" will be used to refer collectively to free-ranging and wild pigs of the family Suidae within the U.S. and its territories. This term also includes escaped domestic pigs and their descendants, as well as Eurasian wild boar, and hybrids between the two lineages. Herein, the terms "feral swine" "feral pigs" or "wild pigs" will also be used interchangeably to refer to these animals.

Establishment in the United States

Wild pigs are found on most continents around the world, and have become invasive in most of their range, including in North America and in the U.S[13]. As alluded to above, the establishment of swine on the North American continent can be exclusively attributed to introductions by man, with subsequent natural dispersal and additional anthropogenic introduction events allowing for significant range expansion[3,14-16].

The earliest introduction of swine to what is now the U.S. is thought to have occurred between 750 and 1000 A.D. during the settlement of Hawaii, when pigs accompanied human settlers from Southeast Asia[3,14]. Later, during his exploration of the New World in 1493, Christopher Columbus is thought to have brought the first European domestic swine to the West Indies, while the Spanish explorer Hernando de Soto is credited with introducing the species to

the continental U.S. with his arrival on the Florida coast in 1539[15]. It was often common practice for colonists of the New World to bring plants, and various livestock with them when establishing colonies to ensure an adequate food supply reminiscent of their home country; domestic swine were additionally often brought because of the low maintenance care they required, as they are generalist omnivores capable of feeding on an extensive range of materials[3,17]. Indeed, explorers would oftentimes release the pigs they brought onto the landscape to forage and feed on available materials, as well as breed and disperse so that additional colonists arriving would also have an adequate food supply[3,18]. Just two years after their second expedition to the New World and release of the first batch of domestic swine, one Spanish colonist observed that the pigs "reproduced in a superlative manner", and in just a few more years, the number of pigs running wild was described as "*infinitos*", while "all the mountains swarmed with them" according two separate accounts[18]. Populations of feral pigs present in what is now Mexico and the Caribbean constitute the oldest populations of wild pigs on the North American continent and continue to occupy at least 11 Mexican states as well as numerous Caribbean and Pacific islands[1,19-22].

Over 300 years later, in the early 1890s, Eurasian wild boar were brought to North America for use in fenced hunting preserves[16]. Shortly thereafter, in the 1900s, other introductions were made onto both fenced and unfenced landscapes in the Southeastern U.S. for additional hunting opportunities[3,16]. Until the 1980s, populations were primarily found in the southern tier of U.S. states as well as some states on the west coast; in 1982, the species was believed to occupy only a small percentage of counties in 17 states[16,23,24]. The 1980s also marked a time when the first wild boar populations were introduced to Canada[25]. By 2016, that number would increase to 35 states within the continental U.S[26,27], and wild boar would also come to

occupy at least six Canadian provinces[1,21]. Within the U.S. today, feral swine populations are recognized in the majority of U.S. states and territories, and numbers across the entire country are approximated in the millions[24,28].

Ecology

Wild pigs are a generalist species that exhibit substantial behavioral plasticity and can readily and quickly adjust their life history strategies in response to their environment[1]. As such, the ecology of wild pigs in the U.S. depends largely upon the region or ecosystem where which they occur; depending on the landscape individuals will often differ in their resource use and feeding habits seasonally as well[29-33]. More generally, past documents of feral swine feeding habits throughout their current known range have shown that individuals are herbivorous most of the time, but will also forage for soil-dwelling invertebrates, and opportunistically prey upon other vertebrate taxa[17,34-38].

During times of herbivory, feral pigs will consume a variety of grasses as well as soft and hard masts (e.g., roots, tubers and fruits); depending on the region and population dynamics, their herbivory may be in conflict with native fauna and result in substantial resource competition[11,39-47]. When available, animals will preferentially select for cultivated crops, and in some cases, this can lead to significant economic losses to agricultural production[42,48]. While foraging for underground plant and animal matter, as well as during exploration of their environment, pigs will engage in rather unique rooting and trampling behaviors, during which the soil is upturned with the hoofs and snout and materials located with olfaction, a rather sensitive sense for the species[1,49]. Rooting may vary in relative intensity and range from superficial sifting of leaf-litter to excavating large areas of substrate[1].

Although plant matter is thought to take up a relatively large proportion of wild pig diets, opportunistic consumption of other vertebrates is particularly common throughout the U.S., and a significant threat to native wildlife populations[1]. As predators, feral swine have been reported to consume the eggs and chicks of ground nesting birds (*Galliform sp*.), eggs and young of various turtle (order Testudines) and American alligator (*Alligator mississippiensis*) nests, white-tailed deer fawns (*Odocoileus virginianus*), numerous species of adult reptiles and amphibians, as well as juvenile domestic livestock[17,34,37,38,50-54]. Predation of herpetofauna, particularly in the southeastern U.S., has been observed to correlate with the breeding season when animals are most vulnerable; the effects of depredation in herptile populations is often difficult to quantify, but may be particularly significant for threatened and endangered species within this taxonomic group[37,50,51]. Similarly, predation of eggs and chicks of ground nesting birds can be equally as detrimental to population sizes[55].

Socially, feral swine, like their wild boar ancestors, are a highly gregarious species, and exhibit a matrilineal group structure often consisting of multiple generations of females and their offspring[1,49]. Sub-adult and adult male pigs are often solitary, however may on occasion form "bachelor groups"[1,49,57,58]. Similarly, yearling females will sometimes leave their original sounder to form "sister groups" to pursue reproductive opportunities, effectively forming new sounders[1,49,58]. Home ranges of sounders as well as solitary males varies considerably and is believed to depend on a suite of abiotic and biotic factors present within a particular system; seasonality, and sounder size are also thought to play a role in home range size[1]. Estimates for sounders of varying sizes have ranged between 1.1 and 43 km^2, while individual male home range estimates range between 1.4 and 58.7 km^2; generally home ranges of males are 3.5-5 km^2 larger than females in the same area[1,59]. Feral swine have a high reproductive capacity, as

females are capable of breeding within their first year of life and are capable of producing more than one litter per year; fetal counts can range from 1-12 and mean live fetuses per sow is five[1,60].

Throughout most of their range, feral swine are considered to be nocturnal, however populations existing in remote areas far away from human settlements and activities exhibit increased activity during the day[1,46,61-63]. Increased diurnal activity may also be observed in some regions where temperatures are high, or during the summer months, as pigs will wallow before moving to shade in the evening[49]. Wallowing is a common behavior in wild and domestic pigs wherein individuals will cover the body in mud to reduce body temperature as well as to protect the skin from harmful insects and ectoparasites[64]. Pig wallows are typically shallow depressions on the ground, often muddy or filled with water from nearby streams or standing water sites[1,65], and are either naturally occurring, or created from pig rooting and trampling activities[1].

While the large ecological range of feral swine make it difficult to generalize some of the ecology of the species in the U.S., their opportunistic nature, generalist feeding habits, and distinct rooting and wallowing behaviors have earned them the classification of "ecosystem engineers" by many[34,66,67]. Opportunistic and active predation of various vertebrate and invertebrate species can negatively affect populations of native wildlife and may be attributed to the decline of populations such that they subsequently are listed as rare, threatened, endangered, or of special concern[34]. Indirect competition through overlapping resource use also can have deleterious effects on native wildlife species; dietary overlap particularly has been documented between feral swine and a number of species[15,40,46,68-73]. Furthermore, feral swine have also been known to scavenge, and therefore may compete with other mammalian and avian scavengers for

carrion[74]. Finally, disturbances of soil profiles and foraging of underground plant matter and invertebrates through rooting and trampling, as well as regular wallowing can alter overall nutrient cycling, as well as invertebrate and plant species composition that subsequently may have effects at the ecosystem level[1,13,67,72,75,76].

Pathogen Transmission and Disease Surveillance Efforts

It has been recently estimated that feral swine are capable of carrying at least 30 viral and bacterial diseases as well as nearly 40 parasites that may also affect humans, domestic animals, and various wildlife, and have been reviewed extensively elsewhere[1,24,77,78]. Most information on wild pig diseases within the U.S., however, originates from surveys done looking for select pathogens, most of which have been studied based on concerns to humans and domestic livestock[1]. Interestingly, wild pigs are generally assumed to be susceptible to the same pathogens as domestic swine, however few of these appear to cause clinical disease in wild pigs[1]. Understanding disease dynamics in feral swine populations, particularly if they are capable of serving as reservoirs, is therefore important for identifying transmission potentials between feral swine and various wildlife, livestock, and humans.

Regular, nationwide disease surveillance conducted by the U.S. Department of Agriculture's (USDA) National Feral Swine Program (NFSP) monitors feral swine populations for the occurrence of select pathogens, primarily those that pose a threat to the nation's domestic commercial swine herds. The NFSP was formally established in 2014 after Congressional funds were appropriated to the USDA for the purposes of managing the damages caused by feral swine, including monitoring populations for disease, however, the USDA began collecting feral swine samples for surveillance purposes starting in 2004[24,79]. In addition to monitoring populations for

select diseases, the NFSP provides national-level support and resources for the removal and where possible, elimination of feral swine populations, as well as management for the physical damage inflicted by the species; the program often works in collaboration with local, state, and tribal agencies to achieve these objectives[24].

Pathogens regularly surveyed for in by the NFSP include pseudorabies virus and swine brucellosis, both of which are endemic in feral swine but have been eliminated from the nation's commercial domestic herds; reintroduction of either of these diseases into the commercial sphere remains a constant threat to U.S. pork production[34]. Historically however, USDA has also monitored populations for prevalence of porcine reproductive and respiratory syndrome, avian influenza, and leptospirosis[24,80-82], also mostly due to concerns for domestic livestock health. The NFSP does, however, provide flexible regional or local support for the surveillance or identification of additional pathogens of concern as they arise[24]. The agency has also dedicated significant resources to monitoring feral swine for select foreign animal diseases predicted to have devastating consequences should they be introduced to the U.S., and include both African and classical swine fever viruses, as well as foot-and-mouth disease[83].

Current Status and Future Concerns

Despite the ongoing management by local, state, and government agencies to reduce feral swine numbers and the subsequent damages caused by this prolific invasive mammal, human mediated movement and natural dispersion continue to expand the range of feral swine in some regions of the U.S[84,85]. Moreover, the list of currently known pathogens carried, transmitted, or vectored by feral swine is continuously evolving, particularly for emerging or reemerging pathogens[86-88]. Continued research is therefore imperative to identify the risks this invasive

species may pose to native wildlife and ecosystems, as well as domestic animals and humans. The NFSP, in conjunction with other governmental authorities throughout APHIS recently shifted towards a new national sampling approach for feral swine surveillance, such that foreign animal diseases such as African and classical swine fever and foot-and-mouth disease are prioritized[79,89]. This shift in priority was primarily made in response to the rapid global spread of African swine fever within the last few years and to allow for rapid containment and preservation of the country's domestic swine herds should a foreign animal disease be introduced into the country[79]. Despite this change, the production diseases pseudorabies virus and swine brucellosis that are endemic in feral swine populations continue to be monitored by the agency at this time[79]. Furthermore, the NFSP continues to collaborate with a range of institutions and government agencies to conduct targeted surveillance at the local or regional level as well as research concerning additional pathogens to allow for flexibility and rapid response to emerging threats[24,79,89].

While feral swine have been identified as components of the epidemiology of numerous pathogens to date, many more have not been studied relative to this invasive species. Of particular interest herein are the three soil-dwelling organisms *Bacillus anthracis*, *Coccidioides spp*., and *Burkholderia pseudomallei*, the causative agents of anthrax, coccidioidomycosis, and melioidosis, respectively. Climate change, globalization, and increasing interactions along the human-wildlife interface continue to threaten human and animal health through the movement and emergence of known and novel pathogens. The remainder of this literature review will focus primarily on the three focal diseases of interest stated above, and detail their respective history, ecology, known epidemiology, as well as knowledge gaps and research opportunities as they

relate to swine and particularly, feral swine. One final section will briefly identify, and review select emerging disease threats as they may relate to feral swine.

ANTHRAX

Discovery and Historical Perspectives

Anthrax is a disease of multiple phenotypes caused by the gram-positive, spore-forming bacterium *Bacillus anthracis*. Considered an ancient illness, there have been many speculations as to when the organism was first described, however most scholars agree that descriptions of disease resembling that of anthrax can be found as early as the years 1000 through 19 B.C.E[90,91]. One of the earliest possible depictions of anthrax may be found in the Bible's Book of Exodus describing the fifth and sixth plagues of Egypt; as such, it has subsequently been suggested that the disease may have originated in Egypt and Mesopotamia[92]. Recent genetic analyses, however, have since proposed the pathogen originated in Sub-Saharan Africa[93,94]. Otherwise, notable writings containing anthrax-like descriptions have been observed in the works of Homer (1000 B.C.E.), Hippocrates (400 B.C.E.), Virgil (70-19 B.C.E.), and Galen (200 A.D.)[90-92]. In fact, Hippocrates, the infamous Greek physician, was the first to coin the term "anthrax" to describe a disease characterized by black-colored skin lesions and blood (e.g., from the Greek word "anthracites" meaning "coal")[95]. Notable epidemics throughout ancient history that have been attributed to anthrax include the Plague of Athens (430-427 B.C.E.), as well as the "Black Bane" of Europe in 1613[90,91,96]; some academics have even ventured that outbreaks of the disease may have contributed to the fall of Rome in 476 A.D[92]. The disease proved a rather substantial illness effecting people and animals through the Middle Ages and the Victorian era; one epidemic in 18th century Europe was estimated to destroy half of the country's sheep, and what is now recognized

as inhalational anthrax, termed “woolsorters’ disease” in Victorian England was a common occupational affliction in mill workers[90,97].

More formal clinical descriptions of anthrax weren’t made until 1752 and 1769 by the French scientists Maret and Fournier, both respectively describing the cutaneous form of the disease[92,98]. Further advances into the biology of the bacterium were followed by Pierre Rayer and Casimir-Joseph Davaine in 1850 when they described small, rod-shaped bodies “about twice the length of a blood corpuscle” in blood samples from an anthrax-infected sheep[90,99]. As they consistently found these organisms in animals exhibiting the disease, Davaine went on to hypothesize in 1863 that the bacilli were the etiologic agents of anthrax and was the first to demonstrate that the disease was transmissible through infected blood[90,91,99]. Davaine’s hypothesis, however, would not be proven definitively until years later when Robert Koch studied anthrax bacilli and used them as a basis to form his Postulates in 1877, proving that *B. anthracis* was the causative agent of anthrax[92,100]. Koch would further document the complete life cycle of the bacteria, observing that under stressful conditions the organism formed spores that appeared resistant to degradation and were capable of surviving for long periods of time in adverse environments[101,102]. The development of Koch’s Postulates have since been considered groundbreaking in the field of microbiology and continues to be used and further adapted as the basis for defining the etiology and transmission of infectious disease[103,104].

Louis Pasteur however, now known for his groundbreaking work on the principles of vaccination, did not believe that Koch’s work was sufficient in explaining the transmission of disease, and sought to further prove the causation of anthrax using vaccination[96]. Years prior while studying the bacterium that causes chicken cholera (now known as *Pasteurella*), Pasteur

observed that laboratory cultures can lose their pathogenicity but still retain "attenuated" pathogenic characteristics over the course of many generations[105]. In 1879, when he began to study *B. anthracis*, he determined that repeated culturing of the organism at suboptimal temperatures (e.g., between 42-43°C) for 15-20 days resulted in attenuation, and subsequently began developing the first anthrax vaccine[105-107]. Shortly thereafter, in 1881, Pasteur conducted a study where which he inoculated a group of cattle with two doses of his vaccine given 12 days apart, each dose containing the attenuated organisms that he grew in his laboratory[90,108]. Cattle were subsequently injected with a culture of fully virulent anthrax; all the vaccinated animals survived, while non-vaccinated control animals died, further proving that *B. anthracis* was the agent causing anthrax[96,108].

Over the next 50 years, Pasteur's anthrax vaccine would become widely used in cattle and sheep throughout Europe and South America and reduced the incidence of disease in livestock significantly[95,106,109,110]. However, with time and further study into the biology of *B. anthracis*, the Pasteur vaccine was observed to have some limitations, namely declines in potency over time and regular use, that resulted in high mortality risks and losses of animals that were vaccinated to the very disease they were being immunized against[106,110]. Improvements to the vaccine were introduced by Max Sterne in the late 1930s and early 1940s after he isolated a stable variant of *B. anthracis* that was naturally avirulent[111]. Use of live spores of Sterne's strain as a vaccination gave reproducible results and decreased the overall risk of livestock mortality such that its use surpassed that of the initial vaccine developed by Pasteur worldwide[106,112]. The Sterne strain of *B. anthracis* continues to be used today as the basis for livestock vaccination[95,110], and the exact mechanisms of virulence utilized by Pasteur and Sterne weren't exposed until the 1980s, nearly a century after administration of the first anthrax vaccines[95].

These mechanisms, uncovered by the efforts of many, are based on the presence of two virulence plasmids harbored by *B. anthracis*, one of which can be cured from the organism after sequential passage at around 42°C, the same temperature used by Pasteur during his experiments[95,113]. Similarly, loss of the second plasmid resulted in reduced virulence and was missing in Sterne's isolates[114,115]. Both plasmids have since been fully sequenced, and termed pXO1 and pXO2, respectively[116]. The pXO1 plasmid, responsible for the bacterium's ability to produce lethal and edema factor toxins, is likely the plasmid that was attenuated in Pasteur's *B. anthracis* strains, while the Sterne strain has been demonstrated as lacking pXO2, the capsule-encoding plasmid ultimately responsible for protecting the bacterium against recognition by host immune cells[117]. It has since been further demonstrated that the presence of toxin components, particularly the protein termed "protective antigen" responsible for the downstream assembly of lethal and edema factor toxins, is the primary target of the host immune system against anthrax bacteria[118]. The efficacy of Sterne's vaccine can therefore now be explained due to the Sterne strain lacking the capsule plasmid and retaining the toxin-encoding plasmid, which contains the materials necessary for susceptible animals to mount an adequate humoral response against *B. anthracis*[119].

Mass use of Sterne's livestock vaccine, combined with improvements in the handling and production of animal products and proper disposal of anthrax-contaminated carcasses, resulted in a worldwide decline in the incidence of disease in humans and domestic animals throughout the 20th century[92,100]. However, several instances of deliberate use of the pathogen by humans as biological weapons continued to prompt the development of a human vaccine, as well as treatments to combat serious disease into the 1970s[92]. Utilizing the techniques developed initially by Robert Koch to grow and produce large stocks of microorganisms, the German army is said to have secretly infected livestock and animal feed with anthrax that were eventually traded to

Allied Nations during World War I[92]. Years later, during and after World War II, Japan, Great Britain, and the U.S. all experimented with using anthrax as a biological weapon; this continued until the signing of a treaty in 1972 banning the possession, use, and development of biological and toxic weapons[92,120]. Despite over 100 nations signing the treaty[92], a deadly outbreak of anthrax in Sverdlovsk, USSR near a Soviet military facility where which over 60 people died, caused worldwide speculation as to whether the outbreak was in fact caused by activities banned by the treaty[90,92]. Further investigation of the outbreak revealed that the source of infection was indeed the military facility, which mistakenly released aerosolized anthrax spores into the surrounding town[90,121]. Most recently, in 2001 in the U.S., 22 people became ill and five people died after highly concentrated powdered material containing anthrax spores were placed in envelopes and mailed to various government officials and members of prominent media outlets[90,122,123].

Despite the ever-present threat of anthrax being utilized as a weapon, the disease has decreased in apparent prevalence drastically throughout history with the advent of the livestock vaccine, improvements in carcass disposal, and safe handling and sanitation of animal products. Particularly in developed countries, anthrax appears to only be a sporadic disease, however, likely continues to be an underappreciated disease for susceptible wildlife that aren't as closely monitored for clinical signs[95,124]. In developing countries, anthrax continues to be a major problem in both people and animals[95,125-127], indicating that further work must be done to combat the disease.

Ecology and Epidemiology

Bacillus anthracis is an endospore-forming obligate pathogen that can be found in soils worldwide[110,128,129]. Ecologically, the dormant spore form of the bacteria is essential to the cycling and persistence of the pathogen in the environment, however vegetative forms of the bacteria can also play a significant role in disease transmission in some instances[110]. In nature, anthrax spores residing in soil profiles or in standing water are typically ingested by susceptible species such as grazing or browsing herbivores; in some cases, animals may also be exposed by inhaling spore-laden dust, or through cutaneous inoculation into skin lesions[110,129]. Once inside a susceptible host, bacteria transform into the vegetative or active form, and pathogenesis is established with the presence of toxins and capsule, two virulence factors encoded on the bacterium's pXO1 and pXO2 plasmids, respectively[113-115,117]. Lethal factor (LF) and edema factor (EF) proteins encoded on the toxin plasmid enter host cells through the mediation of the cell receptor-binding protein protective antigen (PA) encoded on the toxin plasmid, which also enables the bacteria to produce LF and EF toxins and ultimately cause host death[117,130,131]. Lethal factor toxin primarily targets cardiomyocytes and smooth muscle cells, where disruption of normal cellular pathways results in widespread necrosis and hypoxia due to apoptosis[130,132-134]. Edema factor toxin, in comparison, primarily targets hepatocytes and allows for widespread edema and hemorrhaging to occur throughout the body[130,135,136]. Upon host death, vegetative bacilli exposed to atmospheric oxygen sporulate and contaminate the surrounding environment, where they may either stay localized or be further distributed with the assistance of arthropod or vertebrate vectors, or through a suite of abiotic factors before infection of another suitable host takes place[129,137,138]. In some instances, carnivorous species that prey on infected animals or scavenge on the carcasses of animals that have died of anthrax may be exposed to vegetative *B.*

anthracis in addition to sporulated cells[110]. While vegetative forms of the bacteria are not known to survive the gastrointestinal tract of many carnivores, cutaneous and inhalational inoculation with vegetative cells may also result in infection, although little is known about the relative infectivity of vegetative cells compared to spores in these cases[129,137]. Once sporulated, *B. anthracis* is highly resistant to environmental degradation and can survive upwards of 100 years in appropriate conditions[139,140].

While sporulation and subsequent environmental contamination can be a rather localized process where which the highest risk of anthrax transmission is present at carcass sites[141], avian scavengers and hemophagic arthropods in some regions can increase the distance of bacterial spread significantly and thereby intensify the risk of transmission in additional environments[110]. Biting flies in some enzootic zones have been strongly suspected as the cause of extensive outbreaks[142], as they can carry *B. anthracis* from carcass sites either on body parts or within the gut after feeding on infected blood, and subsequently deposit the organism onto surrounding vegetation or browse[110,137,142-145]. Bacteria have additionally been recovered from ticks collected from endemic zones as well as those directly feeding off moribund animals[146-148], and experimental evidence of *Aedes* mosquitos suggest they too may be capable of transmitting infectious *B. anthracis*[149]. Finally, dried blood and viscera adhering to the fur or feathers of scavengers feeding on infected carcasses, or carriage of sporulated cells within the digestive tract can lead to increased spread of infectious *B. anthracis*; vultures (Cathartidae), herring gulls (*Larus argentatus*), and ravens (*Corvus corax*) in particular have been implicated in past field investigations[110,150-152]. How significant a role other taxa may play in the environmental cycling of anthrax, or overall transmission potential, remain understudied.

Anthrax bacteria are capable of surviving in a range of environments, although different genetic lineages or strains have been found to be associated with specific geographic regions, and subsequently have been linked to distinct ecological requirements[153-155]. More generally however, cases have been described on nearly every continent, and although the overall incidence of disease is unknown in most countries, it is largely assumed that the bacteria reside in most regions, with only limited areas considered truly endemic[110,128,129]. Global projections, when available, have estimated that between 20,000 and 100,000 human cases of anthrax occur annually, mostly in poor or rural areas of undeveloped countries[156]. Enzootics are generally described in warmer climates, and outbreaks appear to be more common in areas characterized by alkaline soils rich in calcium and organic content[110,129,157]. Outbreaks also appear to exhibit a seasonality, wherein cases typically occur in dry summer months following prolonged periods of heavy spring rains[110,157], however, this is not always the case. In some instances, the long-lived nature of sporulated *B. anthracis* in the environment can facilitate the re-emergence of disease after years or decades without a single reported case[137,158].

Geographically, regions where anthrax cases are frequent in livestock and humans include much of Africa, Asia, and the Middle East, as well as in parts of Central and South America[129,156,158]. Anthrax is also reported in Europe, and North America, however, is relatively infrequent compared to the number of reports observed in poorer countries; this likely having to do with the level of control and management on domestic and wild animals often present in developed nations[129]. In the continental U.S., case reports in domestic and wild herbivores have suggested western Texas and southwestern Montana to be highly endemic for anthrax, although cases outside these areas sporadically occur[159-161]. Despite worldwide mandatory reporting rules and the data currently available for regions classified as endemic, the disease is globally

underreported in wildlife and livestock[126,162]. Even in developed countries such as the U.S. where anthrax is not generally perceived as a significant threat, political and social ramifications in some regions likely are significant contributors to the underreporting of anthrax die-offs in wildlife and livestock[137]. Otherwise, underreporting of anthrax occurrence in wildlife is probably also due to a lack of detection, as wild populations are often not monitored for disease as closely as domestics[142,162]. Finally, recent ecological niche modeling efforts in the U.S. alone have identified additional landscapes capable of supporting *B. anthracis*[155], suggesting that disease risk is likely present outside of historically defined endemic areas. The poor characterization of bacterial distribution as well as outstanding questions regarding the transmission dynamics and overall ecology of anthrax on the landscape arguably make anthrax a neglected disease of concern to human and animal health.

Human Infection, Pathogenesis, and Transmission

Clinical presentations of anthrax in humans and the subsequent risk of fatality depend largely on the route of exposure, to which there are currently four described. Most common is the cutaneous form of anthrax, where an individual is exposed to *B. anthracis* spores through an abrasion in the skin, most often taking place through handling contaminated animals or their products[110,163]. Cutaneous anthrax accounts for 95% of human cases worldwide and presents a relatively low risk of mortality[164]. This form is quite often self-limiting and characterized by the presence of a typically painless black lesion referred to as an "eschar" at the site of inoculation[110,157,165]. Eschars will typically begin as a pruritic papule between 2-6 days after exposure, which then develops to surround vesicles, which ulcerate and form a necrotic center[157,166]. Regional lymphadenopathy and low-grade fever may also occur with this form[157]. If

left untreated, approximately 20% of cases will progress to septicemia and become fatal[171]. Next common is the enteric or gastrointestinal form of anthrax, which most often occurs after consumption of materials from infected animals; this form is mostly reported in rural regions of underdeveloped countries and accounts for the remainder of human cases reported worldwide[110,157,167]. Like the cutaneous form, symptoms usually appear 1-6 days after exposure, however, begin as non-specific and include nausea, vomiting, diarrhea, fever, and headache[167]. Should the disease progress, symptoms of acute hemorrhagic diarrhea, nausea, vomiting, abdominal pain, and ascites may arise and be followed by septicemia and death[167]. Without treatment, over 50% of cases of gastrointestinal disease are fatal[168]. Next, respiratory or inhalational anthrax may be acquired from spore inhalation, and is the least common mode of exposure, accounting for a negligible proportion of human cases reported worldwide[158]. Despite being the least common form of disease, inhalational anthrax is the most severe of all those described and is 100% fatal if left untreated[164]. The incubation period for this form ranges from 4-11 days, and subsequent symptoms are non-specific and include fever, sweats, fatigue, nausea or vomiting, and dyspnea[169]; pleural effusion and mediastinal widening are usually observed upon radiological examination of the chest[157,169]. After a variable period ranging from a few hours to days, patients will often progress quickly to sepsis and death unless prompt and aggressive treatment is administered[157,169]. Given the non-specific symptoms at the beginning of disease progression, an accurate diagnosis of anthrax is often not made in time to initiate the proper treatment[166,169]. Lastly, cases of anthrax in drug users after the use of contaminated needles have resulted in the classification of a fourth form of disease, referred to as injection anthrax[168,169]. Symptoms of this form are like that of cutaneous anthrax, however, injection anthrax is likely to carry a higher risk of septicemia, given that the infection begins deep under

the skin where the injection occurred[168]. As numerous other bacterial infections may cause injection site infections in these cases, proper diagnosis and treatment may be difficult to attain before a patient experiences sepsis[168]. Regardless of exposure route, secondary meningitis can occur in all described forms of disease and is usually fatal[164,166].

Bacterial culture of blood, skin lesions, spinal fluid, and/or respiratory secretions is considered the gold standard for anthrax diagnosis, although testing for antibodies or toxins in the blood may also be utilized[168]. Upon proper diagnosis, several options are available for treating the disease, and include a variety of antibiotics and antitoxin[168]. The bacterium is susceptible to most antibiotics, however the compounds most widely used are either penicillin G or amoxicillin; in cases where a person is allergic to penicillin, doxycycline or ciprofloxacin can be used as alternatives[131,157]. Antibiotic treatments are administered intravenously or orally for patients with inhalational and cutaneous anthrax, respectively; gastrointestinal anthrax is also treated with intravenous antibiotics[131]. In patients with severe edema, intravenous hydrocortisone may be administered if there is a threat of the airway being obstructed[157]. In cases of systemic anthrax, the CDC recommends antibiotics in conjunction with antitoxin to treat patients; currently there are three FDA-approved antitoxin products for use, all being either monoclonal or polyclonal IgG that work by binding PA and prevent the downstream assembly of LF and EF toxins[170]. Treatment for uncomplicated forms of disease or those that are diagnosed quickly typically last three to seven days, while more severe disease is treated for 10 to 14 days with extensive follow-up[157]; in the case of a bioterrorism attack, where the inoculating dose is likely higher than that occurring from a natural exposure event, 60 days of treatment is recommended[171].

A vaccine is available for humans, however, is not recommended for use in the general population, and is instead only administered to military personnel or laboratory workers who may be occupationally exposed[171]. Like the vaccine used in livestock, the U.S. vaccine, Anthrax Vaccine Adsorbed, elicits a humoral response against PA and contains materials originating from an avirulent strain of *B. anthracis* lacking pXO1[171]. Otherwise, in cases when exposure to anthrax is highly suspected but symptoms have not yet presented, prophylactic treatment with antibiotics may be considered, however should only be administered for a maximum of seven days[157]. Finally, there is no evidence of person-to-person transmission of anthrax, therefore it is largely accepted that the disease is transmitted through contact with contaminated animals or animal products. Eschars, however, should be covered and standard precautions taken in regard to handling blood and other body secretions[110,157].

Non-Human Host Range

Anthrax is a multispecies disease capable of infecting most homoeothermic animals, yet relative susceptibility varies greatly between taxa. In general, herbivorous species, particularly ruminants and hindgut-fermenters, are most susceptible to infection and will often succumb to disease shortly after being exposed[157]. In contrast, carnivorous and omnivorous mammals, as well as scavenging species are more resistant to infection[110,137]. The LD_{50} values for non-human animals may range from < 10 spores in herbivorous species to over 10^7 spores in more resistant taxa[110]. Of note, however, most of these published LD_{50} values have been determined by parenteral laboratory inoculation, and likely do not represent the levels of infectious material or inoculation route animals may be exposed to naturally. More natural routes of exposure such as inhalational or gastrointestinal have been estimated to be much higher (e.g., in the order of tens

of thousands), even for species regarded as highly susceptible[142]. This disparity in LD_{50} values between routes of inoculation has been repeatedly demonstrated in susceptible laboratory species, including guinea pig, mouse, and rabbit, and may have to do with the non-invasive nature of *B. anthracis*, meaning it must gain access to subepidermal tissue before infection can occur[110,126,142]. As access to subepidermal tissue requires crossing the epithelium barrier, it has been suggested that more cells are necessary to evade the myriad of immune barriers present and gain access to the more vulnerable tissues below[110]. More definitive environmental evidence is also not readily available for livestock, as well as many other ruminant species[142], however James Keepie, in a letter to Max Sterne in 1971 stated that the LD_{100} value for sheep infected parenterally is between 75 and 225 spores[110].

In most ruminant species, as well as hindgut fermenters like those in the family Equidae, anthrax is an acute disease almost always resulting in death[110,137]. Generally, after an average incubation period of 3-5 days, animals will exhibit staggering gait and recumbency; in some instances, fever, excitation or lethargy, dyspnea, and septicemia will also occur, however in most cases convulsions followed by sudden death are the result[110,128,157,172,173]. Once symptoms begin, death is acute and often occurs within 48 hours[174]. Herbivorous animals that have died of anthrax will appear to be in otherwise good body condition, and carcasses will usually exhibit bloating, severe rigidity in the limbs, and bloody discharge from natural orifices[110,157]. Bloody discharge that is observed commonly is dark and does not clot[157]. In stark comparison, naturally occurring anthrax in higher trophic level mammals, particularly carnivores and scavengers, is relatively rare[110]; anecdotally reported exceptions to this trend however include mink (*Neovison vison*) and cheetah (*Acinonyx jubatus*), which appear more susceptible than other carnivores[142]. Despite their relative resistance to developing clinical disease, there have occasional reports of symptoms

in a range of captive and free-ranging carnivores, and mortality events are possible[110,137]. Carnivores, as well as some omnivorous species like suids will usually exhibit subacute to chronic forms of disease, in contrast to the acute and often severe forms displayed by herbivores. Clinical signs in these more resistant species often include oedematous swellings in the face, neck, and ventral parts of the body, and infection may either remain localized or progress to septicemia and subsequent death[137]. Symptoms in these cases typically extend for more than three days before either recovery or death, and animals that recover frequently exhibit measurable antibodies against the bacterium[124,175,176].

Rodents, mainly guinea pigs (*Cavia porcellus*), mice (*Mus spp.*), rats (*Rattus spp*.) and rabbits (*Oryctolagus spp.)* are often used for vaccine testing as well as to model human disease in research, and vary in their relative susceptibilities, both by route of inoculation and by the bacterial strain they're exposed to[164,166]. Despite the abundance of data on these commonly used laboratory species, the incidence of anthrax in wild rodents has rarely been investigated[173]. One large experimental infection study investigated the susceptibility of a number of wild rodents to intracutaneous inoculation with several different doses of virulent *B. anthracis* spores and found varying levels of susceptibility[177]. These results suggest that more work may be required to evaluate the incidence and role that wild rodents may play in anthrax epidemiology.

There are some reports of epizootics and individual cases of anthrax occurring in avian species within the literature, most of which contain little to no details or references[110,173,178]. Given an absence of evidence of avian anthrax cases and die-offs, most references to birds in general have therefore suggested that they too are resistant to disease[110,173]. One apparent exception to this have been ostriches (*Struthio spp*.), as cases have been reported in both farmed

and wild animals repeatedly[179-182]. It has broadly been hypothesized that the reason many avian species are resistant to infection may be their relatively high body temperatures, which often exceed 40°C, and consequently exceed optimal temperature for *B. anthracis* growth[110,173,183,184]. That ostriches appear to be an exception may therefore be explained by their relatively lower body temperatures compared to most other birds[110,173,180].

Like avian species purportedly being resistant to anthrax due to their high body temperatures, reptiles and amphibians have also been referred to as resistant taxa, however their resistance may be attributed to their relatively low body temperatures compared to mammals[110,173]. While no natural infections have been described, anthrax has been experimentally induced in some reptile and amphibian species after their body temperature was raised[173,178]. Similarly, fish are also believed to be resistant, as no natural cases have been described for these animals[173]. However, some have speculated that carnivorous fish consuming contaminated meat may be capable of carrying the bacteria for short periods of time and may, under the right conditions, transmit the pathogen to humans[110].

Anthrax and Swine

Like carnivores and other omnivorous species, suids have been described as rather resistant to anthrax[110,126]. Herd outbreaks may occasionally occur, however are no longer reported with relative frequency; outbreaks occurred at relatively high rates in the 1950s, but have since been attributed to postwar husbandry practices, particularly feeding pigs food waste that inadvertently contained bones and meat from contaminated anthrax carcasses[110]. The earliest published LD_{50} values for pigs exposed via parenteral and inhalational routes via laboratory inoculation are 10^9 and 2.7×10^7 spores, respectively[126]; further experimental data are scarce.

Regarding gastrointestinal exposure, one large study conducted by Redmond and colleagues in 1997 exposed 50 domestic pigs to 10^7-10^{10} *B. anthracis* Ames spores in feed and observed only two deaths attributed to the disease[185]. The remainder of individuals in Redmond's study were observed out to 21 days post exposure and exhibited only mild and transient clinical signs[185], further demonstrating that pigs are highly resistant to fatal anthrax.

Clinical signs in pigs, when observed, most often include local edema and swelling in the neck, face, and/or ventral parts of the body[110,172,186,187]. In cases of pharyngeal or gastrointestinal anthrax where animals have been exposed to significant bacterial loads, lethargy, shivering, constipation and diarrhea may be seen[185]. Absence of blood clotting and rigor mortis, as well as splenomegaly are the most substantial necropsy findings[172,186], and in more severe cases, anthrax may be misdiagnosed in swine as acute classical swine fever, African swine fever, or malignant pharyngeal edema[188]. Otherwise, chronic infection has also been reported in swine[189,190]. In one notable report in a U.S. slaughterhouse, meat inspectors occasionally observed anthrax lesions in various organs of otherwise healthy pigs brought to slaughter approximately nine months after a documented anthrax epidemic in the same region[110,191]. It has therefore been reasoned that chronic infection is more common in pigs than the more serious acute or fatal forms of the disease[142,189]. Lastly, it has been hypothesized by some that pigs may exhibit a "carrier state" where which individuals harbor the organism and be capable of transmitting infection, however, will not display overt symptoms of disease[110,192,193]. Minimal reports of a carrier state exist for pigs[192], and research further investigating this aspect of anthrax disease and transmission potential is limited, as a carrier state has not been largely demonstrated for most species impacted[110].

Finally, while there has been little necessity for serologically examining animals for anthrax exposure historically, due to most of the animals effected and of concern to humans being livestock that either died or had the disease as evidenced by herd history[110], there is increasing research examining seroprevalence in anthrax resistant species, as well as various wildlife residing in regions with recurring enzootics[124,175,179]. The presence of antibodies specific to *B. anthracis* in local animals may be useful in identifying areas where the bacterium is persisting, or in new regions where it may have since spread and could become an emerging or re-emerging disease[124]. One study that examined the serology of wild boar in the Ukraine and mapped results to known livestock operations found evidence of exposure occurring in animals up to 35 km away from livestock operations with reported anthrax[194]. These results suggest either that animals were exposed to the disease at or around livestock facilities and migrated elsewhere, or that the environmental range of anthrax is larger than was originally thought for the region[194]. Given that Ukrainian wild boar are a widespread and abundant species throughout the country, investigators of this work have suggested that they likely could serve as biosentinels for anthrax contamination and risk. Despite similar population sizes and dynamics of wild or feral swine in the U.S., research investigating their sentinel utility and contribution to bacterial spread is lacking.

COCCIDIOIDOMYCOSIS

Discovery and Historical Perspectives

Coccidioides immitis and Coccidioides posadasii are dimorphic fungi and the etiologic agents of coccidioidomycosis, also known as San Joaquin Valley fever or, more commonly, "valley fever". Belonging to the phylum Ascomycota within the order Onygenales, *Coccidioides*

spp. are saprotrophic, although unlike many other members of Onygenales with close associations with plants, are also capable of degrading keratin, and may subsequently cause skin disease in humans and animals[195-197]. Discovered in 1893 in Buenos Aires by Alejandro Posadas[198], the agent was initially believed to be a protozoan, as skin biopsy specimens from a local patient displayed *Coccidia*-like organisms[199,200]. The patient in question was a 36-year-old male that presented with a large purple, fungal-like mass distributed across much of the right cheek, several ulcerative growths on the nose as well as one on the arm "resembling a cauliflower", and numerous papules throughout the extremities and trunk[199]. An Argentinian soldier, the patient was said to have experienced these dermatological problems for a total of nine years, seven of which included symptoms of recurrent fever and progressive cutaneous lesions[201]. One year following Posadas's initial case report, two additional patients, this time in California in the United States, presented with similar lesions at two San Fransisco hospitals[202-204]. In one case, the patient (a 40-year-old male immigrant from Portugal and a manual laborer in San Joaquin Valley, California) died after three years of progressive symptoms, and subsequent autopsy revealed several nodules within the lungs, adrenals, lymph nodes, liver, peritoneum, prostate, spleen, and testes[199]. Further microscopy of these nodules again revealed the presence of *Coccidia*-like protozoans[202]. Regarding the second case, a farmer, also an immigrant from Portugal, presented with coccidioidal granulomas on the face; however, in contrast with the first patient, had a more rapid progression of symptoms, and died just a few weeks after being admitted to the hospital[202]. The organism was subsequently named by Emmet Rixford and T. Casper Gilchrist primarily for its morphologic similarity to *Coccidia* (e.g, *Coccidioides*, meaning "resembling *Coccidia*")[202]. Due to additional differences in the clinical observations of both Californian patients, the organism isolated from the first patient was designated as *Coccidioides*

pyogenes (*pyogenes* meaning, "pus-producing"), while that from the second patient *Coccidioides immitis*, (*immitis* meaning, "not mild")[205].

Correct identification of *Coccidioides* as a fungus did not take place until four years later by William Ophüls and Herbert Moffitt, who demonstrated that infected tissue cultures consistently yielded molds, and that the organism was dimorphic, changing from the filamentous growth form of the mold into the "protozoan-like" form in animal tissues[206,207]. Injection of molds into the ear veins of rabbits further resulted in the same "typical tubercle-like" nodules described in human patients within the lungs, spleen, and kidneys, however unlike the human cases observed at the time, animals did not exhibit any negative symptoms after inoculation[206]. Ophüls further documented with subsequent work the various morphologies and life cycle of the organism, and through observations from other human patients and additional experimental inoculation studies of various animals, proposed "coccidioidal granuloma" as the name for the disease in 1905[208]. This term persisted until 1929, after a Stanford medical student studying *Coccidioides* experienced a laboratory exposure, inadvertently inhaling the pathogen in its mold form and becoming ill nine days later[205,207]. Despite the grave projections of colleagues after a diagnosis of coccidioidal granuloma was made[205,207], the student made a full recovery, and displayed a rather benign form of the disease in contrast to the fatal forms described to date. Additional cases of this nature occurring in the San Joaquin Valley were later described by Dr. Ernest Dickenson, who also proposed the term "coccidioidomycosis" to replace "coccidioidal granuloma" to describe and include all forms of the disease caused by *Coccidioides*[205,207,209]. It has since been determined that most forms of human *Coccidioides* infections are asymptomatic or mild[200,210].

Additional contributions to our current understanding of *Coccidioides* came from the work of Dr. Charles Smith in the late 1930s, after being tasked with describing the epidemiology of coccidioidomycosis by Stanford University[205]. Building upon the epidemiological work performed by the Kern County Health Department[211], Smith performed a 17-month study in California where which patients exhibiting characteristic skin lesions were tested using the recently developed coccidioidin skin test, a diagnostic involving injecting a preparation of the mycelial phase of *Coccidioides* under the skin and observing for a reaction[199,205,212,213]. Through these and subsequent studies, Smith concluded that *Coccidioides* was chiefly transmitted by inhalation of fungal spores, primarily through infected soils, and that a positive skin test result coincided not only with past exposure to the organism, but also with protection from further infection[199,213]. Use of the same skin test by Maddy, as well as Edwards and Palmer in the late 1950's later helped to define the larger endemic range of *Coccidioides* as being concurrent with the Lower Sonoran Life Zone[214,215]. The resulting distribution maps generated by Edwards and Palmer in 1957 continue to be used today for defining the spatial distribution of *Coccidioides* in the United States[216-218].

Finally, nearly a century after initial description of the pathogen, Fisher and colleagues confirmed that the genus *Coccidioides* is made up of two species, however discovered that rather than differences in clinical presentation, genetic polymorphisms are the basis distinguishing the two species[205,219-221]. The second species was re-classified from *C. pyogenes* to *C. posadasii*, in honor of Andrew Posadas[219]. Despite the marked genetic differences warranting the designation of two separate species, *C. immitis* and *C. posadasii* are otherwise morphologically identical to one another, and their predicted proteins over 90% homologous[195,222]. Besides minor differences in growth characteristics[218,221], the largest difference between the two species appears to be their

relative geographic distributions, with *C. immitis* being primarily found in the deserts of Central and Southern California, while *C. posadasii* possesses a larger range, which includes the desert regions of Nevada, Arizona, New Mexico, and West Texas in the United States, as well as parts of Mexico and Central and South America[222-224]. No significant differences in disease phenotype have been observed in either mild or severe cases caused by either species, although differences in virulence have been recorded between strains[197,225].

Ecology and Epidemiology

Coccidioides spp. are currently thought to reside primarily in ecosystems within the Lower Sonoran Life Zone, which is largely defined by high salinity soils and xeric conditions[226]. Creosote bush (*Larrea tridentata*), mesquites (*Prosopis spp*.), Palo Verde (*Parkinsonia spp.),* and yucca (*Yucca spp*.) are the major flora that define this region, while a variety of burrowing rodents, including several species of kangaroo rat (*Dipodomys spp*.), pocket mouse (*Perognathus spp.*), as well as the desert woodrat (*Neotoma lepida*) range throughout[214,227]. Mean annual air temperatures within the Lower Sonoran can range from -0.5 to 24.4°C, with extreme lows and highs of -40°C and 45°C; the surface temperature of soils can range from below freezing to over 80°C[228]. Annual precipitation can range from 5 to 50 cm and is characterized by regional patterns of discrete rainy and dry seasons[228].

Within these extreme climatic conditions, *Coccidioides* propagates in moist soils during, or shortly after rainy seasons as mycelia, or molds, and will form asexual reproductive spores known as arthroconidia as they mature[196,207,222]. Environmental growth in the mycelial form defines the saprobic life cycle, where the fungus survives on dead or decaying organic matter[229,230]. As the seasons shift and soil profiles dry out, arthroconidia will disperse through

windborne mechanisms when soil is disturbed, either through direct manipulation, or weather events such as dust storms[229-231]. Arthroconidia may then either form a new mycelium if deposited into soils with appropriate growth conditions or transform into a spherule if it infects a susceptible host[196,222,232]; infection of hosts by fungal spores is thought to occur chiefly through the respiratory route[229]. Formation of spherules in the tissues of hosts then begins the parasitic life cycle of the pathogen, where which growth and maturation of the spherule gives rise to hundreds of uninucleate endospores, which are fungal offspring that may disseminate throughout the body and give rise to additional spherules[196,232]. Endospores that disseminate to additional parts of the body contribute to the formation of disseminated coccidioidomycosis and in some cases can cause host death[224]. Once a host dies, viable endospores present within the tissues can form new mycelia in the environment[222]. Hosts with non-disseminated forms of the disease however may also help to establish new mycelia in the environment, should they be subclinical and possess spherule-containing granulomas within their tissues[196,222,232,233].

Given the close relationship of *Coccidioides* with desert soils through much of its life cycle, burrowing animals, and particularly desert rodents, are thought to play a role in fungal ecology. The specifics of this role, however, are debated within the literature, and much of the natural host and ecology of this pathogen remains undescribed[197]. Some have speculated that these animals are merely accidental hosts[196,223], and field investigations conducted after past human outbreaks have revealed that isolates are more likely recovered from infected soil or rodent middens, rather than rodents themselves[234,235]. The evidence generated from these field investigations suggest that soil is the primary reservoir for the fungus, and that fungal distribution in the environment could likely be determined by studying soils and climatic conditions. Others, however, have proposed a more involved system where small mammals are a

major reservoir for *Coccidioides*, with the pathogen maintaining a presence in the environment through the infection of animal hosts[196]. Termed the "endozoan, small-mammal-reservoir hypothesis", this theory posits that the fungus persists as spherules within host granulomas, and convert to mycelia after host death, either from disseminated coccidioidomycosis, or from other natural causes[196,236]. Mycelia that return to the environment in this manner survive on the materials of their dead hosts within soils and produce arthroconidia that are available to infect additional hosts and begin the cycle again[196,237]. Evidence supporting this hypothesis includes recent comparative genomic analyses suggesting *Coccidioides* to have evolved to be associated primarily with their hosts, due to the presence of several metabolic genes capable of degrading and consuming materials in live and dead hosts[195]. While debate continues regarding whether the primary ecological reservoir for *Coccidioides* is the soil or small mammals[214], the role that larger desert mammals play in the life cycle of the fungus is significantly understudied, and an animal reservoir remains to be definitively identified[238].

Further confounding our understanding of *Coccidioides* ecology today is the environmental distribution of the pathogen. Despite the Lower Sonoran being identified as the primary environmental range of *Coccidioides*, reports and isolations of the fungus have been made outside of this endemic region within the last two decades and include the more temperate region of eastern Washington state[214,239,240]. Many attempts have been made to define the soil characteristics necessary for fungal growth and presence, however no single parameter or group of conditions yet have displayed a definitive pattern throughout all known sites of fungal contamination, and reliable isolation of *Coccidioides spp.* from the environment remains a problem[222,228,241,242]. Furthermore, while there appear to be distinct geographical differences in the distribution of *C. immitis* and *C. posadasii* in the environment, distribution within soil

profiles is not homogenous for either species, even in regions with high incidence of human valley fever and considered disease "hot spots"; this further complicates any conclusions that may be drawn regarding the ecological requirements for either species[238,241,243].

While directly identifying the presence of *Coccidioides* in the environment remains an elusive problem, human case reports and regional prevalence data can serve as compelling evidence of zones of endemicity. Additionally, larger patterns of human infection have assisted in our current understanding of the risk factors associated with exposure and the development of severe coccidioidomycosis. In agreement with much of the historical work performed by Edwards and Palmer in the 1950s, case report data available today continue to reflect that the highest prevalence of valley fever in the U.S. occurs in the desert southwest, particularly in Arizona and California[244-246]. The most recent data available for 2019 indicates that at least 9,000 cases of valley fever were reported in California, while over 10,000 cases occurred in Arizona[247]; this equates to approximately 97% of all valley fever cases reported in the U.S. coming from both these states[245,246]. An additional 640 cases were reported from other states, the majority of which included Nevada, New Mexico, and Utah, however, other states throughout the continental U.S. well outside the suspected endemic region of the Lower Sonoran reported cases as well[247,248]. Despite the data available, comprehensive epidemiological study of coccidioidomycosis is somewhat challenging, as the disease is not reportable in all states[249], making it very likely that the true prevalence is underestimated in the U.S. Data from the states that do report the disease however have indicated valley fever cases have been steadily increasing after regular reporting began in the 1990s[200,250,251]. In California alone, incidence has reportedly increased almost 800% from 2000 (2.4 cases per 100,000 people) to 2018 (18.8 cases per 100,000)[244,246,252,253]. Similar increases have been observed in Arizona, and case reports from

additional states throughout the U.S. have been on the rise as well[246]. Reports of valley fever and isolations of *Coccidioides* from environments outside of historic regions of endemicity, coupled with increasing case reports in the desert southwest indicate that coccidioidomycosis is a reemerging disease in the U.S., and that further work is necessary to define the ecology and epidemiology of the organism.

Lastly, while any person who resides in or travels to a *Coccidioides* contaminated region can become infected after exposure to fungal spores, several trends have emerged in endemic regions regarding relative exposure risk and probability of developing severe disease. The most notable risk factors for valley fever cases that have been observed to date include sex, age, immune status, and ethnicity[244]. Overall risk for coccidioidomycosis has generally been higher in males than females[244,252,253], as well as higher in people of African, Asian, Hispanic, and Filipino decent[254,255]. Highest incidence has moreover been documented in older individuals, particularly in adults aged 40-49 and ≥65 years in California and Arizona, respectively[256-259]; these individuals are also at higher risk for developing symptomatic disease[256,259]. Similarly, race, in addition to displaying differing case prevalence, also can impact the probability of developing severe or disseminated coccidioidomycosis, with non-white individuals displaying rates of dissemination ranging 2-10 times higher than white persons[210,254,255,260]. While the pathogenetic basis of these differences in relative incidence and proclivity for developing severe disease is poorly understood, they likely are the result of a suite of physiological, biological, and societal factors. For instance, as the exposure to arthroconidia requires intense contact with infected soil, those who work in agriculture or construction are at increased risk of exposure; historically these occupations tend to have a strong male dominance and may be responsible for some of the sex discrepancies observed[244,254,255]. In contrast, some studies that have reported increased risk in

individuals of African descent developing disseminated disease even when there are no apparent differences in exposure compared to their Caucasian counterparts, suggesting other factors are likely at play[210,223,261]. While some genes and blood groups have been suspected to increase the risk of developing sever valley fever[255], societal factors such as access to healthcare and socioeconomic status may also be of significance[244,254,255]. The increased probability of comorbidities and immunosuppression in older individuals, coupled with a higher likelihood of these individuals seeking medical attention have been suspected as the driving factors behind observed age discrepancies[257,259].

Human Infection, Pathogenesis, and Transmission

The infectious dose of *Coccidioides* in humans is currently unknown, however is presumed to be very small[259], as past studies in primates have suggested that inhalation of as few as ten arthrospores can result in severe infection[262]. Infection is thought to occur primarily through the respiratory route and the disease is not considered contagious, however rare cases of percutaneous infection as well as inoculation after receiving an organ transplant have also occurred[207,263,264]. Most instances of percutaneous inoculation have occurred in laboratory workers after accidental hypodermic injection[207]; however, one case of transmission was reported in an individual bitten by a cat with disseminated disease[265]. One additional case of zoonotic transmission evidently occurred when an individual inhaled endospores during the necropsy of a horse with disseminated disease[265]. Otherwise, infection is typically established after inhalation of arthroconidia into the lungs, where they may then lodge into the terminal bronchioles[264]. Once inside the lungs, the presence of increased CO_2 and phagocytic cells within the host will initiate the morphologic conversion of arthroconidia into an endospore-containing

spherule[211,266]. Maturation of the spherule within the lungs or other tissue is complete within approximately 48 hours, after which the spherule may rupture to release the endospores contained within[267]. Each endospore that is released migrate either to surrounding tissue, or in some cases, disseminate throughout the body, and can produce additional spherules[268].

Overall disease presentation ranges from acute or chronic progressive pneumonia, pulmonary nodules and cavities, or disseminated extrapulmonary non-meningeal disease and/or meningitis[264,269]. Symptoms of primary pulmonary coccidioidomycosis typically show within one to four weeks after exposure, while disseminated forms of the disease can present with symptoms months to years later[265]. Infection is asymptomatic in roughly 60% of human cases[210,226]; most remaining cases are mild and usually resolve without medical intervention and are limited to the lungs[200,265]. Less than 5% of infections result in fungal dissemination throughout the body[265], and infection can disseminate to spleen, liver, brain, and bone, however, has been known to spread to other tissues as well[200]. Case fatality rate in disseminated coccidioidomycosis ranges and depends largely on the effected tissues and the course of treatment[265]. Meningitis, for example, is one of the most severe presentations of disseminated disease and is often fatal without prolonged or lifelong therapy; meningitis can occur in 30-50% of patients with disseminated disease[265].

Definitive diagnosis of coccidioidomycosis is possible with identification of the organism in culture or histological examination of patient tissues or body fluid specimens, although these can be difficult to obtain, as they can require invasive procedures[223]. Additionally, isolation of the organism can be challenging as it is often not distributed evenly throughout tissues[223,270]. Histologically, the presence of mature spherules containing endospores can be visualized and is

considered pathognomonic, however, if not observed by a pathologist familiar with the disease, may also be mistaken for other fungal pathogens such as *Histoplasma*, *Cryptococcus*, or *Blastomyces*[223]. Otherwise, a presumptive diagnosis radiologically can be made upon observation of pulmonary nodules or pulmonary cavitation in the lungs[207]. Serological tests may also be utilized for diagnosis, especially in symptomatic cases where a person has never been exposed to *Coccidioides*, or obtaining biopsy specimens is not possible. In these cases, it has been demonstrated that increasing IgG antibody titers are highly correlated with the severity of disease in humans[265]. Furthermore, detection of antibodies in cerebrospinal fluid is often the only way to diagnose coccidioidal meningitis, as biopsies are extremely invasive, and organisms rarely found in these cases[265]. Use of serology alone for diagnosis is limited in that a positive test does not discriminate between past or recent infections, and in the case of severe forms of disease requires serial samples to be collected to establish a trend of increasing antibody titer. Low test sensitivity, and a high rate of false-positive tests also diminish the use of serology alone to diagnose infection[271,272].

No vaccine is currently available to prevent coccidioidomycosis, however antifungal treatment is available for patients experiencing chronic pulmonary or disseminated disease. Antifungal treatments proven efficacious against *Coccidioides* infection include amphotericin B deoxycholate, lipid formulations of amphotericin, ketoconazole, fluconazole, and itraconazole[245,264]. Any of these treatments may be administered to patients experiencing infection with either species of *Coccidioides*, as they are both comparably susceptible to antifungal drugs[273]. Treatment regimen and duration largely depends on the severity of infection, and while efficacious, may require long-term or life-long use with regular follow-ups with a physician[245,264].

Non-Human Host Range

Other than humans, *Coccidioides spp*. appear able to infect most other vertebrate species, with varying levels of subsequent disease[197]. Mammals in particular appear to share a similar level of susceptibility to that of humans, however, disease presentation is also quite variable[274]. The domestic dog (*Canis lupus familiaris*) seems to have a susceptibility and range of illness most like humans, and in the southwestern U.S. also exhibit similar rates of subclinical infection[274]. Similarly, skin lesions are common in humans and domestic cats (*Felis catus*), although cats are more commonly severely ill with disseminated forms of disease compared to either humans or dogs[274]. Otherwise, as alluded to above, various species of desert rodent, as well as some armadillos (family *Dasypodidae*) have often been reported with subclinical lesions in various tissues, and isolations of *Coccidioides spp*. have been made from active burrows[222,227,236,241,275]. Positive serology in many of these rodents have also indirectly implicated them as being susceptible to infection[214,241]. Several bat species (order Chiroptera) and collared peccary (*Pecari tajacu*) occurring in the known ecological range of the fungus have similarly been shown susceptible[276,277]. Observational studies in several livestock species suggest that clinical infection is rare in horses, cattle, sheep, and swine, however *Coccidioides*-associated lesions, when observed, are usually incidentally detected during slaughter or meat processing, and likely do not reflect true incidence[265,274,278,279].

Less commonly, cases of mild and severe coccidioidomycosis have been identified in a myriad of captive exotics and include the Bengal tiger (*Panthera tigris tigris*), red kangaroo (*Macropus rufus*), koala (*Phascolarctos cinereus*), rhino (*Diceros bicornis*), as well as various non-human primates[232]. Isolation of *Coccidioides* and histological observation additionally have

been made in domestic chicken (*Gallus gallus domesticus*)[280], as well as some captive and wild marine species, including bottlenose dolphin (*Tursiops spp.*)[232,281], suggesting that many avian and aquatic species are also susceptible. Finally, relative thermal regulation strategy does not appear to preclude certain taxa from infection, as some reptiles have been identified as susceptible as well[282,283].

Coccidioidomycosis and Swine

Clinical signs and overt illness associated with *Coccidioides* infection are rare in domestic swine, and lesions when they occur, are often identified incidentally during slaughter or meat processing[265,274,278,279]. One of the earliest descriptions of the disease in pigs was made by Giltner who observed *C. immitis* spherical bodies in the lungs of two pigs after intravenous inoculation with a fungal isolate previously recovered from cattle, confirming swine are susceptible to infection by *Coccidioides*[284]. Later, in 1954, natural infection was observed in a juvenile domestic pig in Santa Paula, California when granulomas were observed in the lungs at necropsy[285]; prior to postmortem examination, the animal was reported as being weak and having a cough[279,285]. Additional skin testing in 65 domestic pigs in Florence, Arizona by Maddy in 1959 revealed 11% positivity, suggesting that swine may be exposed regularly to the fungus in endemic regions[279]. Outside of the U.S., serological studies have indicated that at least 12% of domestic swine in some endemic regions of Mexico were positive for *Coccidioides* antibodies[265].

Despite observations of exposure and active infection in swine throughout the current known range of *Coccidioides*, fungal shedding and transmission dynamics in pigs have not been explicitly examined, and no additional investigations have been performed since initial observations were made in the 1950s. Furthermore, as domestic swine appear to seldom develop

disease, yet have been demonstrated capable of exhibiting a serological response like that of humans in endemic regions, utilizing the exposure status of free-ranging pigs may be a useful tool in studying the endemic range of *Coccidioides* today. Of particular interest herein, feral swine, a species taxonomically identical to domestic swine, have never before been examined for their potential role in *Coccidioides* ecology and epidemiology, despite being extremely prevalent in regions of the U.S. with high or increasing rates of coccidioidomycosis in humans. Free ranging feral swine likely experience higher rates of environmental exposure to *Coccidioides* arthroconidia and fungal hyphae in infected environments when compared to their domestic relatives, who are often housed in indoor facilities or have access to limited outdoor spaces[286].

MELIOIDOSIS

Discovery and Historical Perspectives

The disease now known as melioidosis and its causative agent, *Burkholderia pseudomallei* was first described in 1912 by Alfred Whitmore and C.S. Krishnaswami following a post-mortem they performed on a 40-year-old man in Rangoon, Burma the year prior[287,288]. The man in question had been admitted to the hospital after reporting a fever that lasted seven days; his fever continued for the three days he was in the hospital before he died[287]. During autopsy, it was noted that the patient's thighs were riddled with subcutaneous abscesses, which appeared in conjunction with morphia injection sites; further inspection of the internal organs revealed a "peculiar, cheesy consolidation of the lungs"[287]. Initial examination of smears from diseased sites revealed "non-Gram-staining bacilli the size and shape of *B. mallei*" and an initial diagnosis of glanders was made. Additional patient information and subsequent experiments performed by Whitmore and detailed in this first report, however, were quick to distinguish this pathogen from

that of *B. mallei*, as the patient did not have a history of interactions with equids, the primary host for glanders; furthermore, bacterial culture revealed that the organism in question displayed comparatively rapid growth, and colony morphology distinct from that of *B. mallei*[287,288]. One year later, in 1913, 38 additional cases of this glanders-like disease would be described by Whitmore, who proposed the name *Bacillus pseudomallei* for the species[289]. It was around this time that the terms "Whitmore's disease" and "morphia injector's septicemia" were coined to describe the affliction, as it appeared to be particularly associated with morphine addicts[289-291].

New terminology for the disease was introduced in 1921 by Stanton and Fletcher, when they proposed the term "melioidosis" to describe the disease, as well as a new name for the causative bacterium (*Bacterium whitmori*), after study of the disease in a variety of laboratory animals[292]. The term melioidosis, still used today to describe the disease comes from the Greek "melis" meaning "distemper of asses," and "eidos" meaning "resemblance," and was coined due to its resemblance to glanders[292,293]. The etiologic agent of the disease was then renamed several times throughout the following 70 years, and would be referred to as *Malleomyces pseudomallei, Loefflerella pseudomallei, Pfeifferella whitmori,* and *Pseudomonas pseudomallei* before finally being classified as *Burkholderia pseudomallei* in 1992[294,295].

While the experiments of Stanton and Fletcher in various laboratory animals (e.g., guinea pig, rabbit, white mouse, rat, monkey, sheep and goat) allowed for further description of melioidosis and gave the first indications of non-human host susceptibility, they also alluded to environmental routes of bacterial transmission[292]. In 1918, Stanton was able to establish infection in orally infected animals after feeding them materials containing the infectious organism, and subsequently isolated bacteria from the urine and feces, suggesting that the disease was conveyed

through consumption of contaminated food[292]. The identification of soil and water as the primary habitat for the pathogenic bacterium would not be confirmed until much later, however, after the bacterium was isolated from stagnant water and rice paddies[296-300].

Given the variety of nomenclature used, as well as inaccessibility to medical literature on a regional scale during the early years of *B. pseudomallei* discovery, it is difficult to assess the prevalence or distribution of the disease prior to the late 1940s and early 1950s[301]. It was during this period of war that military physicians began to regularly recognize the disease during the French Indochina war and suspect that soil contaminated with the organism was a source of infection[301]. Similarly, high incidence of melioidosis would be recorded later during the Vietnam war, and a particularly high incidence noted among helicopter winch men lead to the suspicion that bacteria may be aerosolized and inhalation an important mode of transmission[297,301]. Indeed, other notable names for the disease throughout history include the "Vietnam time bomb" as the disease was described in Australian and U.S. soldiers during the Vietnam War; this name also in reference to the nature of the disease being reactivated even after years of latency[290,302]. Finally, as awareness for the disease increased, case reports and confirmations of melioidosis would continue to rise from throughout the tropics throughout the 20th century[293,296], however non-specific clinical signs, as well as latent infections continue to make estimating regions of endemicity, incidence, and environmental distribution difficult today.

Ecology and Epidemiology

Burkholderia pseudomallei is a Gram-negative bacillus known to reside in moist soils and surface waters and is associated with tropical environments, typically between the latitudes of 20°N and 20°S[293]. The bacterium and the resulting disease melioidosis has largely been

regarded as endemic to southeast Asia and northern Australia, where historically human incidence has been best described[293,303], however the larger region of endemicity also includes the subcontinent of India as well as areas of Central and South America[304,305]. An environmental saprophyte, *B. pseudomallei* is believed to infect most people through cutaneous inoculation, as it has been observed that the highest incidence of melioidosis occurs in agricultural workers in Thailand and indigenous people in Australia who often are exposed to contaminated soil or are without protective clothing[306,307]. Concurrently, it has been demonstrated that sites most likely to yield the bacterium in these regions include agricultural areas such as rice paddies and paddocks grazed by animals[308]. While cutaneous forms of melioidosis appear common in historically defined endemic regions, stochastic weather events have also been suggested as a means for the bacteria to become airborne and cause inhalational disease, although the relative impact of this and other routes of transmission are largely unknown[309]. Recent studies out of Australia have reported an increase in pneumonia and severe disease during rainy seasons and after tropical storms[310-312], suggesting that inhalational exposure may be at least seasonally significant for disease spread. Lastly, studies that have recovered *B. pseudomallei* from drinking water have indicated that ingestion may additionally be a significant mode of transmission in regions where water is not properly treated before consumption[309,313,314].

In highly endemic regions, overall incidence of melioidosis can vary widely by larger regions, countries, and year, and has ranged anywhere from 0.19 to 50.2 cases per 100,000 people[315-317]. In addition to agricultural workers and indigenous peoples, other notable high-risk groups that have been identified include those with diabetes, chronic kidney, liver, or lung disease, and excessive alcohol intake[293,296,307,308]. Individuals with diabetes in particular are at the highest risk among those with predisposing factors, as they display a 12-fold higher risk of

melioidosis; diabetes is additionally recorded in greater than 50% of melioidosis patients worldwide[307,318]. It has been hypothesized that these comorbidities are likely associated with melioidosis due to a shared ability of impaired neutrophil function that subsequently diminishes the immune system's response to bacterial infection; indeed, past studies have demonstrated that those with diabetes mellitus, chronic renal failure, or participating in high alcohol consumption as a result have diminished chemotaxis, phagocytosis, oxidative burst, and killing activity[293]. Even so, over 80% of pediatric cases, as well as those in approximately 20% of adults are not associated with any of these high-risk factors but have instead been exposed to high levels of the bacteria[296,319-321], indicating that continued identification of risk factors to infection will be imperative to combatting the disease moving forward.

While several reviews have sought to define the global environmental distribution of *B. pseudomallei*, many have reported this with the caveat that many regions likely endemic for melioidosis lack the diagnostic tools required for proper diagnosis[293,305,322]. Moreover, autochthonous cases as well as isolations of the bacteria in environments well outside of historical regions of endemicity in the last two decades indicate that the range of *B. pseudomallei* is much larger than originally assumed[323-326]. Further complicating attempts to define geographical distribution of this pathogen is the broad clinical spectrum of the disease; colloquially known as the "great mimicker", symptoms of melioidosis are very often mistaken for other infections[296,327], and likely go underdiagnosed and subsequently underreported. The global burden of melioidosis and how likely it may become established in additional environments is not well described, although some recent studies have attempted to predict environmental suitability for the species[328]. Unsurprisingly, most investigations that have sought to define the environmental requirements for this pathogen have found that high rainfall and

temperature, as well as anthrosol and acrisol soil types are strongly correlated with bacterial presence[328-330]. These soil types are defined as being formed from long-term human activity or modification as well as rich in clay; these corresponding with the agricultural areas in tropical areas currently considered endemic for the disease[328]. Despite these apparent associations, autochthonous cases have been reported outside these environments, including in regions of the Middle East, Sub-Saharan Africa, the Caribbean, and Central and South America, and the southeastern U.S within the last two decades[323-326]. Cases associated with the use or consumption of contaminated products imported from highly endemic regions additionally have likely contributed to global pathogen spread and may have since caused contamination of environments far from those normally associated with the disease[303,331-336]. Taken together, misdiagnoses and underreporting of disease, along with increasing evidence of expanding geographical range, make melioidosis an emerging disease of significant concern to human and animal health worldwide.

Human Infection, Pathogenesis, and Transmission

Naturally acquired infections with *B. pseudomallei* in humans are usually the result of cutaneous inoculation through broken skin, inhalation, or ingestion of the organism from the environment[296,309,337]. The minimum infectious dose required for human infection is unknown[301]. Regardless of the route of inoculation, the overall mechanisms of cellular invasion and pathogenesis are largely the same and can present as a spectrum of clinical signs ranging from acute sepsis to chronic or latent disease[296]. In general, *B. pseudomallei* will first attach and replicate in epithelial cells of the mucosa or damaged skin before spreading to additional cell types throughout the body[296]. The process of host cell invasion and intracellular survival is

mediated by a suite of virulence factors including but not limited to, types II, III, V, and VI secretion systems, type IV pili, and the cytotoxin Burkholderia lethal factor I (BLF1)[296,303]. While replicating in the intracellular space, bacteria are additionally capable of causing infected host cells to fuse with neighboring uninfected cells, resulting in the formation of multi-nucleated giant cells (MNGCs) and allowing for the intracellular cycle to continue without the organism ever needing to enter the extracellular space[338,339]. Rather unique to *B. pseudomallei* is its ability to enter, survive, and replicate in a variety of host cell types through possession of many of these virulence factors; the resulting intracellular behavior of the bacterium and formation of MNGCs is currently considered vital for disease pathogenesis, and a major hallmark of infection[338-342]. In addition to cell-to-cell spread, *B. pseudomallei* may also disperse to the bloodstream and cause sepsis, or to immune cells; infection of immune cell types can further facilitate bacterial spread to secondary and tertiary sites after the organism is introduced to the lymphatic system[296,343]. Finally, *B. pseudomallei* may enter a dormant state in some cases, further evading immune surveillance within a host and result in latent infection[296,344-346]; the mechanism(s) by which the organism can evade detection for so long remain to be described[296,347].

Despite what is known regarding the general pathogenesis and unique display of MNGCs in infected individuals, subsequent host cell death is likely the result of a combination of virulence factors expressed by the bacterium, some of which are known while others not yet described[339]. Further complicating generalizations of disease pathogenesis is the fact that *B. pseudomallei* as a species possesses a rather flexible genome[348,349], resulting in high levels of genetic diversity. As such, virulence factors may not be uniformly expressed *in vivo*, and depends largely on the genetic background of a particular bacterium[339]. Moreover, genetic mutations have also been known to occur after infection, as seen in serially collected isolates from patients with

acute melioidosis[350,351]. This high genetic diversity, coupled with a range of clinical presentations that often mimic those of other pathogens can make diagnosis and subsequent treatment for melioidosis challenging. Indeed, the same genetic diversity that allows for varying mechanisms of host cell invasion and intracellular survival also allows for the bacterium to exhibit natural resistance to a range of antibiotics[352,353].

Likely due to its flexible genome and ability to infect most host cell types, clinical manifestations of *B. pseudomallei* infection can be variable and non-specific, and subsequent diagnosis and treatment challenging[296,303,315,354]. In most cases, patients generally present with acute febrile illness accompanied by pneumonia, bacteremia, and/or abscess formation, commonly in the spleen or liver[303,355]. Osteomyelitis and various neurological expressions of disease may also occur in some severe cases[296,315,356]. In cases of cutaneous inoculation, lesions on the skin will appear as firm ulcers or ulcers grey or white in color and may caseate[357]. The average incubation period is nine days; however, it can range from 1-21 days depending on the inoculation route and level of exposure; in instances of chronic or latent infection, symptoms may not appear until months or years later[296,357-359]. Bacteriemia can occur in 40-60% of all melioidosis patients, while septic shock in an estimated 20% of cases; overall illness presents as pneumonia in half of all cases[296]. Untreated septicemia has a mortality rate between 80-90% and can be fatal as early as 24 hours after the onset of symptoms[360,361]. Otherwise, overall case fatality can range from 10-40% worldwide[328].

Bacterial culture from clinical specimens remains the gold standard for diagnosis of melioidosis, however, in some cases *B. pseudomallei* may be mistaken as either a culture contaminant or other bacterial species, including *Bacillus spp*. or *Pseudomonas spp*.[296,331,362]; risk

of misidentification is especially high in non-endemic regions where clinicians may be largely unaware of the disease[363]. Colonies successfully isolated from clinical specimens may be identified with biochemistry combined with microscopic and culture appearance[357]. Upon microscopic examination, bacteria will appear as motile, short, Gram-negative bacilli with bipolar or irregular staining[357]. Serological tests, most often the indirect hemagglutination assay (IHA), may also be helpful for diagnosis yet can be difficult to interpret in endemic regions where most of the population is seropositive[331,357,364]. Furthermore, suboptimal sensitivity and specificity of the IHA are also major limitations to using serology as a reliable diagnostic[357].

While no vaccine currently exists to prevent melioidosis, treatment is available and consists of two phases of intensive antibiotic therapy[296,357,365]. During the initial phase, patients receive intravenous cephalosporin or carbapenem antibiotics, usually ceftazidime or meropenem, respectively[357,365,366]. Intravenous antibiotics are either given six or eight hours apart for 10-14 days, although this phase may last longer for severely ill patients exhibiting extensive pulmonary disease, osteomyelitis, organ abscesses, or neurological symptoms[296,365,367]. Following the initial intravenous therapy is the second phase of treatment, which is comprised of prolonged oral antibiotic therapy for at least three months[296,365]. The purpose of this second phase is to eradicate any remaining *B. pseudomallei* within the body and prevent recurrent disease and consists of two different compounds than those administered during the first phase[296,365]. Careful supervision and monitoring is necessary when transitioning patients from the first to the second phase, as there can be a risk of septicemic relapse[368]; it is often common practice to overlap the intravenous treatments of phase I, with the oral treatments of phase II to assess patient tolerance to the oral compounds and prevent any lapse in treatment pressure to the infectious organism[365]. Common antibiotic choices for the second phase include trimethoprim–sulfamethoxazole, co-amoxiclav, or

doxycycline[296,357,365]. Co-trimoxazole and doxycycline may also be administered together during this phase[369]. Deployment of this two-phase treatment protocol has been demonstrated to reduce the risk of relapse occurring significantly in some regions[370], however, relapse due to prolonged sequestration or dormancy of *B. pseudomallei* within more immune privileged sites of the body are possible, and currently not well understood[365]. The ability of *B. pseudomallei* to form biofilms in tissues likely contributes to treatment failure and eventual relapse, as these complex multicellular communities allow for continued evasion host defenses, as well as limits the penetration of antibiotic treatment[371-374].

Lastly, the bulk of human infections are believed to come from exposure to contaminated soil or water[357,375,376]. Human-to-human transmission of melioidosis is extremely rare, however cases of potential sexual transmission, vertical transmission, and perinatal transmission have been reported in the past[377-383]. Additionally, handling infected products or animal materials have also been attributed to cases of human melioidosis, and represent an additional pathway of transmission[357,384,385]. Zoonotic transmission is currently thought to be relatively rare[386].

Non-Human Host Range

Burkholderia pseudomallei is not host-specific, and host body temperature does not appear to hinder infection; clinical and subclinical disease have therefore been documented in a wide range of non-human animal species[387]. Relative host susceptibility as well as disease presentation, however, varies considerably between taxa, and is reminiscent of the variable clinical signs seen in human infection. As with human melioidosis, the incubation period in non-human animals can range from days to months or years, and symptomatic infection may be acute, subacute, or chronic, as well as either mild or severe[357]. Abscesses in the lungs, spleen,

liver, and various lymph nodes are common, however the bacteria may spread to any organ. Asymptomatic or subclinical infection is common in animals of any taxa, and abscesses containing *B. pseudomallei* are usually not found until after an animal has died or is euthanized[357].

In many mammals, including cats, dogs, camels, and various livestock, clinical signs most often include coughing, nasal discharge, and weight loss[357,384,385,387-391]; these and other symptoms are usually non-specific, and cases where which abscesses have been observed in various organs were not initially suggestive of an infectious disease[357,391]. Epizootics in various livestock, including cattle, horses, pigs, sheep, and goats have been reported, and sheep and goats are considered particularly susceptible to clinical disease[384,390,392-394]. In historically defined endemic regions such as in Thailand and Australia, goats have been described as the species most frequently affected by melioidosis[384,389]. Otherwise, reports of disease have also been made in camels and alpacas, and these animals have also been classified by some as highly susceptible to clinical disease[357,384].

Additional reports of natural infection have also been made in a range of zoo animals, as well as captive reptiles, birds, and marine mammals[357]. For many exotic mammals, generalized lymphadenopathy and respiratory signs are common, while most cases documented in captive marine mammals, including various species of dolphin and the killer whale (*Orcinus orca*) present as acute septicemia with fever inappetence, and anorexia followed by death[357,395-397]. In reptiles and fish, ulcers and necrotic nodules on the skin or internal organs are most often described[332,357,387,398]. Cases of melioidosis or carriage of *B. pseudomallei* have also been reported for several bird species, many of which captive[399-405]. Isolation of bacteria from a wild

peaceful dove (*Geopelia placida*), a ground foraging species sampled at a coastal nature preserve in Australia suggests that carriage may be common in some avian species residing in endemic regions[406].

Despite the large host range and potential for zoonotic transmission, the role that non-human animals play in bacterial epidemiology for *B. pseudomallei* is significantly understudied in comparison to known environmentally mediated mechanisms of transmission[386]. This remains particularly true for animals with close associations with humans, such as livestock or peridomestic wildlife species; wild animals with close associations with humans may further be contributing to pathogen spread by additionally interacting with other wild or domestic hosts as well as humans and may significantly contribute to transmission risk.

Melioidosis and Swine

As with sheep and goats, pigs are thought to be one of the common domestic animal species infected in endemic countries[357,389,407], and there have been several reported cases of melioidosis in domestic swine through the years[392,388,389,392,393,408-410]. Otherwise, the earliest recorded case of melioidosis in a pig appears to be that in Madagascar in 1936 by the French scientist Girard, who isolated the organism from the submaxillary lymph node of a slaughtered pig[411,412]. Larger epizootics in pigs have also been recorded in regions of endemicity, with one of the earliest being that of a group of domestic swine living on Aruba in 1957[408]; epizootics in pigs, however, appear rather rare. Most often, cases of melioidosis have been incidentally observed in domestic swine after an animal has gone to slaughter or market, and lesions inadvertently detected[357,388,389,392,393,408-410,413]. Abscesses attributed to *B. pseudomallei* detected at abattoirs or slaughter facilities have been observed in a variety of organs, although most often are

detected in spleen and either submandibular or gastrohepatic lymph nodes[357,393]. From these observational and often indirect documents of disease, it has been largely accepted that natural infection in pigs is usually chronic and asymptomatic[357,388,389,392,393,408-410,413]. Further supporting this theory are more recent studies that have sampled market pigs in regions endemic for disease and have found that close to 1% of randomly sampled animals may actively carry *B. pseudomallei* in the trachea[414], while serological surveys in the same regions identified at least 6.3% seroprevalence in farmed swine[386,413]. In rare cases or in younger animals, clinical signs that may be observed include progressive emaciation, skin ulcers, diarrhea, coughing with or without nasal discharge, and various neurological signs[357,416].

Empirical evidence of swine susceptibility are available from a few experimental infection studies, which have demonstrated that swine are rather resistant to infection with *B. pseudomallei*[394,417]. Interestingly, in each of the experimental infection studies available, investigators inoculated animals using routes to likely to induce high rates of infection (e.g., intratracheal, IT; intravenous, IV), however, these routes of inoculation are likely not an appropriate model for natural infection. Despite these invasive inoculation routes, as well as relatively high inoculum doses between 10^8 and 10^9 bacilli, and immunosuppression of some animals, most often study pigs developed chronic, generalized infection, rather than acute disease; this further illustrates and confirms that pigs are rather resistant to infection[394,417].

Despite there being many documents of *B. pseudomallei* infection in domestic swine, there are limited reports of the disease in wild pig populations. One study references a surveillance program in Australia implying reduced disease incidence during the 1992 dry season, however no further details or references are provided[384]. Similarly, one earlier study, also

in Australia, sought to evaluate a modified complement fixation test for improved serodiagnosis of melioidosis in pigs, and utilized serum from domestic as well as feral swine, however, did not differentiate results between the two animal populations[418]. This disparity between studies examining melioidosis incidence in domestic and wild pigs is likely due to sampling efficiency and the ease that which domestic swine may be observed in comparison to wild or free-ranging pigs. However, given the propensity of asymptomatic or chronic melioidosis infections in domestic swine, it is very likely that wild pigs possess similar susceptibility given their genetic relatedness[10]. Finally, given the current range of wild and free-ranging pigs worldwide[13], and the overlap this range possesses with many endemic regions and those where which *B. pseudomallei* was recently isolated, further investigation into melioidosis incidence in these animals is warranted.

EMERGING PATHOGENS

While we largely were interested in the role(s) feral swine may be playing in the ecology and epidemiology of the three zoonotic pathogens reviewed above, their broad ecologic range and potential to interact with a suite of other taxa make them a significant threat to the development and subsequent transmission of other emerging and novel pathogens. For this reason, performance of generalized disease surveillance or screening in feral swine populations may be important to identify additional biologic threats, either to humans or other animals.

Ticks are obligate hematophagous arthropods and are considered to be the second most important vector of human diseases after mosquitoes[419,420]. Collectively, ticks are additionally known to parasitize every class of vertebrate in almost every region of the world, with many species being capable of feeding on multiple host species throughout the entirety of their life

cycle[419,420]. Ixodidae or "hard ticks" particularly are thought of as an important family for the transmission of pathogens of significance to human health and exhibit a number of characteristics that greatly enhance their capability as disease vectors[420]. These characteristics include their tendency to feed on a host for relatively long periods of time (e.g., several days), and their multi-host feeding behaviors, effectively increasing the probability of pathogen transmission[421,422]. Notable viral and bacterial pathogens vectored by ticks and transmissible to humans include the causative agents of heartland virus, Powassan virus, Lyme disease, Rocky Mountain spotted fever, and tularemia[423]. These and most other tickborne pathogens require passage in a vertebrate host for survival, and are becoming increasingly important, as they can cause high morbidity and mortality in both humans and animals and may have significant impacts on animal production[424,425]. Furthermore, many tickborne infections in humans, companion animals, and livestock are associated with wildlife reservoirs[425], demonstrating an increased need to monitor some wildlife for bloodborne infections. Lastly, recent investigations have suggested that climate change, as well as human and animal movements have likely contributed to the range expansion of some ixodid ticks in the U.S., suggesting that the relative risk(s) these species pose in terms of disease transmission are likely to increase in the future[426].

Tularemia, a disease caused by the Gram-negative coccobacillus *Francisella tularensis*, is highly infectious and can cause debilitating and potentially fatal febrile illness in humans[427]. The disease has recently been classified as a re-emerging disease globally due to its large and still evolving geographical distribution, large host range, and potential role as an agent of bioterrorism[428]. Composed of three subspecies or biovars and capable of infecting over 250 species of vertebrates and invertebrates, the epidemiology of this pathogen is rather complex[420,429]. Multiple routes of transmission have been recorded, however in North America

the most significant modes of transmission to humans are through contact with contaminated wildlife, particularly lagomorphs, or through the bite of an infected arthropod, including some species of ixodid ticks[420,429]. Despite a strong link to rodents in North America, clinical disease has also been documented in infected domestic swine, and several field surveys have detected antibodies to *F. tularensis* in feral swine throughout the U.S., suggesting they may be contributing to bacterial spread[430,431]. The contribution of feral swine to the persistence of this pathogen throughout their range, however, remains to be described.

Finally, the emergence of novel pathogens continue to threaten the health of many taxa and can be exasperated by processes like globalization and urbanization, as well as human-wildlife interactions. It has additionally been suggested that the majority of emerging diseases in humans have a wildlife reservoir[432]. This was most recently illustrated with the emergence of SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19). First described in Wuhan China during the winter of 2019, this novel respiratory virus is thought to have been introduced to the human population at the Huanan seafood market by an intermediate animal host[433,434]. The virus subsequently and very rapidly adapted to a human host, and after just a four-month period was classified by the World Health Organization as a global pandemic[435]. Despite the rapid development and deployment of several therapeutics as vaccines, SARS-CoV-2 continues to affect the human population today, and concerns remain over the evolutionary trajectory of the virus[436,437]. Furthermore, while it has been largely hypothesized that the animal of origin was likely a bat, detection of the virus in a myriad of other taxa since 2019 has led to added concerns of reverse zoonosis or spillback of the pathogen, as well as what this process might also mean for viral evolution[434,436,437]. One notable example of viral evolution in an animal host leading to the development of a novel variant is the global swine flu pandemic of 2009,

where which two swine influenza viruses reassorted, producing a novel strain capable of causing disease in humans; a domestic pig likely acted as the mixing vessel for this emergence[438,439]. Persistent SARS-CoV-2 infections in novel hosts may similarly lead to further viral adaptations, with the possibility of strains evolving to become more transmissible, pathogenic, or capable of escaping the protection afforded by current vaccines[436,440].

RATIONALE FOR CURRENT STUDY

Wild pigs are found on almost every continent around the world, and have become invasive in most of their range, including in North America and in many regions of the U.S[13]. Within the U.S., wild pigs or "feral swine," are recognized for their significant and largely negative impacts on native wildlife and ecosystems, agricultural output, and human property. In addition to physical damage, however, feral swine also play a role in the persistence and transmission of a myriad of pathogenic organisms, many of which are transmissible to humans and other animals. While many of the diseases that are transmissible by feral swine are relatively well characterized, many more are understudied as they relate to this prolific mammal. Moreover, emerging and re-emerging pathogens whose ecology is unknown or remains to be fully elucidated may be influenced greatly by the presence of feral swine; we hypothesize that this may be the case for the pathogens studied herein. Secondarily, despite the continued efforts of government agencies to monitor populations for disease threats, more generalized screening processes may be necessary to illuminate the biological threats posed by the species moving forward and inform appropriate management strategies. This is particularly important as the list of known pathogens carried or vectored by feral swine continue to grow and subsequently threaten the health of humans and other animals.

As feral swine display close behavioral associations with soils, we were primarily interested in investigating soil-dwelling pathogens, and their potential relationship(s) to feral swine. As implied throughout this chapter, the etiologic agents of anthrax, coccidioidomycosis, and melioidosis are each understudied pathogens as they relate to wild pigs, and additionally have many outstanding questions concerning their respective ecologies. Furthermore, the emergence of novel pathogens such as SARS-CoV-2 in late 2019 have demonstrated a need for continued opportunistic surveillance of wildlife populations, particularly those with close associations with humans. This is similarly the case for many viral and bacterial pathogens that are vectored by many tick species expanding in their geographic range, as many of these ticks can also feed on feral swine. The research presented throughout this work therefore primarily seeks to address the potential role(s) feral swine may be playing in the persistence, maintenance, and spread of *B. anthracis*, *Coccidioides spp*. and *B. pseudomallei*, all of which may also be classified as emerging or re-emerging pathogens in different regions of the U.S. Besides targeting these three focal pathogens, however, we also were interested in performing more generalized disease surveillance to identify any additional threats currently being posed by this invasive mammal, including the tick vectors they may be harboring.

This work is organized predominantly by pathogen, with each chapter being dedicated to one of the three focal pathogens of interest, while one final chapter describes our efforts to generally survey a population of feral swine for a variety of pathogens, including the three major pathogens of interest identified above.

In Chapter 2, we present field and laboratory-based investigations that address the sentinel utility of feral swine for improved anthrax surveillance, given they are a species resistant to disease. The laboratory-based study presented within Chapter 2 additionally assesses the role

that feral swine may play as mechanical vectors of *B. anthracis*, and the implications this may have on the distribution of this pathogen on the landscape.

Chapter 3 then presents the findings of an experimental infection study of feral swine with *Coccidioides*, and the subsequent consequences feral swine presence may have on fungal ecology and spread. Chapter 4 reports on the immune response generated by domestic swine in response to exposure to *B. pseudomallei*, and evaluates the performance of a serological test to examine antibody prevalence in feral swine populations throughout the U.S.

Finally, Chapter 5 details an intensive field-based study where which we screened a population of Texas feral swine residing in an area of high biodiversity and human visitation for all three focal pathogens; ticks that were actively blood feeding on the feral swine sampled in this study were also collected to characterize some of the vectors in the area as well as the pathogens they may be carrying. We additionally utilized general screening methods to opportunistically survey this population for additional viral and bacterial pathogens, including but not limited to, SARS-CoV-2 and *F. tularensis*.

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CHAPTER 2: EVALUATION OF FERAL SWINE AS BIOSENTINELS AND MECHANICAL VECTORS FOR *BACILLUS ANTHRACIS*[1,2]

SUMMARY

Anthrax is a disease that affects livestock, wildlife, and humans worldwide, however its relative impact on these populations remains underappreciated. Despite being a reportable disease in the United States, surveillance is largely passive, and mainly consists of reporting the presence of suspicious animal carcasses. Active management, where present, is largely based on livestock vaccination, however this practice is not standard across regions. Furthermore, environmental isolation is laborious and bacterial distribution across large landscapes difficult to confirm, making risk and disease management challenging. Feral swine (*Sus scrofa*) are an invasive species that rarely succumb to anthrax and whose range is extensive in the southern United States. Behaviorally, feral swine are closely tied to soil, the primary substrate for *B. anthracis*, and past serological surveys have alluded to their potential utility as sentinels for surveillance, yet empirical data to support this is lacking. Moreover, whether feral swine may assist in the spread of anthrax by transporting infectious spores has not been investigated, despite indications of other resistant species being capable of mechanically vectoring viable *B. anthracis*.

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[2] Maison, R. M., Priore, M. R., Brown, V. R., Bodenchuk, M. J., Borlee, B. R., Bowen, R. A., & Bosco-Lauth, A. M. (2023). Feral Swine as Indirect Indicators of Environmental Anthrax Contamination and Potential Mechanical Vectors of Infectious Spores. *Pathogens*, *12*(4), 622.

To address these knowledge gaps, we first conducted a field serosurvey in feral swine originating from regions of known anthrax endemicity, and retrospectively documented bacterial exposure using an in-house enzyme-linked immunosorbent assay (ELISA). The ELISA used herein was developed to document the presence of antibodies against *B. anthracis* protective antigen and was optimized for detecting antibodies of swine origin.

We report feral swine may serve as a biosentinel for anthrax in the United States based on comparative seroprevalence in swine taken from historically defined endemic and non-endemic anthrax regions of Texas, USA. Overall seropositivity was 43.7% (n=478), and logistic regression revealed that county status, age-class, sex, latitude, and longitude were informative for predicting antibody status. However, of these covariates, only latitude was statistically significant (β=-0.153, P=0.047). This suggests anthrax exposure in swine could serve as a proxy for bacterial presence when paired with continuous location data.

To confirm that feral swine seroconvert after exposure to anthrax bacteria, as well as evaluate their potential as mechanical vectors for infectious spores, we next performed an experimental infection study where which feral swine of known exposure status were intranasally inoculated with *B. anthracis* strain Sterne 34F2 spores, and seroconversion and bacterial shedding measured over time. Animals were intranasally exposed to either 10^4 or 10^7 colony forming units of *B. anthracis* Sterne either one or three times to examine differences between single and multiple exposure events. We report that feral swine exhibited measurable antibody responses to *B. anthracis* after intranasal exposure, and that the strength of response correlated positively with both inoculum dose and the number of exposure events experienced. We additionally report that feral swine may assist in the spread of infectious spores on the

landscape after intranasal exposure, as viable *B. anthracis* Sterne was cultured from the nasal passages of most study animals throughout the study period.

Findings from both the field serosurvey as well as experimental infection study may have significant implications for the identification of landscapes contaminated with *B. anthracis*, as well as exposure risk to more susceptible hosts. Observation of anti-anthrax antibodies in feral swine originating across regions of known endemicity suggests that anthrax exposure in swine, particularly when paired with continuous location data, could serve as a proxy for bacterial presence in some regions, and an additional tool for proactive surveillance. This proposed utility of feral swine was further confirmed after seroconversion was observed in study animals after known inoculation with *B. anthracis* Sterne. Finally, isolation of viable *B. anthracis* from the nasal passages of animals experimentally exposed suggests that feral swine may be assisting in the spread of infectious spores on the landscapes they occupy and that are contaminated with anthrax bacteria.

INTRODUCTION

Anthrax, caused by *Bacillus anthracis*, is a zoonotic disease of global importance due to its ecologic and economic impacts to wildlife and free ranging livestock, worldwide distribution, and ability to cause disease even after decades of lying dormant in the environment. *B. anthracis* is a Gram-positive, endospore-forming bacterium and cases of anthrax have been clinically described since the 1700s, although initial descriptions of the disease may include those as early as 1000 B.C.E[1,2]. Genetic studies however, suggest that the geographic origin of *B. anthracis* is sub-Saharan Africa, with subsequent environmental spread occurring after the arrival of humans and domesticated animals[3,4]. Current case report data indicate that enzootic anthrax correlates

with warmer climates, although some cases have been documented above the arctic circle, in Canada, and Northern Siberia[5]. The true incidence of disease remains unknown in many countries, although it is assumed that the bacteria reside in most regions[6]. Additionally, extensive ecological modeling efforts have allowed some predictability in spatial and temporal outbreak risks in several countries[7-10]. Of interest herein, recent modeling efforts have indicated that in the United States (U.S.), the landscapes capable of supporting *B. anthracis* span a north-south corridor encompassing most of the central U.S. as well as southwestern Texas[11].

Thought to affect all mammals to varying degrees, herbivorous species generally exhibit the highest levels of morbidity and mortality when exposed to *B. anthracis*[12,13]. Exposure in animals most frequently occurs after ingesting *B. anthracis* spores from contaminated soil, water, or vegetation, although in some environments biting flies as well as other necrophilic and hemophagic arthropods can also transmit the bacteria and result in infection[14-17]. Inhalation of aerosolized spores is also a possible route of exposure; however, limited evidence to date has supported this as a significant mode of transmission for ruminants in natural settings[18,19]. Spillover to humans most often occurs when contaminated animals or animal products are directly handled or consumed[5].

Once inside a susceptible host, bacteria undergo a transformation into the vegetative form, and pathogenesis is established with the presence of toxins and capsule, two virulence factors encoded on the bacterium's pXO1 and pXO2 plasmids, respectively[20]. Lethal factor (LF) and edema factor (EF) proteins encoded on the toxin plasmid enter host cells through the mediation of the cell receptor-binding protein protective antigen (PA) encoded on the toxin plasmid, which also enables the bacteria to produce LF and EF toxins. These toxins are

ultimately responsible for the subsequent death of the host[20-22]. Upon host death, vegetative bacilli exposed to atmospheric oxygen sporulate and contaminate the surrounding environment, where they may either stay localized or be further distributed with the assistance of arthropod or vertebrate vectors, or a variety of abiotic factors, before infection of another suitable host takes place[17,23,24]. Once sporulated, *B. anthracis* is highly resistant to environmental degradation and can survive upwards of 100 years in appropriate conditions, making environmental decontamination a difficult disease management option[4,25,26]. Humans and other animals that subsequently encounter infected carcasses and animal materials are thus at increased risk of exposure, as infected carcasses that are manipulated or opened can initiate sporulation, and consequently perpetuate the persistence of infectious *B. anthracis* on the landscape.

Despite being considered a disease of antiquity, the relative impacts of anthrax on susceptible animal populations remain largely undescribed[27]. This is particularly true for wild ruminants, as these populations are highly susceptible to fatal anthrax and are not as easily observed for clinical disease as domestic animals, making it likely that true incidence is underestimated[28]. Compounding the unknown incidence in wildlife, ongoing surveillance in highly susceptible domestic populations such as livestock is largely passive or opportunistic and is largely defined by the detection and proper disposal of animal carcasses, those often appearing to be from otherwise healthy individuals[11,29]. Preventative management, where existing, is primarily vaccine-based, although vaccination is not a requirement for livestock owners located in highly endemic regions and is instead often used as a reactionary measure to control disease spread after an outbreak has begun rather than as a preventative action[11,12,30]. Moreover, the current vaccination procedure used for livestock requires animals to be given an annual subcutaneous injection, which is not a viable strategy for managing disease in wild or free-

ranging ruminant populations. Thus, in highly endemic regions of the U.S. such as western Texas, where anthrax enzootics frequently occur, the disease is further perpetuated on the landscape and remains a problem in wild ruminants such as the white-tailed deer (*Odocoileus virginianus*)[8,17].

Humans, suids, and carnivores are considered incidental hosts, and considerably less susceptible than herbivores to lethal infection[17]. While the cause(s) of these variations in susceptibility remain largely unknown, it is likely they are a combination of differences in physiology, behavior, dosage, and transmission route[27]. For instance, carnivores, omnivores, and scavengers all have lower stomach pH than herbivores, which likely kill *B. anthracis* spores or vegetative cells incidentally ingested while foraging[12,31]. Furthermore, some evidence indicates that necrophilic and haemophagic arthropods can contribute to infection[17,32], and surveys of scavengers such as vultures have recovered viable spores from both feces and the surfaces of feathers after they have scavenged on infected carcasses, suggesting that dissemination may also differ by a region's competent vector species[5,33,34].

Given that not all animal species are equally impacted by anthrax, it has been suggested that more resistant taxa may be used as sentinels for either outbreak management or ongoing surveillance efforts, as they often exhibit a high prevalence of antibodies against the bacterium after exposure[27,35]. This trend has been observed frequently in historically endemic regions, such as Africa, where there appears to be little evidence of predators and scavengers dying of anthrax, rather those animals exhibit a high prevalence of antibodies against the bacterium[27]. Following these observations, it has been proposed previously that resistant suid species may be biosentinels for anthrax, such as the Eurasian wild boar (*Sus scrofa*) in Ukraine and feral hog in

the U.S[36]. Of note, while Bagamian and colleagues[36] described serological evidence of exposure in Ukrainian wild boar, no studies to date have formally evaluated exposure in the taxonomically identical feral swine (also classified as *Sus scrofa*) present in the U.S. Introduced initially in the 1500s to states bordering the Gulf of Mexico, populations of feral swine have exploded since the 1980s, and have become established throughout most suitable habitats in the southern U.S[37]. Additionally, pigs are known for their close associations with soil through their distinct rooting and wallowing behaviors, and therefore may be exposed to anthrax spores in contaminated soils at high rates compared to other species[36,38].

Known risks of *B. anthracis* exposure, considered together with an unconfirmed environmental distribution in most regions and unidentified or evolving epidemiological risk factors, make *B. anthracis* a pathogen of continuing human and animal health concern. Furthermore, the presence of resistant wildlife species under certain conditions may contribute to anthrax epidemiology by increasing the risk of exposure to more susceptible species and humans or helping to disseminate infectious spores to new landscapes through mechanical transmission or bacterial shedding[6,34]. Feral swine are known to be opportunistic omnivores that occasionally scavenge carcasses, as well as routinely root in soils for food[38]. These behaviors, coupled with their documented resistance to anthrax, suggest that feral swine may be a good indicator of bacterial presence on the landscapes they occupy. Moreover, pigs, and particularly feral swine, have yet to be examined for the active role(s) they may be playing in anthrax epidemiology beyond simple exposure to the bacterium, particularly if they may be vectors themselves and assist in the spread of infectious spores on the landscape. To address these knowledge gaps, we first examined antibody prevalence from confirmed endemic and non-endemic regions of Texas to determine the potential biosentinel utility of U.S. feral swine for anthrax. We next evaluated a

group of juvenile feral swine for their ability to seroconvert and shed viable *B. anthracis* spores after known intranasal exposure to empirically evaluate sentinel competency, as well as their ability to act as mechanical vectors for infectious spores.

MATERIALS AND METHODS

Development of a Rapid Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Antibodies to *B. anthracis* in Swine

Antigen Preparation

Recombinant protective antigen (rPA) from *B. anthracis* was obtained from the American Type Culture Collection (#NR-3780) as a suspension of 6.5 mg/ml, suspended in 20mM sodium phosphate buffer (pH 8.32) and 150 mM NaCl. Upon arrival, commercial antigen stock was divided into smaller aliquots (100 µl) and stored at -80°C until assay.

Serum Samples

A total of 130 serum samples were utilized for initial assay development and standardization. Sera were subdivided into four distinct groups (I to IV):

(i) **Group I (*n*=3): Samples from one domestic goat, before and after vaccination with Anthrax Vaccine Adsorbed.** Group I samples were obtained from one male domestic goat (*Capra aegagrus hircus*) prior to intramuscular vaccination with Anthrax Vaccine Absorbed (BioThrax™, #NR-2642). Additional serum samples were collected three weeks and five weeks post vaccination, for a total of three samples. Sera from this group was utilized as negative and positive assay controls.

(ii) **Group II (*n*=21): Samples from feral swine removed from confirmed anthrax-positive premises (Texas, USA).** Group II samples were collected from feral swine removed by the United States Department of Agriculture (USDA) personnel from Jim Hogg, Kinney, and Sutton counties in Texas after captive ruminants from several premises were confirmed anthrax positive during the summers of 2018 and 2019[39,40]. Three adult feral swine of mixed sex were removed and sampled from Jim Hogg County on June 5, 2018, following the confirmation of a calf on a nearby premises on June 1, 2018. Ten feral swine of mixed age-class and sex were removed and sampled from Kinney County on July 24, 2019, following confirmation of captive ruminants positive for anthrax on July 16, 2019. Approximately five months later, on December 17, 2019, eight additional feral swine of mixed sex and age were removed and sampled from Sutton County. Feral swine originating from Sutton County were captured from a ranch adjacent to one of the ten premises in the county that experienced an anthrax outbreak during the summer of 2019, and likely had home ranges overlapping the outbreak area.

(iii) **Group III (*n*=83): Samples from an isolated population of feral swine removed from a region non-endemic for anthrax (Guam, USA).** Group III samples were collected from feral swine removed by USDA personnel from Guam during normal feral swine damage management activities. Samples were collected in the years 2017-2018, from a population of feral swine of mixed age-class and sex. Guam is a region with no known reported cases of anthrax in either humans, or susceptible domestic and wild animals[40], and represents a geographically isolated population of feral swine that may be presumed seronegative.

(iv) **Group IV (*n*=23): Samples from isolated population of swine removed from a region non-endemic for anthrax (North Carolina, USA).** Group IV samples were collected from a group of Ossabaw Island breed pigs of mixed sex housed at Colorado State University (CSU) and utilized for an unrelated *Brucella suis* experimental infection study. Animals were purchased from a private producer in North Carolina in August 2018, and directly transported to CSU's animal biosafety level 3 (ABSL-3) facility, where they were housed for the duration of the study. Both North Carolina where these individuals were raised, as well as Ossabaw Island in Georgia, USA where the Ossabaw Island breed originates are regions considered non-endemic for anthrax and represents an additional population of swine that may be presumed seronegative[41]. All serum samples were collected on July 20, 2018, prior to infection with *B. suis* and were passed through a 0.22 µm filter before being taken out of the ABSL-3 for serological analysis.

Assay Optimization

Due to their inherent resistance to anthrax infection, domestic pigs are not as routinely vaccinated as other ruminant livestock species. As such, swine sera to be utilized as antibody positive and negative controls for this assay were unavailable. Instead, control sera included in each assay were obtained from a male domestic goat before and after vaccination with Anthrax Vaccine Absorbed, (Group I). Recombinant protein A/G is known to bind to the constant region of both goat and swine IgG with comparable affinity[42-44] and when used as a secondary detection antibody, allows for both swine and goat sera to be examined on the same assay.

The optimum dilutions for control sera and secondary detection antibody were determined using checkerboard titration[45]. Positive and negative goat sera were diluted in 1% blocking buffer, composed of skim milk powder suspended in sterile phosphate-buffered saline at a dilution range of 1 to 1:3200 (two-fold serial dilution starting from 1, or undiluted serum). Protein A/G-horseradish peroxidase (HRP) (Thermo-Scientific #32490) secondary antibody was diluted at a range of 1:1000 to 1:32000 in 1% blocking buffer, (two-fold serial dilutions). All serum and secondary antibody dilution pairs were tested in duplicate. Signal-to-noise (S/N) ratios were calculated at each dilution by dividing the mean absorbance value of known positive goat sera by that exhibited by negative sera and used to determine optimal assay conditions. Assay conditions resulting in S/N ratios of 10 or above were considered optimal[46] and utilized in the final assay protocol, described in the ELISA section below.

Following assay optimization for control sera, samples from Groups II, III, and IV were assayed and compared to known negative and positive sera from Group I. To confirm antibody recognition across species using protein A/G-HRP as a secondary antibody, select swine sera exhibiting high absorbance values comparable to that exhibited by positive goat sera from Group I were serially diluted at a range of 1 to 1:51200 in 1% blocking buffer (two-fold serial dilutions) and assayed again alongside serially diluted positive goat sera. Absorbance readings for each species were then plotted as a function of sample dilution to generate standard curves.

Cutoff Determination

An assay cutoff value used to distinguish between antibody positive and negative individuals was defined based on Group I sera, which contained samples from a domestic goat before and after anthrax vaccination and were of known antibody status. To conservatively

exclude ≥99.7% of seronegative members from the population of interest from being classified as seropositive, an absorbance value was therefore set at three sample standard deviations (SD) above the mean absorbance value of the seronegative control sera (e.g., +3SD above Group I pre-vaccinated goat sera). This methodology is currently accepted by the World Organization for Animal Health as a legitimate means to establish cutoff values if the diagnostic assay in use is not validated, or if known positive samples are absent for the population of interest[45,47].

ELISA

We utilized an indirect ELISA platform similar to those described previously[35,48-50], with slight modifications to target antibodies of swine origin, and optimized as described under the above Assay Optimization section. Prior to performing all ELISAs, rPA aliquots were thawed, and diluted to 5 µg/ml with carbonate buffer (0.16 g Na_2CO_3, 0.29 g $NaHCO_3$, 100 ml distilled water, [pH 9.6]).

High-binding polystyrene 96-well flat-bottom microtiter plates (Thermo-Scientific, #3455) were coated with rPA from *B. anthracis* diluted in carbonate buffer solution at a concentration of 5 µg/ml per well, and incubated overnight at 4°C. The following day, coating buffer was discarded, and wells washed five times with sterile phosphate-buffered saline (PBS) containing 0.05% Tween 20 (washing buffer). Wells were blocked with the addition of 300 µl of 10% skim milk in PBS and allowed to incubate for 1.5 hours at room temperature. Wells were again washed, followed by the addition of 100 µl of test serum diluted 1:100 in 1% blocking buffer, and incubated for an hour with shaking at room temperature. Following additional washing, 100 µl/well of protein A/G-HRP diluted 1:1000 in blocking buffer was added, and plates further incubated with shaking for 30 minutes. After one final washing step, 150µl of 1-

step ABTS (Thermo-Scientific, #37615) was added, incubated for 15 minutes, and then stopped by the addition of 100 µl of 1% sodium dodecyl sulphate solution. Absorbance was measured at 25°C and 405nm using a microplate reader (BioTek #258653) paired with Gen5 microplate reader and imager software (version 3.09). Samples were considered positive for rPA antibody if their mean absorbance measurements were greater than three times the SD above the mean of the negative control. Individual samples were run in triplicate.

Field Serosurvey of Texas Feral Swine

Study Area

We conducted our investigation in Texas, as anthrax is a reportable disease and is relatively predicable in select regions of the state. Feral swine populations are also present in most counties, offering a unique opportunity to evaluate the species as a biosentinel for *B. anthracis*. Additionally, observations by Texas locals within the state's endemic region have described resurgences of anthrax in areas recently colonized by feral swine, anecdotally suggesting the two events may be related.

Colloquially referred to as the "Anthrax Triangle", regular outbreaks of anthrax occur in portions of Crockett, Edwards, Kinney, Maverick, Sutton, Uvalde and Val Verde, counties, usually in dry summer months following heavy spring rains[51,52]. Conversely, the eastern region of the state does not experience regular outbreaks, despite also being heavily populated with domestic livestock[53]. Furthermore, populations of ranched white-tailed deer in areas of Val Verde, Uvalde and Webb counties are also regularly affected, suggesting wild herbivores in the same region may succumb at similar rates. Here we binarily defined "endemic" anthrax areas to be those counties within the historic Anthrax Triangle on the western side of the state, as

depicted in Figure 2.1. Conversely, we defined "non-endemic" regions simply as those outside of this region, as these counties do not experience the regular, seasonal cases often described in the Anthrax Triangle.

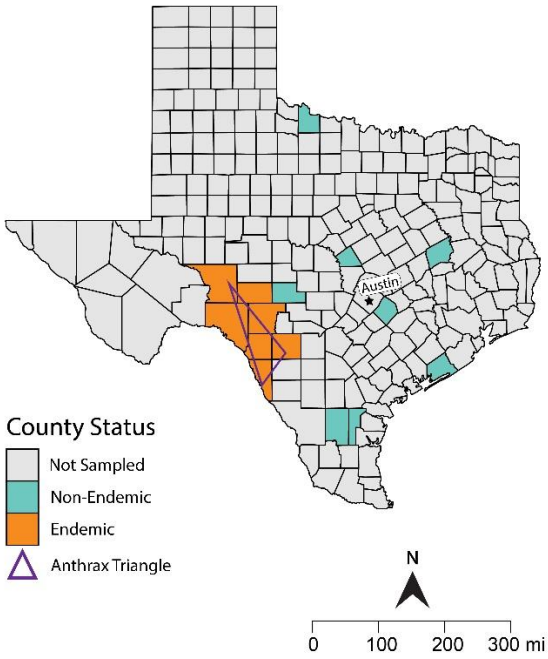

Figure 2.1: Field sampling designations for feral swine sera collected in Texas, USA. The Anthrax Triangle illustrates the region that experiences semi-regular outbreaks of anthrax in both domestic and wildlife species.

Field Sampling

Wildlife Services (WS), a branch within the USDA, routinely remove feral swine off the landscape for damage control and invasive species management, and as part of these efforts, collect samples off a subset of individuals for disease surveillance. Samples not utilized for routine surveillance are archived and can be employed for select retrospective studies. Through these efforts, 478 serum samples obtained from feral swine removed throughout Texas were tested to determine the prevalence of feral swine exposed to *B. anthracis* by measuring

antibodies against PA. The geographic origin of feral swine sera studied here are illustrated in Figure 2.1, and further detailed in Table 2.1. Of note, due to many samples being collected on private property, spatial data is illustrated at the county level to protect personally identifiable information. Sera were collected from 2007-2019, with approximately half of samples originating from seven counties within the endemic Anthrax Triangle (n=243), and half from seven counties considered non-endemic for anthrax (n=235). The seven counties considered non-endemic were randomly selected out of the 246 counties residing outside of the Anthrax Triangle; seven were selected so that the sampling effort was equal between endemic and non-endemic regions. Sampling events took place year-round.

Sera were taken from male and female feral swine classified as either adults or sub-adults based on age estimates by WS field personnel (Table 2.1). Sub-adults were defined as being between 2 months and 1 year of age, while adults were any individual estimated to be over 1 year of age. Samples were not collected from juveniles to avoid confounding serology that could result from the presence of maternal antibodies[54]. All blood samples were collected post-mortem, and serum extracted within 12 hours of clotting and shipped overnight on ice to the National Wildlife Research Center in Fort Collins, Colorado, where they were stored at -80°C until testing.

Table 2.1: Sampling distribution of feral swine sera collected from endemic and non-endemic regions of Texas, USA.

	Sex				
	Male		Female		
Region	Adult	Sub-Adult	Adult	Sub-Adult	**Total**
Endemic	90	16	121	16	243
Non-endemic	113	12	101	9	235
Total	203	28	222	25	478

Serology

All serum samples were thawed on the day of assay and tested for rPA antibodies as described in the ELISA section above. All samples were blindly assayed relative to the origin, sex, and age-class of individual animals until all results were finalized.

Statistics

We examined how the probability of an individual being positive for anthrax antibodies varied by region (endemic vs. non-endemic), sex, age-class (adult vs. sub-adult), latitude, and longitude using logistic regression and mixed-effects models implemented in program R (version 4.0.2)[55]. Region, sex, age-class, latitude, and longitude were examined as fixed effects and sampling year evaluated as a random effect to account for temporal variation in anthrax prevalence and sampling. Since most anthrax cases in Texas originate from the Anthrax Triangle[51,52], region was included as a fixed effect to evaluate whether feral swine residing in known contaminated environments are more likely to be antibody positive than those outside. County centroids were used as a proxy for sampling locations and latitude and longitude were

considered fixed effects to account for spatial trends in anthrax prevalence. Interaction between age-class and sex was also examined to account for potential impacts of age varying by sex.

Support for the inclusion of a random effect (sampling year) was evaluated using Akaike's Information Criterion (AIC) and a likelihood ratio test (LRT; stats package)[55]. As recommended by others[56], we first examined if sample year should be included by comparing a fully parameterized fixed effects model with and without its addition. If the random effect was supported (Δ AIC > 2 compared to the model excluding the random effect), then it was kept in all models and the fixed effects compared. We used LRT as an additional method of evaluating the inclusion of sampling year. The difference in the log likelihoods of the two nested models (i.e., fully parameterized fixed effect model with and without the addition of the random effect) was calculated and if the difference was statistically significant ($\alpha = 0.05$), then the random effect was included in all models.

We compared all combinations of fixed effects covariates using AIC implemented in R package MuMIn[57], the lowest AIC value representing the most parsimonious model. If model uncertainty existed (i.e., more than one competing model within 2 Δ AIC of the top model) we examined the relative support for each covariate by calculating cumulative covariate weights; weights over 0.5 were considered supported[58]. The final model was selected based on the supported covariates regression coefficients used to calculate odds ratios (OR), and 95% confidence intervals for the probability of having anthrax antibodies by covariate. Finally, to assess model fit we calculated Area Under the receiver operator Curve (AUC)[59] using the pROC package[60]; the receiver operator curve (ROC) allowed for performance assessment of the binary classification model identifying individuals as positive or negative. To summarize the ROC

curve, we calculated the AUC - an aggregated measure of the binary classification model performance in which the AUC for a model with no predictive power = 0.5, poor predictive power = $0.5 < AUC < 0.7$, acceptable predictive power = $0.7 \leq AUC < 0.8$, and excellent predictive power = $0.8 \leq AUC < 0.9$ [61].

Experimental Inoculation of Juvenile Feral Swine with *B. anthracis* Sterne

Animals

Wild-caught juvenile feral swine of mixed-sex were captured in Burnet County, Texas, before being transported to CSU. Feral swine sounders were trapped by USDA personnel in Texas using baited corral traps from April 25-27, 2021 during routine feral swine damage management operations. Juvenile swine were preferentially selected from sounders to minimize the chances of previous exposure to anthrax. Juveniles were defined as less than 2 months of age, and age was estimated based on the absence of incisors and permanent canines on the lower jaw[62]. Upon capture, individuals were selected based on these criteria and loaded onto a USDA-designated trailer, wherein they were bed on straw, provided water and food ad libitum and transported overnight to Colorado. Animals were then group-housed in an ABSL-3 facility at CSU, in two rooms (12 ft. x 18 ft) with natural lighting and controlled climate. Swine had access to food and water ad libitum throughout the acclimation and study period.

Animals were allowed to acclimate for six days before being given a thorough physical examination, where an initial blood sample was taken, and individuals screened externally for ectoparasites, sexed, and implanted with thermally sensitive microchips (Lifechips, Destron-Fearing) for identification and temperature measurement. Due to significant numbers of ectoparasites being observed at this time, a thin application of deltamethrin insecticide (Delta

Dust, Bayer) was also applied to the coat of each pig during the initial exam. Blood samples were collected and then examined by ELISA as described in the Materials and Methods section, to screen for previous or recent exposure to *B. anthracis*. Fifteen confirmed seronegative pigs were randomly assigned to one of five study groups (n=3 per group), summarized in Table 2.2. Depending on group assignment, pigs were challenged with *B. anthracis* Sterne spores with either 10^4 CFU/ml or 10^7 CFU/ml, and experienced either one or three inoculations. Three pigs were designated as positive serological controls and were given a single subcutaneous dose of the standard livestock vaccine, equating to 1 ml and 10^4 CFU Sterne spores. Pigs were housed such that individuals experiencing equal numbers of exposures were housed together (Table 2.2). This was done to minimize cross-contamination and ensure that individuals were only exposed to the inoculum level desired. All methods and use of animals described here were performed with CSU's Institutional Animal Care and Use Committee approval (Protocol #1535).

Table 2.2. Study group assignments for juvenile feral swine, exposed to varying amounts of *Bacillus anthracis* Sterne spores. i.n, intranasal; s.c, subcutaneous.

Animal ID	Sex	Times Exposed	Inoculum Dose (CFU/ml)	Route of Inoculation	Animal Room
7634	F	1	10^4	i.n	1
7635	F	1	10^4	i.n	1
7637	F	1	10^4	i.n	1
7618	F	1	10^7	i.n	1
7641	F	1	10^7	i.n	1
7636	M	1	10^7	i.n	1
7619	F	1	10^4	s.c	1
7621	F	1	10^4	s.c	1
7633	M	1	10^4	s.c	1
3915	F	3	10^4	i.n	2
3911	M	3	10^4	i.n	2
3920	M	3	10^4	i.n	2
3913	F	3	10^7	i.n	2
3912	M	3	10^7	i.n	2
3919	M	3	10^7	i.n	2

Spore Preparation

One 10 ml vial of commercial Anthrax Spore Vaccine (Serial #471, Vet License No. 188) containing *B. anthracis* Sterne 34F2 was acquired from Colorado Serum Company. To prepare a new working stock, 40-100 µl of the vaccine was streaked onto a chocolate agar plate using a sterile inoculating loop. After overnight incubation at 37°C, the resulting vegetative *B. anthracis* Sterne was then sporulated as described previously, to prepare a stock of inoculum[63]. Briefly, one colony from the overnight plate culture was inoculated into 10 ml of pre-warmed 2X SG medium[64] and incubated on a shaker at 37°C. A 2.5 ml sample of this preculture was then added to 100 ml of pre-warmed 2X SG medium and shaken at 37°C for 24 hours. After the 24-hour incubation, 400 ml of double-distilled water was added to the culture, and incubation with shaking resumed for an additional 40 hours. After 40 hours, the culture was transferred into three sterile Oakridge tubes and centrifuged in a Sorvall Superspeed centrifuge with an SS-34 rotor at 4-10°C and 4,000 rpm for 20 minutes. The resulting pellets were then washed once with PBS (50 ml per tube) and centrifuged again for 20 minutes at 4,000 rpm before being combined and resuspended in 40 ml sterile PBS. The final spore suspension was then heated to 68°C for 40 minutes to kill any residual vegetative cells present in solution. The final spore solution was then transferred into 1 ml aliquots and stored at -80°C. Stock titer was determined to be approximately $10^{6.9}$ CFU/ml after serial dilution onto Polymyxin B-Lysozyme-EDTA-Thallous acetate agar (PLET) plates (Sigma, #55678).

Animal Challenge

Prepared aliquots of *B. anthracis* Sterne spores were thawed immediately prior to animal challenge and diluted in sterile PBS to concentrations of either 10^4 CFU/ml or 10^7 CFU/ml.

Notably, since the estimated titer of the bacterial stock was slightly lower than that required for the high dose group, inoculum volume for the high dose groups was raised slightly from 1 ml to 1.2 ml to allow for the desired dose to be administered. Diluted *B. anthracis* Sterne spores were then administered to the nares of twelve individuals using a pipette (500µl per nare for low dose groups, and 600µl per nare for the high dose groups) under manual restraint. Three individuals serving as positive controls were given 1 ml of the Anthrax Spore Vaccine subcutaneously as recommended by the manufacturer. For those groups experiencing three exposures, inoculum was prepped twice more as described above and administered once every other day until three total doses were administered.

Sampling and Clinical Observation

We evaluated all feral swine for seroconversion as well as bacterial shedding in nasal passages following single and repeat exposures to *B. anthracis* Sterne. Both blood and nasal swab samples were collected from all individuals on 14-, 28-, 42-, and 56-days post-inoculation (DPI); however, animals that experienced one intranasal exposure event were only observed to 28 DPI before being euthanized. Removing these individuals from the study at 28 DPI was determined based on the average time the vertebrate adaptive immune system takes to mount a complete response to antigen that's introduced to the system for the first time being between 1-2 weeks[65]. Blood (3-5 ml into serum separator tubes) was collected from the anterior vena cava vein under manual restraint, and serum extracted within two hours of collection. All sera were passed through a 0.22 µm filter before being taken out of the ABSL-3 and stored at -80°C pending serological analysis. Nasal swabs were collected by rotating a standard polyester swab (Puritan, #25-806) inside the nasal passage for five seconds while under manual restraint. Swabs

were then broken off into tubes containing 1 ml of PBS with 20% glycerol and stored at -80°C pending cultivation, DNA extraction from suspicious colonies, and confirmation by PCR.

All animals were otherwise observed daily and included an assessment of temperament and the presence or absence of clinical signs of disease, including but not limited to ocular and nasal discharge, ptyalism, coughing/sneezing, dyspnea, diarrhea, lethargy, anorexia, and moribund status. Body temperatures were also observed during days of sample collection when animals were being handled.

Serology

We evaluated all feral swine sera for the presence of anti-anthrax antibodies by ELISA as described in the Materials and Methods section.

Nasal Swab Processing

All nasal swab samples were thawed completely before being placed in a 65°C water bath for 30 minutes to preselect for endospore-forming bacteria[66]. Tubes were then vortexed briefly, and 100 µl of swab media plated onto PLET plates. Plates were then incubated for 18-24 hours before being observed for colonies with characteristic *B. anthracis* morphology[67].

DNA Extraction and PCR

DNA extraction was performed by suspending one colony in 100 µl of TE buffer (10 mM Tris-HCl [pH 7.6], 1mM EDTA [pH 8]) and heating at 95°C for 20 minutes. Multiplex PCR was performed using Thermo Fisher Scientific Platinum Multiplex PCR Master Mix (Cat. #4454268), and capsule (*cap*) and protective antigen (*pag*) primers described previously[68]. A total volume of 25 µl reactions containing 0.2 µM of *cap* and 0.05 µM *pag* primer sets and 1 µl

of template DNA was used. The PCR program consisted of an initial denaturation at 94°C for 1 minute, followed by 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 58°C for 2 minutes, extension at 72°C for 2 minutes; the program was completed with a final extension at 72°C for 10 minutes. Amplified products as well as 1-kilobase ladder (Invitrogen, #10787018) were then loaded onto a 1% agarose gel stained with ethidium bromide and electrophoresed for 1.5 hours at 100 V. Each PCR assay contained three control strains, each exhibiting unique pXO1/pXO2 profiles for quality assurance and are detailed in Table 2.3. Products were considered positive for *B. anthracis* Sterne if they exhibited a band profile consistent with the plasmid content for *B. anthracis* Sterne, constituting a band of the appropriate size for *pag* (364 bp), and no band presence for *cap* (578 bp).

Table 2.3. Plasmid characteristics and associated references for bacterial strains used as controls for multiplex PCR.

Species	Strain [Reference]	Plasmid Content	
		pag	*cap*
Bacillus cereus	UW85 [69]	-	-
Bacillus anthracis	Sterne 34F2 [70]	+	-
Bacillus anthracis	1075.4 [71]	+	+

RESULTS

Development of a Rapid ELISA for Detection of Antibodies to *B. anthracis* in Swine

Assay Optimization and Cutoff Determination

Following checkerboard titration of Group I control sera, the optimal sample sera and secondary antibody concentrations were determined to be 1:100 and 1:1000, respectively. The corresponding S/N ratio calculated from this concentration pairing was 14.

Sample absorbance exhibited by samples from each group utilized during assay development is illustrated in Figure 2.2. The mean absorbance values for Groups I-IV varied, and were 1.12 (SD=1.26), 2.20 (SD=1.64), 0.047 (SD=0.048), and 0.045 (SD=0.038) absorbance units, respectively. Notably, since Group I sera included antibody negative and positive samples, values were further delineated into pre-vaccination and post-vaccination status to compare absorbance values before and after seroconversion (Figure 2.2). Negative control goat serum collected prior to vaccination exhibited absorbance readings ranging between 0.018 and 0.113 (mean=0.08, SD=0.022). Pooled positive serum taken at both 3- and 5-weeks post anthrax vaccination exhibited an absorbance range of 0.26-3.42 (mean=1.62, SD=1.27). The assay cutoff of +3SD above the mean of the negative controls was calculated to be 0.15 absorbance units.

When compared to Group I pre-vaccination sera, both Group I post-vaccination sera and Group II sera displayed absorbance values that were significantly higher ($p<0.0001$, Figure 2.2). Groups III and IV exhibited mean absorbance values comparable to Group I pre-vaccination sera and did not exhibit a statistically significant difference ($p=0.99$, Figure 2.2).

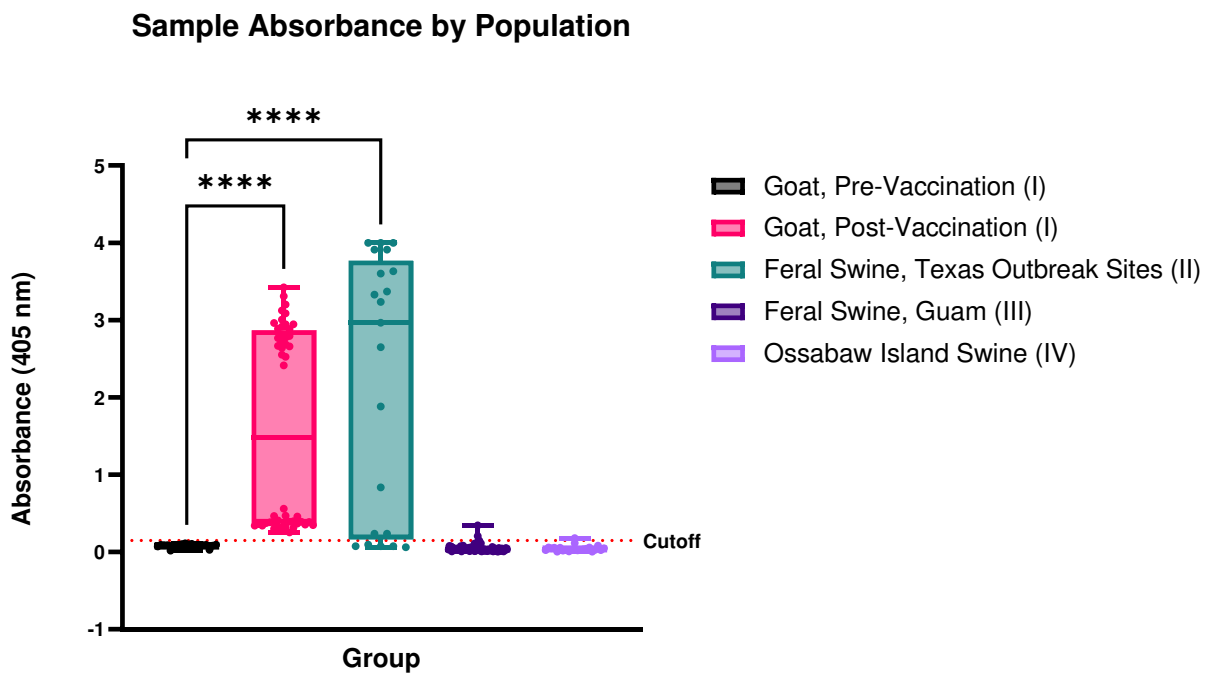

Figure 2.2. Sample absorbance measured at 405 nm for Groups I-IV by ELISA (n=130). Corresponding group number for each population sampled are in parentheses next to group description in the graph legend (I-IV). For more detailed definitions of each group, see Materials and Methods section of text. The dotted red cutoff line represents the calculated assay cutoff between seropositive and seronegative individuals (e.g, +3SD above the mean of the negative control), equal to 0.15 absorbance units. Asterisks represent statistical significance as determined using a one-way ANOVA with Dunnett's test for multiple comparisons, with each population compared to the known seronegative population, or Group I pre-vaccinated goat sera (p<0.0001).

Following the trends observed at the population level for each group of interest as well as the establishment of an assay cutoff, it was presumed that most samples from Group II were positive for rPA antibody (Figure 2.2). To rule out that the observed seropositivity in Group II sera wasn't due to non-specific binding of protein A/G-HRP and confirm antibody recognition across species, standard curves were generated and compared for post-vaccination goat sera and swine sera from Group II identified as positive and are illustrated in Figure 2.3. Sera from each group were serially diluted at a range of 1 to 1:51200 in 1% blocking buffer (two-fold serial dilutions) and assayed by ELISA as described in the Materials and Methods section. The feral

swine sera examined during this experiment was collected from a juvenile female swine removed from Kinney County, Texas in July 2019, and is a representative sample of sera from Group II identified as positive during assay optimization (Figure 2.2). This positive feral swine sample displayed an average absorbance value of 2.97 when examined during assay optimization.

Post-vaccination goat serum undiluted and diluted 1:51200 displayed absorbances ranging from 3.90 to -0.001, respectively (Figure 2.3). In comparison, feral swine serum examined in the same conditions exhibited absorbance readings ranging from 3.66 to 0.02 when diluted 1:100 to 1:51200 respectively. Notably, the feral swine serum examined during generation of standard curves exceeded the range of the ELISA at undilute and 1:50 dilutions, as absorbance values were too high to be registered at 405 nm; therefore, the standard curve generated for the feral swine sera examined starts at a dilution of 1:100 and ends at 1:51200 (Figure 2.3). Despite the missing values for feral swine serum at lower dilutions, the standard curves generated for both species displayed a similar trend of decreased magnitude of absorbance after two-fold serum dilution. At all dilutions, feral swine serum displayed higher absorbance values than goat serum, and on average were 0.49 absorbance units higher (Figure 2.3). Pearson correlation coefficient (r) of 0.99 was calculated between the two curves, indicating a highly positive correlation.

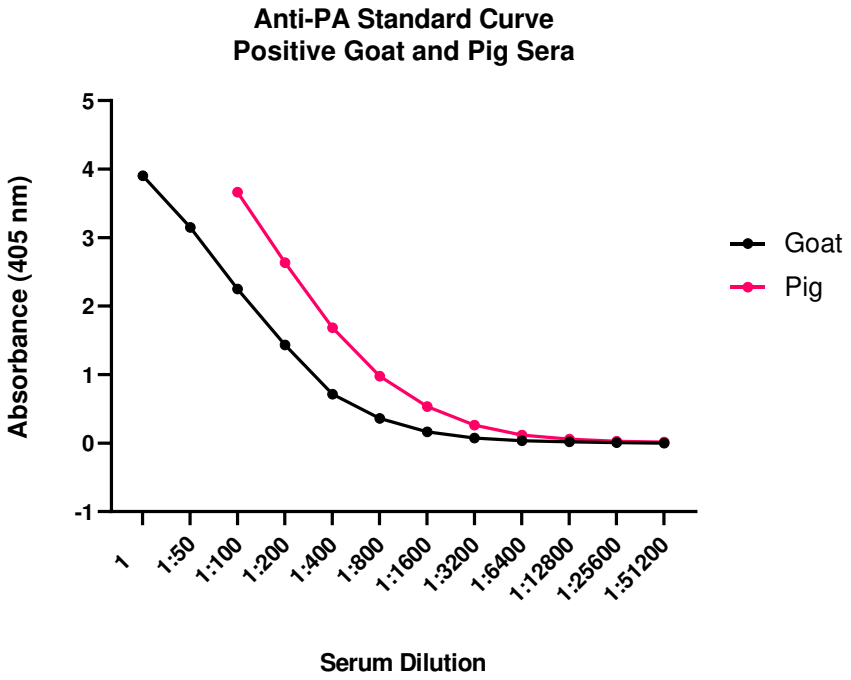

Figure 2.3: Standard curves for goat and pig serum, on indirect ELISA. Goat sera was collected from an AVA-vaccinated goat, 5 weeks post vaccination. Pig sera illustrated here was collected from a juvenile female feral pig removed from Kinney County, Texas in July 2019, and presumed positive after initial screening under optimized assay conditions. Serum from the individual feral swine examined here is representative of seropositive individuals identified in Group II during assay optimization.

Field Serosurvey of Texas Feral Swine

Serology

Of the 478 samples examined, 209 (43.7%) were identified as positive and 269 (56.3%) as negative for anti-PA antibodies. Minimum and maximum absorbance values recorded for the entire sample pool were -0.006 and 3.9, respectively.

Statistics

Basic data structure including anthrax antibody status stratified by covariate and apparent seroprevalence is described in Table 2.4. Raw data revealed that more swine from the endemic region were seropositive (49.49%) compared to the non-endemic region (37.45%); individual sample absorbance by region is further illustrated in Figure 2.4. Seroprevalence was also higher in female swine (48.18%) than in males (38.96%) and more adult swine were seropositive (44.71%) than sub-adults (35.85%). The fully parametrized model failed to converge; therefore, we used the fully parameterized model without longitude and the interaction term (age-class*sex) to evaluate the inclusion of sampling year as a random effect. Sampling year did not improve the predictive power of the model (fixed effects model AIC = 649.87 and mixed-effects model AIC = 648.59; LRT $p = 0.070$); probability of an individual being seropositive was thus best explained by a fixed effects model. There was model uncertainty (7 models were within 2 Δ AIC); therefore, cumulative covariate weights were examined to determine their relative importance. Based on cumulative covariate weights, county status, age-class, sex, latitude, and longitude were informative for predicting antibody status, and thus included in the final model. Odds ratios and 95% confidence intervals were calculated for each predictor variable (Table 2.5); however, only latitude was statistically significant ($\beta = -0.153$, $p = 0.047$). The final model had poor predictive ability (AUC = 0.613) suggesting the presence of unexplained variance in anthrax antibody status.

Table 2.4: Distribution of anthrax seroprevalence in feral swine by region, sex, and age-class.

Predictor Variable	Level	n	Positive	Apparent Seroprevalence (%)
Region	Endemic	243	121	49.49
	Non-endemic	235	88	37.45
Sex	Male	231	90	38.96
	Female	247	119	48.18
Age-class	Sub-adult	53	19	35.85
	Adult	425	190	44.71

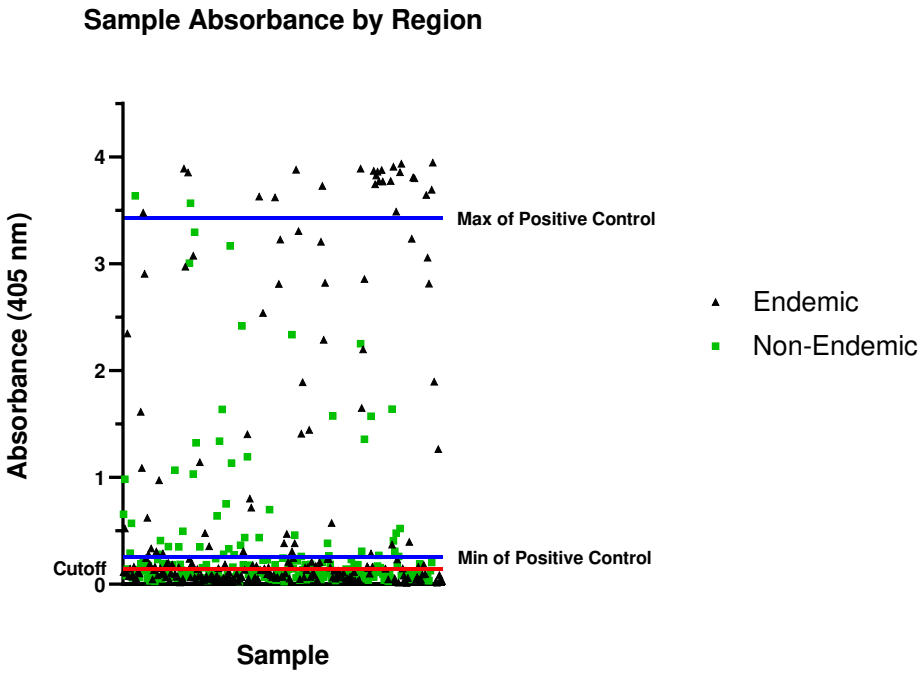

Figure 2.4: Sample absorbance values measured at 405 nm for feral swine sera collected from defined endemic and non-endemic regions of Texas, USA by ELISA (n=478). The red cutoff line represents the calculated assay cutoff between seropositive and seronegative individuals (e.g, +3SD above the mean of the negative control), equal to 0.15 absorbance units. Blue lines delineate the absorbance unit range of the positive assay control.

Table 2.5: Odds ratios and 95% confidence intervals of the probability of having anthrax antibodies by fixed effects covariates.

Covariate	Odds Ratio	95% Confidence Interval
County Status - Endemic	1.035	0.523- 2.054
Age Class - Adult	1.641	0.903- 3.059
Sex - Female	1.398	0.966- 2.026
Latitude	0.858	0.737-0.997
Longitude	0.877	0.702-1.092

Experimental Inoculation of Juvenile Feral Swine with *B. anthracis* Sterne

Clinical Observation

None of the feral swine displayed any outward signs of clinical disease following exposure to *B. anthracis* Sterne intranasally or subcutaneously and maintained stable body temperatures throughout the study period. Moreover, none of the study animals displayed any abnormal behavior(s) compared to the acclimation period.

Serology

Serological results as determined by ELISA are summarized by group in Figure 2.5. All study groups, except those experiencing the lowest exposure does of 10^4 CFU/ml administered once intranasally, seroconverted and displayed anti-PA antibodies, which are depicted as absorbance values above the assay's cutoff of 0.15 absorbance units (Figure 2.5). All other intranasally exposed pigs that did seroconvert exhibited antibody levels and seroconversion rates that positively correlated with their level of exposure. Pigs inoculated with 10^4 CFU/ml exhibited less of a response than those inoculated with 10^7 CFU/ml, and animals experiencing a single exposure being lower than those experiencing three exposures. Pigs in the positive control group who were given the standard livestock vaccine of 10^4 CFU/ml subcutaneously displayed the highest absorbance values throughout the study period and seroconverted the fastest. Antibody

levels in most pigs peaked at 28 DPI and, in most individuals that were kept beyond 28 DPI, started to decline at similar rates between 42 and 56 DPI. The two exceptions to these were pigs in the intranasal 10^4 CFU/ml group exposed three times, and those in the 10^7 single exposure group, who both peaked in absorbance values at 42 and 28 DPI, respectively.

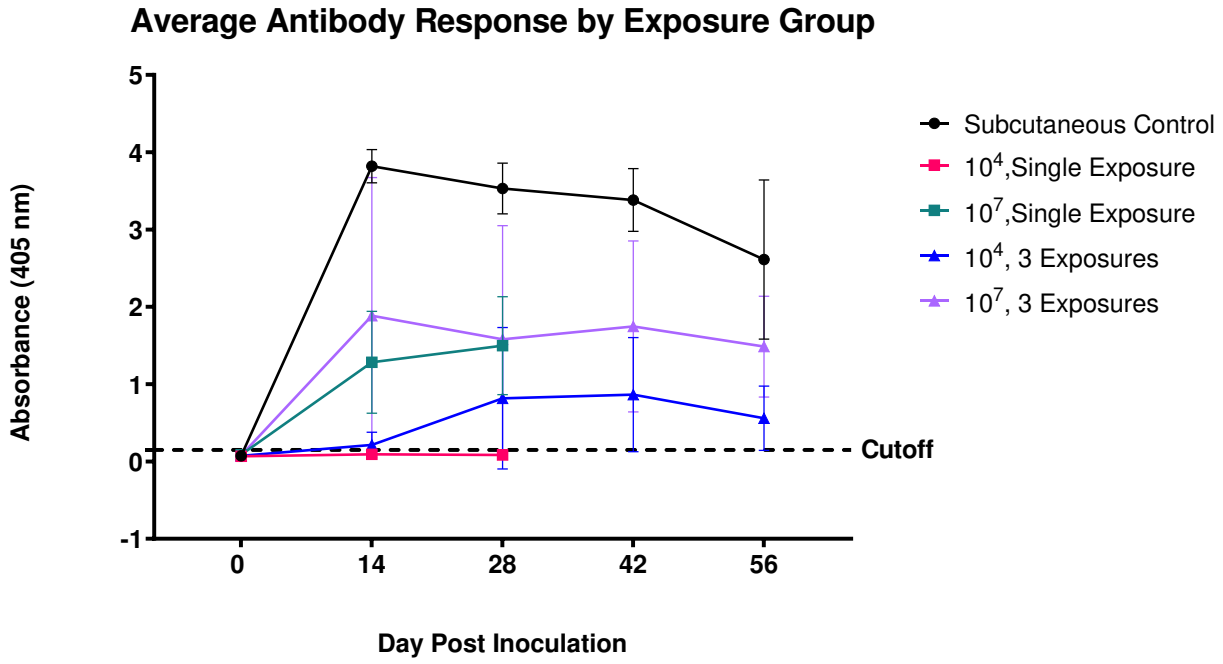

Figure 2.5. Average antibody response in feral swine exposed to various levels of *B. anthracis* Sterne 342 spores over time ± standard deviation for each group. "Subcutaneous Control" refers to swine that were given the standard livestock vaccine for *B. anthracis*, equating to 1 ml of 10^4 CFU/ml of *B. anthracis* Sterne administered once subcutaneously. The dotted cutoff line represents the assay cutoff between seropositive and seronegative animals (e.g., +3SD above the mean of the negative control sera) equal to 0.15 absorbance units.

Nasal Swab Culture and PCR

The results of nasal swab culture and subsequent colony PCR confirmation performed on all intranasally exposed pigs are depicted in Table 2.6. *B. anthracis* Sterne was successfully isolated from the nasal passages of the majority (10/12) of pigs at one or more time points post-

inoculation. For single exposed individuals kept only until 28 DPI, three of six had detectable *B. anthracis* Sterne colonies out to 28 DPI, one in the 10^4 and two in the 10^7 CFU/ml exposure groups; the other three pigs were negative at the time of euthanasia. Regarding the groups exposed multiple times, all six pigs had positive cultures on 14 and 28 DPI. The majority (5/6) of pigs that experienced multiple exposures were positive on 42 DPI, and 2/6 were positive on 56 DPI, one exposed to 10^4 and one 10^7 CFU/ml. While most pigs that displayed positive cultures post-exposure were positive on 14 DPI and either stayed positive or became negative at the next time point, three individuals in single exposure groups (#7634, 7641, and 7636) were negative during this initial sampling point, and instead became positive on 28 DPI. Relative bacterial loads within swab samples also were correlated with exposure level, as more Sterne colonies were observed from samples taken from individuals exposed to higher inoculum doses, as well as those exposed multiple times (Table 2.6). For most swine, bacterial load from nasal swabs also declined substantially over time, with individuals experiencing between 4 and 100-fold reductions in colony counts between time points. Exceptions to this trend were three pigs in single exposure groups, who exhibited Sterne on 14 DPI swab cultures, but either 10 CFU/ml (#7634 and 7641), or 20 CFU/ml (#7636) on 28 DPI. Additionally, one individual (#3911) in the low dose, multiple exposure group experienced a slight increase in bacterial load between 42 and 56 DPI, with 10 CFU/ml detected on 42 DPI, and 30 CFU/ml on 56 DPI.

Table 2.6. Results of nasal swab culture by study group, intranasally exposed swine.

			Nasal Swab Culture[1] +/-, (CFU/ml)			
Animal ID	**Inoculum Dose (CFU/ml)**	**Times Exposed**	**D14**	**D28**	**D42**	**D56**
7634	10^4	1	-	+ (10)	NA	NA
7635	10^4	1	-	-	NA	NA
7637	10^4	1	-	-	NA	NA
7618	10^7	1	+ (80)	-	NA	NA
7641	10^7	1	-	+ (10)	NA	NA
7636	10^7	1	-	+ (20)	NA	NA
3915	10^4	3	+ (220)	+ (50)	+ (50)	-
3911	10^4	3	+ (TN)	+ (60)	+ (10)	+ (30)
3920	10^4	3	+ (390)	+ (110)	-	-
3913	10^7	3	+ (670)	+ (180)	+ (20)	-
3912	10^7	3	+ (270)	+ (20)	+ (80)	-
3919	10^7	3	+ (110)	+ (160)	+ (50)	+ (10)

[1] "+" Indicates *B. anthracis* Sterne was detected after nasal swab culture and confirmed by PCR. "-" Indicates no detectable *B. anthracis* Sterne was isolated. NA: Not applicable. Colony forming units of *B. anthracis* Sterne detected per ml of swab media are indicated in parenthesis. TN: Too numerous to estimate.

DISCUSSION

Development of a Rapid ELISA for Detection of Antibodies to *B. anthracis* in Swine

We chose to develop an indirect ELISA to measure anti-anthrax antibodies in feral swine sera because of the platform's high throughput and relatively short performance times compared to other serologic tests, making it an attractive option for use in future surveillance efforts. Additionally, when optimized, ELISAs are relatively simple to perform, and depending on the materials and reagents utilized, can be extremely cost-effective when screening large quantities of samples[72].

The results obtained during assay development and optimization demonstrate that anti-rPA antibody can be detected in post AVA-vaccinated goat sera, as well as in feral swine sera

collected from individuals removed in and around properties that experienced known anthrax outbreaks in domestic livestock (e.g, Groups I and II). Conversely, pre-vaccinated goat sera (also Group I), as well as most feral swine and Ossabaw swine removed from Guam and North Carolina respectively (e.g., Groups III and IV), were seronegative. Most results from Groups II-IV are unsurprising given that anthrax is observed regularly in Western Texas, while considered non-endemic in both Guam and North Carolina; the sparse human case reports of anthrax in North Carolina that have occurred to date have been attributed to occupational exposure in textile mills and were traced back to imported herbivore materials that were contaminated[73,74]. Despite most of Groups III and IV being seronegative, however, one Ossabaw pig and five Guam feral pigs exhibited absorbance readings higher than that of the assay cutoff of 0.15 absorbance units, and therefore considered seropositive. Of note, the Ossabaw swine identified as positive exhibited an absorbance of 0.181, while the five Guam samples exhibited readings of 0.152, 0.2, 0.205, 0.247, and 0.346. While above the binary assay cutoff value, most of these readings lay in between the maximum absorbance value of the pre-vaccinated goat sera (0.113) and the minimum value of post-vaccinated goat sera (0.255), with the exception being the Guam sample with an absorbance value of 0.346. If using a more continuous scale for results interpretation, samples displaying absorbances above the assay cutoff but between those of the assay's negative and positive controls may instead be considered "suspect" rather than "positive" in some instances, as they lay in between the values displayed by the known positive and known negative serum of Group I. Alternatively, it's possible these seropositive samples may be false positives due to non-specific antibody binding. Despite the potential for false positives, these individuals may have also been exposed to *B. anthracis* in the environment(s) they've encountered throughout their lifetime and are true positives; this may be the case for the swine populations

examined here, as their complete exposure history is unknown, being free-ranging animals. Moreover, the large geographic range identified for *B. anthracis* to date does not preclude the possibility of the organism existing on some level in these anthropogenically defined "non-endemic" regions; that is, environmental contamination may exist at such a level as not to be detectable by traditional case reporting.

Despite the use of similar ELISA protocols described here to measure antibodies against anthrax PA in various wildlife species[35,48-50], the methodology used here is not without some limitations. First, the use of control sera from a genus unlike than that of the population of interest meant that there were restrictions in the detection antibody that could be used; instead of utilizing a more targeted anti-swine IgG, Protein A/G-HRP was chosen so that antibody detection was conserved to both swine and goat immunoglobulin. Use of a more generalist secondary antibody may have skewed results such that more or less antibody was detected in control and test sera. Additionally, existing differences in the individual binding affinities of both Protein A and Protein G have been documented between immunoglobulins of different species[75,76] and may produce different serological results when examined individually across different taxa. Despite this, the recombinant form of Proteins A and G combined offer a unique ability to circumvent some of these differences. Proteins A and G when isolated and used alone are known to differ in their relative affinity to immunoglobulins across species, however when the recombinant form of these two proteins is utilized (e.g, Protein A/G), the binding affinity between swine and goat IgG exhibit more similar characteristics, with comparably strong binding exhibited for both species. This is due to the recombinant form producing a ligand with a broader range of interactions than either parent protein alone[44]. This phenomenon was further confirmed upon observation of a Pearson correlation coefficient of 0.99 between the standard curves of positive goat and suspect

positive swine sera. This relatively high coefficient indicates a high positive correlation between the absorbance values measured between goat and swine sera, and suggest conservation of anti-PA antibody recognition, despite minor differences in absorbance magnitude between the two species. Finally, the use of multiple serological tests may have further helped to validate the serology presented here, however it has been demonstrated previously that the bulk of the humoral immune response against anthrax is to PA, and higher anti-PA antibody titers are correlated with increased levels of protection against disease[77]. Moreover, other studies examining the relative strengths of *B. anthracis* immunogens have illustrated PA to be the strongest, while LF and EF considered relatively moderate immunogens[77]. Finally, PA has been documented to be highly specific for *B. anthracis*[5,78], suggesting antibody responses against the antigen to be indicative of true exposure to anthrax bacteria. For these reasons, we believe the false positive rate due to antigen selection to be negligible, and only evaluated exposure by measuring humoral response against rPA in swine.

Field Serosurvey of Texas Feral Swine

Serologic surveillance in various resistant species have assisted wildlife managers and health officials in identifying areas of high outbreak risk[27] and the surprisingly high seroprevalence to anthrax we identified in feral swine support this strategy. *B. anthracis* spores are present in soil and carcasses of animals that have died from anthrax, but the sampling efforts required to identify contaminated environments and subsequent outbreak risks are often too laborious or expensive to employ, making the use of a biosentinel an appealing option. Additionally, human and animal case reports and mortality data likely underestimate the geographic extent of this pathogen, while exposure data obtained through serosurveillance may

allow for acquisition of multi-dimensional biological information, such as environmental range and relative time of exposure. Swine are resistant to anthrax[17], and with serologic evidence of exposure in taxonomically identical species like the Ukrainian wild boar, feral swine may be a good indicator of bacterial presence throughout their range in the U.S. Feral swine also exhibit relatively small home ranges, between one and five square kilometers[79,80], potentially allowing for high resolution in estimating the geographic extent of contaminated environments.

Data presented here demonstrate that the overall odds of feral swine in Texas possessing anthrax antibodies are different for those inhabiting broadly defined endemic and non-endemic regions, with animals originating within the Anthrax Triangle exhibiting higher odds of being seropositive than those outside. This is not surprising given the regularity of outbreaks in domestic herbivores within this region and supports our preliminary hypothesis that feral swine are being exposed in regions experiencing regular occurrences of the disease. However, that approximately 37% of individuals from non-endemic counties were also seropositive, as well as county status alone not being a significant predictor covariate, suggest that swine are being exposed beyond the confines of the Anthrax Triangle, and subsequently the bacteria may also be present outside of this region. This is further supported by latitude being a statistically significant covariate in our top-performing model instead of county status. While the role that feral swine may play in the overall epidemiology of anthrax is currently unknown, they are recognized to exhibit close relationships with soil[38]; as a result, swine likely experience higher rates of exposure than humans and perhaps some domestic and wild ruminants and may contribute to bacterial spread through biological or mechanical dissemination. Alternatively, this level of exposure may simply reflect bacterial presence irrespective of swine involvement in dissemination, as outbreaks outside of the Triangle are reported occasionally[52]. Although our

statistical analysis was unable to distinguish anthropogenically defined endemic and non-endemic regions, the high apparent seroprevalence observed in feral swine across the state of Texas is still useful information, as exposure data is further indicative of bacterial distribution occurring beyond the confines of the Anthrax Triangle, as is predicted by the ecological modeling efforts of others[8,11].

Interestingly, female swine and adult swine tended to have higher seropositivity, although this relationship was not statistically significant. Higher odds by sex may be due to the inherent dynamics of swine sounders, with groups typically being composed of several females and their offspring; adult and sub-adult males are often solitary, only associating with females during breeding[38]. The likelihood then of observing seropositive females in a *B. anthracis* contaminated region may be higher simply because females traveling together are experiencing the same environmental exposure compared to their solitary male counterparts. The potential age-class bias observed could be explained in part by the sample sizes between these covariates not being equal; more extensive data may be necessary to confirm this association. Lastly, feral swine have been observed to opportunistically feed on carcasses of other animals, as well as prey on some livestock[81-83]. Thus, feral swine may be contributing to anthrax epidemiology through a variety of mechanism(s), including carrying and depositing spores and/or vegetative cells acquired from rooting in soil profiles or by feeding on the carcasses of animals who have succumbed to anthrax.

As with any retrospective, opportunistic serosurvey, the data and subsequent findings presented here are not without limitations. First, the fact that we broadly defined regions as endemic and non-endemic solely based on whether a county was present in the Anthrax Triangle

likely does not account for the continuous or disjointed presence of this bacterium in soils predicted throughout the state[11], and counties that were sampled on the border of the Anthrax Triangle, such as Kimble, might have skewed results with some positive individuals originating from this region. We also did not examine any environmental conditions or weather patterns in conjunction with the regions we defined, which likely are significant factors influencing bacterial distribution and infectivity rates between the sampling years examined and could be the source of the unexplained variance suggested during model evaluation.

Experimental Inoculation of Juvenile Feral Swine with *B. anthracis* Sterne

After exposure to varying quantities of *B. anthracis* Sterne spores, most study individuals successfully seroconverted and displayed measurable antibodies against anthrax PA. Additionally, feral swine appear to be competent hosts and possible vectors of *B. anthracis* spores after intranasal exposure, as viable bacteria were isolated from the nasal passages of most individuals weeks after their initial or final intranasal exposure.

While we observed our study pigs daily for clinical signs of disease, as well as observed body temperatures at each sampling time point, we detected no abnormalities throughout the study period, and no adverse responses after either intranasal or subcutaneous exposure to *B. anthracis* Sterne. This was not surprising given that the Sterne strain is an avirulent strain, and routinely used to vaccinate livestock with little risk[84,85]. Additionally, members of the Suidae family are thought to be relatively resistant to developing anthrax after exposure to fully virulent *B. anthracis*, suggesting that any clinical presentation of disease would be a rare occurrence.

The strength of the antibody response observed in our study pigs was positively correlated with both the dose of the inoculum given, as well as the number of exposures

individuals experienced over time. The seroconversion observed after exposure to known quantities of *B. anthracis* was consistent with the serology observed within our Texas field survey, where varying levels of anti-PA antibodies were detected in wild-caught swine with no known exposure status, as well as that observed from previous studies detecting anti-PA antibodies in wild boar[36]. This evidence supports past hypotheses postulating that swine may be indirect indicators of anthrax contamination. Of note, the only study group that did not seroconvert was the 10^4 CFU/ml single exposure group. This suggests that multiple exposure events, or relatively high environmental contamination may be required for pigs to be useful as sentinels in regions with anthrax and where feral swine removal or invasive species management is taking place. Furthermore, the fact that most of our study pigs were exposed to *B. anthracis* Sterne intranasally and still seroconverted at detectable levels by ELISA validates that inhalational exposure is a legitimate exposure route for swine. The intranasal inoculation of our study pigs was meant to simulate an exposure event from pigs engaging in natural rooting behaviors, and inadvertently inhaling infectious materials from their environment. Currently, inhalational exposure is believed to be the least common route of infection in livestock and wildlife, as anthrax spores tend to cluster together with surrounding organic materials in soil and are not considered easily aerosolized[86]. It is additionally thought that susceptible ruminants are most likely exposed to anthrax via the ingestion of spores; this has been proposed for grazing species like bovids, as their grazing habits involve ripping grass with their tongues, this action more likely facilitating the ingestion of soil, the main substrate for *Bacillus* spores[13]. For browsing ruminants, especially those whose ranges overlap with necrophilic flies, the ingestion of spores from browse is thought to be enabled by the vectoring of spores from infected carcasses to the leaves of vegetation by flies[14-17]. Exposure in resistant carnivorous and

omnivorous species is also thought to be from the ingestion of spores, mainly from feeding on contaminated animals[5]. Unlike grazing and browsing herbivores, pigs interact with soil profiles very differently, most often obtaining food from utilizing their muzzle to upturn substrate, and their sensitive sense of smell to locate suitable roots or grubs[38,87]. This behavior likely facilitates the aerosolization of infectious materials better than does the grazing activities of other species and may be a more likely route of exposure for swine in natural settings. Like predators and scavengers, feral swine also are known to predate on wildlife as well as scavenge upon carcasses, and therefore may also experience gastrointestinal anthrax exposure in some instances[36]. Regarding how these exposure routes might influence resulting serosurveillance efforts, follow-up studies will be needed to better elucidate any differences in the seroconversion of pigs experiencing gastrointestinal versus inhalational anthrax exposure.

In addition to marked seroconversion, pigs that experienced intranasal exposure to *B. anthracis* Sterne also retained observable quantities of inoculum within their nasal passages for varying periods of time after inoculation. Bacterial culture and subsequent PCR confirmation of colonies isolated from nasal swab samples revealed that for some pigs, *B. anthracis* Sterne stayed present at detectable levels weeks after an exposure event occurred. This was particularly true for pigs exposed multiple times, as *B. anthracis* Sterne was recoverable in some individuals for as long as 42- and 56-days following primary inoculation. As pigs have a natural propensity to root in soil, this suggests that in environments contaminated with *B. anthracis*, feral swine may serve as mechanical vectors by inadvertently inhaling soil particles containing spores into the nasal passages, where they may then adhere to the nasal mucosa and persist for long periods of time. Notably for three pigs in the single exposure groups, *B. anthracis* Sterne was not detected in nasal swabs at 14 DPI but was at 28 DPI. This pattern, as well as continued recovery

of bacteria from pigs inoculated multiple times may be explained either by persistence of the inoculum, or by replication of vegetative cells that have colonized the nasal epithelium. Given the lack of clinical abnormalities in any of the pigs, as well as the avirulent nature of the Sterne strain, replication and preservation of vegetative cells in the nasal passages is unlikely. Furthermore, the decreasing colony counts observed over time from nasal swab cultures of intranasally inoculated pigs does not support continued bacterial replication in the nasal passages. This suggests that *B. anthracis* Sterne spores were recovered from the nasal passages, and therefore persistence of the inoculum rather than establishment of bacterial infection in the epithelium. Indeed, it has been suggested by others that species resistant to anthrax predating or scavenging on infected animals can act as carriers by dispersing spores via dried viscera adhered to fur or feathers, or in feces after ingestion of contaminated materials[4,15,34]. Mechanical vectoring of viable spores has also been demonstrated for some arthropods, including various necrophagous flies as well as some mosquitos[15,88,89]. Additional movement of anthrax spores, especially in regions where feral swine are present, could therefore have significant implications for the exposure of more susceptible species, and consequently how long an outbreak may last. This may especially be true in instances where susceptible wildlife and feral swine are in competition for the same food sources, as has been proposed between feral pigs and white-tailed deer in regions of Texas during times of drought[90]. White-tailed deer are also known to visit feral swine wallows and this additional source of interaction likely has further implications for the transmission of diseases like anthrax[91].

This experimental infection study had limitations, chief among them utilization of an avirulent strain of *B. anthracis* for inoculation rather than a wild-type or fully virulent strain. Exposure to *B. anthracis* Sterne in a species already relatively resistant to developing anthrax

may have resulted in an underestimation of the subsequent serological response of each study group, as the Sterne strain does not possess the capsule-encoding pXO2 plasmid, and thus does not interact with the host immune system in the same way fully virulent pXO1 and pXO2 expressing strains do[22,84]. The Sterne strain does express the toxin-encoding pXO1 plasmid, which includes the cell receptor binding protein PA. It has been demonstrated previously that the bulk of the humoral immune response generated to anthrax is against PA, and that higher anti-PA antibody titers are correlated with increased levels of protection against disease[77]. This is further supported by resistant wildlife species in regions endemic for anthrax displaying measurable antibodies against PA[27,35,92-94], and suggests that despite challenge with a non-virulent strain, the antibodies we observed in our feral swine very likely mimic those that are observed in natural systems containing wild-type anthrax. However, comparing antibody levels using a quantitative ELISA platform may be necessary to further elucidate if differences exist in the serology of swine exposed to different strains of *B. anthracis.* Lastly, while recovery of viable spores from the nasal passages after intranasal inoculation suggests the potential for mechanical transmission, we did not sample any of the substrates or surfaces within our animal rooms, and thus cannot confirm that spores were then deposited elsewhere and available for other individuals to then be exposed. Additional experiments are necessary to verify spore deposition onto surfaces or substrates utilized by feral swine after intranasal exposure, and particularly if enough infectious material is deposited to then infect other individuals. Furthermore, while we also collected nasal swab samples from subcutaneously exposed pigs to assess transmission of Sterne from intranasally inoculated pigs housed in the same room, contamination of the swab samples collected on 0 DPI with inoculum confounded any conclusions to be drawn from samples collected at subsequent time points from those individuals. In other words, subcutaneously

inoculated individuals appeared positive for *B. anthracis* Sterne within their nasal passages on the same day that they were inoculated, suggesting that cross-contamination occurred either within the nasal passages of those individuals, or on the swab itself after or during sampling; any positive swab samples appearing subsequent time points therefore could not be interpreted as being the result of mechanical transfer between pigs. Further studies are therefore required to confirm whether spores may be transferred between the nasal passages of exposed and naïve swine.

CONCLUSIONS

Feral swine are a fecund invasive species that often encounter humans and domestic animals, as well as other wildlife species. Past investigations have identified a myriad of pathogens that can be transmitted or carried by these animals[82], and national programs facilitated by the USDA regularly survey populations for diseases of national concern to humans and/or agriculturally important species[37]. Despite the amount of attention feral swine receive for some pathogens, their role in anthrax ecology remains unknown. Anthrax is largely considered a disease of antiquity, yet it remains a significant problem for domestic livestock and wild ruminants worldwide. Moreover, there remain significant knowledge gaps regarding *B. anthracis* environmental distribution and ecology, including the relative role that resistant host species may play in bacterial dissemination[4]. Feral swine are a species resistant to anthrax and are known for their regular interactions with soil, the primary substrate for infectious *B. anthracis*, but have been overlooked for their role in anthrax ecology.

We report that feral swine intranasally exposed to *B. anthracis* Sterne may serve as competent biosentinels, based on detectable antibody in wild-caught swine in endemic regions of

Texas, as well as observed seroconversion in juvenile feral swine after exposure to known levels of anthrax bacteria. Measuring the antibody status of feral pigs may thus be used as a more targeted surveillance tool for determining whether an area poses an exposure risk for susceptible livestock or wildlife; in some cases, this information may assist livestock owners or wildlife managers in determining appropriate actions to mitigate risk or used to support the use of livestock vaccination[27,95]. We additionally report that feral swine exposed intranasally to *B. anthracis* Sterne may serve as mechanical vectors for infectious spores, based on carriage in the nasal passages weeks after a known exposure occurred. Management of feral swine in endemic and emerging anthrax regions may therefore not only include serosurveillance as a tool for determining anthrax contamination status, but also could incorporate removing individuals whose movements may be from regions experiencing outbreaks to avoid mechanical transmission to more susceptible species, as well as humans.

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CHAPTER 3: EVALUATION OF PATHOGENESIS AND IMMUNE RESPONSE TO *COCCIDIOIDES POSADASII* INFECTION IN U.S. FERAL SWINE[3]

SUMMARY

The dimorphic, soil-dwelling fungus *Coccidioides* is the causative agent of coccidioidomycosis, also known as Valley Fever, and is a reemerging pathogen of human health concern. Despite increases in human Valley Fever cases and isolations of the organism outside of its theoretical environmental range, much of *Coccidioides* epidemiology, including complete host range, remains unknown. Feral swine (*Sus scrofa*) are a proliferative invasive species with a large distribution across the United States. Known to disturb soil profiles through their rooting and wallowing behaviors, some feral swine populations also currently overlap regions with high Valley Fever incidence. We report on an experimental infection study wherein we evaluated seven feral swine for susceptibility to *Coccidioides* over a period of 42 days. After intranasal inoculation with 6.2 $\log_{10}$ CFU of *C. posadasii,* no pigs displayed outward signs of clinical disease, nor did any seroconvert and exhibit *Coccidioides* antibodies by AGID testing. Histopathologic evaluation did not reveal the presence granulomatous lesions, and fungal spherules or endospores were not observed in any of the tissue sections examined, although individuals had significant comorbidities, most notably *Metastrongylus* nematodes in the lungs. Despite the absence of lesions and organisms histologically, *C. posadasii* was isolated by culture from the lung and mediastinal lymph node of two pigs, indicating active infection. These results

[3] Maison, R.M.; Kessinger, P.; Priore, M.R.; Han, S.; Powell, D.A.; Moale, H.; Brown, V.R.; Bodenchuk, M.J.; Bowen, R.A.; Bosco-Lauth, A.M. Evaluation of Pathogenesis and Immune Response to *Coccidioides posadasii* Infection in U.S. Feral Swine (*Sus scrofa*). Preprints 2023, 2023071730. https://doi.org/10.20944/preprints202307.1730.v1

suggest that feral swine are mildly susceptible to acute *Coccidioides* infection and may aid in fungal dispersal due to the presence of spherules in tissues post-mortem.

INTRODUCTION

Coccidioides immitis and *posadasii* are largely indistinguishable pathogenic fungi and are the causative agents of coccidioidomycosis, or Valley Fever, a potentially deadly disease in humans[1]. *Coccidioides spp*. primarily rest in soil profiles, with fungal spores or arthroconidia infecting hosts chiefly through the respiratory route. This mode of transmission is thought to be driven primarily by soil disruption and aerosolization of infectious arthroconidia, with large-scale fungal dissemination mediated by extreme weather events like dust storms[2]. Based on the seminal work performed by Edwards and Palmer in the 1950s[3,4], it's believed that in the United States, *Coccidioides* is endemic to the Central Valley of California, southern Arizona, and western Texas, and that desert rodents and armadillos play a role in fungal ecology by providing organic materials within their burrows, as well as being incidental hosts themselves[5,6].

Reports and isolations of *Coccidioides* have been made outside of the defined endemic region within the last decade, suggesting that the organism is expanding geographically, yet despite this, an animal reservoir has not been definitively identified[3,7,8]. Ongoing study of *Coccidioides* distribution and ecology has proven difficult, as distribution within soil profiles is not homogenous even in identified disease "hot spots," and coccidioidomycosis is not a reportable disease in all states[7,9,10]. Furthermore, although attempts have been made to better define environmental suitability, resulting evidence has been inconclusive, and reliable isolation of *Coccidioides* from the environment remains a challenge[7,10-12].

Besides humans, *Coccidioides* appears able to infect most vertebrates with varying levels of disease[13]. Experimental infection studies in wild rodents including Merriam's kangaroo rat (*Dipodomys merriami*) and the desert pocket mouse (*Chaetodipus penicillatus*) have revealed they may be infected but rarely die from coccidioidomycosis, and serological studies have detected antibodies in the serum of kangaroo rats, desert woodrats (*Neotoma lepida*) and deer mice (*Peromyscus maniculatus*), suggesting many desert rodents are potential reservoirs or incidental hosts[3,6]. It has thus been broadly hypothesized that burrowing mammals play a role in fungal ecology due to their association and close interactions with soils that may be contaminated with fungal hyphae[5-7,11]. Similarly, infection in domestic dogs is thought to be more prevalent than in humans because of their behavioral tendency to disrupt soil[14,15].

Observational studies in several livestock species suggest that clinical infection is rare in horses, cattle, sheep, and swine. *Coccidioides*-associated lesions, when observed, are incidentally detected during slaughter or meat processing, and likely do not reflect true incidence[15-18]. Limited reports for domestic swine indicate that they are susceptible to *Coccidioides,* but rarely develop clinical signs or succumb to disseminated disease, and past serological surveys have suggested that at least 12% of domestic swine in some endemic regions can be positive for *Coccidioides* antibodies[15,16]. Like canids, pigs have behavioral tendencies to disrupt soil, such as rooting for food or wallowing for thermoregulation[19]. Despite this, fungal shedding and transmission dynamics in pigs have yet to be fully elucidated.

Feral swine (*Sus scrofa*) are a proliferative invasive species with widespread distribution across the United States and are known for their disruption of soil through rooting and wallowing[20,21]. Taxonomically identical to domestic swine, feral swine have yet to be considered

for their potential role in *Coccidioides* ecology and epidemiology, despite population overlap with regions exhibiting high and low incidence of Valley Fever[22,23]. We thus evaluated a group of feral swine for susceptibility to *C. posadasii* and documented clinical signs, serological response, and fungal shedding in tissues after intranasal exposure.

MATERIALS AND METHODS

Animals

Seven wild-caught juvenile feral swine of mixed-sex were evaluated for susceptibility to *C. posadasii*. Feral swine sounders were trapped by the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (USDA) personnel in Burnet County, Texas using baited corral traps from April 25-27, 2021. Juvenile swine were preferentially selected from sounders to minimize the chance of previous fungal exposure and were defined as less than 2 months of age, where age was estimated based on the absence of incisors and permanent canines on the lower jaw[24]. Upon capture, seven individuals were selected based on these criteria and transported overnight to Fort Collins, Colorado. Animals were then group-housed in an Animal Biosafety Level 3 (ABSL-3) facility at Colorado State University, in one room (12 ft. x 18 ft) with natural lighting and controlled climate. Once in the ABSL-3, swine had access to food and water ad libitum throughout the study period.

Animals were allowed to acclimate for six days before being given a thorough physical examination, where an initial blood sample was taken, and individuals screened externally for ectoparasites, sexed, and implanted with thermally sensitive microchips (Lifechips, Destron-Fearing) for identification and temperature measurement. Due to significant numbers of ectoparasites observed on arrival, a thin application of deltamethrin insecticide (Delta Dust,

Bayer) was also applied to the coat of each pig during the initial exam. All procedures and use of animals were performed with the approval of Colorado State University's Institutional Animal Care and Use Committee (Protocol #1360).

Fungal Challenge

Prior to inoculation, baseline body temperatures were recorded for all individuals (day 0). Wild-type *C. posadasii* strain Silveira spores diluted in phosphate-buffered saline (PBS) were then administered into the nares of six individuals using a pipette (500ul per nare) under manual restraint. One individual was not inoculated and left as a contact control. Immediately following animal challenge, back titration was performed on 1 X GYE medium (1% glucose, 0.5% yeast extract, and 1.5% agar) and confirmed that animals received 6.2 $\log_{10}$ CFU of *C. posadasii*.

Clinical Observations

All animals were observed daily and included an assessment of temperament and the presence or absence of clinical signs of disease, including but not limited to ocular and nasal discharge, ptyalism, coughing/sneezing, dyspnea, diarrhea, lethargy, anorexia, and moribund status. Body temperature was recorded starting the day prior to challenge and continued to seven days post-inoculation (DPI); after day seven temperature was measured again at 14, 28 and 42 DPI. In addition to monitoring for overt clinical signs, we also monitored behavior by observing animals behind double-paned glass, assessed food intake as well as response to provided enrichment, including playing with toys and consuming treats.

Sampling

We evaluated feral swine for acute pathologic changes, fungal dissemination in tissues, and serological immune response after *Coccidioides* exposure. Blood samples (3-5 ml into serum separator tubes) were collected from all individuals on 14-, 28-, and 42-DPI. At each timepoint two animals were also euthanized and tissues harvested for fungal culture and formalin fixation. The exception to this process was 42 DPI, where three individuals were euthanized; this included the last two inoculated pigs as well as the uninoculated contact control. Blood was collected from the anterior vena cava vein under manual restraint, and animals that were euthanized were sedated with 1-2 mg/kg xylazine and 15-20 mg/kg ketamine before being removed from the room and euthanized by captive bolt. During necropsy, approximately 100 mg samples of lung, liver, spleen, and mediastinal lymph node tissue were collected in 1 ml of PBS with two stainless steel balls for fungal culture. Approximately 1-5 g of the same tissues, in addition to kidney, pancreas, small intestine, and heart were collected in 10% neutral-buffered formalin for histopathology.

Serology

Serum was extracted from all blood samples within two hours of collection and passed through a 0.22 μm filter before being taken out of the ABSL-3 and stored at -80°C. Sera were shipped to the Veterinary Medical Diagnostic Laboratory at the University of Missouri, where they were evaluated for the presence of Coccidioides antibodies by porcine agar gel immunodiffusion assay (AGID).

Fungal Culture

All manipulations of viable cultures and tissue specimens were performed within the ABSL-3. Tissues collected for culture were homogenized using a Qiagen Tissuelyser at 25 cps for 5 minutes and 100µl of homogenate was plated onto 1 X GYE medium supplemented with selective fungicide and antibiotics (1% glucose, 0.5% yeast extract, 1.5% agar, and 50ug/ml each of cycloheximide, chloramphenicol, and streptomycin). Plates were allowed to incubate for a maximum of 1 week at 37°C and any suspicious fungal growths were harvested into 1 ml of PBS and stored at -80°C pending DNA extraction and PCR.

DNA Extraction

DNA extraction was achieved by placing approximately 1cm^2 of mycelia in a 2 ml screw-cap tube containing 0.5 mm diameter acid-washed glass beads (Fisher Scientific, #501461310) and 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8.0], 100 mM NaCl, 0.5% sodium dodecyl sulfate, and 50 mM dithiothreitol) and subjected to mechanical disruption by shaking on a Qiagen Tissuelyser at 25 cps for 5 minutes. Samples were then incubated at 65°C for a minimum of 30 minutes before being centrifuged at 10,000 rpm for 2 minutes. Nucleic acids were extracted from the supernatant with buffered phenol:chloroform:isoamyl alcohol pH 8.0 (25:24:1) and again with cholorform:isoamyl (24:1) and precipitated from the aqueous layer with 0.1 volume of 0.2 mM NaCl and 0.1 volume of ethanol. The resulting suspension was then centrifuged at 14,000 rpm for 10 minutes, washed again with ethanol, re-suspended in 300 µL of TE buffer (10 mM Tris-HCl [pH 7.6], 1mM EDTA [pH 8]), with 100ug/mL RNAse A (Thermo Fisher, #EN0531), and incubated at 37°C for 30 minutes to degrade extraneous RNA. DNA of interest was then precipitated out with the addition of 300µl

of 5M LiCl followed by a 10-minute incubation on ice, before being centrifuged for 14,000 rpm for 10 minutes. Supernatants were then transferred to 1.2 ml of ethanol and incubated on ice for an additional 10 minutes and centrifuged one final time at 14,000 rpm for 10 minutes. The resulting pellet was then washed with 70% ethanol and allowed to air dry before being suspended in 100µl of TE buffer. Final suspensions were stored at -20°C until analysis by PCR.

PCR

All suspicious fungal growths isolated from tissues collected at necropsy were confirmed by conventional PCR, using primers targeting the ITS region of *Coccidioides* described previously[25]. Singleplex PCR was performed using Invitrogen PCR Supermix (Cat. #10572014), according to the manufacturer's instructions. Briefly, 50µl reactions consisting of 0.2µM of the ITS primer set and 1µl template DNA was used. The PCR program consisted of initial denaturation at 94°C for 2 minutes, followed by 35 cycles each of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, extension at 72°C for 1 minute, and one cycle of final extension at 72°C for 7 minutes. Amplification products as well as 1-kilobase ladder (Invitrogen, #10787018) were then loaded onto a 1% agarose stained with ethidium bromide and electrophoresed for 1 hour at 100 V. Band presence at 223 bp was considered positive for *C. posadasii* when viewed under ultraviolet light.

Histopathology

Tissues collected for histopathology were fixed in 10% neutral-buffered formalin for 15 days before being transferred to 70% ethanol. Tissues were then processed for paraffin embedding and sectioned for staining with hematoxylin and eosin (H&E). All slides were interpreted by a veterinary pathologist blinded to animal treatments and history.

RESULTS

None of the feral swine exhibited outward clinical signs of disease and maintained stable body temperatures throughout the study period. Body temperature, initial body weight, as well as clinical scoring information is depicted in Table A.1 of the Appendix. None of the animals displayed abnormal behavior compared to the acclimation period. Furthermore, AGID testing of sera collected at 14, 28, and 42 DPI revealed that no pigs seroconverted after experiencing either a primary or (regarding the contact control) secondary exposure event.

Animal demographics, day of termination, and the results of culture and subsequent colony PCR, as well as a summary of histologic findings are depicted in Table 3.1. For a more detailed histological report, see Table A.2 within the Appendix. During necropsy, four pigs (#3916, 7639, 7652, and 7670) displayed grossly noticeable congestion in the lungs, with three of four animals exhibiting regions of congestion diffusely across all lung lobes, and one (#7670) with congestion localized only to the lower respiratory tract in both caudal lobes. No additional gross lesions were observed in any other tissues. Culture of tissue homogenates resulted in four suspect colonies being harvested from the right middle lung lobe of one pig (#7639) and two colonies each from the mediastinal lymph node of two additional pigs (#7670 and 7648). Analysis by PCR however revealed that only those colonies harvested from pigs 7648 and 7639 were *Coccidioides*. Histopathology did not reveal the presence of fungal spherules or endospores in any tissues, yet inflammatory processes were observed in most tissue types for all animals (Table 3.1). Most commonly, interstitial pneumonia, lympho-histiocytic inflammation, lympholysis, and alveolar edema were observed in the lungs. Notably, Metastrongylus nematodes were also detected in the lung sections of three individuals (#3916, 7648, and 7670);

a representative depiction of this is illustrated in Figure 3.1. Due to inadequate sample collection at necropsy, lung tissue was only cultured and not examined by histopathology for pig 7632 (Table 3.1).

Table 3.1. Results of fungal culture, colony PCR, and summary of histologic findings for feral swine inoculated with *C. posadasii.*

Animal ID	Sex	Group[1]	Necropsy Day (DPI)	Fungal Culture (Number of Colonies/100mg of Tissue Type)[2]	Colony PCR (+/-)[3]	Histologic Findings
7652	F	Infected	14	--	NA	Interstitial and perivascular lympho-histiocytic inflammation in lungs. Perivascular lympho-histiocytic inflammation in heart and liver. Lympholysis in spleen.
7670	F	Infected	14	2/Mediastinal Lymph Node	-	*Metastrongylus* nematodes in lungs. Alveolar edema, interstitial pneumonia, bronchitis, and BALT hyperplasia with lympholysis in lungs. Mild hyperplastic arteriosclerosis and scattered individual hepatocellular necrosis in heart.
7620	M	Infected	28	--	NA	Interstitial and perivascular lympho-histiocytic inflammation in lungs, liver, and heart. Lympholysis in spleen and lymph node. Granulomatous lymphoid hyperplasia in lymph node.
7632	M	Infected	28	--	NA	Lungs not examined. Lympholysis and resorbed neutrophils, and mild edema in lymph node. Lympholysis in spleen. Interstitial edema in heart.
3916	F	Uninfected	42	--	NA	*Metastrongylus* nematodes in lungs. Interstitial pneumonia, lympho-histiocytic inflammation, alveolar and interstitial hemorrhage and edema in lungs. Lympho-histiocytic inflammation in kidney and spleen. Myocardial interstitial edema in heart.
7648	F	Infected	42	2/Mediastinal Lymph Node	+	*Metastrongylus* nematodes in lungs; interstitial pneumonia and bronchitis and alveolar edema. Lympho-histiocytic inflammation in lung and liver. Lympholysis in spleen and kidney.

Animal ID	Sex	Group[1]	Necropsy Day (DPI)	Fungal Culture (Number of Colonies/100mg of Tissue Type)[2]	Colony PCR (+/-)[3]	Histologic Findings
7639	M	Infected	42	4/Right Middle Lung	+	Interstitial pneumonia, alveolar edema, and lympho-histiocytic inflammation in lungs. Lympholysis in spleen. Interstitial edema in heart.

[1]"Uninfected" denotes contact-control.
[2]Results are expressed as number of colonies harvested per 100 mg of tissue type indicated; "--" denotes no colonies observed in any tissues examined.
[3]A positive (+) colony PCR indicates confirmation of colonies as *C. posadasii* by conventional PCR. NA: Not Applicable.

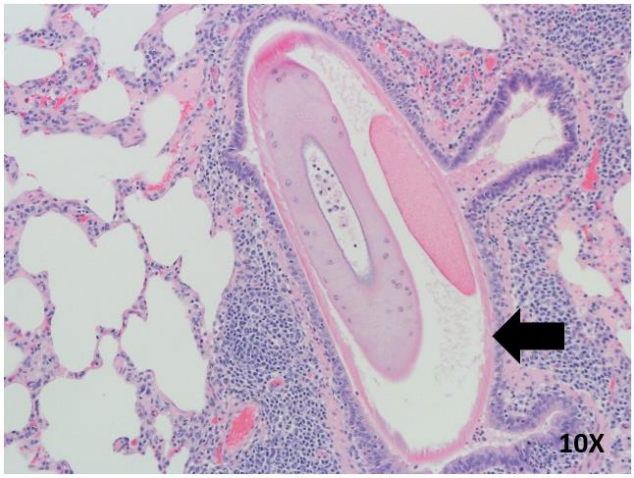

Figure 3.1. Representative lung histology of study pigs infected with parasitic nematodes. Adult *Metastrongylus* nematode inside the bronchiole of the right caudal lung lobe section, H&E-stained tissue; pig #3916, negative contact control. The black arrow delineates the nematode muscular body wall.

DISCUSSION

After intranasal exposure with 6.2 $\log_{10}$ CFU of *C. posadasii,* feral swine, as suggested for their domestic relatives, appear resistant to developing and succumbing to acute coccidioidomycosis. Despite lack of overt clinical signs and the absence of significant lesions at necropsy, *C. posadasii* was cultured from tissues of two pigs, indicating that active infection was achieved. That infection was only detected in two exposed animals, however, was somewhat

surprising given the substantial dose of the inoculum. This low infection rate may be due to the route of inoculation used; by nature of instilling PBS-suspended spores into the nasal cavity, some inoculum could have passed into the oropharynx and been swallowed rather than inhaled into the respiratory tract. Alternatively, it is possible that infection was simply missed by sampling insufficiency, as only a small amount of tissue was cultured, and the organism is not always easy to detect nor evenly distributed in organs[5,26]. The route of inoculation likely mimics a natural exposure event from feral swine rooting within contaminated soil and inadvertently inhaling infectious materials[27]. We additionally examined the likelihood of animal-to-animal transmission by leaving one individual uninoculated, but group-housed with exposed animals, as swine are very gregarious, often making nose-to-nose contact with littermates or other members of their sounder[19]. Despite regular nose-to-nose contact between our animals throughout the study period, we did not detect any signs of *Coccidioides* infection in the contact control, suggesting animal-to animal transmission after intranasal exposure is unlikely in feral swine. This is unsurprising given that coccidioidomycosis is not considered contagious, and transmission events between vertebrates are extremely rare[16,28,29].

Interestingly, none of the pigs developed antibodies to *Coccidioides* as assayed by AGID following inoculation. Lack of seroconversion in animals without associated active infection is not altogether surprising, however no marked change in the serology of the two culture positive pigs through 42 DPI is somewhat puzzling. These results, however, may be explained by the challenges associated with *Coccidioides* serology. While AGID is highly specific and remains the gold standard serological test for both humans and animals[15,30], its relatively low sensitivity can be problematic when testing the serum from individuals with self-limiting infections or those who are immunocompromised[31-33]. Indeed, as seen with many canine coccidioidomycosis cases,

dogs with regular or chronic clinical symptoms can be serologically negative by AGID[31,34]. Given that all study pigs displayed general inflammation across organ systems, seroconversion as well as subsequent serology may have been confounded to some degree by the occurrence of coinfection in some or all of the pigs, particularly those coinfected with detectable parasites[35-37]. Alternatively, the lack of seroconversion as well as fungal bodies in tissues examined histologically may be due in part to the relatively short duration of our study. Primary pulmonary infections in humans are typically symptomatic between one and four weeks after exposure, while disseminated disease can occur months or years later[16]. While the timeline for pathogenesis in pigs may not be the same, follow-up studies will be required to further assess the susceptibility and shedding potential of feral swine experiencing chronic infection with *Coccidioides,* or results of infection past the 42-day period examined here.

We inoculated all study pigs with wild-type *C. posadasii* spores, despite *C. immitis* also known to be present in regions of the United States. It's currently thought that the largest difference between the two species is their relative geographical distribution, with *C. immitis* primarily occurring in Central and Southern California, and *C. posadasii* in the desert regions of Nevada, Arizona, New Mexico, and West Texas[11]. Otherwise, *C. immitis* and *C. posadasii* are morphologically identical, and no clinical differences have been identified between species to date; however, no investigations have assessed phenotypic variation in the context of pathogenicity[11,14]. Additional experiments will therefore be necessary to determine if any differences exist in infectivity and shedding dynamics in suspected reservoir species.

Lastly, all individuals appeared to have considerable comorbidities; notably, half of inoculated individuals possessed nematodes within the bronchioles of the lungs, and all animals exhibited generalized inflammation within most tissues. While nematodes were not detected in

the tissue sections of all animals, the systemic inflammation observed in others is more likely indicative of a parasitic infection, rather than disseminated coccidioidomycosis[5,38-41]. The fact that all animals also exhibited similar pathology may be due to pigs used here originating from the same geographic area, and thus experiencing similar environmental exposures, or direct transmission from infected individuals, either while grouped together while in transportation to Fort Collins or while being group-housed for the duration of the study[42,43].

While it is largely accepted that burrowing mammals contribute to *Coccidioides* ecology because of their interactions with soils that may contain fungal hyphae, the role that additional species play in fungal persistence and spread is underexplored. Moreover, increases in both human coccidioidomycosis cases and isolation of *Coccidioides spp*. from soils outside of historically defined endemic regions have implied that the metrics used to determine endemicity and environmental suitability are outdated[7,44]. Feral swine, like desert rodents, are behaviorally drawn to soil and are additionally known for their opportunistic interactions with humans, domestic animals, and native wildlife, which in some instances can have implications for the transmission of pathogens and disease[21,43]. In the case of *Coccidioides*, we postulate that feral swine, like rodents and armadillos, may be reservoirs and disseminators of the fungus on the landscape, particularly if they experience infection and expire with viable spherules within their tissues. This mode of fungal dissemination could prove significant, as others have proposed that animal carcasses can serve as substrates for the growth of *Coccidioides* mycelia in the soil[45-48]. Based on viable *Coccidioides* being cultured from the tissues of two study pigs post-mortem, feral swine carcasses may therefore pose a risk to human health, particularly for wildlife managers removing feral swine for invasive species management and hunters, who may regularly manipulate feral swine carcasses[49,50]. This risk could also extend to other wildlife or

domestic animals who may scavenge or inspect infected carcasses and come into direct contact with infectious material[45].

Coccidioidomycosis is a potentially severe disease and its causative agent an understudied pathogen[51]. Knowledge gaps concerning *Coccidioides* environmental requirements, host range, and epidemiology currently make mediation of human infection difficult. Additionally, it is likely that changing climates, increased urbanization, and spread of invasive species may be modifying *Coccidioides* epidemiological patterns and increasing the risk of human disease[5,9]. The expansion of invasive species susceptible to and capable of disseminating *Coccidioides* post-mortem such as feral swine may contribute to fungal ecology and should not be overlooked as potential sources for environmental contamination of *Coccidioides spp.* in the United States, particularly in regions where the pathogen is emerging.

CONCLUSIONS

After intranasal exposure to 6.2 $\log_{10}$ CFU *C. posadasii*, feral swine appeared mildly susceptible to infection, although resistant to acute disease, and could potentially serve as a reservoir species for *Coccidioides*. Lack of overt clinical signs as well as gross lesions in internal tissues may allow active *Coccidioides* infection to go unnoticed by wildlife managers or hunters that may be manipulating feral swine carcasses in the field and could facilitate secondary human exposure. Furthermore, death of feral swine with viable spherules or endospores present in tissues may also contribute to fungal dissemination and establishment of *Coccidioides spp*. into novel environments by providing a starting growth medium for fungal establishment into soils. Movement of feral swine from known endemic to non-endemic *Coccidioides* environments could therefore pose a threat to more susceptible vertebrate species, including humans.

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CHAPTER 4: EVALUATION OF A RAPID-ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES TO *BURKHOLDERIA PSEUDOMALLEI* IN U.S. FERAL SWINE

SUMMARY

Burkholderia pseudomallei is the causative agent of melioidosis, a neglected tropical disease that has been emerging worldwide. Historically found in soil and freshwater in Southeast Asia and Northern Australia, case reports of melioidosis and environmental isolations of *B. pseudomallei* have been increasing, both in frequency and geographic distribution. While cases of human melioidosis on the mainland United States (U.S.) have previously been associated with international travel to endemic countries, autochthonous cases have been reported, and recent isolation of *B. pseudomallei* from soil and water samples in the Gulf Coast region of Mississippi have confirmed the bacteria to be locally endemic. It is therefore important to better understand the true and possibly changing distribution of *B. pseudomallei* in North American environments.

Wide-ranging and non-specific clinical signs, as well as diagnostic and treatment difficulties have made estimating the global disease burden difficult, and present significant challenges to combating melioidosis. Furthermore, the role that non-human animals may play in *B. pseudomallei* epidemiology is significantly understudied, despite the bacterium's broad host range and the potential for zoonotic transmission in addition to environmental exposure. Feral swine (*Sus scrofa*) are an invasive species in the U.S. with an extensive ecological range, some of which currently overlap regions with environmental suitability for *B. pseudomallei* as well as in the Gulf Coast region of Mississippi where bacterial presence has been confirmed. Past surveys of farmed swine from endemic countries have detected significant numbers of animals

either seropositive for past bacterial exposure, or actively carrying *B. pseudomallei*, suggesting that they may be useful tools for surveillance. Despite the promise of utilizing serology in swine, improvements in diagnostic specificity and sensitivity are required before this strategy may be utilized to its full capacity, and feral swine have yet to be examined for their surveillance utility in the U.S.

To address these knowledge gaps, we evaluated a previously described indirect enzyme-linked immunosorbent assay (ELISA) utilizing *B. pseudomallei* Bp82-derived whole cell lysate antigen as an alternative serologic test for environmental exposure in swine serum. We assessed assay performance primarily using serially collected serum samples from a group of domestic swine experimentally inoculated with Bp82, a non-virulent laboratory strain of *B. pseudomallei*. Given that goats are also commonly affected livestock in melioidosis-endemic countries, and exhibit clinical signs more often than swine, we also opportunistically evaluated assay performance using sera acquired from 32 domestic goats before and after inoculation with fully virulent *B. pseudomallei* strain Bp511. Secondarily, we examined samples from various populations of free-ranging or feral swine in the U.S., originating from regions of purported high and low probable endemicity for *B. pseudomallei*. Samples were collected from mainland feral swine and originated from California (n=82), and Arizona (n=40), as well as free-ranging Vietnamese pot-bellied pigs from the U.S. territory Puerto Rico (n=40).

To evaluate assay specificity, we assessed the reactivity of all swine and goat sera against *Pseudomonas aeruginosa* PAO1 whole-cell lysate antigen. Antigen from *P. aeruginosa* was chosen to evaluate assay specificity due to the ubiquitous environmental distribution of *Pseudomonas*. For all assays, two cutoff methods were considered to interpret the antibody status

of animals. The first method considered was the standard ELISA cutoff method accepted by the World Organization for Animal Health, defined as being three standard deviations above the mean absorbance value exhibited by negative control sera (+3SD). The second cutoff value considered was based on the equation described by Dixon and Massey (1983) and represented a more statistically rigorous method.

Most experimentally inoculated swine (5/6), and goats (30/32) seroconverted and displayed antibodies against Bp82 whole-cell lysate antigen by ELISA, peaking in magnitude on either 28- or 21-days post-inoculation, respectively. When analyzed against PAO1 antigen, sera from both goat and pig displayed very little cross-reactivity, exhibiting average absorbance values that were below both assay cutoff points, as well as significantly lower than those exhibited by antibody positive samples identified with Bp82 antigen-based assays. Both the cutoff values identified most laboratory animals as seropositive after known inoculation with *B. pseudomallei*, and there was a high level of agreement in overall sample interpretation at most sampling time points. In contrast, examination of sera collected from populations of wild or free-ranging swine across the U.S. revealed comparable absorbance values to both Bp82 and PAO1-derived antigens for most samples, suggesting high antibody cross-reactivity across these two bacterial species in wild-caught animals.

While this ELISA successfully identified seropositive laboratory animals after known inoculation with two strains of *B. pseudomallei*, comparable assay readings across Bp82 and PAO1 antigen preparations in wild-caught animals suggested that high levels of antibody cross-reactivity between bacterial species exists. We therefore cannot advise the use of this platform for serological evaluation of wild swine. As *P. aeruginosa* is a close relative of *B. pseudomallei*,

and ubiquitously distributed in the environment, future serological analysis of free-ranging swine sera will therefore require the use of more targeted antigens to improve assay specificity.

INTRODUCTION

Burkholderia pseudomallei is a Gram-negative, intracellular bacillus and is the causative agent of melioidosis, an emerging infectious disease worldwide. Naturally found in moist soils and surface waters in tropical environments, *B. pseudomallei* is currently thought to be endemic primarily in southeast Asia and northern Australia, where historically incidence has been best described[1,2]. However, within the last two decades, autochthonous cases have been reported in regions of the Middle East, Sub-Saharan Africa, the Caribbean, and Central and South America, suggesting the range of *B. pseudomallei* to be much larger than originally assumed[3-6]. Moreover, recent statistical models have predicted that melioidosis is likely endemic in several additional countries that have never reported the disease, while others contain environments suitable for *B. pseudomallei* growth and persistence, including regions within the United States (U.S.)[7]. Historically, most case reports of melioidosis in the U.S. have been the result of international travel to an endemic country, or importation of contaminated products originating from endemic regions[2,8-13]. Despite human and commercial product movements contributing to the bulk of cases in the U.S., seasonal and stochastic weather events, including tropical storms have been suggested as additional means of the pathogen to disperse to new landscapes, and may be contributing to increasing case reports in historically non-endemic countries[14-16]. Indeed, in the U.S., a case of melioidosis was reported in 2018 in a Texas resident with no known travel history, and later genomic analysis revealed that the isolate grouped with others from additional patients in Texas, suggesting the pathogen to be endemic; however, the organism remains to be isolated

from the local environment[7,17]. Four years later in the Gulf Coast Region of Mississippi, *B. pseudomallei* was successfully isolated from both soil and water samples and subsequently linked to two cases reported in 2020 and 2022 within the same region, confirming the bacteria to be locally endemic[18]. Since environmental isolation of *B. pseudomallei* in Mississippi, our understanding of melioidosis epidemiology continues to evolve in the U.S[19]. True environmental distribution as well as global disease burden remain significant knowledge gaps, and present substantial public health challenges.

Most infections by *B. pseudomallei* are acquired through percutaneous inoculation of contaminated soil or water into penetrating injuries or pre-existing skin abrasions, ingestion of contaminated food or water, or inhalation[20-22]. Colloquially known as the "great mimicker", clinical manifestations of melioidosis are wide-ranging, and symptoms are often difficult to differentiate from other infections[20,23]. In humans, symptoms can range from skin and soft tissue abscesses to acute pneumonia and septicemia, as well as osteomyelitis and various neurological expressions[10,24,25]. Chronic forms of the disease have also been documented, wherein symptoms can surface years to decades after an exposure event[26-28]. Bacterial culture remains the gold standard for diagnosis, however, in some cases *B. pseudomallei* can be misidentified as either a culture contaminant or other bacterial species, including *Bacillus spp*. and *Pseudomonas spp*[2,8,29]. An indirect hemagglutination assay (IHA) is the most common serological test for diagnosis, but high background seropositivity can produce misleading results, especially when utilized in disease-endemic regions[8,30]. Several investigations have sought to improve serological tests by targeting select protein and polysaccharide components expressed by *B. pseudomallei* cells to improve assay specificity with some success[31-34]; however, these materials remain commercially unavailable, and require specialized laboratory equipment to isolate and produce[31,33,35].

Moreover, some investigations have suggested that no one antigen may be best for detecting *B. pseudomallei* antibodies in serum, but rather a combination of antigens are both more sensitive and specific[31,33]. Compounding difficulties in clinical diagnosis, *B. pseudomallei* is intrinsically resistant to a wide range of antibiotics, making the infection difficult to treat once identified[36,37], and overall case fatality rate ranges between 10-40%[7,38,39]. No licensed vaccine is currently available.

Besides humans, *B. pseudomallei* infection has been observed in a wide range of hosts, including various mammalian, marsupial, avian, aquatic, and reptilian taxa[27,40]. As with humans, symptomatic infection when observed can vary widely. In mammals such as cats, dogs, as well as assorted livestock, coughing, nasal discharge, and weight loss are often observed, while many avian species will become lethargic and develop diarrhea[22,41-44]. In reptiles, ulcers and necrotic nodules on the skin or internal organs are most often observed with *B. pseudomallei* infection[22,45,46]. Nonspecific symptoms and signs of illness have additionally been reported in marsupial species as well as various zoo animals, and acute septicemia with fever have been documented in some marine mammals[42,43,45].

While the apparent host range of *B. pseudomallei* is broad, exposure to infected livestock offer one of the most likely sources of transmission to humans, and it's been recognized in endemic countries that agricultural workers face increased risk of bacterial exposure due to either zoonotic or environmental transmission[7,47]. Despite the potential for zoonotic transmission, the role that non-human animals play in *B. pseudomallei* epidemiology is significantly understudied compared to environmentally mediated mechanisms of transmission[31]. Of the livestock species that may be affected by melioidosis, pigs, sheep, and goats are commonly affected in endemic

countries, although pigs appear more resistant to disease[45,41]. In pigs, infection is often chronic and asymptomatic, although abscesses may be present in multiple organs, which may not be observed until an animal goes to market[41,47,48]. Past surveys of domestic swine in endemic countries have revealed close to 1% of animals randomly sampled may actively carry *B. pseudomallei* in the trachea[49], while serological surveys in the same regions identified at least 6.3% seroprevalence in farmed swine[31].

Feral swine (*Sus scrofa*) are taxonomically identical to domestic swine and are an invasive species in the U.S. with an extensive ecological range[50]. Unlike most domestic swine in the U.S. that are often housed in indoor facilities[51], feral swine inhabit a multitude of environments and interact closely with both soil and standing water through their natural behaviors[50,52,53], both of which are the chief environmental reservoirs for *B. pseudomallei*. Because of this, it can be hypothesized that feral swine exposure in regions where the bacteria is present likely occurs at higher rates compared to humans and many domestic species; studying natural exposure in U.S. feral swine may thus help to identify true levels of environmental exposure as well as human risk in regions where melioidosis is endemic or may be emerging[31].

An extensive host-range, as well as diagnostic and treatment difficulties, coupled with unknown environmental distribution and global disease burden have made ongoing surveillance for melioidosis challenging. Furthermore, prior to the isolation of *B. pseudomallei* in Southern Mississippi, the disease was not nationally notifiable in the U.S., and the Centers for Disease Control and Prevention (CDC) received only voluntary reports[17,18,54]. Increased understanding of *B. pseudomallei* ecology, improved diagnostics, as well as enhanced surveillance strategies will

therefore be imperative in combatting melioidosis, especially in regions where the disease is emerging, or contain suitable environments for future bacterial establishment.

To address some of these knowledge gaps, we evaluated an indirect enzyme-linked immunosorbent assay (ELISA) utilizing *B. pseudomallei*-derived whole cell (WC) lysate antigen as an alternative serologic test for environmental exposure of swine, and assessed its performance in samples collected from free-ranging pot-bellied pigs and feral swine residing in three distinct regions of the U.S. To obtain swine sera of known exposure status for use as assay controls, we additionally experimentally inoculated six domestic swine either subcutaneously or intranasally with the avirulent *B. pseudomallei* strain Bp82 and evaluated seroconversion over time. As goats are also commonly effected livestock in melioidosis endemic countries, and exhibit clinical signs more often than swine, we also evaluated assay performance using sera opportunistically acquired from 32 domestic goats before and after inoculation with fully virulent *B. pseudomallei* strain Bp511. Finally, to assess assay specificity, we tested all goat and swine sera against WC lysate antigen prepared from *Pseudomonas aeruginosa*, a closely related bacterial species with an extensive environmental range[55-57].

We report that although this ELISA was successful at identifying seropositive laboratory animals experimentally inoculated with both virulent and avirulent strains of *B. pseudomallei*, high cross-reactivity with *P. aeruginosa* WC lysate antigen was identified during examination of samples collected from wild-caught swine throughout the U.S. Cross-reactivity with a closely related but rather ubiquitous environmental bacteria such as *P. aeruginosa* suggests the assay platform is likely inappropriate for use in field serosurveillance, particularly for samples

originating from free-ranging swine. More targeted antigens will thus be necessary to improve assay specificity before this technique may be utilized to its full capacity.

MATERIALS AND METHODS

Experimental Inoculation of Domestic Swine with Bp82

Animals

All methods and use of animals described here were performed with the approval of Colorado State University's Institutional Animal Care and Use Committee (Protocol #1679). Six female domestic swine approximately three months of age were obtained from Diamond Livestock in Kersey, Colorado, and transported directly to Colorado State University in Fort Collins, Colorado on October 6, 2022. Upon arrival, individuals were group-housed within the university's indoor barn space at the Foothills Campus in one pen (area = 350 ft^2) for one week prior to being moved into CSU's Animal Biosafety Level-3 (ABSL-3) space. Animals were also group-housed within the ABSL-3, in one room (area = 216 ft^2) with natural lighting and controlled climate. All swine had access to food and water ad libitum throughout all acclimation and study periods.

While housed in the indoor barn space, swine were allowed to acclimate for four days before being given a physical examination, where an initial blood sample was collected, and all individuals were implanted with thermally sensitive microchips (Lifechips, Destron-Fearing) for subsequent identification and temperature measurement. Blood (3-5 ml into serum separator tubes) was collected from the anterior vena cava vein under manual restraint, and serum extracted within two hours of collection. After being transferred into the ABSL-3, animals were

allowed to acclimate for one additional week before inoculation with *B. pseudomallei* strain Bp82.

Inoculum Preparation

Pre-existing Bp82 bacterial stocks prepared on August 28, 2022, and stored at -80°C were thawed one week prior to animal challenge, and 10-fold serial dilutions plated onto brain-heart infusion (BHI) agar plates to estimate stock titer. Plates were incubated overnight at 37°C, and stock titer was determined to be approximately $10^{8.5}$ CFU/ml. On the day of animal challenge, fresh aliquots of the same bacterial stock were thawed and diluted in sterile phosphate-buffered saline (PBS) to a concentration of 10^{8} CFU/ml.

Animal Challenge

Prior to inoculation, baseline body temperatures were recorded for all swine, as well as one final pre-inoculation blood sample collected (day 0). Diluted Bp82 was then administered either into the nares using a pipette (500µl per nare; n=3 pigs), or subcutaneously using an 18-gauge, 1-inch needle (1 ml; n=3 pigs). All inoculations were performed under manual restraint and were performed two more times as described above on 15- and 42-days post-inoculation (DPI), so that all individuals were exposed a total of three times.

Sampling and Clinical Observation

We evaluated all domestic swine for seroconversion following three exposures to Bp82. Blood samples were collected from all individuals on 0-, 15-, 28-, and 42-DPI while animals were under manual restraint, as during the initial acclimation period prior to animals being moved into the ABSL-3. All sera collected during the initial acclimation period were extracted

within two hours of collection and stored at -80°C until serological analysis. Sera that was collected after animals were housed within the ABSL-3 were also extracted within two hours of collection, however, were additionally passed through a 0.22 µm filter before being taken out of the ABSL-3 and stored at -80°C.

All animals were observed daily and included an assessment of temperament and the presence or absence of clinical signs of disease, including but not limited to ocular and nasal discharge, ptyalism, coughing/sneezing, dyspnea, diarrhea, lethargy, anorexia, and moribund status. Body temperatures were also recorded during days of sample collection when animals were being handled, as well as following any observed clinical abnormalities. All individuals were euthanized at 56-DPI by captive bolt after sedation with a 1 ml solution of 50 mg ketamine, 50 mg xylazine, and 80 mg telazol. Immediately following euthanasia, a terminal blood sample was collected directly from the heart, and a brief necropsy performed. During necropsy, approximately 100 mg of spleen tissue was collected into tubes containing 2 stainless steel balls and 1 ml of 20% glycerol in PBS for bacterial culture. All other internal organs were inspected grossly for lesions or abnormalities.

Bacterial Culture

All manipulations of viable cultures and tissues specimens were performed within the ABSL-3. Spleen tissue collected for culture were homogenized using a Qiagen Tissuelyser at 25 cps for 5 minutes and 100 µl of homogenate was plated onto Ashdown's selective medium[58,59]. Plates were observed daily for colonies with characteristic *B. pseudomallei* morphology[60], and allowed to incubate for a maximum of 96 hours at 37°C.

Serology

Antigen Preparation

Whole cell lysate antigen was extracted from *B. pseudomallei* strain Bp82, as well as *P. aeruginosa* strain PAO1 using methods described previously[61]. In brief, each respective strain was streaked and grown on BHI plates for 48 hours before being harvested into 30 ml of PBS and vortexed to create a homogenous solution. The resulting cell suspensions were then heat-killed at 80°C for one hour. Heat-killed suspensions were then centrifuged at 10,000 x g for 10 minutes, and the supernatants removed and used as assay antigens. Final antigen suspensions were placed in 1 ml aliquots by strain and stored at -20°C until assay.

To quantify the amount of antigen utilized for assays, we estimated the starting titers of all bacterial stocks used prior to inactivation by heat-killing, as well as the mass of protein present in the final supernatant. To estimate initial stock titers, 10-fold serial dilutions of each bacterial strain were plated onto brain-heart infusion (BHI) agar plates. Plates were incubated overnight at 37°C, and stock titer was determined to be approximately $10^{8.5}$ CFU/ml for Bp82, and $10^{8.7}$ CFU/ml for PAO1. After heat-killed suspensions of each bacterial strain were centrifuged and prior to storage at -20°C, we next estimated total protein content within the harvested WC-lysate supernatants using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, #23225). Total protein content for aliquots of Bp82 and PAO1 antigen preparations was estimated to be 1.14 mg/ml and 0.35 mg/ml, respectively.

Serum Samples

A total of 296 serum samples were examined for antibodies against *B. pseudomallei* Bp82 and *P. aeruginosa* PAO1 WC antigen preparations. Sera of interest were subdivided into five

distinct groups (I to V), and included animals of both known and unknown *B. pseudomallei* exposure status:

(i) **Group I (*n*=118): Samples from 32 domestic goats, before and after inoculation with *B. pseudomallei* strain Bp511.** Group I samples were obtained from 32 adult female domestic goats (*Capra aegagrus hircus*) utilized for an unrelated study evaluating the efficacy of three antimicrobial treatment regimens for melioidosis. Briefly, 32 goats were divided into four groups of eight individuals and challenged with $10^{3.7}$ CFU of *B. pseudomallei* strain Bp511. Each goat received 200 µl of inoculum, half of which was administered subcutaneously, and the other half intradermally. Following inoculation, individuals were either left alone (untreated control); or treated with select antibiotics twice a day. Serum samples from all individuals were collected prior to inoculation, as well as on days 7, 14, and 21 DPI for a total of 118 samples.

(ii) **Group II (*n*=36): Samples from six domestic swine, before and after inoculation with *B. pseudomallei* strain Bp82.** Group II samples were collected from six female domestic swine (*Sus scrofa*) before and after inoculation with *B. pseudomallei* strain Bp82, described in more detail in the above Materials and Methods sections. Briefly, individuals were divided into two groups of three, and challenged a total of three times, either intranasally or subcutaneously with 10^8 CFU of Bp82. Serum samples were collected prior to inoculation, as well as on days 7, 15, 28, 42, and 56 DPI.

(iii) **Group III (*n*=40): Samples from Vietnamese pot-bellied pigs removed from a region endemic for melioidosis (Puerto Rico, USA).** Group III samples were collected from Vietnamese pot-bellied pigs (*Sus scrofa*) of mixed sex and age-class

removed by USDA personnel from Puerto Rico in August 2019. Vietnamese pot-bellied pigs have become an increasingly problematic invasive species on the island, starting in 2015 when an influx of people purchased them as pets, but later released them[62]. This was further exacerbated in 2017 after Hurricane Maria displaced many more from confinement; since then, thousands of pot-bellied pigs have been estimated to roam 67 of the island's 78 municipalities[62,63]. Puerto Rico is a region endemic for melioidosis, with documented human infections as well as environmental isolations of *B. pseudomallei*[13,45,64].

(iv) Group IV (*n*=82): Samples from feral swine removed from a coastal region non-endemic for melioidosis (California, USA). Group IV samples were collected from feral swine removed by USDA personnel from California during routine feral swine damage management activities. Samples were collected in the years 2019 and 2020, from San Luis Obispo (n=60), Santa Clara (n=4), Kern (n=4), and Napa (n=16) counties. Feral swine sampled were of mixed age-class and sex. While considered a coastal region, these counties are considered part of the Upper and Lower Sonoran life zones and are defined by desert ecology[65]. California is additionally a region with no current predicted environmental suitability for *B. pseudomallei*[7] and has had no non-travel related case reports of melioidosis to date. Feral swine residing here thus represent a population that may be presumed seronegative.

(v) Group V (*n*=20): Samples from feral swine removed from an inland region non-endemic for melioidosis (Arizona, USA). Group V samples were collected from feral swine removed by USDA personnel from Arizona during routine feral swine damage management activities. Samples were collected in the years 2018-2020 from

Mohave County. Arizona is a land-locked state in the western U.S., composed mostly of desert ecotypes[65], and is a region with no current predicted environmental suitability for *B. pseudomallei*[7]. To date the state has documented one autochthonous human case of melioidosis in a man in Southern Arizona in 2008, and while the source of exposure was never identified, genomic analysis of the patient's isolate revealed the etiologic agent to be of Southeast Asian origin, suggesting exposure to imported material(s)[66,67]. Feral swine residing here may thus be presumed seronegative.

Assay Optimization

Optimum sample sera and secondary detection antibody for Bp82 assays were determined separately by species (e.g., goat or swine) using checkerboard titration[68]. Pre-inoculation and final post-inoculation serum samples (e.g., 21 DPI for goat sera and 56 DPI for swine sera) were diluted in 1% blocking buffer, composed of skim milk powder suspended in sterile PBS at a range of 1 to 1:2000 (two-fold serial dilutions starting from 1, or undiluted serum). Either protein A/G-horseradish peroxidase (HRP) (Thermo-Scientific #32490) or rabbit anti-pig IgG-HRP (Invitrogen #PA1-28602) secondary antibody was diluted at a range of 1:1000 to 1:32000 in 1% blocking buffer, (two-fold serial dilutions) for respective goat and swine assays. All serum and secondary antibody pairs were tested in duplicate. Signal-to-noise (S/N) ratios were calculated for each dilution by dividing the mean absorbance value of all known post-inoculation sera, collected at the last sampling time point (n=22 for goat assays, 21 DPI; n=6 for swine assays, 56 DPI), by that exhibited by all negative or pre-inoculation sera (n=32 for goat assays; n=6 for swine assays). Assay conditions resulting in S/N ratios of 10 or above were

considered optimal[69] and utilized in the final assay protocol, as described in the ELISA section below.

Antigen for Bp82 assays was coated onto plates at a concentration of 250 ng/ml for initial testing[70], and further optimized following initial sera and secondary antibody optimization described above. To further optimize the S/N ratios of pre- and post-inoculation swine and goat sera (Groups I and II), Bp82 WC lysate antigen was diluted at a range of 0 to 5 µg/ml (2-fold dilutions starting from 5 µg/ml) in carbonate buffer (0.16 g Na_2CO_3, 0.29 g $NaHCO_3$, 100 ml distilled water, [pH 9.6]) and examined again by checkerboard titration with sample sera, diluted at a range of 1 to 1:2000. All sera and antigen dilution pairs were examined in duplicate.

Following assay optimization for animals of known exposure status, the remaining serum samples from Groups I and II, as well as samples from Groups III-V were assayed and compared to pre-inoculation sera by species. To assess the specificity of the assay and determine if cross-reactive antibodies exist between WC lysate preparations of closely related bacterial species, we additionally examined all samples against PAO1 WC lysate antigen, also by ELISA. Assays utilizing PAO1 antigen were performed using the same optimized conditions determined for Bp82 antigen preparations.

Cutoff Determination

Due to the evolving epidemiology of *B. pseudomallei* in the U.S., identifying false positive individuals, especially in regions with no known case reports of melioidosis represent a relatively high risk, as identification may prompt additional investigation by the CDC[71]. We therefore considered two cutoff values for our assay, the first being the standard cutoff determination of three sample standard deviations (SD) above the mean absorbance value of the

seronegative control sera[72] (e.g., +3SD above Group I pre-inoculation sera for goat assays, or Group II pre-inoculation sera for swine assays). This method is often used for ELISAs and is currently accepted by the World Organization for Animal Health to establish cutoff values if the assay in use is not validated[31,68]. Three sample standard deviations above values exhibited by the seronegative control groups conservatively excludes 99.7% of seronegative animals as seropositive, based on the statistical assumptions of this methodology[31,34]. We additionally considered an alternative methodology for establishing assay an assay cutoff that would potentially exclude seronegative animals even more conservatively; this method was based on the equation initially described by Dixon and Massey in 1983[73]:

$$Cutoff = \bar{X} + SDt\sqrt{1 + (1/n)}$$

Where $\bar{X}$ is the average absorbance of independent negative control sera, SD is the standard deviation of negative control sera, n is the number of independent negative controls, and t is the $(1-\alpha)th$ percentile of the one-tailed t-distribution with $v = n$ degrees of freedom[74]. As this method also considers the number of independent control sera utilized in assays as well as confidence levels ranging from 95 to 99.9%, it theoretically represents a more statistically rigorous method that also does not require sophisticated computation[74]. A confidence level of 99.9% was considered for both swine and goat assays examined here. Both cutoff values were determined by species using pre-inoculated goat and swine sera (Group I, n=6; and Group II, n=36) and were conserved across Bp82 and PAO1-based assays.

ELISA (Optimized Protocol)

We utilized an indirect ELISA platform like those described previously[28,61,70,75], utilizing WC-lysate from heat-killed Bp82, as well as heat-killed PAO1 as crude antigens. Slight modifications were made to target antibodies of swine and goat origin, and the assay optimized as described in the above Assay Optimization section. Prior to performing all ELISAs, WC antigen aliquots from either bacterial species were thawed, and diluted to 3 µg/ml with carbonate buffer. Assays were performed separately by antigen and species of interest.

High-binding polystyrene 96-well round-bottom microtiter plates, (Thermo-Scientific, #3655) were coated with heat-killed WC-lysate from either Bp82 or PAO1 diluted in carbonate buffer solution at a concentration of 3 µg/ml per well and incubated overnight at 4°C. The following day, the coating buffer was discarded, and wells washed four times with PBS containing 0.05% Tween 20 (washing buffer). Wells were blocked with the addition of 250 µl of 5% skim milk in PBS and allowed to incubate for 2 hours at 37°C. Wells were then washed again as described above, and 100 µl of sample sera diluted either 1:100 (goat sera) or 1:400 (swine sera) in 1% blocking buffer and incubated at room temperature for 30 minutes. After additional washing, 100 µl/well of either protein A/G-HRP diluted 1:2000 (goat sera), or rabbit anti-pig IgG-HRP diluted 1:10,000 (swine sera) in 1% blocking buffer was added, and plates incubated again for 30 minutes at room temperature. Following one final washing step, 100 µl of 1-Step TMB substrate (Thermo-Scientific, #PI34022) was added and allowed to incubate for 15 minutes at room temperature. The reaction was then stopped with the addition of 100 µl of 1M HCl, and absorbance value read at a wavelength of 450 nm using a microtiter plate reader (BioTek #BT800TS) paired with Gen5 microplate reader and imager software (version 3.12). Goat

samples were considered positive for *B. pseudomallei* antibodies if their mean absorbance values were greater than the assay cutoff for 0.042 absorbance units for the +3SD cutoff method, or 0.047 AU when considering the Dixon and Massay cutoff method. Similarly, swine samples were considered positive for *B. pseudomallei* antibodies if their mean absorbance value was greater than the assay cutoff of 0.23 absorbance units, or 0.15 AU when considering +3SD and Dixon and Massey cutoff methods, respectively. Cutoff values were conserved across assays utilizing Bp82 or PAO1 antigen for each species. Individual samples were run in duplicate.

RESULTS

Experimental Inoculation of Domestic Swine with Bp82

Clinical Observation

None of the study pigs displayed any outward signs of clinical disease following intranasal or subcutaneous exposure to Bp82 and maintained stable body temperatures throughout the study period. One of the pigs exposed subcutaneously (#3710) however, did develop a minor nodule at the injection site; this nodule was observed on 15 DPI, and was estimated to be approximately 20 mm in diameter. Daily temperature measurements were taken for this individual after initial observation of the nodule to determine if any systemic adverse reaction was occurring; temperature remained stable and within normal limits. Palpation of the area did not illicit any pain response from the animal, and no discharge was observed to originate from the site. No additional skin lesions were observed for this individual over the remainder of the study period, and the nodule appeared completely resolved by 30 DPI. Moreover, none of the study animals displayed any abnormal behavior(s) compared to the acclimation period. For

detailed illustration of individual body temperature and clinical notes for each pig, see Table A.3 of the Appendix.

Bacterial Culture

Bacterial culture of spleen homogenates collected at necropsy on Ashdown's medium did not result in any bacterial growth over the course of the 96-hour incubation period, indicating no active infection or replication of Bp82 within any of the study pigs.

Serology

Assay Optimization and Cutoff Determination

Following checkboard titrations of both goat and swine sera with either protein A/G-HRP or rabbit anti-pig IgG-HRP, the optimal sample sera dilution was determined to be 1:100 and 1:400 for goat and pig sera, respectively. Optimal secondary antibody concentrations were 1:2000 for protein A/G-HRP, and 1:10,000 for rabbit anti-pig IgG-HRP. Corresponding S/N ratios calculated from each of these concentration pairings for pre- and post-inoculation goat and swine sera were 38.14 and 11.27, respectively.

Negative and positive goat control sera (e.g., Group I pre- and final post-inoculation samples) exhibited average absorbances of 0.011 (SD=0.010) and 0.42 (SD=0.21), respectively. An assay cutoff of +3SD above the mean of the negative controls was calculated to be 0.042 absorbance units for all subsequent assays examining goat sera. An additional cutoff value of 0.047 absorbance units was determined using the Dixon and Massey method, where $t = 3.37$, derived from the one-tailed students t-distribution table[76] and corresponded to the critical value

for 99.9% confidence. The number of independent negative controls utilized for goat assays (e.g., n in the Dixon and Massey cutoff equation), was 32.

Negative and positive sera utilized for swine assays (e.g., Group II pre- and final post-inoculation samples) exhibited average readings of 0.055 (SD=0.030) and 0.65 (SD=0.21) absorbance units respectively. A cutoff of +3SD above the mean of the negative control sera was calculated to be 0.15 absorbance units. The Dixon and Massey cutoff point for 99.9% confidence was determined to be 0.23 absorbance units, with $t = 6.37$; and $n = 6$.

Group I

Serological results for *B. pseudomallei* by Bp82 WC lysate ELISA for domestic goats experimentally inoculated with Bp511 are illustrated in Figure 4.1 and broken down by treatment group. Most individuals in each group seroconverted and displayed antibodies against Bp82 WC lysate, defined as absorbance values above either 0.042 (+3SD) or 0.047 (Dixon and Massey) AU. Notably, given the proximity of both calculated cutoff values, an average value of 0.045 AU is displayed on Figure 4.1 for simplicity. Average pre-inoculation absorbance value across treatment groups was 0.011 (SD=0.01). Animals classified as seropositive displayed absorbances ranging from 0.045 to 0.74 AU.

The bulk of seroconversion across all treatment groups was observed at 14 DPI, and antibody levels peaked for each group at 21 DPI. Four individuals seroconverted as early as 7 DPI and exhibited absorbance readings of 0.062 (No Treatment), 0.086 (Treatment 2), 0.045 (Treatment 3), and 0.17 (Treatment 3) AU, respectively. At 14 DPI, all remaining study goats except two individuals seroconverted and displayed absorbance levels above that of the more stringent Dixon and Massey cutoff at 14 DPI; those that did not seroconvert displayed

absorbance values below that of both cutoff points and received Treatment 3 (Figure 4.1). Individuals receiving Treatment 2 displayed the highest antibody levels on 14 DPI (mean = 0.29 AU, SD = 0.17), while those receiving Treatment 3 had the lowest (mean = 0.11 AU, SD = 0.083). At 21 DPI, goats in the control group receiving no treatment displayed the highest anti-Bp82 antibody (mean = 0.54, SD = 0.11), although the highest absorbance value was exhibited by one goat receiving Treatment 2 (0.74 AU). Goats receiving Treatment 3 remained the group with the lowest antibody response (mean = 0.11, SD = 0.16).

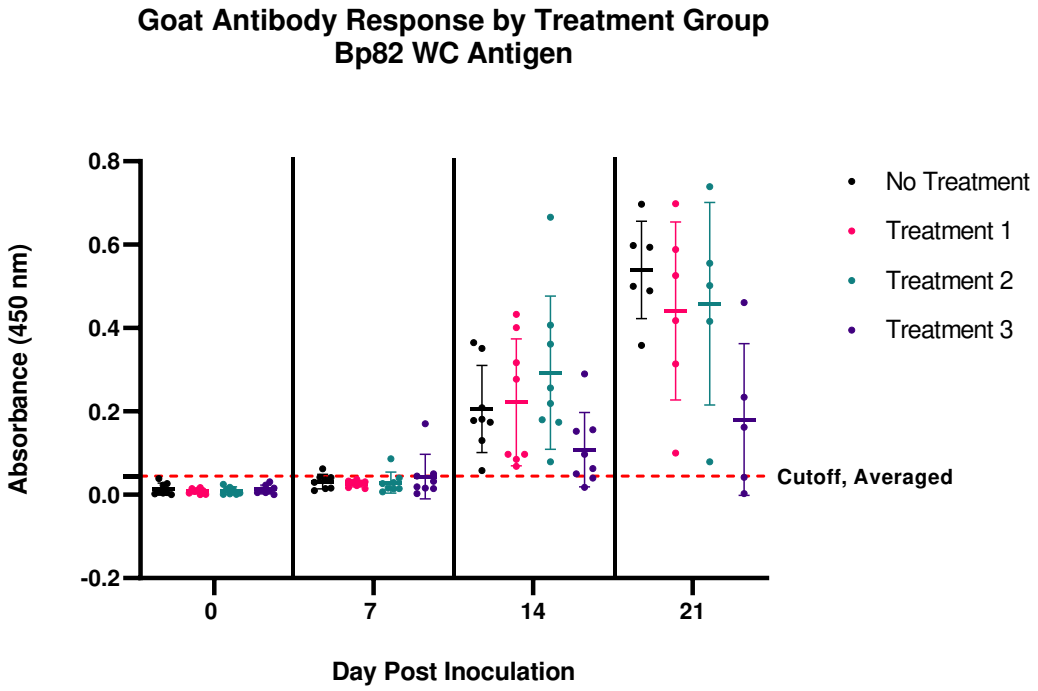

Figure 4.1. Antibody responses to *B. pseudomallei* Bp82 WC lysate antigen over time in domestic goats inoculated with $10^{3.7}$ CFU/ml Bp511, separated by treatment group. The red dotted line denotes the average cutoff value estimated from +3SD and Dixon and Massey methods as described in the text and equates to 0.045 absorbance units.

Corresponding *P. aeruginosa* serological results by PAO1 WC lysate ELISA for Bp511 inoculated goats are illustrated by treatment group in Figure 4.2. On average, absorbance reading from all Group I goat sera (e.g., serum samples from all goats collected pre- and post-inoculation with Bp511) was 0.13 AU higher when examined against Bp82 WC lysate antigen than when examined against PAO1 antigen (Figures 4.1 and 4.2). The majority (26/32) goats displayed absorbance values below that of both cutoff values determined for Bp82 assays and ranged from 0 to 0.039 AU (mean = 0.014, SD = 0.011). Average absorbance for each treatment group was relatively stable throughout the study period, oscillating slightly in value between each sampling point (Figure 4.2). The remaining six individuals displayed absorbances above that of either the +3SD cutoff or both +3SD and Dixon and Massey cutoff values. Goats with absorbances above only the +3SD cutoff value but below the Dixon and Massey cutoff were from the No Treatment control group (n = 2) and Treatments 1 and 2 (n =2 and n=1, respectively). One goat in the group receiving Treatment 1 consistently displayed absorbance values above that of both cutoff values considered and peaked at 0.12 AU on 14 DPI. Finally, one goat from the Treatment 1 group that initially displayed an absorbance value above the +3SD cutoff (0.043) on 0 DPI dropped to levels below both cutoffs for 7 and 14 DPI, before climbing to a level above that of both cutoffs on 21 DPI (0.063).

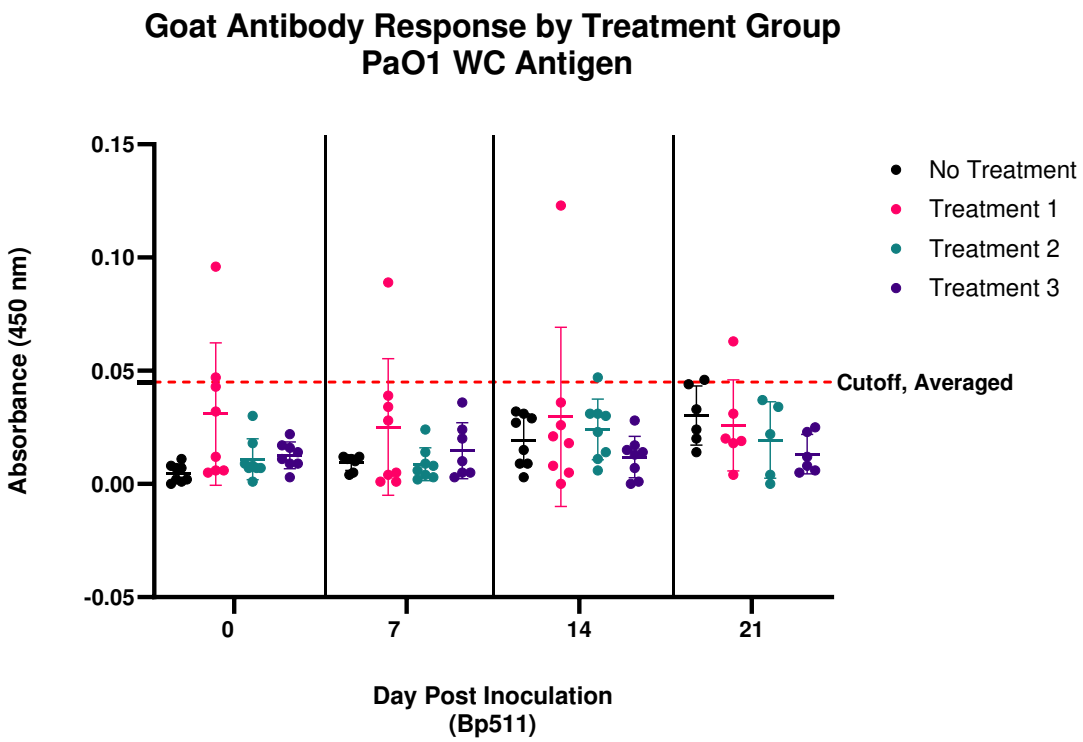

Figure 4.2. Antibody responses to *P. aeruginosa* PAO1 WC lysate antigen over time in domestic goats inoculated with $10^{3.7}$ CFU/ml Bp511, separated by treatment group. The red dotted line denotes the average cutoff value estimated from +3SD and Dixon and Massey methods determined for Bp82 assays as described in the text and equates to 0.045 absorbance units.

Group II

Serological results by Bp82 WC lysate ELISA for Bp82 inoculated swine are illustrated in Figure 4.3. Most individuals appeared to seroconvert and display antibodies against Bp82 WC lysate, depicted as absorbance values above the assay cutoffs of either 0.15 (+3SD) or 0.23 (Dixon and Massey, 99.9% confidence) absorbance units. All study pigs except #1301 exhibited absorbance values above both cutoffs by 28 DPI; pig #1301 never reached levels above that of the Dixon and Massay cutoff, and only exhibited absorbances above that of the +3SD cutoff on 28 and 56 DPI.

While varying slightly by individual, relative antibody response differed by route of exposure, with subcutaneously inoculated pigs seroconverting faster than those intranasally inoculated; subcutaneously inoculated pigs also displayed higher absorbance values than those inoculated intranasally at any given time point post-inoculation (Figure 4.3). The exception to this trend was 56 DPI, where one intranasally exposed pig (#3707) displayed an absorbance value above that of two of the three subcutaneously inoculated swine (#1302 and 3709). Most (4/6) individuals peaked in absorbance on 28 DPI, with the highest anti-Bp82 antibody response exhibited by pig #3709 (0.87 AU), while the lowest peak was displayed by pig #1301 (0.21 AU). The remaining two pigs that did not peak in antibody response on 28 DPI both peaked instead on 56 DPI (#3710, 0.85 AU; #3707, 0.67 AU). Individuals that peaked at 56 DPI represented both subcutaneous and intranasally inoculated groups. Finally, following rechallenge on 15 and 42 DPI, the majority (5/6) individuals displayed increased anti-Bp-82 antibodies at subsequent time points (e.g., 28 and 56 DPI). The exception to this trend was subcutaneously inoculated pig #3709, who displayed decreased antibody levels on 56 DPI after being rechallenged on 42 DPI. Between 28 and 42 DPI, all individuals experienced a decline in antibody response, prior to being rechallenged a second and final time on 42 DPI.

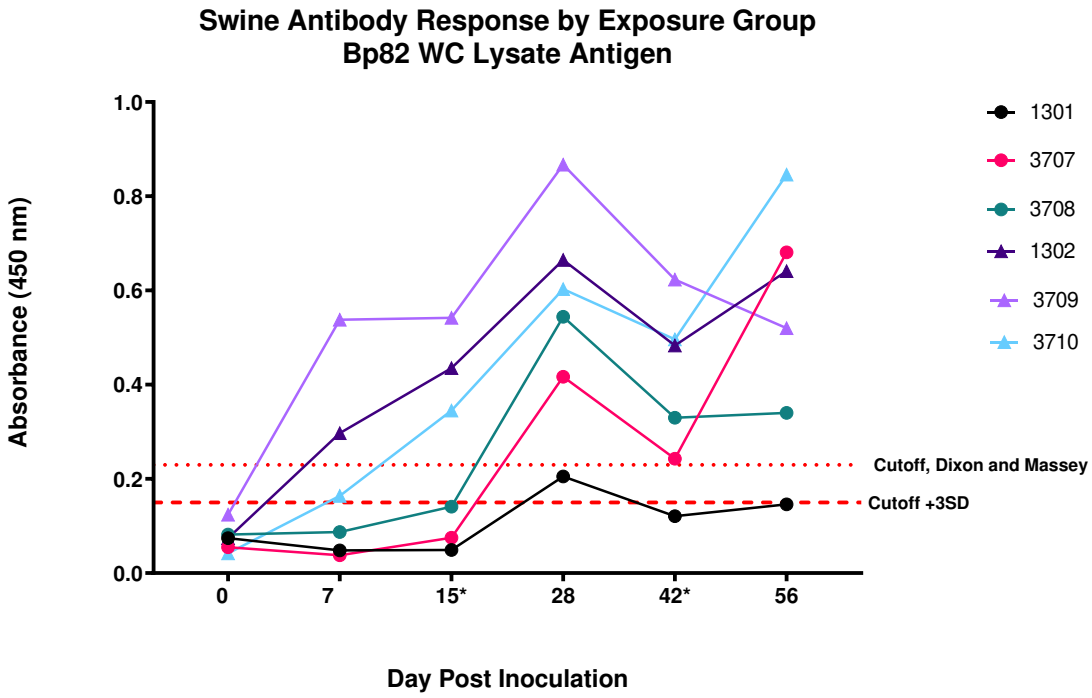

Figure 4.3. Antibody responses to *B. pseudomallei* Bp82 WC lysate antigen over time in domestic swine inoculated with 10^8 CFU/ml Bp82. Circles represent individuals that were intranasally inoculated, and triangles subcutaneously inoculated individuals. Starred timepoints on the x-axis indicate when all swine were re-challenged, (e.g, 15 and 42 DPI). Red dotted lines denote either the +3SD cutoff of 0.15 absorbance units, or the Dixon and Massay 99.9% confidence cutoff, equating to 0.23 absorbance units.

Serological results by PAO1 WC lysate ELISA for Bp82 inoculated swine are illustrated in Figure 4.4. On average, absorbance readings for Group II swine sera (e.g, samples from all swine collected pre- and post-Bp82 inoculation) was 0.23 AU higher when examined against Bp82 WC lysate antigen than against PAO1 WC lysate antigen (Figures 4.3 and 4.4). The majority (5/6) of individuals did not display absorbance values above either of the two cutoffs established for Bp82 assays and oscillated slightly in value throughout the study period. The one exception to this trend was subcutaneously inoculated pig #3709; this individual consistently displayed absorbance values above the +3SD cutoff, and after 15 DPI increased to values above

the Dixon and Massey cutoff point, where they remained until 56 DPI; despite being above either or both cutoff points, raw absorbance values for #3709 was 0.33 AU higher against Bp82 antigen than for PAO1 on average (Figures 4.3 and 4.4). For all intranasally inoculated swine, the average antibody response exhibited against PAO1 antigen throughout the study period was 0.099 AU (SD = 0.025), while subcutaneously inoculated individuals displayed an average absorbance of 0.11 AU (SD = 0.076). Absorbance values for pig #3709 peaked at 0.29 AU on 28 DPI, before declining at a steady rate for the remainder of the study period.

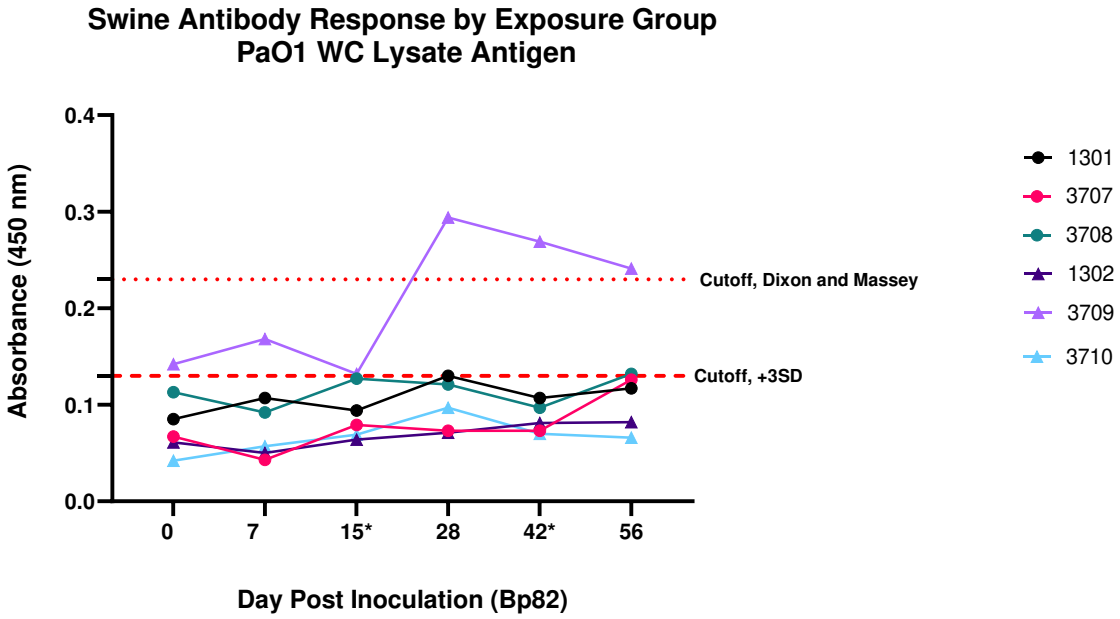

Figure 4.4. Antibody responses to *P. aeruginosa* PAO1 WC lysate antigen over time in domestic swine intranasally or subcutaneously inoculated with 10^8 CFU/ml *B. pseudomallei* Bp82. Circles represent individuals that were intranasally inoculated, and triangles subcutaneously inoculated individuals. Starred timepoints on the x-axis indicate when all swine were re-challenged with Bp82 (e.g, 15 and 42 DPI). For reference, red dotted lines denote either the +3SD or Dixon and Massay 99.9% confidence cutoff points as determined for Bp82 ELISAs, equating to 0.15 and 0.23 absorbance units, respectively.

Groups III-V

Serological results for free-ranging feral swine and Vietnamese pot-bellied pigs against Bp82 and PAO1 WC lysate antigens are depicted in Figure 4.5 and are compared to seronegative and seropositive sera from experimentally inoculated pigs (Group II). Seronegative control sera included pre-inoculation serum samples from Group II domestic swine, as well as post-inoculation sera exhibiting absorbance values below that of both +3SD and Dixon and Massey cutoff points (n = 14 samples). Seropositive control sera were defined as samples collected from study swine post-Bp82 inoculation exhibiting absorbance values above both assay cutoff points (n = 20 samples).

In general, mean absorbance was relatively stable across region of origin and antigen type (Figure 4.5). Average absorbance exhibited against Bp82 WC lysate was 0.19 (SD = 0.076), 0.22 (SD = 0.086), and 0.22 (SD = 0.051) AU for Arizona, California, and Puerto Rico swine populations, respectively. Average absorbance exhibited against PAO1 WC lysate was 0.20 (SD = 0.13), 0.20 (SD = 0.11), and 0.26 (SD = 0.15) AU for Arizona, California, and Puerto Rico swine populations, respectively. A two-way ANOVA with Šídák's multiple comparisons test was performed to examine the effect of antigen type on observed absorbance level for each respective population of free-ranging swine. Two-way ANOVA analysis revealed that the only significant difference in absorbance levels between Bp82 and PAO1 antigens was that exhibited by seropositive assay control sera, with absorbance exhibited against Bp82 WC lysate antigen being significantly higher than that exhibited against PAO1 WC lysate ($p < 0.0001$). No statistically significant difference in sample absorbance was found between antigen types for Arizona ($p =$

1.00), California (p =0.44), or Puerto Rico (p = 0.39) free-ranging swine serum samples, nor between seronegative control sera from Group II domestic swine (p = 1.00).

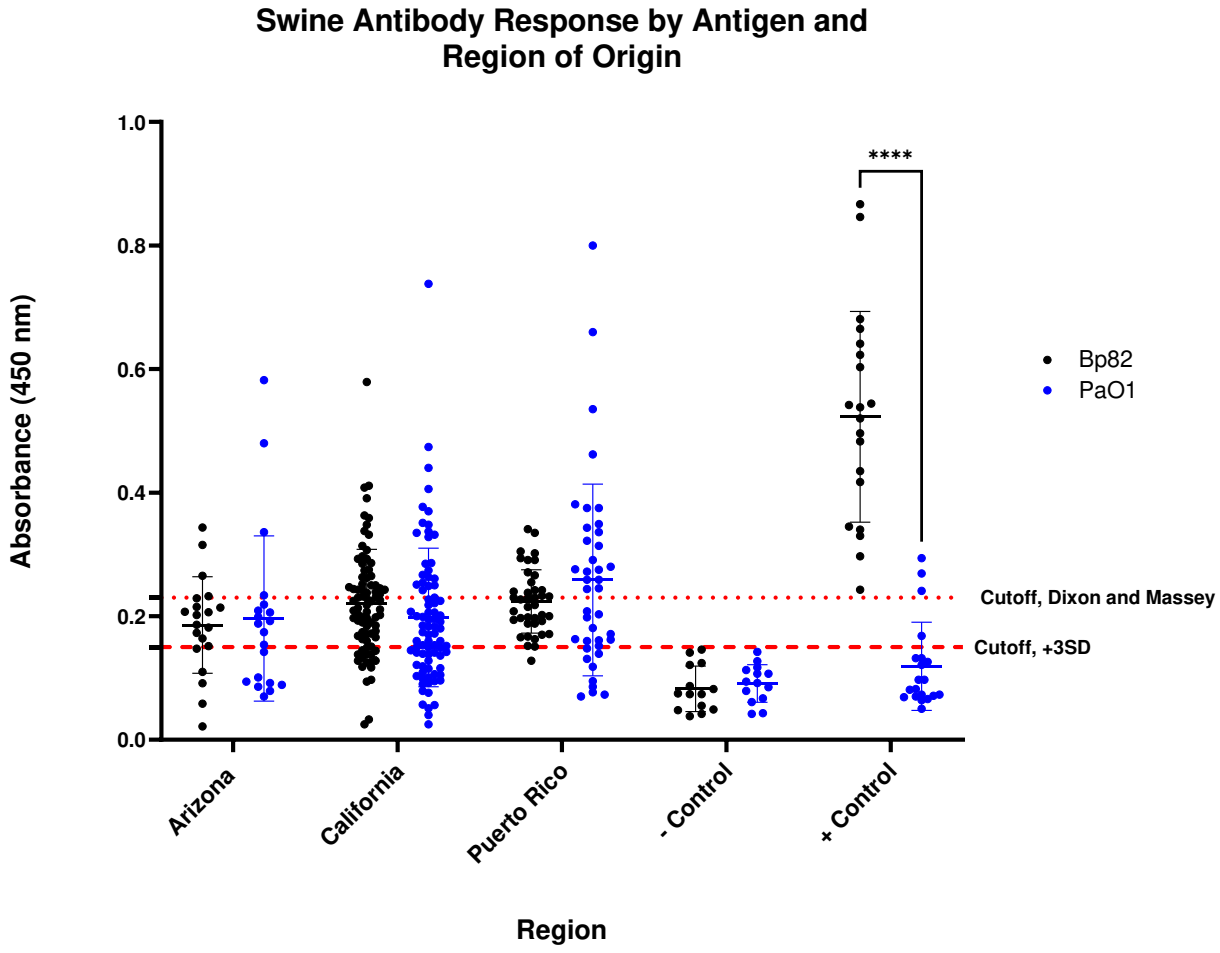

Figure 4.5. Antibody responses to *B. pseudomallei* Bp82 and *P. aeruginosa* PAO1 WC lysate antigens in free-ranging pigs on the mainland U.S. and Puerto Rico. For reference, sera collected from domestic swine inoculated with 10^8 CFU/ml Bp82 were included as known seropositive and seronegative controls. "– Control" denotes all sera collected from domestic swine that read below both +3SD and Dixon and Massey cutoffs over the course of the experimental inoculation study described in the text. "+ Control" denotes sera collected from domestic swine post-inoculation with 10^8 CFU/ml Bp82 and exhibiting absorbance readings above that of both +3SD and Dixon and Massay cutoff points. Red dotted lines denote either the +3SD or Dixon and Massay 99.9% confidence cutoff points as determined for Bp82 ELISAs, equating to 0.15 and 0.23 absorbance units, respectively. Asterisks represent statistical significance as determined using two-way ANOVA with Šídák's multiple comparisons test; absorbance readings were compared across antigen type within each swine population (p<0.0001).

DISCUSSION

This study sought to evaluate an ELISA utilizing crude WC lysate antigen to examine swine sera for previous or recent exposure to *B. pseudomallei*. As feral swine are a prolific invasive species in the U.S., incorporating serosurveillance for melioidosis in regions where animals are being removed for invasive species or damage management may help to indirectly detect bacterial presence, and subsequently identify sites where finer scale environmental sampling is warranted. Given that swine have behavioral tendencies to disrupt soil and utilize standing water sites through rooting and wallowing, bacterial exposure in contaminated environments also may occur at higher rates compared to humans or other taxa that inhabit the same environments. Sampling feral swine may thus offer a unique opportunity to evaluate the environmental distribution of *B. pseudomallei* in regions of the U.S. where our epidemiological understanding of the pathogen is unknown or evolving.

An ELISA was chosen as an alternative serological test to IHA due to the relative simplicity of the assay protocol as well as its high throughput capacity. Moreover, the assay utilized here was previously demonstrated in human sera to have significantly higher sensitivity and specificity than IHA, as well as comparable sensitivity and specificity to the more targeted 6-deoxyheptan capsular polysaccharide (CPS) and O-polysaccharide (OPS) antigens purified from *B. pseudomallei*[70]. Both OPS and CPS have been formerly demonstrated as promising antigens for serodiagnosis of melioidosis in regions nonendemic for the disease, however purification of these polysaccharides often requires the use of specialized equipment and can be labor intensive[31,33,35]. In contrast, preparation of WC lysate antigen mixtures is a relatively simple procedure, and requires minimal equipment compared to the purification of protein or

polysaccharide antigens, thus offering an economical alternative for testing large quantities of samples. Additionally, use of a WC lysate antigen mixture may better mimic the complete antigenic profile of *B. pseudomallei*, and therefore better classify seropositive individuals than any single antigen alone[31,33,36]. In addition to assessing this ELISA for its ability to correctly characterize animals of known exposure status, we also considered two methods for establishing assay cutoffs, and examined how they may affect the interpretation of samples of known and unknown antibody status. Two cutoff values of different stringency were evaluated due to the high cost associated with falsely identifying seropositive animals, as *B. pseudomallei* is a Tier-1 select agent[77], and regions of endemicity in the U.S. are still being established and largely unknown. Lastly, we tested all sera against WC lysate antigen mixtures derived from *P. aeruginosa* PAO1, to determine if cross-reactive antibodies may exist between closely related bacterial species, as many surface proteins and membrane polysaccharides are conserved between *B. pseudomallei* and *P. aeruginosa*[55,83]. Despite the potential for high antigen conservation, no study to our knowledge has examined whether antibody cross-reactivity exists between these two species, particularly with sera examined against crude WC lysate antigen mixtures as described for the ELISA protocol evaluated here.

The results obtained during initial assay optimization demonstrated that domestic goats and swine exhibit measurable antibodies against bacterial WC lysate following known exposure to *B. pseudomallei* (e.g., Groups I and II). While the goat sera examined here originated from a species different than that of the primary population of interest, they represented an additional cluster of animals of known exposure status for assay evaluation. The strain of *B. pseudomallei* goats were exposed to (Bp511) was also derived from a virulent field isolate and contrasted from the avirulent laboratory strain (Bp82) used during pig inoculation as well as the source of coating

antigen for assays. Examining serology in goats pre and post exposure to a wild-type strain of *B. pseudomallei* therefore allowed for evaluation of Bp82-derived WC lysate antigen performance and served as a point of comparison for the swine sera subsequently examined. After testing of serum from experimentally exposed goat and swine, we found that despite the minor protocol differences across species, similar patterns and levels of seroconversion were detected in goats challenged with Bp511 and pigs that were challenged with Bp82, suggesting strong epitope conservation between bacterial strains. These patterns ultimately support the use of Bp82 as a source of WC lysate antigen to classify animals as being exposed to wild-type or more virulent strains. Finally, the serological response of all animals experimentally inoculated with *B. pseudomallei* was significantly lower at all sampling timepoints when examined against PAO1 antigen, and most goats and swine displayed absorbance values below that of both cutoff values considered for corresponding Bp82-based assays. Six exceptions to this trend however were observed for inoculated goats, and one exception for inoculated swine, where animals displayed absorbance values above either or both assay cutoff values under the PAO1-based ELISAs. While the absorbance values observed for these individuals were still significantly lower than those obtained against Bp82 antigens, they were above either or both assay cutoff values considered, suggesting some antibody cross-reactivity between Bp82 and PAO1 antigens.

As our populations of interest were U.S. feral and free-ranging swine, we experimentally inoculated a group of domestic swine with 10^8 CFU of Bp82 and collected serial serum samples to measure seroconversion as well as obtain relevant assay controls for use when examining pig sera of unknown exposure status. Secondarily, we also evaluated the effects of exposure route on seroconversion and relative antibody levels, and inoculated pigs either subcutaneously or intranasally to simulate percutaneous and inhalational routes of *B. pseudomallei* exposure. While

all animals were given the same dose of inoculum, the relative strength of the antibody response differed between exposure routes, with pigs challenged subcutaneously seroconverting faster and displaying higher antibody levels than those challenged intranasally. This trend continued for the majority (5/6) of study animals after re-inoculation on 15 and 42 DPI, and all but one individual experienced increased antibody response following additional exposure events. Most animals challenged subcutaneously additionally displayed absorbance values above both assay cutoffs considered, and particularly above that of the more stringent Dixon and Massay cutoff value, further suggesting a more robust antibody response than intranasally inoculated pigs. In contrast, only two of the three intranasally exposed individuals appeared to seroconvert and display absorbance values above that of the more stringent assay cutoff; one individual did clear the lower +3SD cutoff on 28 DPI, however remained below both assay cutoff values at all other sampling time points.

We chose to evaluate two methods for establishing assay cutoff values to correctly identify seropositive and seronegative individuals given the high risk associated with identifying seropositive individuals in a country historically non-endemic for melioidosis. Concerning the interpretation of animals of known exposure status, we found that most samples identified as seropositive were above both estimated cutoff values and considered positive by either method; this pattern was consistent across goat and swine-based assays. Interestingly however, the cutoff values themselves established for assays examining goat sera were closer together in value, appearing to converge around 0.04 AU, while the values estimated for assays using domestic swine sera as controls were more separated in absolute value, signifying less agreement between the two cutoff methods considered. The calculated +3SD and Dixon and Massay cutoff values for assays examining swine sera were separated by 0.08 AU, in contrast to the 0.005 AU

separating corresponding cutoff values for goat assays. Differences in the magnitude of assay cutoff values may have to do with the number of known negative controls utilized for respective goat and swine assays ($n = 32\ and\ 6$, respectively). Despite accounting for similar levels of confidence, the Dixon and Massay cutoff methodology, unlike that for the +3SD, accounts for the number of independent negative controls utilized for interpretation (e.g, n)[31,68]; higher n values as used during assays examining goat sera may therefore have resulted in values closer in value, and therefore even higher agreement between the cutoff values for swine assays.

While we observed our study pigs daily for clinical signs of disease, we detected no clinical abnormalities for the majority (5/6) of individuals throughout the study period after subcutaneous or intranasal exposure to Bp82. This was not surprising given that Bp82 is an attenuated laboratory strain, demonstrated to be avirulent in both immunocompetent and immunodeficient animals[79]. Moreover, suids appear to be relatively resistant to developing observable melioidosis after exposure to fully virulent *B. pseudomallei*, suggesting that any clinical presentation of disease would be a rare event. While one study pig subcutaneously challenged (#3710) developed a small nodule at the site of injection, no other clinical abnormalities were observed for the individual through the remainder of the study period, and body temperature remained within normal limits. Small nodules are common for many intramuscular and subcutaneous vaccinations given in mammals[80,81], and the minor reaction observed here after injection of avirulent bacterial strain may have been similar in nature. Finally, following euthanasia, we performed a brief necropsy and collected spleen tissue from all study animals to confirm that subclinical infection was not present, and that bacterial replication was not occurring within the body. The lack of any abscesses in the internal organs as well as lack of *B. pseudomallei* isolation from tissue homogenates after bacterial culture is not surprising

given that the Bp82 strain utilized for inoculation is incapable of efficient replication and dissemination to the spleen, one of the common tissue tropisms for melioidosis infected animals[79,82].

Following initial assay optimization and testing of laboratory exposed swine, we next sought to validate this ELISA for use in field samples by examining sera collected from swine originating from three distinct regions of the U.S. to assess environmental exposure to *B. pseudomallei* (e.g., Groups III-V). We chose to test serum collected from feral swine removed from Arizona and California, which are likely to be unsuitable for endemicity, as well as Vietnamese pot-bellied pigs in Puerto Rico, where melioidosis endemicity can be confidently inferred; these are also regions that have established swine management programs where which animals are regularly being sampled and removed off the landscape[50]. Assay of field samples using the optimized ELISA protocol revealed that swine across the U.S. and in the U.S. territory of Puerto Rico possess similar anti-Bp82 antibody levels. Further analysis of these samples against PAO1 antigen however, indicated that animals displayed similar values against non-B. *pseudomallei* derived antigen mixtures, and subsequent statistical analysis further supported a non-significant difference in serology by antigen type. Taken together, these results imply that significant antibody cross-reactivity exists between Bp82 and PAO1 WC lysate antigens; therefore, a confident serological interpretation of these samples could therefore not be reached using the optimized ELISA protocol examined here.

The results and subsequent statistical analysis of these field samples were somewhat surprising given that very little agreement was observed between antigen types of experimentally inoculated goat and swine sera tested during assay optimization. Secondarily, the ELISA protocol

examined here was previously demonstrated to have high specificity in human sera examined in regions of known melioidosis endemicity; the results described for these previous studies additionally juxtaposed those we obtained here from pigs also originating from similarly characterized regions. Contrasting levels of agreement between Bp82 and PAO1 antigens in laboratory and field swine however may be explained by the level of bacterial exposure each group likely experienced before being sampled, as well as the taxonomic relationship of those species to *B. pseudomallei*. The swine utilized here for experimental inoculation with Bp82 were all juveniles housed indoors immediately after being obtained for study, and therefore were likely exposed to a limited collection of bacterial species in their environment. In contrast, the free-ranging swine populations removed from the landscape and sampled for analysis here likely were exposed to a myriad of bacterial species within their respective environments by nature of existing solely outdoors. Furthermore, as most field animals sampled were of sub-adult or adult age-classes, the number and load of bacterial species individuals likely encountered over the course of their lifetime may be exponentially higher than that of our laboratory animals. While Arizona and California are states that currently are not known to harbor *B. pseudomallei*, they instead are known to harbor other species of the *B. cepacia* complex (Bcc)[78,83,84], as well as other genetic relatives to *Burkholderia spp.*, including *P. aeruginosa*[83]. High levels of exposure to related and ubiquitously distributed environmental bacteria may therefore explain why comparable absorbance values against crude WC lysate antigen mixtures of Bp82 and PAO1 were observed in animals sampled in the field, as these species are known to share many of the same molecular components that could be recognized and bound by host immunoglobulins. Inconsistent specificity values across species may similarly be explained by levels of natural exposure levels to related bacterial species. Members of the *Suidae* family are recognized for

their propensity to disrupt soil profiles, as well as for their need to wallow to thermoregulate[53], both of which are not behaviors exhibited by humans and other wild mammals present in the U.S. Higher assay sensitivity in previous studies examining human sera under this ELISA protocol may thus be plausible given that humans typically are not manipulating their environment(s) in the same way swine are, and therefore not exposed to the same microorganisms.

This study and the findings presented here are not without limitations. Most predominantly, while we examined seroconversion in goats inoculated with wild-type *B. pseudomallei*, we utilized a non-virulent laboratory strain to inoculate our study pigs as well as the source of WC lysate coating antigen for ELISAs. Use of an avirulent strain to expose animals already relatively resistant to developing clinical melioidosis may have resulted in an underestimation in the subsequent serological response of our study animals. Additional inactivation and use of a laboratory strain for assay coating antigen may too have underestimated pathogen-specific antibodies present in serum samples, as some of the immunogenic materials exhibited by virulent *B. pseudomallei* strains could be significantly different than those exhibited by Bp82. Despite these, the results obtained during assay optimization suggest that the antigenic profile between virulent and avirulent *Burkholderia* strains are likely comparable, as we observed high reactivity to Bp82-derived antigen mixtures in goats exposed to wild-type *B. pseudomallei.* Future investigations, however, should examine whether similar antibody levels are exhibited in WC lysate preparations derived from other wild-type strains, particularly in swine sera. Finally, although previous reports on the ELISA evaluated implied comparable specificity between *B. pseudomallei* WC lysate to the more targeted OPS or CPS purified antigens, we did not examine differences in assay performance between our WC lysate mixture

and any subset of purified protein or polysaccharide antigens. Additional experiments are therefore required to confirm whether assay specificity differs between the crude WC lysate antigen-based ELISA examined here and CPS, OPS, or other purified materials for samples of swine origin.

CONCLUSIONS

Burkholderia pseudomallei is an emerging pathogen within the U.S. of significant public health concern. Increasing case reports of melioidosis and novel environmental isolations worldwide, together with a broad host-range and diagnostic difficulties continue to make risk management challenging[2-6,8,27,29,30,40]. Past investigations have alluded to pigs being a useful tool for identifying environmental persistence for this pathogen, particularly since they appear resistant to developing acute or clinical melioidosis but are likely exposed at high levels in contaminated environments[31,49]. Recent reports concerning diagnostic capabilities for melioidosis have additionally suggested that an ELISA utilizing WC lysate derived from *B. pseudomallei* cells as coating antigen may be both sensitive and specific for measuring recent or past bacterial exposure[28,61,70,75], although no study has evaluated this methodology for use in testing swine serum.

We present here that goats inoculated with virulent Bp511 and domestic swine inoculated with avirulent Bp82 both seroconvert and display antibodies against Bp82 WC lysate antigen on ELISA. Anti-*B. pseudomallei* antibodies additionally are relatively specific in laboratory exposed animals, as test sera did not react to *P. aeruginosa* PAO1 WC lysate preparations. Furthermore, while we considered two methods for the establishment of assay cutoff values, there was high agreement regarding results interpretation between both methods considered, despite differences

in the sample size of seronegative control sera utilized for goat and swine-based assays likely contributing to the level of cutoff consensus for swine-based assays. Despite the promising assay results obtained from laboratory-exposed animals, however, examination of sera collected from free-ranging swine in various regions of the U.S. revealed that high levels of antibody cross-reactivity exist between WC lysate preparations of Bp82 and PAO1, suggesting inadequate assay specificity; this trend likely is related to the range and load of genetically related bacterial species free-ranging feral swine are exposed to within their environments compared to captive and laboratory animals. Testing and development of more targeted antigens or antigen mixtures will therefore be necessary before ELISA-based serosurveillance may be utilized in U.S. feral and free-ranging swine populations.

The true environmental extent of *B. pseudomallei* in the U.S. remains unclear, as well as whether feral swine may be significantly contributing to the spread of this pathogen on the landscapes where bacteria and swine presence overlap. Nonetheless, the findings presented herein ultimately justify future efforts to improve melioidosis serology, particularly for animals such as feral swine that may inhabit endemic landscapes and that are routinely in close contact with the environmental reservoirs for this pathogen. Improvement of diagnostics in non-human animals will be vital for generalist pathogens such as *B. pseudomallei*, as zoonotic transmission in addition to environmental exposure will likely play a significant role in the persistence and spread of bacteria to additional environments.

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CHAPTER 5: FIELD SURVEY OF FERAL SWINE FOR ZOONOTIC AND EMERGING PATHOGENS, ARANSAS NATIONAL WILDLIFE REFUGE

SUMMARY

Feral swine (*Sus scrofa*) are the most prolific and destructive invasive ungulate found in the United States, with the highest estimated population residing in Texas. Classified as 'ecosystem engineers', feral swine can significantly modify the environments they inhabit by altering soil chemistry and plant community structure, reducing the abundance of native vertebrate and invertebrate species, and can contribute to the transmission of pathogens and disease. Despite universal recognition as an invasive species, management strategies to control for feral swine can differ largely across state lines, and the desire for recreational hunting opportunities in some regions continue to challenge population control efforts. Notwithstanding the labors of state and federal agencies to remove feral swine from landscapes and monitor populations for disease, expanding urbanization and persistent human encroachment into natural habitats continue to increase the risk of infectious agent spillover. Furthermore, pathogens that are emerging or not routinely investigated as they relate to feral swine may be overlooked for the relative threat(s) they could pose to other wildlife, domestic animals, or humans.

To address this issue of feral swine as pathogen reservoirs, we performed necropsies on 31 feral swine removed from the Aransas National Wildlife Refuge on the southeastern coast of Texas, a region with high biodiversity as well as high levels of human visitation. Various tissue samples, as well as serum and nasal swabs were collected during necropsy and evaluated using bacterial and fungal culture, serology, and plaque assay to determine if animals were actively infected or exposed to various viral, bacterial, and fungal pathogens. Ticks were additionally

collected from the exterior of animals before being identified to species and screened for the presence of viral pathogens to assess transmission of tickborne pathogens in the region. Three ixodid tick species (*Dermacentor variabilis*, *Amblyomma cajennense*, and *Amblyomma maculatum*) were harvested from 30/31 of the feral swine sampled, with many pigs possessing multiple species of each sex on their extremities; none of the ticks sampled appeared to be carrying infectious virus based on results of cytopathic effects assays. We additionally detected no SARS-CoV-2 or the presence of *Bacillus anthracis* in the nasal passages of any pigs, nor did we detect any pathogenic bacterial or fungal pathogens within tissue homogenates; the bacteria isolated from culture were attributed to sample contamination by various enteric and environmental species. A subset (24%) of pigs sampled, however, were positive for anthrax antibodies, suggesting past or recent exposure to *B. anthracis*. Finally, most pigs, while displaying no gross lesions at necropsy, demonstrated marked eosinophilia across most tissues when examined histologically, suggesting that most individuals were experiencing a parasitic infection. A subset of swine lung and liver sections had detectable lesions associated with active or recent infection with *Metastrongylus* nematodes.

While there was no evidence of active infection with bacterial, fungal, and viral pathogens in the feral swine sampled at Aransas National Wildlife Refuge, this study demonstrated a high degree of parasitic infection, in concordance with other studies that have observed these in both wild and domestic swine. Moreover, detection of anthrax antibodies in a subset of individuals suggests the presence of *B. anthracis* along the southeastern coast of Texas; these results agree with past ecological models suggesting bacterial presence outside of the state's historical endemic region on the western side of the state. Continued anthrax serosurveillance in feral swine may be useful for identifying areas of high disease risk for

susceptible ungulate species managed on the refuge. Finally, while the sampling strategy and laboratory techniques utilized for the ticks collected here had limitations, the results generated further confirm that feral swine are hosts for a range of ixodid ticks in Texas, including species that are known to pose risk to the health of livestock, wildlife, and humans. Future studies should continue to characterize the tick burdens of feral swine throughout their range, as well as estimate the prevalence of both viral and bacterial tickborne pathogens to better characterize disease transmission on the landscape, particularly in regions with high biodiversity or human presence. Research that particularly examines incidence of infection by various pathogens, and how it may relate to levels of parasitic coinfection may be useful for future understanding of disease dynamics in feral swine at Aransas National Wildlife Refuge and throughout the U.S.

INTRODUCTION

Feral swine (*Sus scrofa*) are the most abundant free-ranging invasive ungulate found within the United States (U.S.) today[1,2]. First brought to the West Indies by Christopher Columbus in the late 1400s, the first primary introduction of the species to the continental U.S. is thought to have occurred later in 1539, when the Spanish explorer Hernando de Soto arrived on the Florida coast[3]. Domestic pigs were regularly included on expeditions to the New World as a familiar and low maintenance source of food, as they are generalists and omnivores capable of feeding on an extensive range of materials[4,5]. Left behind on the landscape as explorers moved across the continent, free-ranging farming practices implemented by additional European settlers further permitted the spread and establishment of the first feral swine populations[5]. Later, in the 1900s, European wild boar (also *Sus scrofa*) were imported and released onto landscapes for additional recreational hunting opportunities[4,5]. The resulting hybridization of free-ranging

domestic swine with European wild boar has since given rise to a continuum of population genetics, most notably resulting in a striking range of phenotypic variation between individuals within regional populations and even within sounders[3,6]. Despite the vast morphological differences that may be observed between individuals, all lineages continue to be classified as "feral swine" by state and federal agencies and are considered an invasive species within the U.S[4]. Local classifications of feral swine by state agencies range from 'game animals', to 'exotic animals', and even 'outlaw quadrupeds'; these differing legislative classifications across state lines have resulted in vastly different management strategies across the U.S[7]. Today, estimations of feral swine numbers across the entire country are approximated at nearly 7 million individuals[8].

A generalist diet, coupled with persistent foraging behavior is primarily what makes feral swine successful throughout their wide ecological range, and a significant threat to native flora and fauna. Past documents of feral swine feeding habits and diet have revealed that throughout their range individuals are herbivorous most of the time and will feed on a variety of grasses as well as soft and hard masts, including roots, tubers, and fruit; consumption of specific flora varies seasonally, and in some cases can lead to resource competition with other herbivorous wildlife[1,9-11]. When available, individuals will often preferentially select for agricultural crops, which can result in significant economic losses[1,12,13]. In addition to foraging for plant matter, animals will also feed on a variety of soil-dwelling invertebrates[4,14]. Access to both plant and animal matter in soil profiles is accomplished through the species' unique rooting and trampling behaviors, where which soil is disturbed and upturned with the hoofs and snout, and food materials located through olfaction[15]. As active predators, individuals have been known to consume an extensive range of vertebrates, including but not limited to, the eggs and chicks of

ground nesting birds (*Galliform sp*.), white-tailed deer fawns (*Odocoileus virginianus*), various reptiles and amphibians, and juvenile domestic livestock[4,6,16,17]. Direct predation, as well as the indirect altering of plant species composition and nutrient cycling from modifying soil profiles in ecosystems where feral swine have been introduced have resulted in the decline of numerous species, many of which have since been listed as rare, threatened, endangered, or of special concern[6].

Feral swine have furthermore been demonstrated capable of carrying an estimated 30 viral and bacterial diseases, and nearly 40 parasites that may also affect humans, domestic animals, and various wildlife[18,19]. Individuals may harbor or shed bacteria and parasites that can contaminate surrounding environments, most notably shared water sources or vegetable crops harvested and subsequently consumed by humans[20,21]; examples of such bacterial and parasitic organisms include those from the genus *Cryptosporidium*, *Giardia*, *Trichinella*, and *Salmonella*, as well as *Leptospira*[1,8,19]. In some instances, pathogenic bacteria isolated from feral swine have been directly linked to human outbreaks after consumption of contaminated crops and include species such as *Escherichia coli* strain O157:H7, an increasingly antimicrobial resistant strain currently classified by the Centers for Disease Control and Prevention as an urgent threat to public health[21-25]. Additional zoonotic bacterial pathogens that may be asymptomatically carried by feral swine include anthrax (*Bacillus anthracis*), tularemia (*Francisella tularensis*), and plague (*Yersinia pestis*); each of these organisms are capable of causing fatal disease in humans and are also classified as Tier 1 Select Agents of bioterrorism[26-30]. Despite the recognition that individuals can carry these organisms, the complete and relative role(s) that populations of invasive swine may play in the overall epidemiology of many of these remains underestimated. Regular, national disease surveillance conducted by the U.S. Department of Agriculture's

(USDA) National Feral Swine Program monitors populations for the occurrence of select pathogens, primarily those that may pose a significant threat to the health of the nation's domestic commercial swine herds, and include such pathogens as pseudorabies virus and swine brucellosis[6,18]. The agency also dedicates significant resources to monitoring feral swine for foreign animal diseases predicted to have devastating consequences should they be introduced to the U.S[31]. Despite the targeted efforts of the USDA to monitor populations for disease threats, the list of known pathogenic organisms carried, transmitted, or vectored by feral swine is continuously evolving, particularly for emerging or reemerging pathogens[32-34]. Finally, the appearance of novel pathogens, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019 demonstrate the need for continued and opportunistic investigation of wild populations for zoonotic organisms, especially those residing in anthropogenically-modified habitats or with close associations with humans, as is often the case with feral swine[35]. Adaptive surveillance as well as more comprehensive or general screening processes may thus be required to fully elucidate the biological threats posed by the species in the future.

Wild pigs have existed in Texas for over 300 years, and like many of the feral swine present across the U.S. are descended from hybrids of escaped or released domestic swine and Eurasian wild boar released for hunting[36,37]. Natural range expansion as well as human-mediated movements of animals after their primary introduction have since allowed the species to occupy most of the region's available habitat, with all but one of the state's 254 counties being occupied by feral swine[4,6,38,39]. Today, Texas continues to be the state with the largest documented feral swine population, with estimates suggesting the presence of approximately 2.6 million individuals[4,8]. The state also possesses several major ecological regions, and ranks high in biodiversity, endemism, as well as the number of native species presumed or possibly extinct

within its borders[40,41]. While many factors contribute to the decline of local and regional biodiversity, the presence of invasive feral swine throughout nearly the entire state continue to present one of the most significant threats to the native flora and fauna of Texas[36,42].

Given the unknown pressures posed by feral swine for some pathogens, as well as the combined high biodiversity score of the state and large populations of feral swine present, we conducted a general survey of feral swine removed from a popular and highly biodiverse wildlife refuge in Texas to assess the carriage and exposure of animals to various bacterial, fungal, and viral pathogens not normally investigated under the USDA's current disease surveillance program. To evaluate the potential for disease transmission to humans as well as other wildlife within the same region, we collected a multitude of samples from individual pigs and examined them for the presence of infectious bacterial, fungal, and viral pathogens, as well as gross and microscopic lesions caused by infectious organisms. Specifically, we primarily focused on pathogens of zoonotic concern, including but not limited to *Bacillus. anthracis*, *Burkholderia. pseudomallei*, *Coccidioides spp*., and *Francisella. tularensis*. Secondarily, we also collected, identified, and evaluated a subset of adult ticks present on individuals at the time of sampling to assess the presence and subsequent transmission potential of tickborne pathogens that may be occurring at the refuge. Finally, we also collected serum to assess whether individuals were exposed previously to *B. anthracis*, the causative agent of anthrax, and a concern for wild and domestic ungulates residing throughout the state.

We did not detect any pathogenic bacterial, fungal, or viral infections in any of the swine sampled here, nor did we detect any infectious virus present in any of the ticks that were actively blood feeding on any of the pigs. Signs of active or recent infection with nematode parasites

were present throughout the tissues, and antibodies against anthrax-causing bacteria were detected in the serum of seven individuals, indirectly suggesting the presence of *B. anthracis* in the region. Despite lack of evidence of active infection with any of the zoonotic pathogens examined here, we further confirm feral swine to be competent hosts for three Ixodid tick species at Aransas National Wildlife Refuge, and potential targets for future anthrax surveillance studies in the region.

MATERIALS AND METHODS

Study Area

Aransas National Wildlife Refuge (ANWR) is composed of five discrete units totaling approximately 115,000 acres of diverse habitats along the southeastern coast of Texas[43], as depicted in Figure 5.1. Located near Austwell on the Blackjack Peninsula, ANWR was established in 1937 primarily to preserve the wintering grounds of the endangered Whooping Crane (*Grus americana*). Today, it additionally serves as a sanctuary for a multitude of migratory birds and other wildlife, including the endangered Kemp's Ridley Sea turtle (*Lepidochelys kempii*)[44,45]. Several shallow bays surround the main unit of the refuge, and strong winds irregularly push water from the bays onto the mainland, creating a unique ecocline from salt to freshwater marsh habitats[43,46]. Live oak (*Quercus virginiana*), black-jack oak (*Quercus marilandica*), red bay (*Persea borbonia*), and various coastal grasses define the region's major inland flora[47]. Other common fauna of the region include American alligator (*Alligator mississippiensis*), coyote (*Canis latrans*), collared peccary (*Pecari tajacu*) and white-tailed deer (*Odocoileus virginianus*)[43]. Despite being a site predominantly charged with the protection of fish, wildlife, and their associated habitats however, consumptive use of the refuge, including

hunting and fishing, is permitted within some of ANWR[48]; non-consumptive activities such as hiking, and biking, may also take place on designated trails[43].

Invasive feral swine first appeared in the region of the current ANWR boundaries beginning in the late 1800s, after release of domestic swine by European colonizers[4,47]. Approximately 100 years later, European wild boar were released for additional hunting opportunities, and allowed for hybridization between the two lineages[47]. Today, the USDA regularly partners with refuge personnel to assist in the removal of feral swine off all units of the refuge. The presence of feral swine at the ANWR is often marked by patches of various sizes with no vegetation present, as individuals will uproot plant matter and disturb natural soil profiles in search of below-ground forage and soil invertebrates[49].

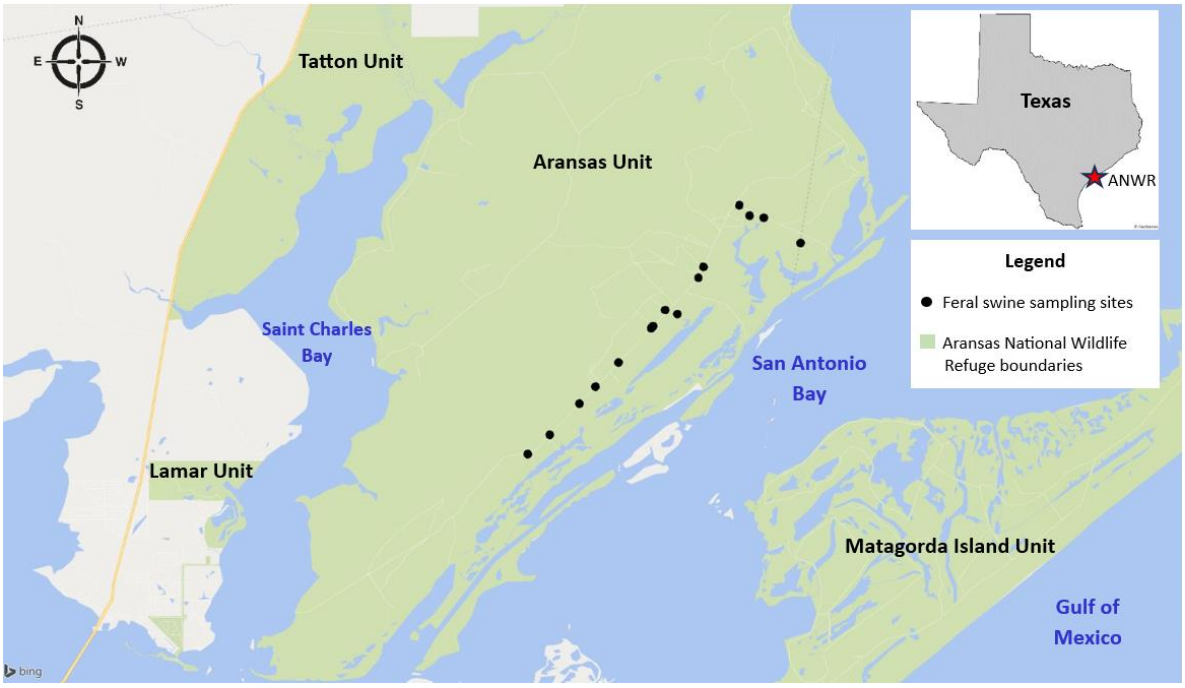

Figure 5.1: Feral swine sampling sites at Aransas National Wildlife Refuge (ANWR), Texas, USA. Map created using Microsoft Excel (2016).

Field Sampling

We partnered with the USDA's Wildlife Services aerial operations program during regular feral swine removal activities to collect both demographic data and various biological samples from 31 feral swine removed from the mainland of the ANWR (Figure 5.1). Sampling took place on May 3-4, 2022, along the southeastern portion of the Aransas Unit where public access is not permitted. Feral swine were flushed out of thick stands of oak and dispatched adjacent to refuge access roads via helicopter by USDA personnel, before being necropsied by a separate team on the ground. Demographic data collected for each pig included the GPS coordinates associated with where each pig was dispatched, as well as individual age-class and sex. Juveniles were defined as individuals with an estimated weight less than 23 kg, while adult swine were conversely classified as being over 23 kg. Classification of age by weight was used for expediency in the field and has been demonstrated to be highly correlated with age classification using standard dentition methods used by the USDA[50,51].

Various samples were collected during swine necropsy. First, when present, 3-5 adult ticks were collected from the exterior, (e.g., head and ears, neck, belly, and groin regions) of each pig during a brief external inspection; ticks were immediately placed in 15 ml empty falcon tubes. Two nasal swab samples were then collected, and each placed in tubes containing 1 ml of either PBS with 20% glycerol for later bacterial culture, or BA-1 medium (Tris-buffered MEM containing 1% bovine serum albumin) supplemented with antibiotics for subsequent viral isolation. After external samples were collected, a full necropsy was performed, and the internal organs inspected for the presence of obvious lesions or signs of disease. Whole blood (3-5 ml in serum separator tubes) was collected directly from the heart. In addition to tissue surrounding

any lesions, approximately 3-5 g of lung, liver, spleen, kidney, parotid lymph node tissue was collected and placed into individual sterile whirl-pack bags. All samples were kept on ice before being shipped overnight to Fort Collins, Colorado for further processing on May 5, 2022.

Serology

We evaluated all feral swine sera for the presence of anti-anthrax antibodies by ELISA, as previously described[29,52]. In brief, high-binding polystyrene 96-well flat-bottom microtiter plates (Thermo-Scientific, #3455) were coated with recombinant protective antigen (rPA) from *B. anthracis* diluted in carbonate buffer (0.16 g Na_2CO_3, 0.29 g $NaHCO_3$, 100 ml distilled water, [pH 9.6]) solution at a concentration of 5 µg/ml per well, and incubated overnight at 4°C. The following day, coating buffer was discarded, and wells washed five times with sterile phosphate-buffered saline (PBS) containing 0.05% Tween 20 (washing buffer). Wells were blocked with the addition of 300 µl of 10% skim milk in PBS and allowed to incubate for 1.5 hours at room temperature. Wells were again washed, followed by the addition of 100 µl of test serum diluted 1:100 in 1% blocking buffer, and incubated for an hour with shaking at room temperature. Following additional washing, 100 µl/well of Protein A/G-horseradish peroxidase (Thermo-Scientific #32490) diluted 1:1000 in blocking buffer was added, and plates further incubated with shaking for 30 minutes. After one final washing step, 150µl of 1-step ABTS (Thermo-Scientific, #37615) was added, incubated for 15 minutes, and then stopped by the addition of 100 µl of 1% sodium dodecyl sulphate solution. Absorbance was measured at 25°C and 405nm using a microplate reader (BioTek #258653) paired with Gen5 microplate reader and imager software (version 3.09). Samples were considered positive for rPA antibody if their mean absorbance measurements were greater than three times the SD above the mean of the negative control. The

known negative and positive control sera utilized for each assay were obtained from a male domestic goat (*Capra aegagrus hircus*) before and after vaccination with Anthrax Vaccine Absorbed (BioThrax™, #NR-2642, Lansing, MI, USA), also described previously[29]. Individual samples were run in triplicate.

Virology

Nasal swab samples collected in BA-1 medium were processed for SARS-CoV-2 viral isolation by double overlay plaque assay as previously described[53]. In brief, 12-well plates containing confluent monolayers of Vero E6 cells were inoculated with 100 µl of serial 10-fold dilutions of sample media and allowed to incubate for 1 hour at 37°C and 5% CO_2. Following incubation, wells were overlaid with 0.5% MEM supplemented with antibiotics/antifungal agents and 2% fetal bovine serum (FBS). A second overlay containing neutral red dye was added 24 hours after the first overlay, and viral plaques were counted at 48, 72, and 96 hours.

Histopathology

Samples of feral swine tissues were fixed in 10% neutral-buffered formalin for 18 days before being transferred to 70% ethanol. Tissues were then processed for paraffin embedding and sectioned for staining with hematoxylin and eosin (H&E). All slides were interpreted by a veterinary pathologist blinded to all study and corresponding animal information.

Tick Identification and Processing

Ticks were morphologically identified by species and sex using the Centers for Disease Control and Prevention's pictorial key to U.S. tick species[54]. After identification, all ticks were divided longitudinally, and one half placed in 900 µl of BA-1 medium (Tris-buffered MEM

containing 1% bovine serum albumin) supplemented with gentamicin, amphotericin B, and penicillin/streptomycin; the other half was placed in an empty 2 ml tube and stored at -80°C for any later analysis required. All ticks and tick halves were pooled by the animal they originated from throughout identification and subsequent processing.

To screen for the presence of viral pathogens, tick halves placed in BA-1 medium were homogenized for 5 minutes using a Qiagen Tissuelyser at 25 cps to create a homogeneous suspension. A fixed volume (100 µl) of tick suspensions were then inoculated in duplicate onto a confluent monolayer of Vero E6 cells in 12-well culture plates (Corning, #07-200-82). Two wells designated as positive control wells contained 100 µl of a Vero cell passage 2 stock of SARS-CoV-2 (WA-01 isolate originally from BEI Resources, [NR-52281, Lot 700033175]). Samples were then incubated at 37°C with a 5% CO_2 environment for 30 minutes and were rocked gently every 10 minutes. Following adsorption, 1.5 ml liquid overlay consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FBS and antibiotics was added into each well. Plates were then incubated at 37°C with 5% CO_2 for a total of seven days and monitored daily for cytopathic effect (CPE) using a light microscope.

Bacterial and Fungal Culture

We utilized both general and selective methods of bacterial and fungal culture to identify pathogenic organisms present within feral swine tissues. All manipulations of viable cultures and tissue specimens were performed within an ABSL-3 to ensure personal safety should any highly virulent organisms or select agents be identified upon processing.

Approximately 100 mg samples of lung, liver, spleen, kidney, and parotid lymph node tissue were collected into 1 ml of PBS with two stainless steel balls for bacterial and fungal

culture. Tissues were then homogenized using a Qiagen Tissuelyser at 25 cps for 5 minutes and 100 µl of homogenate was first plated onto brain-heart infusion (BHI) agar plates to generally screen tissues for the presence of bacterial pathogens. Plates were allowed to incubate for 24 hours at 37°C before being observed for colony growth. To additionally screen for the presence of *B. pseudomallei*, *Coccidioides spp*., *F. tularensis*, and *B. anthracis*, and to eliminate the possibility of environmental or enteric contaminants outcompeting them on non-selective agar, we additionally plated a subset of tissue homogenates and nasal swabs onto selective media. To screen for active *B. pseudomallei* infection, an additional 100 µl of spleen homogenate was plated onto Ashdown's selective medium[55], and incubated for a maximum of 96 hours at 37°C. To screen for active *Coccidioides* infection, an additional 100 µl of lung tissue was plated onto 1 X GYE medium supplemented with selective fungicide and antibiotics (1% glucose, 0.5% yeast extract, 1.5% agar, and 50ug/ml each of cycloheximide, chloramphenicol, and streptomycin). Plates were allowed to incubate for a maximum of 1 week at 37°C before any suspicious fungal growths were harvested into 1 ml of PBS and stored at -80°C pending DNA extraction and PCR. Finally, to better screen for *F. tularensis*, an additional 100 µl of liver homogenate was plated onto cysteine heart agar (CHA) with 2% hemoglobin (Alpha Biosciences, #C03-122), and allowed to incubate for 72 hours at 37°C.

To screen for *B. anthracis* spore carriage in the nasal passages, all nasal swab samples collected in 20% glycerol in PBS were thawed completely before being placed in a 65°C water bath for 30 minutes to preselect for endospore-forming bacteria[56]. Tubes were then vortexed briefly, and 100 µl of swab media plated onto Polymyxin B-Lysozyme-EDTA-Thallous acetate agar (PLET) plates (Sigma, #55678). Plates were then incubated for 18-24 hours before being observed for colonies with characteristic *B. anthracis* morphology[57].

Bacterial DNA Isolation and 16S rDNA Sequencing

Bacterial growth on general BHI media as well as GYE, CHA, and PLET selective media, when observed, was noted for each sample, and 1-2 representative colonies of each observed morphology type were harvested off select plates into 100 µl of TE buffer (10 mM Tris-HCl [pH 7.6], 1mM EDTA [pH 8]). Colony suspensions were then heated at 95°C for 20 minutes to extract bacterial DNA. The 16S rRNA genes were then amplified by PCR using universal primers 27F 5′-AGAGTTTGATYMTGGC-3′ and 1492R 5′-TACGGYTACCTTGTTACGA-3′ described previously[58]. Reactions were performed using Invitrogen PCR Supermix (Cat. #10572014), according to the manufacturer's instructions. Briefly, 25 µl reactions containing 10 µM mixtures of forward and reverse primers and 1 µl of crude bacterial extract was used. Reaction conditions included an initial denaturation cycle of 94°C for 2 minutes, followed by 30 cycles each of denaturation at 94°C for 15 seconds, annealing at 48°C for 30 seconds, extension at 72°C for 90 seconds. Amplification products were then purified by enzymatic cleanup using ExoSAP-IT (Applied Biosystems, # 78200) according to the manufacturer's instructions. Pre-mixed purified PCR products and forward primer were then submitted to Azenta Life Sciences for Sanger sequencing. Finally, the resulting sequences were analyzed in GenBank using the NCBI BLASTn program[59] (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and identified to the genus level.

Bacillus anthracis-Targeted PCR

Colonies identified as *Bacillus sp.* after 16S, as well as any suspicious colonies observed from nasal swab plating onto PLET were examined on multiplex PCR to confirm whether the species isolated was *B. anthracis*. Multiplex PCR was performed using Thermo Fisher Scientific

Platinum Multiplex PCR Master Mix (Cat. #4454268), and capsule (*cap*) and protective antigen (*pag*) primers described previously[60]. A total volume of 25 µl reactions containing 0.2 µM of *cap* and 0.05 µM *pag* primer sets and 1 µl of template DNA was used. The PCR program consisted of an initial denaturation at 94°C for 1 minute, followed by 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 58°C for 2 minutes, extension at 72°C for 2 minutes; the program was completed with a final extension at 72°C for 10 minutes. Amplified products as well as 1-kilobase ladder (Invitrogen, #10787018) were then loaded onto a 1% agarose gel stained with ethidium bromide and electrophoresed for 1.5 hours at 100 V. Products were considered positive for *B. anthracis* if they exhibited a band profile consistent with the plasmid content for *B. anthracis*, constituting bands of the appropriate size for *pag* (364 bp), as well as *cap* (578 bp).

Fungal DNA Extraction and Coccidioides-Targeted PCR

DNA extraction on fungal growths observed on GYE medium was performed as described previously[61]. Briefly, approximately 1cm^2 of mycelia was placed in a 2 ml screw-cap tube containing 0.5 mm diameter acid-washed glass beads (Fisher Scientific, #501461310) and 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8.0], 100 mM NaCl, 0.5% sodium dodecyl sulfate, and 50 mM dithiothreitol). Mycelia suspensions were then subjected to mechanical disruption by vortexing on a Qiagen Tissuelyser at 25 cps for 5 minutes. Samples were then incubated at 65°C for a minimum of 30 minutes before being centrifuged at 10,000 rpm for 2 minutes. Nucleic acids were extracted from the supernatant with buffered phenol:chloroform:isoamyl alcohol pH 8.0 (25:24:1) and again with cholorform:isoamyl (24:1) and precipitated from the aqueous layer with 0.1 volume of 0.2 mM NaCl and 0.1 volume of

ethanol. The resulting suspension was then centrifuged at 14,000 rpm for 10 minutes, washed again with ethanol, re-suspended in 300 µL of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA [pH 8]), with 100ug/mL RNAse A (Thermo Fisher, #EN0531), and incubated at 37°C for 30 minutes to degrade extraneous RNA. The DNA of interest was then precipitated out with the addition of 300µl of 5M LiCl followed by a 10-minute incubation on ice, before being centrifuged for 14,000 rpm for 10 minutes. Supernatants were then transferred to 1.2 ml of ethanol and incubated on ice for an additional 10 minutes and centrifuged one final time at 14,000 rpm for 10 minutes. The resulting pellet was then washed with 70% ethanol and allowed to air dry before being suspended in 100µl of TE buffer. Final suspensions were stored at -20°C until analysis by PCR.

Conventional PCR, using primers targeting the ITS region of *Coccidioides* was then performed as described previously[62]. Singleplex PCR was performed using Invitrogen PCR Supermix (Cat. #10572014), according to the manufacturer's instructions. In brief, 50µl reactions consisting of 0.2µM of the ITS primer set and 1µl template DNA was used. The amplification program consisted of initial denaturation at 94°C for 2 minutes, followed by 35 cycles each of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, extension at 72°C for 1 minute, and one cycle of final extension at 72°C for 7 minutes. Resulting products as well as 1-kilobase ladder (Invitrogen, #10787018) were then loaded onto a 1% agarose stained with ethidium bromide and electrophoresed for 1 hour at 100 V. Band presence at 223 bp was considered positive for *Coccidioides* when viewed under ultraviolet light.

RESULTS

Field Sampling

The relative location of each feral swine removed and sampled from ANWR is depicted in Figure 5.1, while the remaining demographic data associated for all individuals, including age-class and sex, is illustrated in Table 5.1. Most (20/31) of the feral swine removed and necropsied were adults, while nine were identified as juveniles. Due to a recording error, we were unable to identify the age-class of two individuals. Sex was more evenly distributed, with 17 males and 11 females collected; approximately equal numbers of each sex were represented across the two age-classes of interest (Table 5.1). Three individuals were of unknown sex, also due to recording error.

During necropsy, most individuals appeared in healthy condition, although more often it appeared that females were of poorer body condition than males, primarily presented as generally thinner in exterior appearance, as wells as less fat deposition both subcutaneously and intramuscularly. Gross inspection of the viscera revealed no gross lesions or signs of disease for most pigs, regardless of age-class and sex. Eight of the 31 feral swine necropsied displayed observable discoloration or lesions in some organs, most commonly in the liver. Five pigs (TX-006, 010, 012, 026, and 033) displayed liver discoloration, predominantly seen as mottled and pale in color. Four of those pigs (TX-006, 010, 026, and 033) in addition to displaying discoloration, also contained liver lesions, which included sections of firm or fibrotic tissue; one pig (TX-010) displayed a single, white-colored abscess. In addition to liver abnormalities, two pigs (TX-006 and 026) displayed either cystic or white focal lesions on the kidneys, respectively.

Lung tissue appeared edematous or mottled for two pigs (TX-006 and TX-011), respectively. Finally, a single abscess was observed on the spleen of one individual (TX-006).

Serology

Serological status of all swine for antibodies against *B. anthracis* by ELISA are depicted in Table 5.1. Of note, due to the high environmental temperatures experienced during field collection days and the subsequent high rate of blood coagulation in animal carcasses, we were unable to collect viable blood samples from two of the 31 pigs. Of the 29 remaining samples available for serological examination, seven (24%) were positive by ELISA for anti-anthrax antibodies, exhibiting absorbance units above the assay cutoff of 0.15 absorbance units. Excluding one individual with unidentified age class and sex, all seropositive pigs were classified as adults, with sex represented equally (Table 5.1).

Table 5.1: Pig demographics and results of tick species identification, anthrax serology, and histopathology for feral swine removed from Aransas National Wildlife Refuge, TX USA.

Animal ID	**Sex[1]**	**Age-Class[2]**	**Tick Species Collected[3] (M/F)**			**Anthrax Serostatus[4] (+/-)**	**Histological Lesion Score by Tissue[5]**				
			Ac	*Am*	*Dv*		Lung	Liver	Spleen	Kidney	LN
TX-001	M	Juvenile	0/0	0/0	0/0	–	0	1	0	0	0
TX-002	F	Adult	0/1	0/0	0/0	–	1	NS	0	1	1
TX-003	F	Adult	0/1	0/0	0/2	–	0	3	0	2	2
TX-004	M	Adult	0/1	0/0	0/2	–	0	1	0	1	1
TX-005	F	Adult	0/2	0/0	0/1	–	0	2	0	1	2
TX-006	F	Adult	0/0	0/0	0/3	+	0	1	0	3	3
TX-007	M	Juvenile	2/0	0/0	0/0	–	0	0	0	1	2
TX-008	U	Unknown	1/1	0/0	0/1	+	1	4	0	1	2
TX-009	M	Adult	0/0	0/0	1/3	–	0	1	1	0	1

Animal ID	Sex[1]	Age-Class[2]	Tick Species Collected[3] (M/F)			Anthrax Serostatus[4] (+/-)	Histological Lesion Score by Tissue[5]				
			Ac	*Am*	*Dv*		Lung	Liver	Spleen	Kidney	LN
TX-010	M	Adult	0/0	0/0	0/3	−	0	5	1	0	1
TX-011	M	Adult	0/1	0/2	0/0	−	5	3	1	1	NA
TX-012	M	Adult	0/0	0/0	2/3	−	0	2	1	NA	2
TX-013	F	Adult	1/0	0/0	1/1	−	1	4	1	0	2
TX-014	M	Juvenile	0/0	0/0	0/3	−	0	2	NA	0	1
TX-015	F	Juvenile	0/0	0/0	1/3	NA	3	3	1	0	2
TX-016	M	Juvenile	0/1	0/1	0/1	−	5	5	1	0	2
TX-017	M	Adult	0/0	0/1	1/1	+	0	1	1	2	1
TX-018	F	Adult	0/0	1/1	0/0	−	5	3	2	1	2
TX-019	M	Adult	0/0	0/2	2/0	−	1	4	0	2	NA
TX-020	M	Adult	1/0	0/0	1/1	+	0	1	0	2	1
TX-021	M	Adult	2/0	0/0	0/1	−	1	5	0	0	1
TX-023	F	Juvenile	0/0	0/0	2/1	NA	0	2	1	0	1
TX-024	U	Adult	0/0	0/0	0/2	−	0	3	0	1	1
TX-025	F	Adult	0/0	0/1	2/0	+	2	3	1	0	1
TX-026	M	Juvenile	1/0	0/1	0/1	−	0	NS	1	0	1
TX-027	M	Adult	0/0	1/0	1/1	+	0	0	0	0	2
TX-030	U	Unknown	0/0	0/1	1/1	−	5	2	0	0	0
TX-031	M	Juvenile	0/0	0/0	3/1	−	4	4	1	0	3
TX-032	F	Juvenile	0/0	0/0	1/1	−	0	NS	0	0	2
TX-033	F	Adult	0/0	0/2	1/0	+	5	4	1	2	2

[1]Pig Sex; "M" indicates Male, "F" indicates Female, and "U" indicates unknown due to recording error.
[2]Estimated age class of feral swine based on relative body size and dental profile as described in the text. Pigs with "Unknown" age-class designations represent recording errors.
[3]Number of ticks collected from individual feral swine by species and sex. "*Ac*" *Amblyomma cajennense*; "*Am*" *Amblyomma maculatum*; "*Dv*", *Dermacentor variabilis*. "M", Male; "F", Female.

[4]Results of analysis of feral swine sera for antibodies against *B. anthracis* by ELISA. Positive (+) and negative (−) symbols indicate the presence or absence of anthrax-specific antibodies, respectively. “NA”, not available.
[5]Histologic lesion score by tissue type collected. All scores are on a scale of 0 to 5. 0 = within normal limits, 1 = minimal change, 2 = mild change, 3 = moderate change, 4 = severe change, 5 = very severe change in large area of tissue. “NA”, not available. “NS”, no score provided.

Virology

Examination of nasal swab samples by double overlay plaque assay revealed that none of the feral swine sampled on ANWR were shedding detectable quantities of infectious SARS-CoV-2 virus at the time of sampling.

Histopathology

Relative lesion score by animal and tissue type is illustrated above in Table 5.1 and are on a scale of zero to five. Despite the internal organs of most pigs appearing healthy upon gross inspection at necropsy, most tissue sections submitted for histopathology were assigned scores above zero, or outside of normal limits. Overall, liver tissue sections were scored above zero most often, followed by parotid lymph node, lung, kidney, and spleen, respectively. Lung and liver tissues displayed the highest individual scores, each with a max score of five, followed by both parotid lymph node and kidney with a maximum score of three, and finally spleen, with a maximum score of two across all swine. Liver tissue additionally displayed the highest average score across animals (mean = 3); all other tissue types presented the same average lesion score across individual pigs (mean = 1). The average total lesion score for each pig across all tissue types was six, (min = 1, TX-001; max = 14, TX-033). Student’s two tailed t-test revealed that total lesion score did not differ significantly between individuals of different age-classes (p = 0.57), or sex (p = 0.25).

Despite insignificant differences in the scores of individual pigs across demographics, there were major trends in lesion presentation that surfaced across all pigs and tissue types examined. Throughout most tissue types given a lesion score above zero, marked eosinophilia was observed, with variable evidence of current or recent parasitic infection. Most notably, both the lung and liver displayed the most severe eosinophilia, as well as direct evidence of parasitic infection. Notable lesions associated with lung tissue sections are depicted in Figure 5.2. Despite only 14 pigs receiving lesion scores above zero for the lung, the majority of those receiving scores presented with either mild to severe infiltration of eosinophils within alveolar spaces and interstitial tissues or pneumonia. In five pigs, the presence of embryonated and non-embryonated nematode eggs, or adult nematodes was identifiable in the lung section examined (Figure 5.2).

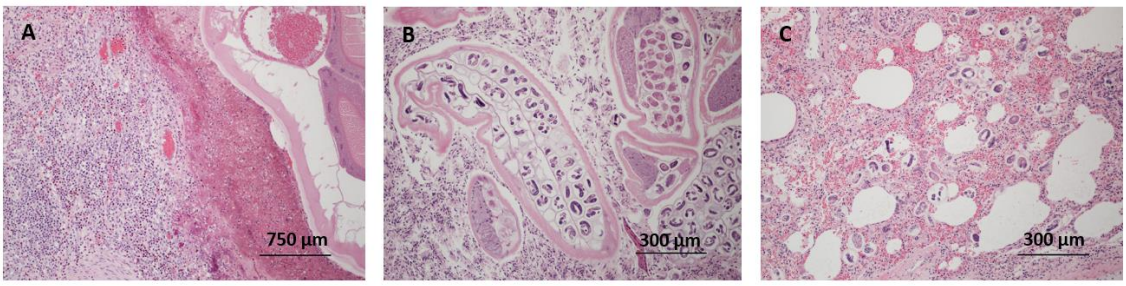

Figure 5.2: Histological evidence of parasitic infection in the lungs of feral swine removed from Aransas National Wildlife Refuge, TX, USA. A) Adult Metastrongyle in the bronchiole, accompanied by inflammatory cells, mucus, and cellular debris, as well as inflammatory infiltrates in the peribronchial parenchyma; pig TX-011. B) Adult Metastrongyles characterized by smooth cuticle, coelomyarian muscles, a large intestine and embryonated eggs both within the reproductive tract and free in the bronchiolar lumen, accompanied by mucus exudate, exfoliated epithelial cells and leukocytes; pig TX-016. C) Embryonated eggs within the alveolar spaces, accompanied by inflammatory cells, mucus, and cellular debris; pig TX-030.

Corresponding histological changes associated with liver tissue sections are depicted in Figure 5.3. Like the lung, most pigs displayed a wide range of mild to severe eosinophilia

throughout, however, unlike that for lung tissue sections, liver tissue sections for the majority (26/31) of pigs were scored above normal limits. Most commonly, varying degrees of interlobular fibrosis and parenchymal necrosis were observed. Notably, the liver specimen of one pig (TX-031) presented with poorly preserved profiles of one or more nematodes as well as scattered non-embryonated nematode eggs, however definitive identification was not possible. In the liver sections of other pigs, lesions suggestive of the presence of ascarid larva migrans were found (Figure 5.3). These lesions were characterized predominantly by pronounced or severe accumulation of eosinophils in the surrounding parenchyma or within portal tracts (Figure 5.3).

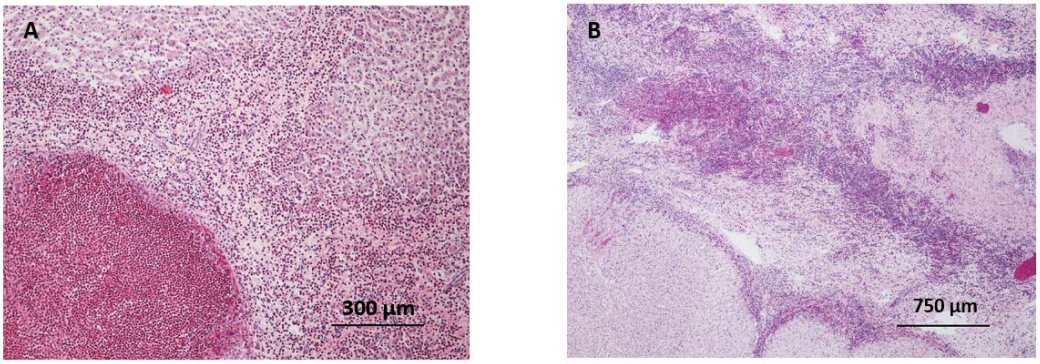

Figure 5.3: Histological evidence of parasitic infection in the liver of feral swine removed from Aransas National Wildlife Refuge, TX, USA. A) Suggestive ascarid migration tract with severe focal accumulation of eosinophils packed within a portal tract; pig TX-033. B) Suggestive ascarid migration tract with pronounced accumulation of eosinophils within surrounding parenchyma; pig TX-016.

Most of the remaining tissues examined, while also displaying marked eosinophilia, were less severe in presentation relative to either lung or liver sections. Regarding the parotid lymph node, all 27 feral swine that scored above zero displayed mild to moderate eosinophilia within most or all compartments of the node, as well as a well-developed paracortical zone. Kidney tissue for pigs scored outside of normal limits (14/31) was typically associated with mild to

moderate infiltration of mononuclear cells (e.g., lymphocytes and monocytes) into the cortex. Spleen tissue for individuals given lesion scores (14/31) demonstrated mild to moderate numbers of eosinophils throughout the white and red pulp. A comprehensive histological report for each pig can be found in Table A.4 of the Appendix.

Tick Identification and Processing

Ticks that were collected identified to species are illustrated in Table 5.1 and correspond to the individual pig they were harvested from. In the field, all pigs displayed rather high tick burdens (approximately 10-50 ticks observed per animal), and an average of three ticks were successfully collected from each individual; only one pig (TX-001) did not have any observable ticks on the auxiliaries. The tick species harvested from the remaining 30 feral swine in order of frequency were *Dermacentor variabilis* (59/90), *Amblyomma cajennense* (16/90), and *Amblyomma maculatum* (15/90). Tick burdens for over half (17/30) of pigs consisted of multiple species, with two individuals possessing all three tick species within their respective tick pools (Table 5.1). Of the tick pools consisting of two species (15/30), seven of those contained both *A. cajennense* and *D. variabilis*; seven contained both *A. maculatum* and *D. variabilis*, and one pool from pig TX-011 contained both *A. cajennense* and *A. maculatum.* The remaining (13/30) individuals only had one species harvested within their pool, with the majority (10/13) of those being *D. variabilis*. Finally, most (65%) of all ticks collected were female, however most (26/30) pigs possessed ticks of both sexes within tick pools; of those 26, 14 pigs possessed both sexes of the same species.

After inoculating tick homogenates onto Vero cell culture, none of the sample pools displayed CPE in the seven days they were under observation. In contrast, positive control wells

inoculated with SARS-2 WA-1 exhibited CPE beginning four days after inoculation and progressed in severity to the end of the observation period. Most tick-borne viral pathogens that lead to human disease are cytopathic on Vero cells, therefore, lack of CPE suggests that these known pathogens were not present in the ticks collected here.

Bacterial and Fungal Culture

Initial plating of all tissue homogenates resulted in a high recovery of bacterial organisms, as most samples displayed colony growth after 24 hours incubation on BHI agar. A subset (30/152) tissue samples plated onto BHI did not display any bacterial growth after 24 hours and appeared truly sterile. Colonies observed on the remaining samples consisted of multiple morphologies, and mixed populations were consistently observed across tissue types and individual pigs. Major colony morphologies observed included punctiform (≤1 mm diameter) circular colonies shiny and white in color, small (1 mm diameter) circular colonies matte white in color, as well as larger (2-5 mm diameter) circular colonies milky brown in color. Notably, due to similar colony profiles being exhibited across individual pigs as well as tissue types, colonies were not harvested from every tissue of each feral swine, but rather as subset selected by morphology across each tissue type. Representative colonies of each morphology type observed were therefore harvested from a subset of feral swine for each tissue type and subsequently identified to genus by Sanger sequencing (n = 69).

A subset of nasal swab and tissue samples that were plated onto selective media for the growth and identification of *B. anthracis*, *B. pseudomallei*, *Coccidioides*, and *F. tularensis*, also displayed colony growth. The exceptions to this were spleen homogenates that were plated onto Ashdown medium for isolation of *B. pseudomallei*, and liver homogenates plated onto CHA for

F. tularensis isolation; no suspicious colonies were observed for either tissue type when plated on selective media. Otherwise, nasal swabs from eight feral swine displayed suspicious bacterial growth on PLET, and lung homogenates from four feral swine exhibited suspicious fungal growth on GYE and were harvested for *B. anthracis* and *Coccidioides* targeted PCR, respectively.

16S Sequencing and Targeted PCR

Amplification of 16S rRNA genes and subsequent Sanger sequencing of the colonies harvested from tissues plated onto BHI revealed high frequency of environmental and commensal bacterial species. Taxa that were identified in order of frequency were *Escherichia* (15/69), *Streptococcus* (11/69), *Bacillus* (10/69), *Ralstonia* (8/69), *Enterobacter* (5/69), *Shigella* (3/69), *Macrococcus* (2/69), *Serratia* (2/69), and *Staphylococcus* (2/69). The remaining 11 isolates were identified as belonging to *Actinomycetes*, *Campylobacter*, *Cellulosimicrobium*, *Janibacter*, *Leclercia*, *Micromonospora*, *Pasteurellaceae*, *Proteus*, *Rhodanobacter*, *Saccharomonospora*, and *Streptomyces* groups, respectively. Of note, repeated sequencing of one isolate from pig TX-030 only allowed for identification to the family level (e.g., *Pasteurellaceae*).

The eight suspicious colonies harvested from nasal swab samples plated onto PLET, as well as the ten isolates from various tissue homogenates identified as *Bacillus sp.* after Sanger sequencing were processed further by *B. anthracis* targeted PCR. All colonies were anthrax negative. Furthermore, all four suspicious fungal isolates harvested from lung tissue homogenates plated onto GYE medium were negative on *Coccidioides* specific PCR. The results of Sanger sequencing for all colony morphologies harvested from initial tissue plating onto BHI,

as well as the subsequent results of pathogen-specific PCR when applicable, are detailed further in Table A.5 of the Appendix.

DISCUSSION

This field investigation sought to identify bacterial, fungal, and viral pathogens that feral swine may be actively carrying or previously exposed to in a remote region of Texas with high levels of biodiversity and human activity. While present in several U.S. states and territories, past examinations of the invasive feral swine present throughout Texas alone have revealed that they may be contributing to the spread of numerous pathogenic organisms, many zoonotic in nature[63-65]. High-risk activities such as hunting and subsequently handling feral swine carcasses and consuming contaminated meat or viscera, particularly those improperly prepared, have historically been identified as one of the primary drivers of disease transmission between feral swine and humans[66-70]; these have also been presumed rare events based on reporting[19]. Although direct transmission from feral swine to humans through these mechanisms may occur rarely, disease transmission through a third host species are also possible and can increase the risks posed to humans[19]. This has been especially demonstrated for domestic animals and livestock, who in addition to being in close contact with humans, may encounter feral swine or their materials at higher rates in the environment[6,71]. Of further concern are expanding urbanization and continued human encroachment into natural habitats, as well as climate change impacting ecosystem structure and function; each of these processes present the potential for drastic changes in the current ecology and epidemiology of some pathogens and hosts[72-76].

The ANWR represents a rather pristine environment, yet also is plagued by populations of invasive feral swine that constantly threaten the health and population structure of resident

wildlife, some of which are classified as species of conservation concern. High biodiversity, coupled with regular consumptive and non-consumptive use of the refuge by humans may additionally permit for increased disease transmission, either direct or indirect. Furthermore, species such as feral swine will likely contribute to changing disease dynamics significantly, given their inherent opportunistic behaviors and generalist lifestyle; these characteristics are likely to place them in a range of environments, as well circumstances for inter and intraspecies contacts to occur[35]. Finally, coastal marine ecosystems are one of the most threatened systems by anthropogenic climate change, with drastic changes in relative species composition and interactions, as well as physiologic stress likely to result in widespread consequences for human and animal health and welfare[77,78].

Examination of serum collected from feral swine removed from ANWR revealed that a subset of individuals were previously exposed to anthrax-causing bacteria at some point within their range (Table 5.1). This was somewhat surprising, given that currently anthrax is believed to be endemic primarily within the 'Anthrax Triangle', a discretely defined region located on the opposite side of the state[79]. Despite the absence of regular case reports of anthrax in susceptible animals outside of the Triangle region, past serosurveillance studies performed in Texas feral swine have indirectly suggested the presence of *B. anthracis* outside of the Triangle based on antibody detection in animals removed throughout the state[52]. Additionally, ecological modeling efforts have predicted presence of *B. anthracis* across Texas, with low levels of the bacteria predicted in the region of ANWR[80].

In addition to examining sera for the presence of antibodies, we also collected nasal swab samples to investigate whether viable *B. anthracis* could be isolated from the nasal passages; this

was decided due to past evidence suggesting that feral swine intranasally exposed to varying amounts of anthrax may be capable of harboring viable spores in the nasal mucosa for long periods of time[29]. Evaluation of the nasal swab samples collected from ANWR swine, however, revealed that none of the pigs sampled were actively carrying bacteria within their nasal passages. This is not remarkable given the predicted low anthrax contamination of the ANWR region, as well as the relatively low seroprevalence identified in the swine that were sampled here. One reason for this could be that seropositive animals exposed intranasally cleared any residual spores within the nasal passages prior to being sampled. While it's not clear how long anthrax antibodies last after an exposure event for many taxa[81], the amount of time where such antibodies may be detectable in the serum may be much longer than that of detectable residual *B. anthracis* in the nasal passages[29,82]. Another likely reason why no bacteria were detectable within the nasal passages of these pigs may have been the route of exposure animals experienced. Despite inhalational exposure through the nasal passages hypothesized to be the most common route of anthrax exposure in feral swine[29,52], cutaneous, and gastrointestinal exposures are also viable routes of transmission for anthrax and may be responsible for the subsequent serology displayed in the swine examined here; should any of the seropositive pigs examined here have been exposed through these alternate pathways, no bacteria would have been detectable within the nasal passages.

Besides evaluating the contents of the nasal passages for *B. anthracis*, we also collected an additional nasal swab sample to examine for active infection and shedding of SARS-CoV-2 and did not identify any animals that were shedding infectious virus. Reverse zoonosis, or spillback, from human populations to wildlife is a concern for the establishment of novel wildlife reservoirs and driving any subsequent evolution for SARS-CoV-2[83-86]. This may particularly be

of concern for species that are abundant and live in close association with humans[87]. While many domestic and wild mammals have been found susceptible and capable of viral replication and shedding[85], past experimental inoculation studies using domestic pigs have suggested that they are not susceptible to SARS-CoV-2[88]. It was therefore not surprising that viral isolation was negative for all the animals sampled here.

Field necropsy of animals did not reveal any obvious signs of disease based on the absence of gross lesions for most individuals. Further examination histologically, however, demonstrated marked eosinophilia was present across most tissue types for the majority of pigs. Eosinophilia is most often associated with parasitic infections[89,90] and is likely what most of the swine sampled here were experiencing, rather than active infection with any bacterial, fungal, or viral pathogen. The fact that most individuals were experiencing current or recent parasitic infection was further supported by the observation of various nematode structures within the lungs, as well as lesions suggestive of nematode migration in the liver of many individuals. Despite speciation of the nematodes present in these feral swine being difficult within the limited tissue sections analyzed, presence of organisms of the family *Strongyloidea*, and the genus *Metastrongylus* were highly suspected. *Metastrongylus spp*. are lungworms commonly found in domestic and wild pigs, as well as peccary throughout the world, and require earthworms as intermediate hosts for transmission[89]. Infection with *Metastrongylus spp*. in swine are not often associated with any observable clinical signs; however, dyspnea, coughing and weight loss have been recorded in infected domestic swine[89,91,92]. Weight loss due to active lungworm infection may have been occurring in some of the female feral pigs sampled here, as we anecdotally observed females to be in poorer overall body condition than males during necropsies. However, given that eosinophilia and lungworms were observed in the tissue sections of pigs of both sexes,

an alternative explanation for this disparity may be the physical stress experienced by female swine of active reproductive status, including severe body reserve depletion during lactation[93,94]. Given that most of the pigs were of adult age-class, it is likely that these females give birth to multiple litters throughout their lifespans, and experienced declines from the stress associated with the physical demand of reproduction and lactation, rather than any particular disease process. While *Metastrongylus* species are not considered to be zoonotic[95], there have been some reports of possible human cases of infection with *M. elongatus*[96], and *M. salmi*[97], implying a small risk is present for those handling feral swine carcasses.

Given that feral swine may asymptomatically harbor many pathogenic organisms, we next sought to identify whether individuals were actively carrying harmful bacterial or fungal organisms within various tissues. Plating tissue homogenates first onto non-selective agar demonstrated potentially high bacterial loads throughout the body, however subsequent Sanger sequencing of a subset of isolates revealed the colonies observed to be common environmental and/or commensal enteric organisms. Identification of these species suggests that tissue samples were more likely contaminated, either from environmental organisms due to the non-sterile necropsy conditions in the field, or from invading enteric organisms due to animals dispatched from a helicopter with a firearm, as many individuals experienced “gut shots”, and therefore destruction of the gastrointestinal barrier. As we were also interested in the isolation of a few select pathogens however, we additionally plated select tissues onto more selective media to eliminate the possibility of small numbers of pathogenic organisms present being outcompeted by environmental or enteric contaminants. Tissue and selective agar pairings were determined based on the chief tropisms exhibited by each pathogen of interest[98-100]. Culturing for these pathogens was chosen based on their chief environmental substrate being soil, as well as

concerns that feral swine may be contributing significantly to their spread or emergence[26,29,61,107]. After plating onto selective agar however, we did not find any evidence of active infection with *B. anthracis*, *B. pseudomallei*, *Coccidioides*, or *F. tularensis*, suggesting the threat(s) posed by these pathogens within this region to be nonexistent or relatively low at this time. While not completely surprising given the relatively small sample size of swine sampled here in relation to the number of animals likely present throughout ANWR, lack of isolation of these organisms may be explained by the low contamination or true absence of these from the region, based on current epidemiological data available for each of these pathogens[80,101-103].

The absence of pathogenic bacterial and fungal organisms from the swine here as demonstrated through general and selective culturing was somewhat surprising, given the level of subclinical stress these pigs were experiencing and particularly, the frequency of parasitic infection we observed throughout the tissues examined. The tissue damage and subclinical stress associated with severe parasite infections might also allow for secondary infections or coinfections resulting in increased disease severity, as has been demonstrated for swine coinfected with *Metastrongylus spp.* and *Mycobacterium bovis*[104]. In other studies, also regarding *M. bovis* and *Metastrongylus* co-infections in wild boar, it's been proposed that infection with *Metastrongylus* nematodes may alter the immune response such that the formation of characteristic *M. bovis* lesions in infected animals is altered; this may be concerning for hunters or individuals handling pig carcasses and assuming an animal to be healthy based on the absence of characteristic lesions[105]. In contrast, others examining the seroprevalence of various respiratory pathogens in Ugandan wild boar discovered that lung lesions in swine coinfected with *Metastrongylus spp.* and viral or bacterial pathogens causing pneumonia can have synergistic effects on the lung lesions observed in pigs at slaughter[106]. Future studies should

therefore examine samples more closely to discern whether other pathogenic organisms may be present in the form of a coinfection, as characteristic lesions may be absent or present differently in some tissues, and severe lung lesions may perhaps be mistaken for a severe parasitic infection alone.

We lastly obtained small tick pools from each pig to characterize any active viral infections that may be occurring between actively feeding adult ticks and feral swine at ANWR. We additionally identified all ticks by species and sex to identify any prevalent intraspecies interactions between tick vectors and feral swine hosts. Tick pools harvested from ANWR feral swine included American dog tick (*D. variabilis*), cayenne tick (*A. cajennense*), and Gulf Coast tick (*A. maculatum*) in order of frequency and included comparable numbers of males and females. While collection of these species was not altogether surprising given that the respective ranges of all three of these include the region containing ANWR, the absence of additional species known to be present in the region, particularly the lone star tick (*Amblyomma americanum*) and deer tick (*Ixodes scapularis*) was interesting. The absence of these additional species from the pigs we sampled may be explained by the relative seasonality of tick activity. In fact, past surveys of feral swine throughout Texas have suggested that optimum times of adult lone star tick blood feeding on feral swine are between March and April, while the deer tick is most often observed on feral swine hosts between January and March[51]. As our observations are also somewhat limited by sample size, however, it is also possible that we missed detecting additional tick species due to the small numbers we harvested off of each individual pig. Otherwise, despite our small sample size, observation of *D. variabilis*, *A. cajennense*, and *A. maculatum* on the swine sampled on ANWR appears to be in concordance with others who have studied tick burdens on feral swine throughout southern Texas to date[47,51].

Finally, an initial and superficial analysis of tick homogenates inoculated on cell culture failed to reveal cytopathic effects, suggesting lack of active viral transmission at the time of sampling. While incidence of tickborne viruses may have been low at the time of sampling, it is important to note our sampling strategy and subsequent laboratory processing procedures were far from comprehensive. First, as we observed while in the field, most of the pigs we sampled had relatively high tick burdens, yet time and labor constraints only allowed us to collect between 3-5 individuals from each pig; a more comprehensive sampling strategy such as environmental sampling using tick drags, or a larger sample of ticks off the feral swine removed here, may have allowed for higher detection rates of infectious virus and subsequently a better estimation of incidence and transmission potential. Secondly, we utilized a simple CPE assay to generally screen tick pools for infectious viruses and observed our Vero cells for seven days. Viral pathogens that may be slower growing, or incapable of causing CPE on Vero cells may have been missed during laboratory analysis, therefore underestimating viral presence. Finally, due to time constraints and backordered laboratory reagents, we were unable to screen tick pools for the presence of bacterial pathogens such as *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, and *Rickettsia spp*., all zoonotic and potentially deadly pathogens in humans. Analysis of tick pools for these and other bacterial pathogens may have altered the results and subsequent conclusions regarding tickborne pathogen transmission at ANWR during our sampling period by underestimating pathogen burden in the ticks sampled.

Despite absence of viral, bacterial, and fungal infection in the animals we sampled, tick-related results generated here largely confirm that feral swine are competent host for a range of ixodid ticks in Texas, including species that are also known to pose risk to the health of livestock, wildlife, and humans. Future studies should therefore continue to characterize the tick burdens of

feral swine throughout their range, as well as estimate the prevalence of both viral and bacterial tickborne pathogens to better characterize disease transmission on the landscape, particularly in regions with high biodiversity or human presence. Larger sample sizes, as well as seasonal tick collections may also provide better insight into the risks posed from tickborne pathogens at ANWR. As a subset of pigs also had detectable anti-anthrax antibodies, follow-up studies should additionally utilize serosurveillance with paired environmental samples or increased observation of susceptible ungulate species to better characterize *B. anthracis* at ANWR. As the incidence of anthrax in wild ungulates is likely underestimated, future studies to investigate the impact of the disease on the white-tailed deer present at ANWR may assist with population management decisions. Anthrax surveillance and public awareness campaigns may additionally be warranted during times of the year when consumptive use of the refuge is occurring, such as hunting of white-tailed deer or feral swine. As most of the feral pigs sampled here observed to be recently or actively infected with *Metastrongylus* parasites, further investigating coinfection rates with common parasites and various viral and bacterial pathogens may give additional insights into the disease dynamics within feral swine populations, and subsequently, transmission dynamics on the landscape.

CONCLUSIONS

Feral swine in the U.S. are a destructive and prolific invasive species that are primarily known for causing significant ecological harm within their host environments[1,2]. While the physical damage they may inflict upon ecosystems is substantial, populations of feral swine can also pose risks to wild and domestic animals, as well as humans through the transmission of various pathogens[18,19]. In some regions, threatened or endangered species of conservation

concern may be most severely impacted. Finally, changing climates, as well as increasing interactions between wildlife and humans continue to pose risks for the evolution and subsequent transmission of emerging or reemerging pathogens[32-34]. In the case of Aransas National Wildlife Refuge, we report that exposure to anthrax-causing bacteria, as well as the presence of multiple Ixodid tick species utilizing feral swine for blood meals may be indicative of larger processes taking place in this system, particularly pathways of indirect transmission. As disease transmission dynamics will evolve with changing environmental conditions, indirect transmission either through contact with materials that have been contaminated by feral swine, or interactions with arthropod vectors that have taken an infectious bloodmeal from feral swine may present increased risks to human and animal health. Landscapes where feral swine are present should therefore continue to be considered for increased disease surveillance during feral swine removal events to further characterize risk and inform management strategies seeking to benefit human and animal health.

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CHAPTER 6: CONCLUDING REMARKS AND FUTURE DIRECTIONS

CONCLUSIONS

The purposes of the research presented here were two-fold. Primarily, we were interested in examining the potential pathway(s) that feral swine may be contributing to the persistence and spread of various zoonotic pathogens emerging or re-emerging in the United States. Concurrently, we also sought to evaluate how feral swine might be utilized as additional tools for the surveillance and further study of pathogens whose full ecological range is evolving or have yet to be fully described. Through a review of the literature, we identified three primary pathogens fitting these conditions and with similar knowledge gaps as they relate to feral swine: *Bacillus anthracis*, *Coccidioides*, and *Burkholderia pseudomallei*. Of note, each of these microorganisms are closely tied to soils through their respective life cycles, a relationship that is shared by feral swine, who behaviorally manipulate soil profiles often. Available information regarding the susceptibility of domestic pigs additionally suggested that feral swine are likely resistant to developing disease after exposure to each of these organisms. We consequently hypothesized throughout this work that feral pigs are likely exposed to these and other soil-dwelling pathogens at high rates, and that they may be useful tools for targeted surveillance due to a potential resistance to developing clinical disease.

In Chapter 2 we demonstrate that feral swine exposed intranasally to varying amounts of *B. anthracis* develop a measurable immune response to the bacteria, and that this response might be utilized in the field for proactive anthrax surveillance. It was further suggested through experimental inoculation that feral swine intranasally exposed may act as mechanical vectors for

B. anthracis, if exposed to high enough quantities. Similarly, in Chapter 3, after being intranasally exposed to high levels of *Coccidioides* spores, viable fungal cells were isolated from select tissues of a subset of pigs post-mortem. These results suggest that in some cases, feral swine may act as reservoirs for *Coccidioides*, and may disseminate the fungus to additional environments should they expire with viable spherules in their tissues. High rates of parasitic infection (e.g., *Metastrongylus spp.*) were also identified for the majority of pigs used for this study and may have influenced fungal recovery rates as well as the overall pathogenesis observed.

The experimental inoculation of domestic swine with *B. pseudomallei* described in Chapter 4, also like that described for anthrax, demonstrated that pigs develop a measurable immune response against the bacteria after intranasal exposure, however, more specific antigens are necessary to accurately identify seropositive feral swine in field settings. Finally, the general survey of a subset of swine removed from Aransas National Wildlife Refuge described in Chapter 5 identified a subset of individuals previously exposed to *B. anthracis*, indicating that the bacteria may be present and subsequently effecting wild ungulates present at the refuge. There was no evidence of active infection with any of the pathogens of interest or those additionally screened for, including the causative agents of COVID-19 and tularemia, respectively. Interestingly, as for the *Coccidioides* study pigs of Chapter 3, a high rate of parasitic infection was identified for most of the Aransas pigs, further suggesting that *Metastrongylus* infection is common in feral swine.

The transboundary nature of infectious diseases demonstrates an increasing need to be prepared for emerging or re-emerging pathogens[1]. Identification of disease risk relies on knowledge of a pathogen's ecology and overall epidemiology, which include the environmental

requirements and distribution of organisms on the landscape. The presence of competent hosts, particularly those that may subsequently amplify, or vector infectious agents additionally contribute to the persistence of pathogenic organisms[2]. Moreover, cross-species spillover is a distinguishing feature of zoonotic pathogens, of which many have been documented and can place an immense burden on public health[1,3-6]. While many physical and biological barriers must be broken before a successful spillover event[1], processes like climate change, urbanization, and increasing human-wildlife interactions may accelerate the pathways to cross-species transmission[2]. Feral swine present a unique opportunity to study the ecology of emerging and reemerging pathogens as well as the risk of cross-species spillover, as they are a highly adaptive species whose activities and behaviors likely place them in situations where they may acquire and transmit pathogenic organisms. Particularly for the three soil-dwelling organisms studied here, a high probability of exposure in feral swine that manipulate soils through rooting or wallowing activities may make them good sentinels for identifying contaminated areas, as demonstrated for anthrax. In the same vein, surveying feral swine populations for these organisms may also help to re-estimate their true geographic range, given that they exist rather ubiquitously in some regions of the U.S. but exhibit relatively small home ranges; this would be extremely useful not only for *B. anthracis*, but also *Coccidioides* and *B. pseudomallei*, as both of these appear to be expanding in their environmental range. Lastly, the generalist lifestyle and opportunistic nature of feral swine facilitates their interaction with a wide range of taxa, and therefore may increase the probability of cross-species transmission events or harboring infected parasites, including ixodid ticks.

While additional research is still necessary to address some of the questions posed within this work, the results generated here contribute to the collective knowledge not only concerning

pathogens that may be carried or vectored by feral swine, but also to the broader ecological knowledge of *B. anthracis*, *Coccidioides*, and *B. pseudomallei*.

FUTURE DIRECTIONS

Throughout this work, we partnered with the United States Department of Agriculture (USDA), and specifically the agency's existing feral swine damage management and disease surveillance activities throughout the country to answer our research questions. Given that the infrastructure exists for yearly disease surveillance in feral swine, there is a possibility for continued observation of pathogen distribution across both space and time after the completion of the work described here. Continued serosurveillance for anthrax, as well as collection of nasal swab samples from pigs in or around contaminated areas may help to identify risk to more susceptible species, as well as inform management strategies, such as removing individuals from outbreak areas in order to avoid mechanical transmission of spores. Similarly, in regions with feral swine and with high incidence of human or animal coccidioidomycosis, fungal culture of tissue samples collected from removed pigs may help to identify areas contaminated with *Coccidioides*; this tool may also be deployed in regions with feral swine and where autochthonous human cases are reported. Identification of feral swine in the field with active *Coccidioides* infection, and extrapolation of that individual's home range, may additionally help identify landscapes worth sampling for isolation of the fungus from the environment.

Separate from continued surveillance, additional work will be necessary to improve the specificity of the serological test examined here to properly identify wild pigs with antibodies to *B. pseudomallei*. Utilization of the seropositive animals generated during the experimental inoculation study of Chapter 4 may be useful for these endeavors. Otherwise, obtaining samples

for bacterial culture from feral swine removed from regions of known endemicity may alternatively help to assess how far the bacteria may be spreading on the landscape. This may be particularly useful for regions like the Gulf Coast of Mississippi where environmental presence of *B. pseudomallei* was recently confirmed, however regional extent of the bacteria is unknown.

Finally, while we did not identify any feral swine at the Aransas National Wildlife Refuge actively infected with bacterial or fungal organisms based on the selective and general culturing methods used, high incidence of parasitic infection was identified, particularly with *Metastrongylus* nematodes. This observation was in concordance with other studies that have observed this in both wild and domestic swine; a relatively high incidence of *Metastrongylus* infection was also identified in the study pigs of Chapter 3. Given that other investigations have identified changes in the immune response as well as lesion characteristics in swine coinfected with *Metastrongylus spp.* and various viral or bacterial species, future studies should examine how parasitic coinfection changes the pathology and subsequent transmission dynamics in swine for these and other pathogens.

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APPENDIX

Table A.1: Initial body weight, temperature, and clinical scores and comments for juvenile feral swine experimentally inoculated with *Coccidioides posadasii*.

Animal ID	Sex	Group	Parameter[1]	Day Post Inoculation											
				-1	0	1	2	3	4	5	6	7	14	28	42
3916	F	Control	Weight	10.21	-	-	-	-	-	-	-	-	-	-	-
			Temp	39.77	103	102.2	102.6	101	101	99.5	101.6	102.4	100.6	98.9	100.1
			Clinical Score	0	0	0	0	0	0	0	0	0	0	0	0
			Notes												
7648	F	Infected	Weight	9.8	-	-	-	-	-	-	-	-	-	-	-
			Temp	-	101	99.7	99.1	101.4	99.9	100.6	101.2	100.6	100.6	102.6	101.2
			Clinical Score	0	0	0	0	0	0	0	0	0	0	0	0
			Notes	No temp											
7652	F	Infected	Weight	15.47	-	-	-	-	-	-	-	-	-		
			Temp	40	103	99.1	98.7	99.3	100.1	101.2	100.3	99.1	100.3		
			Clinical Score	0	0	0	0	0	0	0	0	0	0		
			Notes												
7670	F	Infected	Weight	19.96	-	-	-	-	-	-	-	-	-		
			Temp	40.78	105	100.6	102.6	102.8	100.8	103	101.4	101.8	100.6		
			Clinical Score	0	0	0	0	0	0	0	0	0	0		
			Notes												

Animal ID	Sex	Group	Parameter[1]	Day Post Inoculation -1	0	1	2	3	4	5	6	7	14	28	42
7620	M	Infected	Weight	14.52	-	-	-	-	-	-	-	-	-	-	
			Temp	40.22	103	100.1	102.8	100.6	100.8	100.6	101.4	101.4	99.7	102.4	
			Clinical Score	0	0	0	0	0	0	0	0	0	0	0	
			Notes												
7639	M	Infected	Weight	7.53	-	-	-	-	-	-	-	-	-	-	-
			Temp	41.61	101	101.8	102.4	102	101.4	100.8	100.1	100.1	101.2	104.2	102
			Clinical Score	0	0	0	0	0	0	0	0	0	0	0	0
			Notes											Lungs felt congested	
7632	M	Infected	Weight	11.61	-	-	-	-	-	-	-	-	-	-	
			Temp	40.11	101	98.7	99.7	99.7	100.1	98.1	99.7	99.1	99.7	101.6	
			Clinical Score	0	0	0	0	0	0	0	0	0	0	0	
			Notes												

[1] Weight (kg); Temp = Temperature (°C); Clinical Score, scale of 0 to 3, 0 = No signs of disease; 1 = Mild; 2 = Moderate; 3 = Severe.

Note: Body weight was only measured one day prior to inoculation (Day -1), due to difficulties in restraining animals during sampling times. All individuals observed to increase in size over the course of the study period despite body weights not being formally recorded after Day -1; taken together with an absence in clinical signs, all animals presumed to be of appropriate body condition. Grey cells on Day 42 post inoculation indicate animal was euthanized at prior time point and no longer part of study.

Table A.2: Histologic findings from tissues collected from juvenile feral swine experimentally inoculated with *Coccidioides posadasii*.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
7670	Mediastinal Lymph Node	4	--	--	4	0	8	Resorbed eosinophilic-histiocytic inflammation. Edema with no lymphadenitis.
	Heart	0	--	--	0	0	0	Mild hyperplastic arteriosclerosis, scattered individual hepatocellular necrosis (acute catecholamine response).
	Lung 1	4	5	4	4	3	20	Lobe not identified. Interstitial pneumonia and bronchitis, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic subacute marked with perivasculitis, alveolar edema, moderate interstitial edema, hemorrhage, BALT hyperplasia with lympholysis.
	Lung 2	4	0	4	4	4	16	Lobe not identified. Interstitial pneumonia vs. atelectasis, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic subacute marked with perivasculitis, moderate

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
								interstitial edema, hemorrhage, BALT hyperplasia with lympholysis.
7670	Lung 3	4	0	2	4	2	12	Lobe not identified Interstitial pneumonia vs. atelectasis, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic subacute marked with perivasculitis, moderate interstitial edema, hemorrhage, BALT hyperplasia with lympholysis.
	Lung 4	4	2	4	4	2	16	Lobe not identified. Interstitial pneumonia vs. atelectasis, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic subacute marked with perivasculitis, moderate interstitial edema, hemorrhage, BALT hyperplasia with lympholysis.
	Lung 5	3	5	2	3	3	16	Bronchitis erosive eosinophilic necrotizing with luminal *Metastrongylus*

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
								nematodes. Interstitial pneumonia vs. atelectasis, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic subacute marked with perivasculitis, moderate interstitial edema, hemorrhage, BALT hyperplasia with lympholysis.
7670	Lung 6	3	5	3	3	2	16	Lobe not identified upon submission. Bronchitis erosive eosinophilic necrotizing with luminal Metastrongylus nematodes. Interstitial pneumonia vs. atelectasis, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic subacute marked with perivasculitis, moderate interstitial edema, hemorrhage, BALT hyperplasia with lympholysis.
	Kidney	2	--	--	2	2	6	Pelvic perivascular and interstitial nephritis. Lympho-histiocytic eosinophilic, chronic mild.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
7670	Liver	0	--	--	0	0	0	Hydropic change diffuse mild, mild perivascular EMH.
	Spleen	2	--	--	0	0	2	Lympholysis scattered mild, white pulp normal. Red pulp contracted with moderate EMH, moderate nodular reticuloendothelial hyperplasia.
	Pancreas	0	--	--	0	0	0	None.
	Duodenum	2	--	--	2	0	4	Duodenitis. Lympho-histiocytic eosinophilic, chronic mild.
7620	Mediastinal Lymph Node	4	--	--	4	0	8	Abscessing/granulomatous lymphadenitis, lymphoid hyperplasia marked with mild lympholysis.
	Heart	2	--	--	0	2	4	Interstitial and perivascular lympho-histiocytic inflammation.
	Thymus	2	--	--	0	0	2	Thymic involution diffuse moderate.
	Lung 1	2	0	0	2	2	6	Lobe not identified. Interstitial and perivascular lympho-histiocytic inflammation.
	Lung 2	3	0	0	3	3	9	Lobe not identified. Interstitial and perivascular lympho-histiocytic inflammation.
	Lung 3	5	0	1	5	4	15	Lobe not identified. Interstitial,

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
								peribronchiolar, perivascular lympho-histiocytic inflammation. Interstitial edema marked; BALT hyperplasia moderate, alveolar hemorrhage moderate.
	Lung 4	3	0	0	3	3	9	Lobe not identified. Interstitial and perivascular lympho-histiocytic inflammation.
7620	Lung 5	1	0	0	2	1	4	Lobe not identified upon submission. Pleural mesothelial hyperplasia mild. Lympho-histiocytic interstitial perivascular inflammation.
	Kidney	2	--	--	0	0	2	Glomerulonephritis segmental hyaline mild.
	Liver	1	--	--	0	1	2	Lympho-histiocytic perivascular inflammation.
	Spleen	1	--	--	0	0	1	Lympholysis scattered mild.
	Pancreas	0	--	--	0	0	0	None.
	Duodenum	0	--	--	0	0	0	None.
7652	Mediastinal Lymph Node	4	--	--	4	0	8	Abscessing/granulomatous lymphadenitis, lymphoid hyperplasia severe with lympholysis mild.
	Heart	2	--	--	0	2	4	Interstitial and perivascular lympho-histiocytic inflammation.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
7652	Thymus	2	--	--	0	0	2	Thymic involution diffuse moderate.
	Lung 1	2	0	0	2	2	6	Lobe not identified. Interstitial and perivascular lympho-histiocytic inflammation.
	Lung 2	3	0	0	3	3	9	Lobe not identified. Interstitial and perivascular lympho-histiocytic inflammation.
	Lung 3	5	0	1	5	4	15	Lobe not identified. Interstitial, peribronchiolar, perivascular lympho-histiocytic inflammation. Interstitial edema marked; BALT hyperplasia moderate, alveolar hemorrhage moderate.
	Lung 4	3	0	0	3	3	9	Lobe not identified. Interstitial and perivascular lympho-histiocytic inflammation.
	Lung 5	1	0	0	2	1	4	Lobe not identified. Pleural mesothelial hyperplasia mild. Lympho-histiocytic interstitial perivascular inflammation.
	Kidney	2	--	--	0	0	2	Glomerulonephritis segmental hyaline mild.
	Liver	1	--	--	0	1	2	Lympho-histiocytic perivascular inflammation.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
	Spleen	1	--	--	0	0	1	Lympholysis scattered mild.
	Pancreas	0	--	--	0	0	0	None.
	Duodenum	0	--	--	0	0	0	None.
7632	Mediastinal Lymph Node 1	2	--	--	0	0	2	Lympholysis follicular mild multifocal. Resorbed histiocytes; neutrophils mild, edema mild.
	Mediastinal Lymph Node 2	4	--	--	0	0	4	Lympholysis follicular mild multifocal. Resorbed histiocytes; neutrophils mild with marked blood resorption.
	Heart	1	--	--	0	0	1	Interstitial edema multifocal mild.
	Kidney	3	--	--	3	3	9	Pelvic, perivascular lymphonodular inflammation with lympholysis, submucosal lympho-histiocytic pyelitis.
	Liver	2	--	--	0	0	2	Hydropic change diffuse mild. Mild perivascular EMH.
	Spleen	1	--	--	0	0	1	Lympholysis scattered mild. White pulp normal, red pulp contracted with moderate EMH.
	Mesentery	2	--	--	0	0	2	Arteriopathy, hyperplastic, chronic, mild.
	Intestine	2	--	--	2	0	4	Eosinophilic-histiocytic enteritis mild.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
7639	Mediastinal Lymph Node	4	--	--	4	3	11	Lymphadenitis lympho-histiocytic, suppurative, necrotizing, chronic. Marked with severe hemorrhage and multifocal granulomas with giant cells and suppurative necrosis. No microorganisms detected with H&E stains.
	Heart	1	--	--	0	0	1	Interstitial edema multifocal mild.
	Lung, Left Cranial Lobe	4	1	2	4	3	14	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative subacute marked with perivasculitis. Alveolar edema and hemorrhage, marked interstitial edema, BALT hyperplasia with lympholyis.
7639	Lung, Left Caudal Lobe	4	1	2	4	4	15	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative subacute marked with perivasculitis. Alveolar edema, moderate interstitial edema, hemorrhage, mild type II pneumonocyte hyperplasia. BALT

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
								hyperplasia with lympholysis.
	Lung, Right Cranial Lobe	5	1	4	5	3	18	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative subacute severe with perivasculitis, alveolar edema, moderate interstitial edema, hemorrhage. Mild type II pneumonocyte hyperplasia, BALT hyperplasia with lympholysis.
7639	Lung, Right Middle Lobe	5	1	4	5	4	19	Interstitial pneumonia multifocal extensive, peribronchiolar and subpleural. Lympho-histiocytic chronic severe with discrete granulomas with suppurative inflammation. Distinct spherules not detected.
	Lung, Right Caudal Lobe	4	2	4	4	3	17	Interstitial pneumonia, peribronchiolar and subpleural. Lympho-histiocytic suppurative subacute marked with perivasculitis, alveolar edema, moderate interstitial edema hemorrhage. BALT

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
								hyperplasia with lympholysis.
	Lung, Accessory Lobe	5	1	3	5	3	17	Interstitial pneumonia, peribronchiolar and subpleural. Lympho-histiocytic suppurative subacute severe with perivasculitis alveolar edema, moderate interstitial edema, hemorrhage. BALT hyperplasia with lympholysis.
7639	Lung	5	1	3	5	3	17	Lobe not identified upon submission. Interstitial pneumonia, peribronchiolar and subpleural. Lympho-histiocytic suppurative subacute severe with perivasculitis, alveolar edema, moderate interstitial edema, hemorrhage. BALT hyperplasia with lympholysis.
	Liver	2	--	--	0	0	2	Hydropic change diffuse mild. Mild perivascular EMH.
	Spleen	1	--	--	0	0	1	Lympholysis scattered mild. White pulp normal; red pulp contracted with moderate EMH.
	Pancreas	0	--	--	0	0	0	None.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
	Duodenum	2	--	--	2	0	4	Enteritis lymphoplasmacytic eosinophilic, chronic mild with globule leukocytes.
7648	Mediastinal Lymph Node	5	--	--	5	4	14	Lymphadenitis lympho-histiocytic, suppurative, eosinophilic, necrotizing, chronic, marked with follicular hyperplasia.
	Heart	2	--	--	0	2	4	Myocardial perivasculitis; lympho-histiocytic chronic mild with mild interstitial edema.
	Lung, Left Cranial Lobe	3	2	2	3	3	13	Bronchitis, interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative subacute moderate with perivasculitis, alveolar edema, moderate interstitial edema, hemorrhage. BALT hyperplasia with lympholysis.
7648	Lung, Left Caudal Lobe	4	4	4	4	3	19	Bronchitis, alveolitis, interstitial pneumonia, peribronchiolar, subpleural, lympho-histiocytic, suppurative, eosinophilic. Marked with bronchiolar and alveolar *Metastrongylus* larvae and larvated eggs.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
7648	Lung, Right Cranial Lobe	4	2	2	4	3	15	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative, eosinophilic, subacute. Marked with perivasculitis, alveolar edema, moderate interstitial edema, hemorrhage. BALT hyperplasia with lympholysis.
	Lung, Right Middle Lobe	4	1	3	4	4	16	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative subacute severe with perivasculitis, alveolar edema, moderate interstitial edema, hemorrhage. BALT hyperplasia with lympholysis.
	Lung, Right Caudal Lobe	5	1	3	5	3	17	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic, subacute marked with perivasculitis, alveolar edema. Moderate interstitial edema, hemorrhage. BALT

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
								hyperplasia with lympholysis.
	Lung, Accessory Lobe	5	1	3	5	4	18	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative eosinophilic, subacute severe with perivasculitis, alveolar edema. Moderate interstitial edema, hemorrhage. BALT hyperplasia with lympholysis. Granulomas peribronchiolar.
	Kidney	3	--	--	0	3	6	Pelvic, perivascular lymphonodular inflammation with lympholysis, submucosal lympho-histiocytic pyelitis.
	Liver	3	--	--	0	3	6	Perivasculitis bridging lympho-histiocytic eosinophilic, suppurative, chronic moderate with mild portal-portal bridging fibrosis.
7648	Spleen	2	--	--	0	0	2	Lympholysis scattered mild. White pulp normal, red pulp contracted with moderate EMH. Moderate nodular reticuloendothelial hyperplasia.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
	Pancreas	2	--	--	0	2	4	Ductitis, lympho-histiocytic chronic mild.
	Duodenum	3	--	--	3	0	6	Duodenitis, lympho-histiocytic eosinophilic chronic moderate with rare suppurative crypt necrosis.
3916	Mediastinal Lymph Node	4	--	--	4	2	10	Lymphadenitis lympho-histiocytic, suppurative, eosinophilic, necrotizing, chronic. Marked with follicular hyperplasia, resorbed inflammation, and edema. Hemorrhage multifocal and moderate lympholysis.
	Heart	1	--	--	0	0	1	Minimal myocardial interstitial edema.
	Lung, Left Cranial Lobe	4	0	3	2	2	11	Interstitial pneumonia, peribronchiolar and subpleural, lympho-histiocytic suppurative subacute moderate with alveolitis. Marked alveolar and interstitial edema and hemorrhage.
	Lung, Left Caudal Lobe	3	1	1	3	1	9	Interstitial pneumonia, lympho-histiocytic eosinophilic chronic. Moderate peribronchiolar, alveolar, and interstitial hemorrhage and edema.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
3916	Lung, Right Cranial Lobe	4	1	2	2	1	10	Pulmonary hemorrhage and edema. Multifocally extensive, acute marked with multifocal mild type II pneumonocyte hyperplasia. Mild lympho-histiocytic interstitial pneumonia.
	Lung, Right Middle Lobe	3	1	1	3	2	10	Interstitial pneumonia, lympho-histiocytic eosinophilic chronic moderate peribronchiolar. Alveolar and interstitial hemorrhage and edema.
	Lung, Right Caudal Lobe	4	5	3	3	2	17	Bronchitis with a myriad of adult and larval *Metastrongylus* nematodes. Severe BALT hyperplasia, marked lympho-histiocytic, suppurative, eosinophilic interstitial pneumonia peribronchiolar and subpleural with mild histiocytic alveolitis and edema.
	Lung, Accessory Lobe	3	1	1	3	1	9	Interstitial pneumonia lympho-histiocytic eosinophilic chronic moderate peribronchiolar. Alveolar and interstitial hemorrhage and edema marked.

Animal ID	Organ	Lesion Scores*						Comments
		Lesion Extent	Bronchitis	Alveolitis	Interstitial Inflammation	Perivasculitis	Total Score	
3916	Kidney	2	--	--	1	2	5	Pelvic perivascular and interstitial nephritis, lympho-histiocytic, eosinophilic chronic mild.
	Liver	0	--	--	0	0	0	Hydropic change diffuse mild. Mild perivascular EMH.
	Spleen	2	--	--	0	0	2	Lympholysis scattered mild. White pulp normal. Red pulp contracted with moderate EMH, moderate nodular reticuloendothelial hyperplasia.
	Pancreas	0	--	--	0	0	0	None.
	Bile Duct, Common	3	--	--	3	0	6	Periductular inflammation, lympho-histiocytic eosinophilic moderate.
3916	Duodenum	2	--	--	2	0	4	Duodenitis, lympho-histiocytic eosinophilic chronic mild.

--: Not applicable.
BALT: bronchus-associated lymphoid tissue.
EMH: extramedullary hematopoiesis.
H&E: hematoxylin and eosin.
*Scoring: Scale of 0 to 5. 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe.
Histopathology by Dr. Sushan Han, Colorado State University.

Table A.3: Initial body temperature and clinical notes for juvenile domestic swine experimentally inoculated with *Burkholderia pseudomallei* Bp82.

Animal ID	Inoculation Route	Parameter[1]	Day Post Inoculation[2] 0*	7	15*	25	26	27	28	42*
1301	Intranasal	Temp	35.83	35.72	37.61	-	-	-	37.17	37.05
		Clinical Notes	-	-	-	-	-	-	-	-
3707	Intranasal	Temp	38.44	37.5	39	-	-	-	38.67	38.89
		Clinical Notes		-	-	-	-	-	-	-
3708	Intranasal	Temp	38.67	38.11	38.78	-	-	-	38.89	39
		Clinical Notes		-	-	-	-	-	-	-
1302	Subcutaneous	Temp	38.44	37.39	39.44	-	-	-	40	39.11
		Clinical Notes		-	-	-	-	-	-	-
3709	Subcutaneous	Temp	38.56	38.22	39.44	-	-	-	39	37.83
		Clinical Notes	-	-	-	-	-	-	-	-
3710	Subcutaneous	Temp	36.72	36.06	37.72	37.83	36.72	38.33	37.61	37.28
		Clinical Notes		-	Minor abscess observed at site of infection, approx. 20mm diameter.	-	-	-	-	Abscess completely resolved.

[1] Temp = Temperature (°C); "-" denotes no temperature taken.
[2] Starred values indicate days when animals were challenged with 10^8 CFU/ml Bp82.

Table A.4: Histologic findings from tissues collected from feral swine removed from Aransas National Wildlife Refuge, Texas, USA.

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-001	NA	(0)	(0)	Focally minimal infiltration of MNC in sinusoids. The cause is not apparent. (1)	(0)
TX-002	Large numbers of eosinophils are scattered throughout all compartments of node. (1)	There is multifocal mild to moderate interstitial MNC infiltration. Parenchyma otherwise wnl. (1)	(0)	Multifocal mild MNC infiltration	Minimal to mild interstitial eosinophilia, but no frank inflammation. (1)
TX-003	Pronounced eosinophilia in all compartments of lymph node. (2)	In a moderately extensive area of the cortex there is multifocal to coalescing infiltration of MNC, interstitial fibrosis and sclerosis of glomeruli. Dx: chronic interstitial glomerulonephritis, mild. (2)	(0)	Generalized, marked interlobular fibrosis with corresponding atrophy of hepatic lobules. There are large numbers of eosinophils admixed with varying numbers of MNC surrounding portal triads and in septae. Also mild to moderate bile ductule proliferation and increase in sinusoidal leukocytes. (3)	(0)
TX-004	Mild eosinophilia (1)	There are a couple of foci in cortex with mild to moderate interstitial infiltration of MNC. Adjacent tubules appear wnl. (1)	(0)	Focally, in a narrow track, stretching from a portal triad to a central vein, there is mild infiltration of MNC with few adjacent hepatocytes undergoing degeneration. A cause is not apparent. (1)	(0)
TX-005	Pronounced eosinophilia throughout all compartments. Large numbers of secondary follicles and the PC is expanded. (2)	Focally in cortex moderate interstitial infiltration of MNC. Adjacent tubules appear wnl. (1)	(0)	Mild infiltration of MNC & rare eosinophils around portal triads and to a lesser extent in interlobular septae, which have mild to moderate fibrosis. Occasional small clusters of MNC + eosinophils within sinusoids. (2)	(0)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-006 TX-006	The node appears very enlarged with pronounced expansion of the "medullary" draining reticulum and to a much lesser extent of PC and follicle compartments. Large numbers of eosinophils and pigment-laden, foamy macrophages within the reticulum. (3)	The pelvis appears partially effaced by marked fibrosis, partially centered on a focus of central necrosis, surrounded by a rim of MNC admixed with fibroblasts. Neovascularization radiates towards the lesion (suggestive or organization). The cause is not apparent. Elsewhere in pelvis there is subepithelial, marked infiltration of MNC and eosinophils. This too may be surrounding a focus of necrosis right at the cut edge of specimen (?). Cortical renal parenchyma wnl. (3)	(0)	Mild to moderate interlobular fibrosis. Minimal MNC infiltration surrounds a few portal triads. (1)	(0)
TX-007	Marked eosinophilia throughout all compartments. (2)	Few, scattered, tiny foci of mild interstitial MNC-infiltration in cortex. (1)	(0)	(0)	(0)
TX-008	Very pronounced hyperplasia of secondary follicles and expansion of the PC. Large numbers of eosinophils throughout all	Multifocal lymphofollicular aggregates present in the deep cortex. Renal parenchyma otherwise wnl. (1)	(0)	Multifocal to coalescing, very severe infiltration of eosinophils and MNC accompanied by fibroblast proliferation and deposition of ECM associated with multiple foci of parenchymal necrosis (likely result of larva migrans). Outside of the more severely affected areas there is very pronounced eosinophil infiltration in	Very mild interstitial (and/or vascular) eosinophilia. (1)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
	compartments. (2)			the portal tracts and variable sinusoidal leukocytosis. (4)	
TX-009 TX-009	Pronounced eosinophilia throughout all compartments. (1)	(0)	The white pulp (WP) is quiescent. Moderate numbers of eosinophils within the WP. (1)	Mild MNC infiltration around portal triads and mild fibrosis of interlobular septae. (1)	(0)
TX-010	The PC is markedly expanded & appear with prominent PCV. Marked eosinophilia throughout all compartments (1)	(0)	The WP appears quiescent. Moderate eosinophilia in both WP and red pulp (RP). (1)	Very severe interlobular fibrosis with corresponding atrophy of hepatic lobules. In places the fibrotic areas encompass grossly visible foci in which neovascularization, bile duct hyperplasia and hypertrophy, clusters of hemosiderin-laden macrophages and lymphoid aggregates are present (likely sequelae to larva migrans). Throughout there is also mild eosinophil infiltration in interstitial and parenchyma. (5)	(0)
TX-011	NA (only salivary gland on slide)	There are few scattered foci of mild interstitial MNC infiltration. Adjacent nephrons are wnl. (1)	Moderate numbers of eosinophils throughout RP and PALS. (1)	Moderate to marked infiltration of eosinophils around portal triads and in interlobular septae with an admixture of fewer MNC. There is variable degrees of fibrosis, but marked in some triad areas, where bile ductule hyperplasia is also apparent. Hepatocytes appear mildly swollen, with “wispy” cytoplasm (likely glycogen accumulation). (3)	There is multifocal to coalescing, moderate to severe peribronchial, peribronchiolar and septal fibrosis, multifocal moderate to severe interstitial and intra-alveolar infiltration of eosinophils, MNC and hemorrhage. Within one of the more severely affected areas are the profile of a decomposing nematode, characterized by a smooth cuticle, broad lateral chords, platomyarian muscles and a prominent brushborder in the intestine (likely a “true” strongyle

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-011					sp.), surrounded by a rim of cellular debris. Another focus contains only highly eosinophilic debris surrounded by inflammatory cells including few multinucleated giant cells (MuNGC) (5)
TX-012	Moderate to marked occurrence of eosinophils throughout all compartments, but particular in sinuses. (2)	NA	Mild eosinophilia in both RP and WP. (1)	Multifocal areas of marked fibrosis with neovascularization and mild to moderate bile duct proliferation. Interlobular septae are otherwise unremarkable. There is mild, multifocal MNC infiltration with rare eosinophils present in portal tracts. (2)	(0)
TX-013	Moderate to marked occurrence of eosinophils throughout all compartments, but particular in sinuses. (2)	(0)	Mild eosinophilia in both RP and WP. (1)	Diffusely there is pronounced interlobular fibrosis and infiltration of MNC and fewer eosinophils in portal tracts. In places the fibrosis has character of scarring as for Tx-012. Focally within two such areas is a small granuloma with central dark eosinophilic, amorphous material surrounded by MuNGC and MNC. (4)	A small subpleural artery appears thrombosed and is accompanied by mild MNC infiltration in surrounding interstitium. The cause is not apparent. (1)
TX-014	Mild to moderate eosinophilia, notably in the subcapsular sinuses. (1)	(0)	NA (slide labelled spleen contains a liver section)	Multifocal, randomly scattered, mild MNC infiltration in portal tracts and within lobular sinusoids. (2)	(0)
TX-015	PC appears quiescent and there are only few secondary follicles apparent. Marked	(0)	Mild eosinophilia. (1)	Multifocal, randomly scattered, moderate MNC infiltration in portal tracts and within lobular sinusoids. Mild eosinophilia and interlobular fibrosis. (3)	Focally in the interstitium of two large bronchioles and associated arteries/veins/lymphatics there is moderate to marked infiltration of MNC and eosinophils, including large numbers of lymphocytes in

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-015	eosinophilia in the reticular/sinus areas. (2)				the lymphatic vessels (active recirculation). Also, diffusely mild to moderate MNC and eosinophil infiltration in alveolar septae. Dx: interstitial pneumonia, mild, diffuse. (3)
TX-016	Large numbers of secondary follicles present and well-developed PC. Pronounced eosinophilia throughout all compartments. (2)	(0)	Large numbers of eosinophils throughout both WP and in particular, RP. (1)	Very severe infiltration of eosinophils and fewer MNC throughout all portal tracts and with particular high cell density in an area of fibrosis surrounding a severely damaged large vessel undergoing organization with ingrowth of fibroblasts between necrotic debris and infiltrating eosinophils and macrophages. Within the affected area is also a small granuloma with central dark eosinophilic, amorphous material surrounded by MuNGC and MNC. Hepatocytes appear glycogen-rich. (5)	Within large bronchioles are multiple profiles of adult nematodes characterized by a smooth cuticle, coelomyarian muscles, a large intestine and embryonated eggs both within the reproductive tract of the worms and free in the bronchiolar lumen amongst abundant exudate of mucus, exfoliated epithelial cells and effete leukocytes. There is multifocal lymphofollicular proliferation in the peribronchiolar interstitium as well as associated with many smaller bronchioles. There is mild to moderate eosinophil infiltration throughout alveolar septae and interstitial tissues. In a focally extensive area there is alveolar atelectasis, clusters of MuNGC, free embryonated eggs and hypertrophy of alveolar pneumocytes. Dx: bronchopneumonia, chronic-active, severe, with intralesional metastrongyles (adults & eggs). (5)
TX-017	Specimen sub-optimally	Multifocal to coalescing, moderate to severe	Moderate numbers of	Mild to moderate eosinophil and MNC infiltration in portal tracts. (1)	(0)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-017	preserved (DNA-smearing etc), but no frank pathology is apparent. Moderate numbers of eosinophils present. (1)	interstitial infiltration of lymphocytes, plasma cells and fewer monocytes. Adjacent nephrons appear unaffected except for mild displacement. (2)	eosinophils throughout RP and WP. (1)		
TX-018	Many secondary follicles & well-developed PC. Marked eosinophil infiltration throughout all compartments. (2)	Scattered, tiny foci of interstitial MNC infiltration in cortex. Nephrons wnl. (1)	There is hypertrophy of the WP and mild to moderate hemosiderosis in RP. Moderate numbers of eosinophils scattered throughout RP and WP. (2)	Moderate to severe interlobular fibrosis with accompanying neovascularization, bile ductule proliferation and infiltration of MNC + eosinophils, variably causing atrophy of hepatic lobules. (3)	Multifocal to coalescing, moderate to severe, interstitial infiltration of MNC and eosinophils in peribronchiolar interstitia and extending into alveolar septae to varying degrees. In a focally extensive area there is complete effacement of alveolar spaces due to severe infiltration of MNC + eosinophils, hypertrophy of pneumocytes and alveolar atelectasis. Throughout the area and elsewhere are large numbers of scattered embryonated nematode eggs, variably associated with/surrounded by MuNGC and macrophages. Adult worm(s) not apparent in section. (5)
TX-019	NA (only salivary gland on slide)	Focally within the cortex there is pronounced interstitial infiltration of eosinophils and fewer MNC. Adjacent tubules & glomeruli appear wnl. (2)	(0)	Diffusely severe interstitial fibrosis with corresponding atrophy and loss of hepatic lobules. Within the fibrotic areas there are multiple small granulomas with a central core(s) of eosinophilic amorphous material and necrotic cellular debris, surrounded by macrophages, including MuNGC,	Focally mild to moderate infiltration of eosinophils in association with BALT. Otherwise wnl. (1)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
				and small numbers of lymphocytes and eosinophils. (4)	
TX-020 TX-020	Many secondary follicles & well-developed PC. Moderate eosinophil infiltration throughout all compartments. (1)	Multifocal interstitial MNC infiltration in deep cortex and a few smaller foci in the outer cortex. Some infiltrates impinges on and displace tubules, but nephrons otherwise appear wnl. (2)	(0)	Minimal to mild infiltration of MNC and eosinophils in portal tracts. (1)	(0)
TX-021	Many secondary follicles & well-developed PC. Moderate eosinophil infiltration throughout all compartments. (1)	(0)	(0)	Very severe fibrosis with complete effacement of parenchyma in 1/3 of specimen. In the remainder pronounced thickening of interlobular septae with corresponding reduction of lobules. Within the diffusely fibrotic area are few small clusters of degenerating hepatocytes surrounded by heavy eosinophil and MNC infiltration. These cell types also infiltrate septae throughout the sample. There are several small granulomas with eosinophilic amorphous centers surrounded by macrophages, MuNGC and small numbers of lymphocytes and eosinophils. Dx: hepatitis, chronic-active, eosinophilic and fibrosing, severe. (5)	Focally marked interstitial MNC infiltration inclusive marked vascular leukocytosis. The cause is not apparent. (1)
TX-022	Many secondary follicles & well-developed PC. Moderate eosinophil infiltration	(0)	(0)	Diffusely moderate thickening of interlobular septae due to fibrosis and mild MNC + eosinophil infiltration. Corresponding mild to moderate atrophy of lobules. (3)	Multifocal mild interstitial fibrosis and infiltration of MNC, mostly associated with small bronchioles and/or vasculature. (2)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
	throughout all compartments. (1)				
TX-023 TX-023	Many secondary follicles & well-developed PC. Moderate eosinophil infiltration throughout all compartments. (1)	(0)	The WP appears quiescent. Moderate to large numbers of eosinophils in RP. (1)	Diffusely mild thickening of interlobular septae due to fibrosis and mild MNC + eosinophil infiltration. Corresponding mild to moderate atrophy of lobules. (2)	(0)
TX-024	Moderate eosinophil infiltration throughout all compartments. Also moderate lipocyte infiltration. (1)	Multifocal, mild to moderate MNC infiltration in interstitium of cortex, particularly deep cortex. (1)	(0)	Diffusely mild to moderate thickening of interlobular septae due to fibrosis and mild MNC + eosinophil infiltration. Corresponding mild to moderate atrophy of lobules. (3)	(0)
TX-025	Moderate eosinophil infiltration throughout all compartments. (1)	(0)	Well-developed WP. Moderate eosinophilia, most prominent in transition zone between PALS and RP. (1)	Very pronounced eosinophil infiltration in interlobular septae and around portal triads, variably accompanied by MNC-infiltration and mild-to-moderate fibrosis. (3)	Focally consolidation with alveolar atelectasis and moderate interstitial infiltration of eosinophils, macrophages, and fewer lymphocytes. The cause is not apparent. Elsewhere mild infiltration of eosinophils and MNC in peribronchiolar interstitia and alveolar septae. (2)
TX-026	Appears quiescent. Moderate numbers of eosinophils within PC and primary follicles, but virtually	(0)	Mild eosinophilia. (1)		(0)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
	absent from sinus system. (1)				
TX-027 TX-027	Many secondary follicles & well-developed PC. Marked eosinophil infiltration throughout all compartments. (2)	(0)	WP quiescent. (0)	(0)	(0)
TX-030	(0)	(0)	(0)	Multifocal, mainly surrounding portal triads, there is mild interstitial infiltration of eosinophils and MNC. Variable, but generally mild interlobular fibrosis. (2)	Several transverse sections of adult nematodes, characterized by a smooth cuticle, coelomyarian muscles, a large intestine and both non-embryonated and embryonated eggs in the reproductive tract, are present in large bronchioles. Additionally large numbers of nematode eggs, both embryonated and non-embryonated are scattered throughout alveolar spaces and smaller bronchioles, but there appears to be only mild to focally moderate interstitial infiltration of MNC and eosinophils associated with these. A few small bronchioles appear obstructed by cellular debris and abundant mucus. (5)
TX-031	There is marked fibrosis of the lymph node capsule, extending into the nodal septae.	(0)	WP quiescent. There are abundant eosinophils throughout both RP and WP. (1)	The specimen is poorly preserved, but there appears to be moderate to very marked infiltration of eosinophils and MNC in interlobular septae and periportal areas, variably accompanied by minimal to moderate	Appears as described for TX-030, except there are fewer free eggs, but they are associated with marked alveolar histiocytosis. (4)

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-031	In addition, there is multifocal deposition of amorphous amyloid-like material or there is hyalinization of collagen in some areas (requires special stain with Congo Red to differentiate). The PC and follicles are well-developed. Large numbers of eosinophils are present throughout the parenchyma and within fibrotic areas. (3)			fibrosis. Right at the cut edge of one of two specimens are poorly preserved profiles of one or more nematodes as well as scattered non-embryonated nematode eggs. Identification is not possible. (4)	
TX-032	There are coalescing areas of amyloid deposition in the medullary drainage areas and moderate eosinophilia. (2)	(0)	WP appears quiescent. (0)	Moderate to very marked eosinophil infiltration in periportal areas and interlobular septae, with milder MNC infiltration and variable fibrosis accompanying the infiltrates. Corresponding moderate to marked atrophy of hepatic lobules.	(0)
TX-033	Many secondary follicles & well-developed PC. Marked eosinophil infiltration	Multifocal to occasionally coalescing interstitial MNC infiltration in cortex, variably displacing some tubules, but nephrons in	WP hypertrophy and high cellularity in RP, including large numbers of pigment-laden	Diffusely there is moderate to severe fibrosis of interlobular septae and perivascular/periductal areas with corresponding atrophy of hepatic lobules. Throughout there is only mild MNC and eosinophil infiltration	Effacing ~20% of the lung parenchyma in the specimen are four coalescing lesions characterized by a core of necrotic debris, effete leukocytes and other proteinaceous material

Animal ID	Parotid Lymph Node	Kidney	Spleen	Liver	Lung
TX-033	throughout all compartments. (2)	general wnl. Multifocal subcapsular foci of amyloid deposition or deposition of hyalinized collagen (Congo red stain required for differentiation). Mild MNC infiltration but no associated frank inflammation. (2)	macrophages but only small numbers of eosinophils. (1)	except for one area where there is very severe focal accumulation of eosinophils in an apparent tract (or degenerate vessel?) and extension of the inflammatory infiltrates into adjacent portal tracts. (4)	surrounded by a rim of variable width composed of mixed inflammatory leukocytes (MNC & eosinophils) and a discontinuous fibrous capsule. The inflammatory infiltrates variably extend into the interstitia, vessels, small bronchioles and alveolar septae. Parasite remnants, fungi or bacteria are not discernible in the H&E section, hence a cause cannot be established. Dx: bronchopneumonia, necrotizing and suppurative with abscess formation, chronic-active, severe. (5)

NA: Not available.
BALT: Bronchus-associated lymphoid tissue.
Dx: Diagnosis.
PALS: Periarteriolar lymphoid sheath.
PC: Paracortical zone.
MNC: Mononuclear cells (i.e., lymphocytes and monocytes).
MuNGC: Multinucleated giant cells.
RP: Red pulp.
WP: White pulp.
(x): score on a scale of 0 to 5. 0 = within normal limits (wnl), 1 = minimal change, 2 = mild change, 3 = moderate change, 4 = severe change, 5 = very severe change in large area of tissue
Histopathology by Dr. Helle Bielefeldt-Ohmann, University of Queensland.

Table A.5: Results of Sanger sequencing and targeted PCR for *Bacillus. anthracis* and *Coccidioides spp*., tissue and nasal swab samples collected from feral swine removed from Aransas National Wildlife Refuge, TX, USA.

		Taxa Recovered by Tissue (16S)[2]					
Animal ID	**Nasal Swab PCR (+/-)[1]**	**Lung**	**Liver**	**Spleen**	**Kidney**	**LN**	**Additional Analysis[3]**
TX-001		NT	*Escherichia*	NT	Sterile	NA	
TX-002		NT	*Enterobacter* *Leclercia* *Ralstonia*	NT	*Streptococcus*	*Bacillus*	LN colony *B. anthracis* negative
TX-003		NT	Sterile	*Bacillus*	NT	NA	Spleen colony *B. anthracis* negative
TX-004		NT	*Escherichia*	NT	NT	NT	
TX-005		NT	*Staphylococcus* *Serratia*	Sterile	Sterile	NT	
TX-006		Sterile	Sterile	NT	Sterile	*Macrococcus*	
TX-007		NT	*Escherichia* *Shigella*	NT	NT	NT	
TX-008		*Escherichia* *Shigella*	*Bacillus* *Escherichia* *Ralstonia*	NT	NT	NT	Liver colony *B. anthracis* negative. Fungal colonies from lung *Coccidioides* negative.
TX-010		NT	Sterile	NT	Sterile	NT	

Animal ID	Nasal Swab PCR (+/-)[1]	Taxa Recovered by Tissue (16S)[2]					Additional Analysis[3]
		Lung	Liver	Spleen	Kidney	LN	
TX-011		NT	*Escherichia*	*Bacillus*	NT	*Streptococcus*	Spleen colony *B. anthracis* negative
TX-012		NT	Sterile	Sterile	NA	Sterile	
TX-013		NT	*Escherichia*	NT	*Escherichia*	NT	
TX-014		NT	*Campylobacter* *Rhodanobacter* *Proteus*	*Bacillus*	Sterile	*Bacillus*	Spleen and LN colonies *B. anthracis* negative
TX-015		NT	*Escherichia* *Shigella*	Sterile	Sterile	NT	
TX-016		NT	*Escherichia*	NT	NT	*Escherichia*	
TX-017	–	*Actinomycetes* *Cellulosimicrobium* *Micromonospora* *Streptomyces* *Saccharomonospora*	Sterile	Sterile	Sterile	NA	Fungal colonies from lung *Coccidioides* negative
TX-018	–	NT	*Bacillus* *Escherichia*	NT	NT	*Streptococcus*	Liver colony *B. anthracis* negative
TX-019		NT	*Enterobacter* *Ralstonia*	*Bacillus*	Sterile	*Streptococcus*	Spleen colonies *B. anthracis* negative
TX-020		NT	*Ralstonia*	Sterile	NT	NT	
TX-021		NT	*Ralstonia*	NT	NT	Sterile	
TX-022	–	NT	*Escherichia*	*Streptococcus*	NT	NT	Fungal colonies from lung

Animal ID	Nasal Swab PCR (+/-)[1]	Taxa Recovered by Tissue (16S)[2]					Additional Analysis[3]
		Lung	Liver	Spleen	Kidney	LN	
							Coccidioides negative
TX-023	–	*Escherichia*	*Janibacter* *Ralstonia* *Staphylococcus*	Sterile	NT	Sterile	
TX-024		NT	*Escherichia*	NT	NT	NT	
TX-025	–	*Streptococcus*	*Escherichia* *Streptococcus*	NT	NT	*Streptococcus*	
TX-026		*Bacillus*	*Ralstonia*	Sterile	Sterile	NA	Lung colonies *B. anthracis* negative
TX-027	–	NT	Sterile	Sterile	Sterile	Sterile	
TX-030		NT	*Pasteurellaceae**	NT	NT	NT	
TX-031		*Streptococcus*	*Enterobacter* *Serratia*	NT	NT	*Streptococcus*	
TX-032		NT	*Streptococcus*	NT	NT	*Macrococcus*	Fungal colonies from lung *Coccidioides* negative
TX-033	–	NT	*Bacillus*	*Enterobacter*	NT	NT	Liver colonies *B. anthracis* negative

[1]Results of B. anthracis targeted PCR for suspicious colony growths identified during nasal swab culture on PLET. Blank cells represent samples where no colonies were observed.

[2]Results of Sanger sequencing of 16S rDNA genes of pure bacterial colonies harvested from tissue homogenates plated onto BHI. All taxa are reported to the genus level. NA: Not available; this was due to the tissue either not being collected and/or proper volume not available for bacterial culture. NT: Not tested; colonies observed on BHI, however not included in subset that was analyzed for sequencing.

[3]Results of subsequent targeted PCR analysis on isolates identified as *Bacillus sp.* via Sanger sequencing, or *Coccidioides*-suspect colonies harvested from lung homogenates plated onto GYE.
*Isolate put through repeated Sanger sequencing protocols; sequencing only able to identify to the family level rather than genus level.