DISSERTATION

PATHOGENESIS AND IMMUNOLOGICAL RESPONSE OF *YERSINIA PESTIS* IN CARNIVORES

Submitted by

Laurie Ann Baeten

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2019

Doctoral Committee:

Advisor: Richard A. Bowen
Co-advisor: Kristy L. Pabilonia

Kathryn P. Huyvaert
Susan VandeWoude
Thomas Gidlewski

ABSTRACT

PATHOGENESIS AND IMMUNOLOGICAL RESPONSE OF *YERSINIA PESTIS* IN CARNIVORES

Yersinia pestis is the causative agent of plague. The pathogen is endemic in rodent populations in the western United States where humans and other mammals become infected with this highly virulent organism when exposed to infected rodents or their fleas. Coyotes (*Canis latrans*) have been used in animal-based surveillance efforts for the detection of plague foci for over 40 years. Coyotes are likely exposed via flea bite or oral routes and are presumed to be refractory to the development of clinical disease. Historical data have been useful in establishing models of the geographic distribution of *Y. pestis* in the landscape. Because the pathological and immunological response of coyotes to *Y. pestis* infection had not been thoroughly characterized, I conducted experimental inoculations of captive-reared, juvenile coyotes (n=12) with *Y. pestis* CO92 via oral and intradermal routes. No clinical signs of disease were observed, and minimal changes were noted in body temperature, and white blood cell counts during the 7 days following exposure. Gross pathology was unremarkable and minimal histopathological changes were noted at days 3 and 7 post-inoculation. The innate immune response was characterized by a brief peak in acute phase proteins between day 2 and 5 after oral exposure but little to no response was noted in the intradermal exposure group. The humoral response to *Y. pestis* fraction 1 capsular protein (anti-F1) was significantly different between inoculation groups in magnitude and duration of antibody production. The anti-F1 titers were measured by passive hemagglutination assay (PHA) and animals inoculated by the intradermal route peaked at day 10 post-inoculation (range = 1:32 to 1:128) with titers remaining stable through day 84. In contrast, orally inoculated animals developed higher titers (range

=1:125 to 1:1024) that remained stable through day 42. Re-challenge at day 98 post-inoculation using the same dosage and routes did not result in changes in clinical behavior, body temperature, or white blood cell counts. Anti-F1 titers in the oral challenge group produced a striking increase (up to 1:4096) within three days, whereas there was minimal to no increase in antibody response noted in the intradermal challenge group. Using western blot, the antibody profile against known immunogenic *Y. pestis* antigens was evaluated. In pre-infection samples, antibodies against components of the *Yersinia* type III secretory system (LcrV, YopD, YopH, YpkA) were detected indicating a possible mechanism for acquired resistance to plague. Post-inoculation samples were found to contain antibodies against all of the antigens in the testing profile (F1, LcrV, Pla, Pst, YopD, YopH, YpkA). Serum samples generated from this experimental trial were used to develop an enzyme-linked immunosorbent assay (ELISA) using recombinant *Y. pestis* antigens where isotypic immunoglobulin production (IgG, IgM) was subsequently compared to PHA. The assay was subsequently optimized for use in testing of whole blood collected from free-ranging coyotes using Nobuto filter paper strips. ELISA was also evaluated for use in the testing of other wildlife species commonly encountered in plague endemic regions. Information gathered from this experimental work may provide additional insight into the use of coyotes for plague serosurveillance programs.

ACKNOWLEDGEMENTS

Funding for this project was provided by USDA-APHIS-WS-National Wildlife Disease Program, Department of Biomedical Sciences, Colorado State University; Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases; Centers for Disease Control and Prevention; Department of Veterinary Science, University of Wyoming, and Biomedical Research Training Grant (T32 NRSA 7072-9). The procedures used in the housing, handling and sampling of coyotes were reviewed and approved by the Institutional Animal Care and Use Committee at Colorado State University (protocol 10-1751). The author extends a special thank you to the USDA-APHIS-WS-NWRC-Predator Field Station for providing training, support and the captive-bred coyotes used in this study. The authors is also greatly appreciative that the Veterinary Diagnostic Laboratories, Colorado State University and USDA-APHIS-WS-National Wildlife Disease Program provided use of laboratory equipment and space.

This work would not have been possible without the assistance from many people. The author is grateful to all of the following individuals for their support and technical expertise; Paul Gordy, Jeret Benson, Angela Bosco-Lauth, Elisa French, Airn Hartwig and Natalie Cleton who provided assistance with animal care; Elisa French, Valerie Johnson, Jennifer Higgins, Barbara Rose, and Nicole Marlenee for technical assistance; Tonie Rocke for helpful discussions; Ryan Pappert, John Young and Martin Schriefer for assistance with serological testing; Christina Weller, Kristy Pabilonia and Cynthia Hirota for laboratory assistance; Sarah Bevins for graphics assistance, James zumBrunnen for the statistical analysis, Christopher Paddock for the immunohistochemistry, and Terry Spraker for his histological expertise. The author would also like to thank those individuals who graciously provided materials for this work; specifically Kenneth Gage for *Y. pestis* isolates, Giulia Vernati and Gerald Andrews for western blot analyses; tissues and cells lines were provided by Doug Thramm (DH82), Steven Dow (J774),

Susan LaRue (canine), and USFWS-Black-footed Ferret Conservation Center (prairie dog), Richard Bowen (feline, domestic ferret) and finally Sarah Bevins and Hank Edwards for access to historical surveillance samples.

The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: *Yersinia pestis* F1-V Fusion Protein, recombinant from *Escherichia coli*, NR-4526; *Yersinia pestis* Fraction 1 Capsular Antigen, recombinant from *Escherichia coli*, NR-44223, and *Yersinia pestis* LcrV Protein, recombinant from *Escherichia coli*, NR-32875.

I wish to express my heartfelt appreciation to all of the individuals that made this journey possible. First, I would like to thank my co-advisor, Kristy Pabilonia, for starting me on this journey. Her constant enthusiasm and encouragement have made me a stronger person. My advisor, Richard Bowen, for agreeing to take me on as a graduate student. His scientific drive is commendable and inspiring. His encouragement and support throughout the scientific process was never ending. I thank my colleague and committee member, Tom Gidlewski for entrusting this project to me. I appreciated the opportunity to work with his staff and attempt to answer their scientific questions. My committee members, Sue VandeWoude and Kate Huyvaert whom I admire greatly for their thoughtful scientific knowledge and expertise, they both made me strive to be a better scientist. My dearest friend, Elisa French, for always being there when I needed something be it technical assistance, access to supplies, or just an ear to help me through the day. My fellow colleagues, Pauline Nol, Jenny Powers, Lynn Creekmore, Christie Mayo and Colleen Duncan, for setting an example for me to follow and helping me to see the light at the end of the tunnel. My fellow graduate students, specifically Brandy Burgess, Britta Wood, Greta Krafsur, Valerie Johnson, Danielle Adney and Jennifer Higgins for help getting me through classes, consultations on experimental design, as well as constant commiseration through the years. My family, for always believing in me, no matter what I did. Finally, my husband, Ron

Chambers, for allowing me the opportunity to follow this career path. Without his constant support and love, I would not have been able to make this journey.

TABLE OF CONTENTS

CHAPTER 1 - LITERATURE REVIEW

Plague has been described as one of the most catastrophic zoonotic diseases in history. Outbreaks of plague have resulted in significant social, political and economic breakdowns over the last millennium [1-5] and remain a worldwide threat in modern times throughout Asia, Africa and the Americas [6-8]. Currently, the World Health Organization reports an average of approximately 1000 human cases annually with a global distribution. The reemergence of plague in areas of India [9-11] and Madagascar [12] in the mid-1990s and North Africa in the early 2000s [13-15] have also elevated public health attention in the modern era. As such, concern for the spread of this pathogen by both natural or nefarious routes [16-18], that led the Centers for Disease Control and Prevention (CDC) to place the disease on the 'Critical Biological Agents' list, developed in 1999 [19].

EPIDEMIOLOGICAL HISTORY OF PLAGUE: Although evidence of its presence exists in biblical records [20], plague was first described in the 6th century [21]. It probably originated in Ethiopia or Egypt followed by rapid spread throughout the Middle East and Mediterranean basin. With recurrent emergence in 8-12 year cycles, it eventually spread to Europe and Central Asia. This period is historically classified as the Justinian Plague (6th- 8th century; 541-622 AD). With mortality rates reported to be as high as 50-60%, it is hypothesized that plague contributed to the fall of the Rome Empire [2, 22]. Recent genomic analysis indicates that the organism designated as the Antiqua biovar*,* described as the causative agent of the Justinian pandemic, is unique from subsequent pandemic isolates and probably extinct [23].

Historic descriptions of the Black Death pandemic (14th to 19th century; 1330-1700s) indicate that plague likely emerged in the steppes of Asian and moved along the silk trade routes to Europe [5, 24]. Mortality rates were reported to be high with estimates indicating the death of over one-quarter of European populations during 2-5 year outbreak cycles. Described as

Mediavealis biovar, the pathogen's emergence resulted in the development of 'preventive medicine and public health measures through government actions' with the institution of sanitation and quarantine measures as well as travel bans [1]. The Black Death era was directly responsible for the increase in medical education, sharing of medical literature and epidemiological records as well as the use of personal protective clothing [1, 22].

Disease events during the 20th century have been described as the Modern Plague period (1894-1950s) [25, 26]. The origin of the 20th century pandemic was speculated to be located in the Yunnan province of China, resulting in the spread to Hong Kong and Canton by way of war movements followed by transport to the Americas by the shipping trade [6, 26, 27]. This third pandemic period occurred during the modern age of 'scientific, laboratory-based medicine' with the acknowledgment of public health inequities (outbreaks tending to occur in poor urban environments) and the emergence of international scientific communications resulting in international quarantine rules, disease reporting, and implementation of rodent control programs [2]. While the *Antigua* and Medievalis biovars are still isolated in parts of Africa and Central Asia, respectively; it was determined that a new biovar, Orientalis*,* is responsible for the establishment of enzootic foci in a variety of rodent-flea sylvatic cycles on all continents [2].

PLAGUE IN THE UNITED STATES: Plague was first reported in 1899 in port cities along the Pacific and Gulf coasts when rats and their fleas were transported from Hong Kong via the shipping trade [6, 26, 28]. Plague remained established in domestic rats in San Francisco with evidence of spread to native rodents such as the California ground squirrel (*Otospermophilus beecheyi*) in the early 1900s [28]. Through a series of outbreaks between the 1920s and 1960s, plague moved eastward, followed by northern spread, resulting in the establishment of endemic foci with a variety of rodent-flea cycles that remain today throughout the western states [29]. Human cases continued to occur in rural areas with Native American Indian reservations in the southwestern United States accounting for one-third of the cases through the 1980s, where

exposure to peri-domestic rodents was identified as the primary risk factor [30]. Subsequent spread within Montana, Nebraska, Kansas, and the Dakotas was documented in the 1990s [6, 31]. Historically, plague was limited to areas west of the 100th meridian but a new outbreak area was identified in South Dakota in 2008 near the 103rd meridian [32]. Endemic rodent hosts in the United States include prairie dogs (*Cynomys* spp.), ground squirrels (*Ammospermophilus* spp. and *Spermophilus* spp.), chipmunks (*Tamias* spp.), woodrats (*Neotoma* spp) and mice (*Peromyscus* spp) [33, 34], with *Oropyslla montanus* as the predominant flea vector [35-38].

Modern-day case reports (1990-2010) indicate that *Y. pestis* causes approximately 7 human cases of plague per year in the United States, with an estimated mortality rate of 16% [7, 8]. The majority of the cases are reported in Arizona, California, Colorado, and New Mexico, and occur between February and August. It is suggested that endemic foci have increased with the urbanization of rodent species and anthropogenic expansion into natural landscapes [39]. Recent epidemiological assessments have identified higher risks of human disease associated with exposures to outdoor cats and to fleas harbored on dogs [40, 41]. The occurrence of pneumonic plague in human has also increased, which is of concern due to the increased potential for human-to-human transmission [42].

A spike in human plague cases in 2006 (n=13) in the United States [43] coincided with predicted changes in climatic conditions by disease models [44]. The causative organism of plague is highly resilient and has proven its adaptability to highly diverse ecological conditions in its historical progression and establishment within various ecological niches worldwide [29]. Clearly, there is significant concern for geographic spread of *Y. pestis* and adaptation to new flea species and/or rodent reservoirs, in step with the predicted changes in climatic conditions [44-46].

ETIOLOGY: *Yersinia pestis* was identified simultaneously by Alexandre Yersin and Shibasaburo Kitasato in 1894 during the Hong Kong epidemic [47]. *Yersinia* species were originally classified as *Pasteurella*, but later determined to be more genetically similar to *Escherichia* and *Salmonella* species and reclassified to the Enterobacteriaceae family. The bacteria is a non-motile, non-spore forming, oxidase negative, gram negative coccobacillus. As a facultative anaerobe, it is capable of intracellular and extracellular replication within mammalian hosts [48, 49]. While optimum growth at 21-28°C contributes to its survival and replication within arthropod vectors, it is capable of growth at a range of temperatures from 6-37°C. The regulation and expression of virulence factors based upon temperature and environmental conditions demonstrate its resiliency and ease in adaptation [50, 51].

There are 18 species in the genus *Yersinia* but only four are considered to be pathogens (Table 1.1). *Yersinia pseudotuberculosis* and *Y. enterocolitica* are enteric pathogens that are capable of rapid invasion of Peyer's patches and results in mesenteric lymphadenitis. The most virulent of the *Yersinia* species is *Y. pestis*, which has been found to be most genetically similar to *Y. pseudotuberculosis*, evolving 5000-12,000 years ago [52-54]. The fourth pathogen is *Y. ruckeri*, an opportunistic pathogen of fish.

Table 1.1 Vertebrate pathogens in the genus *Yersinia*

Organism	**Host**	**Disease**
Yersinia enterocolitica	Mammals, birds	Yersiniosis; enterocolitis
Yersinia pseudotuberculosis	Mammals, birds	Gastroenteritis, mesenteric lymphadenitis
Yersinia pestis	Mammals	Plague
Yersinia ruckeri	Salmonids	Enteric red mouth

Y. pseudotuberculosis causes self-limiting gastroenteritis in humans. The organism is cold-adapted and can survive within the environment. The organism causes disease following fecal-

oral inoculation, after which it invades the gastrointestinal mucosa and targets the mesenteric lymph nodes. Invasion is assisted by uptake into M cells in the intestinal tract. There is usually no systemic spread to organs. However, individuals with iron overload disease or other liver pathology can develop severe, lethal systemic infections [55]. *Y. pseudotuberculosis* is frequently isolated from asymptomatic mammals and birds. Many carnivores are relatively resistant to infection (including cats and dogs). Clinical disease in mammals ranges from sudden death to ill thrift and/or diarrhea, and can be stress induced resulting in enterocolitis and mesenteric lymphadenitis with occasional splenitis or hepatic necrosis. If intestinal mucosal lesions exist (e.g. from endoparasites) animals may be predisposed to septic or reproductive disease. Sheep are the most common species infected (causing abortion, mastitis, orchitis/epididymitis) and cases of abortion have been reported in cattle [55].

As a clonal derivative of *Y. pseudotuberculosis, Y. pestis* evolved to become a vector-borne pathogen by the gain of genes enabling flea transmission and the ability to spread from lymphoid tissues in mammalian hosts [52, 53], followed by the silencing of genes facilitating enteric transmission [56, 57]. *Y. pestis* diverged from *Y. pseudotuberculosis* with the acquisition of two plasmids (pFra and pPla) that have been linked to flea transmission and dissemination within the mammalian tissues [58]. In mammalian hosts, the organism gains entry through flea bite, mucosal surfaces, inhalation, or ingestion. It can be found intracellularly within phagocytes and extracellularly during the early phase of infection. Upon entry, *Y. pestis* rapidly infects draining lymph nodes near the site of inoculation causing severe lymphadenitis. In later stages of infection, it is predominantly an extracellular organism disseminated within blood, lymphoid tissue and organs. If left untreated, infection can progress to cause death due to endotoxic shock and coagulation dysfunction [59].

ARTHROPOD VECTORS OF PLAGUE: The role of fleas in the plague transmission cycle was proposed by Ogata and Simond as early as 1897 [60] and a comprehensive report

summarizing this epidemiological evidence was prepared in 1906 [61-63]. It is estimated that 80 flea species are capable of harboring *Y. pestis* [26]. The Oriental rat flea (*Xenopsylla cheopis)* is the classical vector and its hosts, the domestic black rat (*Rattus rattus*) and brown rats (*Rattus norvegicus*), are the most common reservoir species of *Y. pestis* worldwide [6]. This flea species prefers moderately warm, moist climates and generally infests burrow-nesting rats in urban areas. Additional *Xenopsylla* species are found as common vectors in other areas of the world. In Africa, India, and South America, *X. brasiliensis* infests rural rats living above ground (roofs/walls) and does not tolerate high temperatures. The primary vector in Indonesia and Southeast Asia is *X. astia* while *X. vesabilis* is found in the Pacific Islands. Another flea of domestic rats, *Nosopsyllus fasciatus*, is found worldwide in cool, temperate climates [26]. The human flea (*Pulex irritans)* has a wide host range and may have contributed to the rapid spread of plague during the Black Death [64-66]. Other fleas identified in domestic environments include those of the cat (*Ctenocephalides felis*) and dog (*C. canis*), but they have been found to be fairly poor vectors, likely due to differences in feeding behaviors. Rock squirrels, ground squirrels (species in Sciuridae family.*),* and their fleas (*Oropsylla montanus*) are the most common sources of human infection in the United States [31, 38, 67, 68]. Reports on the vector efficiency of *Oropsylla* species have varied by study, but it does readily feed on humans. Other arthropod vectors such as ticks and lice have been shown to harbor *Y. pestis* but little has been done to investigate their role in natural pathogen transmission [26, 69-72].

The primary mode of bacterial transmission by fleas is regurgitation of infectious blood during feeding. The mechanisms of biological transmission of plague were not elucidated until 1914 when proventricular blockage in *X. cheopis* was first described [73]. After ingestion of *Y. pestis* by means of a blood meal, red blood cells are disrupted by spiny processes within the flea midgut, releasing the hemin and other iron products used for bacterial growth. Replication of *Y. pestis* in the midgut is noted after two days when bacteria colonize the spines of the

proventriculus, the valve between the esophagus and midgut of the flea. The blockage of the proventriculus occurs as bacteria aggregate in a *Y. pestis* induced biofilm combined with hemin at three to nine days postingestion [74, 75]. As *X. cheopis* is a capillary feeder, the blocked fleas will feed repeatedly with regurgitation of *Y. pestis* into the intradermal site, during which up to 10^4 organisms may be injected [76]. Interestingly, this biofilm formation is temperature dependent with fairly rapid clearance of bacteria through the flea intestinal tract occurring at temperatures above 28˚C resulting in a decrease in blockage and bacterial burden [77].

For many years, it was thought that transmission efficiency in fleas was related to the ability of *Y. pestis* to modulate biofilm formation and induce blockage. However, through investigations of the 230 flea species identified on mammals in plague-endemic regions of the United States, 31 species have been evaluated for their ability to transmit *Y. pestis* [78]. The transmission efficiency of each flea species was found to be dependent upon multiple factors including proventricular size and structure, feeding behaviors, life cycles, and environmental requirements, as well as preferred mammalian host characteristics [37]. Recent studies have determined that, in many species, early phase transmission by unblocked fleas occurs with equivalent efficiencies as those noted in blocked fleas [65, 79-82]. However, large differences in investigative models exist making inference between studies difficult [37].

Most flea species are host specific but cross-species infestation has been documented in plague-endemic areas [83, 84]. More cross-species movement by fleas likely occurs during epizootics due to high mortality rates in preferred hosts, which may account for movement between rodent colonies and the increased risks for human exposures. However, this has not been found to account for the persistence of organism during or between enzootic phases [85-87].

DIVERSITY OF MAMMALIAN HOSTS*:* Since Yersin made the connection between rats and plague in 1897 [21], over 250 mammalian species in 73 genera have been reported to be infected with *Y. pestis* [26, 27]. Susceptibility to development of disease varies widely among genera, with rodents representing the most diverse group in their response to infection. Described by Baltazard in 1947 as enzootic hosts, some rodent species appear to develop a moderately resistant response to infection, with mild symptoms and low mortality rates. These hosts likely perpetuate the rodent-flea transmission cycle of *Y. pestis* in endemic areas [27, 88-98]. Spillover of fleas from these enzootic reservoirs can occur resulting in transmission to highly susceptible rodents. These animals then act as amplifying hosts contributing to epizootic events and associated disease in humans.

The most common amplifying rodent species associated with *Y. pestis* in the United States include prairie dogs (*Cynomys* spp.) [67, 94, 97], ground squirrels (*Spermophilus* spp.) [35, 96, 99, 100], rock squirrels (*Otospermophilus* spp.), chipmunks (*Tamias* spp), woodrats (*Neotoma* spp.) [101], deer mice (*Peromyscus* spp.), voles (*Microtus* spp.) and tree squirrels (*Sciurus* spp.) [39, 102].

Lagomorphs *(Sylvilagus* spp. and *Lepus* spp.) have been found to be susceptible and have been a common source of exposure for humans [26, 88]. Members of the mustelid family vary in their susceptibility with black-footed ferrets (*Mustela nigripes*) almost extirpated by this disease [103]. Other mustelids, such as domestic ferrets (*Mustela putorius furo*) and Siberian polecats (*Mustela eversmanii*), show variable susceptibility [104, 105], and mortalities in badgers (*Taxidea* spp.), skunks (*Mephitis* spp.), and mongoose (*Herpestes auropunctatus*) have not been documented [106, 107].

Felids appear to vary in their susceptibility but have been reported as one of the most common sources of human infection in the United States during the last century [41, 108-110].

A 33% mortality rate was observed in experimental infection trials in domestic cats [100, 111-113]. High mortality rates have been attributed to plague in translocated Canada lynx (*Lynx canadensis*) [114], two cases have been reported in bobcats (*Lynx rufus*) with one associated with human bubonic plague [115, 116] and pneumonic plague in a mountain lion (*Puma concolor*) was associated with a human mortality [42]. In contrast, canids and other carnivores, such as fox (*Vulpes* spp.), raccoons (*Procyon* spp.), skunks (*Mephitis* spp.), and black bears (*Ursus americanus*) appear to be resistant to the development of clinical disease [111, 117, 118].

Livestock species vary in their susceptibility to plague, but have been associated with transmission to humans. Human cases occur when exposed to infected fleas or by ingestion of meat from infected ruminants, such as camels and goats [119-122]. However, swine and equids appear to be resistant to the development of disease [100]. Wild ungulate mortalities have been reported with unique clinical presentations such as ocular manifestations [123-125].

VIRULENCE FACTORS OF *YERSINIA PESTIS*: Virulence factors are defined as factors that promote bacterial survival, growth and/or transmission by directly affecting the host through adherence, thwarting host defense responses, disrupting cellular metabolism, and/or acquiring essential nutrients from the host. The best characterized virulence factors identified for *Y. pestis* are listed in Table 1.2. The calcium dependence plasmid (pCD1, *Y. pestis;* pYV, enteric *Yersinia*) is highly conserved between all pathogenic *Yersinia* species. Two plasmids unique to *Y. pestis*, pFra and pPla, have been shown to contribute to the differences in its virulence and clinical manifestations of disease compared to the enteropathogenic *Yersinia* species. Contributing to the virulence and adaptability, *Y. pestis* and *Y. pseudotuberculosis* have the ability to modify gene expression and phenotype based upon the different temperature and environmental conditions experienced between the flea vector and mammalian host.

Table 1.2. Virulence Factors of *Yersinia pestis* §

Virulence Factor	Action	Induction	Encoded	References
Hemin storage locus	Biofilm formation	26°C	Chr	[126-128]
Murine toxin**	Phospholipase D; promotes survival in flea midgut	26°C	pFra	[129, 130]
PhoP-PhoQ regulatory system	Inhibits phagocytosis, promotes intracellular survival	26°C	Chr	[131-134]
Pigmentation locus	Phosphoglutamase; LPS and outer surface changes; iron uptake, ripA	26°C	Chr	[135, 136]
Attachment invasion locus (Ail)	Adhesin, serum resistance, inhibits neutrophil recruitment and inflammation	37°C	Chr	[137-143]
Endotoxin	Outer membrane protein; associated with septic shock	37°C	Chr	[144, 145]
F1 capsular antigen**	Gel-like capsule, Inhibits phagocytosis	37°C	pFra	[6, 146]
Low calcium response V antigen (LcrV)	T3SS Translocon, TLR2 agonist, inhibits phagocytosis, induces macrophage apoptosis	37°C	pCD1	[147-153]
pH 6 antigen (Psa)	Fibrillar structure, intracellular expression, adhesin, bacterial Fc receptor	37°C	Chr	[140, 154-158]
Plasminogen activator (Pla)**	Omptin protease; dissemination in mammalian host tissues, serum resistance	37°C	pPla	[135, 137, 159-163]
Type III secretory system	Inhibition of host innate immune responses	37°C	pCD1	[164-168]
YadBC	Adhesin, invasin	37°C	Chr	[137, 169, 170]
Yersiniabactin	Iron uptake system, siderophore system	37°C	Chr	[171-173]

§As described through 2013; **unique to Y. pestis, Chr = chromosomal, p = plasmid

The shared plasmid pCD1, contains the genes that encode the *Yersinia* type III secretory system (T3SS), which includes the low calcium response V antigen (LcrV) and *Yersinia* secretion apparatus (Ysc), and delivers the *Yersinia* outer proteins (Yops) (Table 1.3) [164-166, 168]. The genes are temperature regulated with the highest expression noted at 37˚C under low calcium conditions. These virulence factors generate the key elements responsible for *Yersinia's* evasion of the host immune defense systems. The T3SS is a needle-like structure referred to as an injectisome. The injectisome is composed of the flagellar basal body apparatus (scaffolding proteins; YscCDJ), an export apparatus (YscRSTUV), an ATPase complex (YscNKL), and YscF, which is the needle polymer that connects to the pore complex (LcrV, YopBD) which in turn generates the pore through the bacterial outer membrane [150, 164, 174]. After the binding of *Y. pestis* to a host cell, which is facilitated by adhesin (Ail) [138], the T3SS utilizes a translocon (LcrV) to form a pore in the host cell membrane. Injection of Yop effector proteins into the cytosol of the host cells occurs in stages and is regulated by YopK. Over 25 effector proteins have been identified with multiple targets that alter cellular processes such as cytoskeleton, phagocytosis, platelet aggregation, and production of pro-inflammatory cytokines [164, 166, 168]. All of these factors contribute to the inhibition of the host immune response which allows the establishment of organism and replication within lymphoid tissue in susceptible species.

The pH6 antigen (Psa) is a chaperone/usher fimbria structure conserved between *Yersinia* species [158, 175]. Expression reaches its highest levels during the early stationary phase of growth at 35-41°C under acidic conditions (pH 5.0-6.7) [154]. Psa acts as an adhesin with strands or aggregates presented on the outer membrane. Binding to host lipoproteins masks recognition by host phagocytes [157, 176] and its expression within the phagolysosome may facilitate bacterial escape from macrophages [154, 157].

Table 1.3 *Yersinia* Outer Proteins (Yop)

	Description	Function
YscC,D,J	Basal body apparatus	Scaffolding proteins
Ysc R,S, T, U, V	*T3SS apparatus complex	Export apparatus
YscF	T3SS apparatus complex	Needle complex
Ysc N,K,L	T3SS apparatus complex	ATPase complex
LcrV	Low calcium V antigen	Translocon,
YopB, D	T3SS translocation apparatus	Pore formation
YopE	GTPase activating protein	Inhibit phagocytosis, alter actin cytoskeleton
YopT	Cysteine protease	Inhibit phagocytosis, alter actin cytoskeleton
YpkA (YopO)	Serine/threonine kinase	Inhibit phagocytosis, alter actin cytoskeleton
YopH	Protein tyrosine phosphatase	Inhibit phagocytosis
YopJ	Cysteine protease	Inhibits MAPK & NF-ƙB signaling pathways
YopK	T3SS effector protein	Regulates translocation
YopM	T3SS effector protein	NK depletion
YopN	T3SS regulator protein	regulates translocation apparatus

*T3SS = *Yersinia* type III secretory system

The low calcium response V antigen (LcrV) is encoded on the shared pCD1 plasmid and is expressed at 37°C when bacteria are grown in the absence of exogenous calcium or nucleotides [149, 177]. LcrV has been shown to be a multifunctional protein and identified as the first major immunogen linked to virulence [147, 148]. Its major structural function is its role as the translocon in the T3SS pore formation allowing the delivery of Yops [150]. In addition, LcrV can be detected within 2-5 hours after infection within the mammalian host as a secretory product that has immune modulating effects. It has been found to be associated with the inhibition of the proinflammatory process by down-regulation of tumor necrosis factor α (TNFα) and gamma interferon (IFNɣ) as a result of TLR2 mediated interleukin 10 (IL10) inductions [152, 153, 178-182]. The downregulation results in inhibition in macrophage phagocytosis, and

induction of apoptosis of infected macrophages. Interestingly, these effects appear to be masked by the production of F1, which occurs at approximately 24 hours post-infection [152].

The acquisition of the plasminogen activator (Pla) by horizontal transfer appears to be the key virulence factor contributing to the evolution of *Y. pestis*. Pla functions are responsible for systemic dissemination in mammalian hosts contributing to the characteristic necrosuppurative lymphadenopathy seen in bubonic plague [52]. Pla is a plasmid-encoded gene unique to *Y. pestis*. It encodes an outer membrane protease of the omptin family that has a beta barrel structure that requires an interaction with rough lipopolysaccharide (LPS) for full activity [183]. The protease activates host plasminogen and binds to laminin [161, 162, 184, 185]. The resultant localized proteolysis, fibrinolysis, and extracellular matrix destruction allows the spread of organism from inoculation sites and associated lymph nodes contributing to secondary septicemia and associated endotoxic shock. There appears to be no difference in expression of Pla between flea and mammalian hosts, and synthesis continues during calcium deficient growth restriction, however, fibrinolytic activity may be higher at 37°C [186]. Pla has also been shown to cleave complement (C3), aid in delivery and degradation of Yops, possess adhesin properties, inactivate cationic antimicrobial peptides, and bind to surface receptors on macrophages and dendritic cells [140, 159, 161, 183, 187].

Fraction 1 capsular antigen (F1) was originally thought to be a component of the capsule but has been found to be similar to fimbrial structures found in the Enterobacteriaceae family [188-190]. It is produced by *Y. pestis* grown at 35-37°C and is also associated with calcium deficient growth restriction. This gel-like matrix consists of linear fibers in a bilayer with an internal hydrophobic region around the bacterial cell [191]. The *caf1* operon is encoded on the pFra plasmid thus unique to *Y. pestis,* but acquired horizontally and assembled and exported through a chaperone/usher system [192]. F1 is a major immunogen and highly conserved among *Y. pestis* strains but may not be required for virulence [193, 194]. F1 is produced in large

quantities and free antigen can be detected in tissues [189, 193, 195, 196]. It contributes to virulence by masking bacterial surface receptors thus preventing opsonization by complement and inhibits phagocytosis of bacteria by neutrophils and macrophages [146]. It continues to be the major antigenic component in *Yersinia* vaccines to date [197-203]

The lipopolysaccharide (LPS) structure of *Y. pestis* has been found to change within host-specific environments. At 21-27°C, it produces a typical hexa-acylated lipid A which stimulates a Toll-like receptor 4 (TLR4) response with downstream induction of pro-inflammatory cytokines (TNFα, IL1, IL6, IL8) and host innate cell activation. However, in bacteria grown at 37°C, the lipid A structure is tetra-acylated, often referred to as rough LPS indicating changes in the O-antigen structures. The tetra-acylated lipid A form does not generate a TLR4 stimulatory response contributing to the inhibition of host innate immune functions including decreased macrophage activation, cytokine production, dendritic cell activation, and maturation. As such, downstream effects are seen in cell-mediated and adaptive immune responses [144, 204-209]. It is hypothesized that a frame-shift mutation in O antigen during *Y. pestis'* evolution from *Y. pseudotuberculosis* may contribute to the ability to reach high bacteremia levels in the mammalian host assuring subsequent flea transmission [210]. Interestingly, there may also be some modifications to LPS during transit through the flea that may contribute to the inhibition of the host innate response during transmission.

Both *Y. pseudotuberculosis* and *Y. pestis* have been associated with fleas. However, infection with *Y. pseudotuberculosis* commonly results in flea mortality due to toxic reactions in the hindgut that generate a diarrhea-like response in the insect [211, 212]. This is linked to insecticidal toxin (Tc) genes and the production of urease [213]. In its evolution, *Y. pestis* has lost the function of genes associated with Tc, urease, motility, and energy metabolism and transport systems that are required for gastrointestinal survival by enteric *Yersinia.* These modifications and acquisition of pFra have allowed *Y. pestis* to survive and replicate within the

flea midgut. The *ymt* gene located within pFra is responsible for the production of phospholipase D, which protects the organism from midgut digestion [129] along with transcription regulators, such as *phoP*. Biofilm formation, which is conserved between *Y. pseudotuberculosis* and *Y. pestis*, is required for aggregation and proventricular accumulations within the midgut. However, pseudogene vestiges of some genes involved in the complex pathway (Rcs and phosphodiesterase enzymes) have allowed *Y. pestis* to colonize the midgut, thereby contributing to transmission by regurgitation.

Prior to passage from the flea, *Y. pestis* lacks some of the virulence factors that are only expressed at 37˚C which may predispose it to rapid clearance within the host. However, initial studies indicate that extracellular matrices (ECM) surrounding *Y. pestis* while in the flea midgut may provide temporary protection from host innate immune responses. Host blood components or other ECM components may mask or block bacterial antigens until expression of virulence factors upregulated at 37C can occur [75, 76, 214, 215].

CLINICAL MANIFESTATION OF *YERSINIA PESTIS* INFECTION IN MAMMALIAN SPECIES: *Yersinia pestis* is able to induce three forms of clinical disease in mammalian species: bubonic, septicemic and pneumonic plague, all of which may be systemically progressive in some individuals [6]. The route of exposure to *Y. pestis* most frequently occurs by way of a flea bite. However, it can also gain entry through skin and mucosal surfaces such as the alimentary or respiratory tracts. Oral exposures in carnivores have been recognized since the Black Death epidemic but recognition of pharyngeal and gastrointestinal disease in humans was not documented until the 1980s where disease was linked to human consumption of plague-infected animals, such as goats and camels [119-122, 216-218].

The route of exposure generally dictates the clinical presentation, progression, and severity of disease in the mammalian host. Bubonic plague is the most common form of disease in

humans and results from *Y. pestis* gaining entry through flea bite or mucosal surfaces [6]. Within 2-6 days infected individuals will experience severe pyrexia and other flu-like symptoms such as malaise, lethargy, and inappetence. As *Y. pestis* is trafficked from inoculation sites to draining lymph nodes, bacterial replication occurs resulting in progressive lymphadenopathy (bubo). These buboes are most commonly detected in axillary and groin regions depending upon the initial site of exposure and are characterized by necrosuppurative inflammation and nodal architectural destruction [219, 220]. If infection is left untreated, severe, or occurs in an immunocompromised individual, progression to the septicemic form of the disease may follow.

Primary septicemic disease is possible when inoculation occurs by a direct hematogenous route or secondary bacteremia occurs in later stages of bubonic infection when high bacterial loads are released from necrotic lymph nodes. Bacterial entry into the bloodstream subsequently results in rapid infiltration of organs (primarily liver, spleen and bone marrow) and classic endotoxic response. Subsequent septic shock, disseminated intravascular coagulation and vasculitis results in organ failure, gangrenous extremities, and rapid death.

Pneumonic plague is the most severe form of disease in all mammalian species. It can manifest as primary disease through inhalation of infectious material. It is estimated that in 12% of untreated bubonic cases septic distribution to the lungs can occur resulting in secondary pneumonic disease. This disease manifestation is very common in domestic felids [112, 221, 222] and disease progression is similar to that reported in humans, rodents, and non-human primates [108, 135, 193, 223-225]. Infection within the lungs is described as biphasic with an initial pre-inflammatory phase and later pro-inflammatory phase. After inhalation, bronchopneumonia develops within the first 36 hours where *Y. pestis* and proteinaceous effusions are detectable in small airways. Over the next 48 hours, there is rapid progresses to lobar pneumonia, which is associated with pulmonary congestion, hemorrhage, and alveolar

flooding. Mortality rates are 100% unless treatment is initiated during the early phase of disease.

In cases of oral ingestion, the primary targets for bacterial infiltration include the palatine tonsil, and pharyngeal and cervical lymph nodes [119, 121, 122, 226]. Disease progression is similar to that of the bubonic form with progressive lymphadenopathy. It has been found that secondary pneumonic plague is a common sequela as a result of aspiration due to necrosis of pharyngeal lymphoid tissues.

HOST INNATE IMMUNE RESPONSE: The development of clinical disease is directly related to the battle between the proliferation of *Y. pestis* within the mammalian host and its elimination by the host innate immune response. Key to its defenses, *Y. pestis* is capable of both extracellular and intracellular survival and proliferates in a variety of cell types including epithelial and endothelial cells [161, 227] but its primary targets are macrophages, neutrophils, and dendritic cells. It establishes itself through a biphasic disease progression. The early phase of infection establishes a localized immunosuppression at the inoculation site and within draining lymph nodes. Using multiple virulence factors, it is able to resist innate responses, subvert host cell signaling and modulate the host inflammatory response. In later stages of disease, there is a switch to a pro-inflammatory state when host immune functions are activated by disseminated organisms and contributes to the pathological changes associated with disease. Subtle differences in pathogenesis have been noted depending upon the host species, route of exposure and bacterial growth conditions [228]. Descriptions below detail the current scientific knowledge (through 2013) of bubonic disease progression in rodent models following flea bite intradermal transmission.

When *Y. pestis* proliferates in the flea midgut, modifications occur that prime specific virulence factors. Whether these are associated with temperature regulated mechanisms or

biofilm-associated anti-phagocytic properties are not clearly defined [75]. Despite apparent resistance to complement-mediated lysis after passage through the flea, it appears that neutrophils can be effective in phagocytic destruction of *Y. pestis* at inoculation sites, but this elimination is likely time and dose-dependent [229].

Once replication of *Y. pestis* occurs, in as early as two to five hours, expression of virulence factors sets the stage for bacterial proliferation. Modification of its lipopolysaccharide structure varies by temperature with hexa-acylated lipid A noted at 26˚C but converted to a tetra-acylated lipid A during growth at 37˚C [206, 208, 230]. This results in a dampening of the host toll-like receptor (TLR4) response. This, in turn, creates localized immunosuppression due to poor activation of macrophages and dendritic cells as well as suppression of proinflammatory cytokine secretions (TNFα, IL1, IL6, IL8) [205, 207]. In addition, temperature-induced expression of F1, Ail, Pla, Psa, and YadBC contributes to inhibition of subsequent neutrophilic phagocytosis via adhesin expression, masking of surface proteins or receptors, and conferred serum resistance.

Uptake by macrophages at the inoculation site is thought to facilitate passage of *Y. pestis* to draining lymph nodes [136, 231] and the resultant permissive intracellular survival provides an escape from the host immune response during transport [136, 232]. Intracellular survival within the macrophage phagosomes occurs by preventing fusion with lysosomes [229, 232] and subsequent proliferation at 37˚C generates bacterial loads with full expression of the virulence factors, T3SS and YOPs [233]. Due to the lack of TLR4 activation signaling to the macrophage, and alterations in mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-ƙB) signaling by YopJ and YopE, release of fully virulent bacteria occurs by apoptosis, resulting in a noninflammatory death contributing further to the immunosuppressive state during early infection [234, 235]. In the later stage of *Y. pestis* infection, macrophages are found to be in an activated state and cell death mechanisms switch to pyroptosis, which induces an inflammatory

state and contributes to the hallmark necrosuppurative tissue necrosis within lymphoid tissues [234, 235].

The proliferation of *Y. pestis* at 37˚C results in expression and activation of the T3SS, where initial production of LcrV represses functions in multiple host cell pathways [149, 179-182, 232, 236]. Its secretion exploits TLR2 activation and the production of IL10 as well as suppresses TNFα and IFNɣ production resulting in suppression of the inflammatory response [178, 236-239]. It also functions as the translocon for the injection of YOPs proteins into subsequent host cells [165, 168, 233]. Yops are mostly found in phagocytic cells of the innate immune system and function to alter cell functions (Table 1.3). The most important functions include induction of apoptosis in infected macrophages and decreasing pro-inflammatory responses by infected cells.

The full effects imposed on other innate immune cells by *Y. pestis* has not been well characterized. However, it is known that dendritic cells are targeted early at inoculation sites where T3SS effectors alter cytoskeletal function and temper antigen presentation processes [207, 240-244]. Natural killer cell depletion is noted to be related to the severity of disease due to decreased IFNγ secretion and subsequent effects on reactive nitrogen intermediates [245, 246].

HOST ADAPTIVE IMMUNE RESPONSE: Cellular mediated responses against *Y. pestis* are poorly defined. It appears that Y. pestis may regulate cytokine induction to reduce a host adaptive response. This may be due to suppression of T cell activation and induction of programmed cell death. The host's ability to secrete IFNγ and IL10 by T cells may play a role in protection via the activation of antimicrobial factors from neighboring phagocytic cells [247-251]. These effects may be related to the ability of Stat 4 phosphorylation and Th1-mediated immune mechanisms [252]. Cell-mediated responses are a key experimental focus in defining the

pathogenesis of pneumonic plague to aid in the development of effective vaccines that could provide protection against the release of *Y. pestis* in a weaponized form.

Historically, immunization against plague was through the use of killed whole cell vaccines. These vaccines are no longer produced due to significant side effects and short-lived protection [253-255]. The live attenuated vaccine EV76 is still in use in some countries [256]. Many of the previously described virulence factors of *Y. pestis* are expressed in the bacterial membrane and have been found to be immunogenic [257-259]. Several subunit vaccines have been developed using F1, LcrV and or a combination of the two antigens for use in both non-human mammals and humans [197, 198, 200, 203, 260-265]. Antibody production against several other antigens has been shown to impart partial protection against infection including YopD, YopH, YopO and YpkA in animal models [197, 266, 267]. Antibodies developed against these antigens likely opsonize extracellular bacteria allowing for rapid clearance. These antibodies may also inhibit the T3SS system and dampen the effector proteins. Surprisingly, F1 and LcrV have recently been shown to be poor T cell immunogens, suggesting a shift in focus to investigate mechanisms of cell-mediated responses during pneumonic Y. pestis infections [268].

CONCLUSION

Plague has been described as having an 'infinite variety of manifestations of the disease resulting from the profound differences in host, vectors, and environmental conditions in different areas' [26]. The pathogenesis of *Y. pestis* results from a battle between the exploitation of the host resources and the ability to overcome the host defenses for its survival. Its adaptation to new ecological niches over the last millennium is a testimony to its resiliency. In the last twenty years many technological advancements in immunological techniques and identification of specific protein and genomic structures of *Y. pestis* have begun to improve our knowledge of the mechanisms that this pathogen uses to establish infection, evade the mammalian host immune system, and persist in the environment [51, 56, 196, 257-259, 269-

275]. These advances should expand the scientific community's' abilities to develop innovative treatments and vaccines to combat the persistence of this historical pathogen.

LITERATURE CITED

1. Mullett, C.F., *The bubonic plague in England; a problem in public health.* Bull Hist Med, 1946. 20(2): p. 299-309.

2. Echenberg, M., *Pestis-redux: The initial years of the third bubonic plague pandemic, 1894-1901.* Journal of World History, 2002. 13(2): p. 429-449.

3. Fritz, C.L., et al., *Surveillance for pneumonic plague in the united states during an international emergency: A model for control of imported emerging diseases.* Emerging Infectious Diseases, 1996. 2(1): p. 30-36.

4. Ganapati, M., *India's pneumonic plague outbreak continues to baffle.* British Medical Journal, 1995. 311: p. 706.

5. Russell, J.C., *Effects of pestilence and plague, 1315-1385.* Comparative Studies in Society and History, 1966. 8(4): p. 464-473.

6. Perry, R.D. and J.D. Fetherston, *Yersinia pestis - Etiologic agent of plague.* Clinical Microbiology Reviews, 1997. 10(1): p. 35-+.

7. Butler, T., *Review Article: Plague Gives Surprises in the First Decade of the 21st Century in the United States and Worldwide.* American Journal of Tropical Medicine and Hygiene, 2013. 89(4): p. 788-793.

8. Butler, T., *Plague into the 21st Century.* Clinical Infectious Diseases, 2009. 49(5): p. 736-742.

9. Ramalingaswami, V., *The plague outbreaks of India, 1994 - A prologue.* Current Science, 1996. 71(10): p. 781-782.

10. John, T.J., *Emerging & re-emerging bacterial pathogens in India.* Indian Journal of Medical Research, 1996. 103: p. 4-18.

11. Carniel, E., *world situation of Y-pestis infections.* Medecine Et Maladies Infectieuses, 1995. 25: p. 675-679.

12. Laventure, S., et al., *An epidemic of urban plague in Majunga, western coast of Madagascar.* Bulletin De La Societe De Pathologie Exotique, 1998. 91(1): p. 85-86.

13. Cabanel, N., et al., *Plague Outbreak in Libya, 2009, Unrelated to Plague in Algeria.* Emerging Infectious Diseases, 2013. 19(2): p. 230-236.

14. Bitam, I., et al., *New Rural Focus of Plague, Algeria.* Emerging Infectious Diseases, 2010. 16(10): p. 1639-1640.

15. Bertherat, E., et al., *Plague reappearance in Algeria after 50 years, 2003.* Emerging Infectious Diseases, 2007. 13(10): p. 1459-1462.

16. Bossi, P. and F. Bricaire, *Plague, a potential biowarfare act.* Presse Medicale, 2003. 32(17): p. 804-807.

17. Christopher, G.W., et al., *Biological warfare - A historical perspective.* Jama-Journal of the American Medical Association, 1997. 278(5): p. 412-417.

18. Wheelis, M., *Biological warfare at the 1346 Siege of Caffa.* Emerging Infectious Diseases, 2002. 8(9): p. 971-975.

19. Inglesby, T.V., et al., *Plague as a biological weapon - Medical and public health management.* Jama-Journal of the American Medical Association, 2000. 283(17): p. 2281-2290.

20. Kohler, W. and M. Kohler, *Plague and rats, the "Plague of the Philistines", and: what did our ancestors know about the role of rats in plague.* International Journal of Medical Microbiology, 2003. 293(5): p. 333-340.

21. Zietz, B.P. and H. Dunkelberg, *The history of the plague and the research on the causative agent Yersinia pestis.* International Journal of Hygiene and Environmental Health, 2004. 207(2): p. 165-178.

22. Little, L.K., *Life and Afterlife of the First Plague Pandemic.* Plague and the End of Antiquity: the Pandemic of 541-750, 2007: p. 3-32.

23. Riehm, J., et al., *Yersinia pestis DNA from Skeletal Remains from the 6th Century AD Reveals Insights into Justinianic Plague.* International Journal of Medical Microbiology, 2013. 303: p. 79-80.

24. Byrne, J.P., *The black death*. 2004, Westport, Conn.: Westport, Conn. : Greenwood Press.

25. Barnes, A.M., *Epidemiology of Plague and other Yersinioses.* Journal of the American Veterinary Medical Association, 1975. 167(9): p. 862-862.

26. Pollitzer, R., *Plague*, W.H. Organization, Editor. 1954: Geneva. p. 698.

27. Barnes, A.M., *Surveillance and Control of Bubonic Plague in the United States*. Symposium Zoological Society of London. Vol. 50. 1982, London, England. 237-270.

28. Adjemian, J.Z., et al., *Initiation and spread of traveling waves of plague, Yersinia pestis, in the western United States.* Am J Trop Med Hyg, 2007. 76(2): p. 365-75.

29. Mann, J.M., et al., *Endemic human plague in New-Mexico - risk-factors associated with infection.* Journal of Infectious Diseases, 1979. 140(3): p. 397-401.

30. Cully, J.F., L.G. Carter, and K.L. Gage, *New records of sylvatic plague in Kansas.* J Wildl Dis, 2000. 36(2): p. 389-92.

31. Survey, U.S.G., *Ouch, Taking a shot at plague: vaccine offers hope for endangered ferrets in plague outbreak.* 2008.

32. Eskey, C.R. and V.H. Haas, *Plague In The Western Part Of The United States Infection In Rodents, Experimental Transmission By Fleas, And Inoculation Tests For Infection.* Public Health Reports, 1939. 54(32): p. 1467-1481.

33. Link, V.B., *Plague in the United States of America.* Public Health Reports, 1955. 70(3): p. 335-336.

34. Smith, C.R., et al., *Plague studies in California: a review of long-term disease activity, flea-host relationships and plague ecology in the coniferous forests of the Southern Cascades and northern Sierra Nevada mountains.* Journal of Vector Ecology, 2010. 35(1): p. 1-12.

35. Gage, K.L., R.S. Ostfeld, and J.G. Olson, *Nonviral Vector-Borne Zoonoses Associated With Mammals In The United-States.* Journal of Mammalogy, 1995. 76(3): p. 695-715.

36. Eisen, R.J., L. Eisen, and K.L. Gage, *Studies of vector competency and efficiency of North American fleas for Yersinia pestis: state of the field and future research needs.* J Med Entomol, 2009. 46(4): p. 737-44.

37. Craven, R.B., et al., *Reported Cases Of Human Plague Infections In The United-States, 1970-1991.* Journal of Medical Entomology, 1993. 30(4): p. 758-761.

38. Malberg, J.A., et al., *Use of A Public Telephone Hotline to Detect Urban Plague Cases.* Zoonoses and Public Health, 2012. 59(7): p. 498-504.

39. Gould, L.H., et al., *Dog-associated risk factors for human plague.* Zoonoses Public Health, 2008. 55(8-10): p. 448-54.

40. Gage, K.L., et al., *Cases of cat-associated human plague in the Western US, 1977-1998.* Clin Infect Dis, 2000. 30(6): p. 893-900.

41. Wong, D., et al., *Primary pneumonic plague contracted from a mountain lion carcass.* Clin Infect Dis, 2009. 49(3): p. e33-8.

42. Prevention, C.f.D.C.a., *Human plague - four states, 2006: Morbidity and Mortality Weekly Report*. 2006.

43. Enscore, R.E., et al., *Modeling relationships between climate and the frequency of human plague cases in the southwestern United States, 1960-1997.* Am J Trop Med Hyg, 2002. 66(2): p. 186-96.

44. Stapp, P., M.F. Antolin, and M. Ball, *Patterns of extinction in prairie dog metapopulations: plague outbreaks follow El Nino events.* Frontiers in Ecology and the Environment, 2004. 2(5): p. 235-240.

45. Parmenter, R.R., et al., *Incidence of plague associated with increased winter-spring precipitation in New Mexico.* Am J Trop Med Hyg, 1999. 61(5): p. 814-21.

46. A, Y., *La peste bubonique a Hong Kong.* Ann Inst Pasteur, 1894. 8: p. 662-667.

47. Gyles, C.L. and C. Ebooks, *Pathogenesis of bacterial infections in animals*. 4th ed.. ed. 2010, Ames, Iowa: Ames, Iowa : Wiley-Blackwell.

48. Butler, T., *Plague and other Yersinia infections*. 1983, New York: New York : Plenum Medical Book Co.

49. Williams, S.K., et al., *Effects of low-temperature flea maintenance on the transmission of Yersinia pestis by Oropsylla montana.* Vector Borne Zoonotic Dis, 2013. 13(7): p. 468-78.

50. Li, B., et al., *Use of protein microarray to identify gene expression changes of Yersinia pestis at different temperatures.* Can J Microbiol, 2011. 57(4): p. 287-94.

51. Achtman, M., et al., *Microevolution and history of the plague bacillus, Yersinia pestis.* Proceedings of the National Academy of Sciences of the United States of America, 2004. 101(51): p. 17837-17842.

52. Achtman, M., et al., *Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis.* Proceedings of the National Academy of Sciences of the United States of America, 1999. 96(24): p. 14043-14048.

53. Prentice, M.B. and L. Rahalison, *Plague.* Lancet, 2007. 369(9568): p. 1196-1207.

54. Bergman, M.A., R. Chafel, and J. Mecsas, *Yersinia*, in *Pathogenesis of Bacterial Infections in Animals*, C.L. Gyles, J.L. Prescot, and G. Songer, Editors. 2010, Wiley-Blackwell: Hoboken, NJ. p. 309-323.

55. Parkhill, J., et al., *Genome sequence of Yersinia pestis, the causative agent of plague.* Nature, 2001. 413(6855): p. 523-527.

56. Chain, P.S.G., et al., *Insights into the evolution of Yersinia pestis through whole-genome comparison with Yersinia pseudotuberculosis.* Proceedings of the National Academy of Sciences of the United States of America, 2004. 101(38): p. 13826-13831.

57. Carniel, E., *Evolution of pathogenic Yersinia, some lights in the dark.* Genus Yersinia: Entering the Functional Genomic Era, 2003. 529: p. 3-12.

58. Vetter, S.M., et al., *Biofilm formation is not required for early-phase transmission of Yersinia pestis.* Microbiology, 2010. 156(Pt 7): p. 2216-25.

59. Simond, P., *La propagation de la Peste.* Ann Inst Pasteur, 1898. 12: p. 625.

60. *Reports on Plague Investigations in India.* Journal of Hygiene, 1907. VII: p. 415.

61. *Reports on Plague Investigations in India.* Journal of Hygiene, 1906. VI: p. 435.

62. *Reports on Plague Investigations in India.* Jounral of Hygiene, 1905. VI: p. 422.

63. Burroughs, A.L., *Sylvatic Plague Studies - The Vector Efficiency Of 9 Species Of Fleas Compared With Xenopsylla-Cheopis.* Journal of Hygiene, 1947. 45(3): p. 371-&.

64. Eisen, R.J., et al., *Early-phase transmission of Yersinia pestis by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics.* Proc Natl Acad Sci U S A, 2006. 103(42): p. 15380-5.

65. Blanc, G. and M. Baltazard, *Recherches sur le mode de transmission naturelle de la peste bubonique et septicemique.* Archives de L'institue Pasteur du Maroc, 1945. III(5): p. 173-227.

66. Ubico, S.R., et al., *A plague epizootic in the white-tailed prairie dogs (Cynomys leucurus) of Meeteetse, Wyoming.* J Wildl Dis, 1988. 24(3): p. 399-406.

67. Anderson, S.H. and E.S. Williams, *Plague in a complex of white-tailed prairie dogs and associated small mammals in Wyoming.* Journal of Wildlife Diseases, 1997. 33(4): p. 720-732.

68. Girard, G., *Plague.* Annu Rev Microbiol, 1955. 9: p. 253-277.

69. Thomas, R.E., R.H. Karstens, and T.G. Schwan, *Experimental-Infection Of Ornithodoros Spp Ticks (Acari, Argasidae) With Yersinia-Pestis.* Journal of Medical Entomology, 1990. 27(4): p. 720-723.

70. Ayyadurai, S., et al., *Body Lice, Yersinia pestis Orientalis, and Black Death.* Emerging Infectious Diseases, 2010. 16(5): p. 892-893.

71. Houhamdi, L., et al., *Experimental model to evaluate the human body louse as a vector of plague.* Journal of Infectious Diseases, 2006. 194(11): p. 1589-1596.

72. Bacot, A.W. and C.J. Martin, *Observations of the Mechansism of the Transmission of Plague by Fleas.* Jounral of Hygiene, 1914. 13: p. 423-439.

73. Douglas, J.R. and W.C. M, *Sylvatic plague studies II. The fate of Pasteurella pestis in the flea.* Journal of Infectious Diseases, 1943. 72: p. 18-30.

74. Jarrett, C.O., et al., *Transmission of Yersinia pestis from an infectious biofilm in the flea vector.* J Infect Dis, 2004. 190(4): p. 783-92.

75. Jarrett, C.O., et al., *Flea-borne transmission model to evaluate vaccine efficacy against naturally acquired bubonic plague.* Infect Immun, 2004. 72(4): p. 2052-6.

76. Cavanaugh, D.C., *Specific Effect Of Temperature Upon Transmission Of Plague Bacillus By Oriental Rat Flea, Xenopsylla-Cheopis.* American Journal of Tropical Medicine and Hygiene, 1971. 20(2): p. 264-+.

77. Hubbard, C.A., *Fleas of Western North America: their relation to public health*. 1947, Ames, IA: The Iowa State College Press.

78. Eisen, R.J., et al., *Demonstration of early-phase transmission of Yersinia pestis by the mouse flea, Aetheca wagneri (Siphonaptera: Ceratophylidae), and implications for the role of deer mice as enzootic reservoirs.* J Med Entomol, 2008. 45(6): p. 1160-4.

79. Eisen, R.J., et al., *Temporal dynamics of early-phase transmission of Yersinia pestis by unblocked fleas: secondary infectious feeds prolong efficient transmission by Oropsylla montana (Siphonaptera: Ceratophyllidae).* J Med Entomol, 2007. 44(4): p. 672-7.

80. Eisen, R.J., et al., *Early-phase transmission of Yersinia pestis by unblocked Xenopsylla cheopis (Siphonaptera: Pulicidae) is as efficient as transmission by blocked fleas.* J Med Entomol, 2007. 44(4): p. 678-82.

81. Vadyvaloo, V., et al., *Analysis of Yersinia pestis gene expression in the flea vector.* Adv Exp Med Biol, 2007. 603: p. 192-200.

82. Engelthaler, D.M. and K.L. Gage, *Quantities of Yersinia pestis in fleas (Siphonaptera: Pulicidae, Ceratophyllidae, and Hystrichopsyllidae) collected from areas of known or suspected plague activity.* J Med Entomol, 2000. 37(3): p. 422-6.

83. Stapp, P. and D.J. Salkeld, *Inferring host-parasite relationships using stable isotopes: implications for disease transmission and host specificity.* Ecology, 2009. 90(11): p. 3268-73.

84. Webb, C.T., et al., *Classic flea-borne transmission does not drive plague epizootics in prairie dogs.* Proc Natl Acad Sci U S A, 2006. 103(16): p. 6236-41.

85. Tripp, D.W., et al., *Flea abundance on black-tailed prairie dogs (Cynomys ludovicianus) increases during plague epizootics.* Vector Borne Zoonotic Dis, 2009. 9(3): p. 313-21.

86. Stapp, P., et al., *Evidence for the involvement of an alternate rodent host in the dynamics of introduced plague in prairie dogs.* J Anim Ecol, 2009. 78(4): p. 807-17.

87. Poland, J.D. and A.M. Barnes, *Plague*, in *CRC Handbook Series in Zoonoses*, J.H. Steele, Editor. 1979, CRC Press: Boca Raton, FL. p. 515-597.

88. Gage, K.L. and M.Y. Kosoy, *Natural history of plague: perspectives from more than a century of research.* Annu Rev Entomol, 2005. 50: p. 505-28.

89. Holdenried, R. and S.F. Quan, *Susceptibility Of New-Mexico Rodents To Experimental Plague.* Public Health Reports, 1956. 71(10): p. 979-984.

90. Bacon, M. and C.H. Drake, *Comparative Susceptibility Of Various Species Of Mice Native To Washington To Inoculation With Virulent Strains Of Pasteurella-Pestis.* Journal of Infectious Diseases, 1958. 102(1): p. 14-22.

91. Cully, J.F. and E.S. Williams, *Interspecific comparisons of sylvatic plague in prairie dogs.* Journal of Mammalogy, 2001. 82(4): p. 894-905.

92. Cully, J.F., et al., *Dynamics of plague in a Gunnison's prairie dog colony complex from New Mexico.* Journal of Wildlife Diseases, 1997. 33(4): p. 706-719.

93. Quan, S.F., V.I. Miles, and L. Kartman, *Ecological Studies Of Wild Rodent Plague In The San-Francisco Bay Area Of California .3. The Natural Infection Rates With Pasteurella-Pestis In 5 Flea Species During An Epizootic.* American Journal of Tropical Medicine and Hygiene, 1960. 9(1): p. 85-90.

94. Pauli, J.N., et al., *A plague epizootic in the black-tailed prairie dog (Cynomys ludovicianus).* J Wildl Dis, 2006. 42(1): p. 74-80.

95. Thomas, R.E., et al., *Susceptibility to Yersinia-pestis in the Northern Grasshopper Mouse (Onychomys-Leucogaster).* Journal of Wildlife Diseases, 1988. 24(2): p. 327-333.

96. Meyer, K.F., et al., *Sylvatic plague studies IV Inapparent, latent sylvatic plague in ground squirrel's in Central California.* Journal of Infectious Diseases, 1943. 73: p. 144-157.

97. Rayor, L.S., *Dynamics Of A Plague Outbreak In Gunnison prairie Dog.* Journal of Mammalogy, 1985. 66(1): p. 194-196.

98. McCoy, G.W., *The relation of animal experimentation to our knowledge of plague.* Journal of the American Medical Association, 1910. 55: p. 186-190.

99. Williams, J.E., M.A. Moussa, and D.C. Cavanaugh, *Experimental Plague in the California ground-squirrel.* Journal of Infectious Diseases, 1979. 140(4): p. 618-621.

100. McCoy, G.W. and C.W. Chapin, *Susceptibility of certain domestic animals to plague infection with particular reference to that of ground squirrel origin.* Journal of Infectious Diseases, 1911. 9(3): p. 276-281.

101. McCoy, G.W. and F.C. Smith, *The susceptibility to plague of the prairie dog, the desert wood rat, and the rock squirrel.* Journal of Infectious Diseases, 1910. 7(3): p. 374-376.

102. Hudson, B.W., et al., *Serological and bacteriological investigations of an outbreak of plague in an urban tree squirrel population.* Am J Trop Med Hyg, 1971. 20(2): p. 255-63.

103. Williams, E.S., et al., P*lague in a Black-footed ferret (Mustela nigripes).* Journal of Wildlife Diseases, 1994. 30(4): p. 581-585.

104. Castle, K.T., et al., *Susceptibility of the Siberian polecat to subcutaneous and oral Yersinia pestis exposure.* J Wildl Dis, 2001. 37(4): p. 746-54.

105. Williams, E.S., et al., *Experimental-infection of domestic ferrets (Mustela-putorius-furo) and Siberian polecats (Mustela-eversmanni) with Yersinia-pestis.* Journal of Wildlife Diseases, 1991. 27(3): p. 441-445.

106. Messick, J.P., G.W. Smith, and A.M. Barnes, *Serologic testing of badgers to monitor plague in southwestern Idaho.* Journal of Wildlife Diseases, 1983. 19(1): p. 1-6.

107. Higa, H.H., W.T. Matsuura, and W.H. Watanabe, *Plague antibody response in the mongoose.* Hawaii Med J, 1971. 30(2): p. 92-4.

108. Werner, S.B., et al., *Primary plague pneumonia contracted from a domestic cat at South Lake Tahoe, Calif.* JAMA, 1984. 251(7): p. 929-31.

109. Kaufmann, A.F., et al., *Public health implications of plague in domestic cats.* J Am Vet Med Assoc, 1981. 179(9): p. 875-8.

110. Gerhold, R.W. and D.A. Jessup, *Zoonotic diseases associated with free-roaming cats.* Zoonoses Public Health, 2013. 60(3): p. 189-95.

111. Rust, J.H., et al., *The role of domestic animals in the epidemiology of plague. I. Experimental infection of dogs and cats.* J Infect Dis, 1971. 124(5): p. 522-6.

112. Eidson, M., J.P. Thilsted, and O.J. Rollag, *Clinical, clinicopathologic, and pathologic features of plague in cats: 119 cases (1977-1988).* J Am Vet Med Assoc, 1991. 199(9): p. 1191-7.

113. Gasper, P.W., et al., *plague (Yersinia-pestis) in cats - description of experimentally induced disease.* Journal of Medical Entomology, 1993. 30(1): p. 20-26.

114. Wild, M.A., T.M. Shenk, and T.R. Spraker, *Plague as a mortality factor in Canada lynx (Lynx canadensis) reintroduced to Colorado.* J Wildl Dis, 2006. 42(3): p. 646-50.

115. Tabor, S.P., *The occurrence of plague (Yersinia-pestis) in a bobcat from the trans-pecos area of Texas.* Southwestern Naturalist, 1986. 31(1): p. 135-136.

116. Poland, J.D., A.M. Barnes, and J.J. Herman, *Human bubonic plague from exposure to a naturally infected wild carnivore.* Am J Epidemiol, 1973. 97(5): p. 332-7.

117. Barnes, A.M., *Plague In The United-States - Present And Future*. Fourteenth Vertebrate Pest Conference, ed. L.R. Davis and R.E. Marsh. 1990. 43-46.

118. Shepherd, G.G., *The response of raccoons (Procyon lotor) to experimental infection with Yersinia pestis*. 1973, Colorado State University.

119. Christie, A.B., T.H. Chen, and S.S. Elberg, *Plague in camels and goats: their role in human epidemics.* J Infect Dis, 1980. 141(6): p. 724-6.

120. Leslie, T., et al., *Outbreak of gastroenteritis caused by Yersinia pestis in Afghanistan.* Epidemiology and Infection, 2011. 139(5): p. 728-735.

121. Bin Saeed, A.A., N.A. Al-Hamdan, and R.E. Fontaine, *Plague from eating raw camel liver.* Emerging Infectious Diseases, 2005. 11(9): p. 1456-1457.

122. Arbaji, A., et al., *A 12-case outbreak of pharyngeal plague following the consumption of camel meat, in north-eastern Jordan.* Annals of Tropical Medicine and Parasitology, 2005. 99(8): p. 789-793.

123. Thorne, E.T., Et Al., P*lague in a free-ranging mule deer from Wyoming.* Journal of Wildlife Diseases, 1987. 23(1): p. 155-159.

124. Jessup, D.A., et al., *Ocular lesions of plague (Yersinia-pestis) in a black-tailed deer (Odocoileus-hemionus-columbianus).* Journal of Zoo and Wildlife Medicine, 1989. 20(3): p. 360-363.

125. Edmunds, D.R., et al., *Ocular plague (Yersinia pestis) in mule deer (Odocoileus hemionus) from Wyoming and Oregon.* J Wildl Dis, 2008. 44(4): p. 983-7.

126. Cornelis, G.R., *The type III secretion injectisome.* Nature Reviews Microbiology, 2006. 4(11): p. 811-825.

127. Cornelis, G.R., *The Yersinia YSC-YOP 'type III' weaponry.* Nature Reviews Molecular Cell Biology, 2002. 3(10): p. 742-752.

128. Viboud, G.I. and J.B. Bliska, *Yersinia outer proteins: Role in modulation of host cell signaling responses and pathogenesis.* Annual Review of Microbiology, 2005. 59: p. 69-89.

129. Cornelis, G.R., *How Yops find their way out of Yersinia.* Molecular Microbiology, 2003. 50(4): p. 1091-1094.

130. Dewoody, R.S., P.M. Merritt, and M.M. Marketon, *Regulation of the Yersinia type III secretion system: traffic control.* Frontiers in Cellular and Infection Microbiology, 2013. 3.

131. Mueller, C.A., et al., *The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles.* Science, 2005. 310(5748): p. 674-676.

132. Kolodziejek, A.M., C.J. Hovde, and S.A. Minnich, *Yersinia pestis Ail: multiple roles of a single protein.* Frontiers in Cellular and Infection Microbiology, 2012. 2.

133. Perry, R.D., et al., A *low-ca-2+ response operon encodes the V-antigen of Yersinia-pestis.* Infection and Immunity, 1986. 54(2): p. 428-434.

134. Price, S.B., et al., T*he Yersinia-pestis V-antigen is a regulatory protein necessary for ca-2+-dependent growth and maximal expression of low-ca-2+ response virulence genes.* Journal of Bacteriology, 1991. 173(8): p. 2649-2657.

135. Burrows, T.W. and G.A. Bacon, *The basis of virulence in Pasteurella-pestis - an antigen determining virulence.* British Journal of Experimental Pathology, 1956. 37(5): p. 481-&.

136. Burrows, T.W., *Antigen Determining Virulence in Pasteurella-pestis.* Nature, 1956. 177(4505): p. 426-427.

137. Une, T. and R.R. Brubaker, *Roles of v-antigen in promoting virulence and immunity in Yersiniae.* Journal of Immunology, 1984. 133(4): p. 2226-2230.

138. Noel, B.L., et al., *Yersinia pestis Can Bypass Protective Antibodies to LcrV and Activation with Gamma Interferon To Survive and Induce Apoptosis in Murine Macrophages.* Clinical and Vaccine Immunology, 2009. 16(10): p. 1457-1466.

139. Weeks, S., et al., *Anti-V antigen antibody protects macrophages from Yersinia pestis-induced cell death and promotes phagocytosis.* Microbial Pathogenesis, 2002. 32(5): p. 227-237.

140. Sing, A., et al., *A hypervariable N-terminal region of Yersinia LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence.* Proceedings of the National Academy of Sciences of the United States of America, 2005. 102(44): p. 16049-16054.

141. Sing, A., et al., *Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression.* Journal of Experimental Medicine, 2002. 196(8): p. 1017-1024.

142. Pouliot, K., et al., *Evaluation of the role of LcrV-Toll-Like receptor 2-mediated immunomodulation in the virulence of Yersinia pestis.* Infection and Immunity, 2007. 75(7): p. 3571-3580.

143. Abramov, V.M., et al., *Attachment of LcrV from Yersinia pestis at dual binding sites to human TLR-2 and human IFN-gamma receptor.* Journal of Proteome Research, 2007. 6(6): p. 2222-2231.

144. Pendrak, M.L. and R.D. Perry, *Characterization of a hemin-storage locus of Yersinia-pestis.* Biology of Metals, 1991. 4(1): p. 41-47.

145. Forman, S., et al., *Identification of critical amino acid residues in the plague biofilm Hms proteins.* Microbiology-Sgm, 2006. 152: p. 3399-3410.

146. Bobrov, A.G., et al., *Insights into Yersinia pestis biofilm development: topology and co-interaction of Hms inner membrane proteins involved in exopolysaccharide production.* Environmental Microbiology, 2008. 10(6): p. 1419-1432.

147. Hinnebusch, B.J., et al., *Role of Yersinia murine toxin in survival of Yersinia pestis in the midgut of the flea vector.* Science, 2002. 296(5568): p. 733-735.

148. Hinnebusch, J., et al., *Murine toxin of Yersinia pestis shows phospholipase D activity but is not required for virulence in mice.* International Journal of Medical Microbiology, 2000. 290(4-5): p. 483-487.

149. Oyston, P.C.F., et al., *The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in Yersinia pestis.* Infection and Immunity, 2000. 68(6): p. 3419-3425.

150. O'Loughlin, J.L., et al., *Yersinia pestis Two-Component Gene Regulatory Systems Promote Survival in Human Neutrophils.* Infection and Immunity, 2010. 78(2): p. 773-782.

151. Bozue, J., et al., *The role of the phoPQ operon in the pathogenesis of the fully virulent CO92 strain of Yersinia pestis and the IP32953 strain of Yersinia pseudotuberculosis.* Microbial Pathogenesis, 2011. 50(6): p. 314-321.

152. Hitchen, P.G., et al., *Structural characterization of lipo-oligosaccharide (LOS) from Yersinia pestis: regulation of LOS structure by the PhoPQ system.* Molecular Microbiology, 2002. 44(6): p. 1637-1650.

153. Welkos, S., et al., *Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of Yersinia pestis in non-human primate and mouse models of pneumonic plague.* Vaccine, 2002. 20(17-18): p. 2206-2214.

154. Pujol, C., et al., *Replication of Yersinia pestis in interferon gamma-activated macrophages requires ripA, a gene encoded in the pigmentation locus.* Proceedings of the National Academy of Sciences of the United States of America, 2005. 102(36): p. 12909-12914.

155. Leo, J.C. and M. Skurnik, *Adhesins of Human Pathogens from the Genus Yersinia*, in *Bacterial Adhesion: Chemistry, Biology and Physics*, D. Linke and A. Goldman, Editors. 2011. p. 1-15.

156. Hinnebusch, B.J., et al., *Role of the Yersinia pestis Ail protein in preventing a protective polymorphonuclear leukocyte response during bubonic plague.* Infect Immun, 2011. 79(12): p. 4984-9.

157. Felek, S., T.M. Tsang, and E.S. Krukonis, *Three Yersinia pestis Adhesins Facilitate Yop Delivery to Eukaryotic Cells and Contribute to Plague Virulence.* Infection and Immunity, 2010. 78(10): p. 4134-4150.

158. Yamashita, S., et al., *Structural Insights into Ail-Mediated Adhesion in Yersinia pestis.* Structure, 2011. 19(11): p. 1672-1682.

159. Tsang, T.M., S. Felek, and E.S. Krukonis, *Ail Binding to Fibronectin Facilitates Yersinia pestis Binding to Host Cells and Yop Delivery.* Infection and Immunity, 2010. 78(8): p. 3358-3368.

160. Bartra, S.S., et al., *Resistance of Yersinia pestis to complement-dependent killing is mediated by the Ail outer membrane protein.* Infection and Immunity, 2008. 76(2): p. 612-622.

161. Knirel, Y.A., et al., *Structural features and structural variability of the lipopolysaccharide of Yersinia pestis, the cause of plague.* Journal of Endotoxin Research, 2006. 12(1): p. 3-9.

162. Aussel, L., et al., *Novel variation of lipid A structures in strains of different Yersinia species.* Febs Letters, 2000. 465(1): p. 87-92.

163. Du, Y.D., R. Rosqvist, and A. Forsberg, *Role of fraction 1 antigen of Yersinia pestis in inhibition of phagocytosis.* Infection and Immunity, 2002. 70(3): p. 1453-1460.

164. Welkos, S., et al., *V antigen of Yersinia pestis inhibits neutrophil chemotaxis.* Microbial Pathogenesis, 1998. 24(3): p. 185-196.

165. Lindler, L.E. and B.D. Tall, *Yersinia-Pestis pH-6 Antigen Forms Fimbriae and is Induced by Intracellular Association with Macrophages.* Molecular Microbiology, 1993. 8(2): p. 311-324.

166. Lindler, L.E., M.S. Klempner, and S.C. Straley, *Yersinia-Pestis pH-6 Antigen - Genetic, Biochemical, and Virulence Characterization of a Protein Involved in the Pathogenesis of Bubonic Plague.* Infection and Immunity, 1990. 58(8): p. 2569-2577.

167. Huang, X.Z. and L.E. Lindler, *The pH 6 antigen is an antiphagocytic factor produced by Yersinia pestis independent of yersinia outer proteins and capsule antigen.* Infection and Immunity, 2004. 72(12): p. 7212-7219.

168. Makoveichuk, E., et al., *pH6 antigen of Yersinia pestis interacts with plasma lipoproteins and cell membranes.* Journal of Lipid Research, 2003. 44(2): p. 320-330.

169. Price, S.B., M.D. Freeman, and K.S. Yeh, *Transcriptional analysis of the Yersinia-pestis pH-6-antigen gene.* Journal of Bacteriology, 1995. 177(20): p. 5997-6000.

170. Sodeinde, O.A., et al., A *surface protease and the invasive character of plague.* Science, 1992. 258(5084): p. 1004-1007.

171. Sebbane, F., et al., *Role of the Yersinia pestis plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague.* Proc Natl Acad Sci U S A, 2006. 103(14): p. 5526-30.

172. Lahteenmaki, K., M. Kukkonen, and T.K. Korhonen, *The Pla surface protease/adhesin of Yersinia pestis mediates bacterial invasion into human endothelial cells.* Febs Letters, 2001. 504(1-2): p. 69-72.

173. Lahteenmaki, K., et al., *Bacterial plasminogen receptors - in-vitro evidence for a role in degradation of the mammalian extracellular-matrix.* Infection and Immunity, 1995. 63(9): p. 3659-3664.

174. Korhonen, T.K., et al., *Fibrinolytic and coagulative activities of Yersinia pestis.* Frontiers in Cellular and Infection Microbiology, 2013. 3.

175. Cornelis, G.R. and F. Van Gijsegem, *Assembly and function of type III secretory systems.* Annual Review of Microbiology, 2000. 54: p. 735-774.

176. Mikula, K.M., R. Kolodziejczyk, and A. Goldman, *Yersinia infection tools-characterization of structure and function of adhesins.* Frontiers in Cellular and Infection Microbiology, 2013. 3.

177. Forman, S., et al., *yadBC of Yersinia pestis, a new virulence determinant for bubonic plague.* Infection and Immunity, 2008. 76(2): p. 578-587.

178. Perry, R.D., et al., *Yersiniabactin from Yersinia pestis: biochemical characterization of the siderophore and its role in iron transport and regulation.* Microbiology-Uk, 1999. 145: p. 1181-1190.

179. Gehring, A.M., et al., *Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by Yersinia pestis.* Chemistry & Biology, 1998. 5(10): p. 573-586.

180. Forman, S., et al., *Yersinia ironomics: comparison of iron transporters among Yersinia pestis biotypes and its nearest neighbor, Yersinia pseudotuberculosis.* Biometals, 2010. 23(2): p. 275-294.

181. Liu, F.Z., et al., *Effects of Psa and F1 on the adhesive and invasive interactions of Yersinia pestis with human respiratory tract epithelial cells.* Infection and Immunity, 2006. 74(10): p. 5636-5644.

182. Payne, D., et al., *The pH 6 antigen of Yersinia pestis binds to beta 1-linked galactosyl residues in glycosphingolipids.* Infection and Immunity, 1998. 66(9): p. 4545-4548.

183. Suomalainen, M., et al., *Using every trick in the book: The pla surface protease of Yersinia pestis*, in *Genus Yersinia: from Genomics to Function*, R.D. Perry and J.D. Fetherston, Editors. 2007. p. 268-278.

184. Lahteenmaki, K., et al., *Yersinia pestis Pla has multiple virulence-associated functions.* Genus Yersinia: Entering the Functional Genomic Era, 2003. 529: p. 141-145.

185. Beesley, E.D., et al., *Pesticins .3. Expression of coagulase and mechanism of fibrinolysis.* Journal of Bacteriology, 1967. 94(1): p. 19-+.

186. Suomalainen, M., et al., *Temperature-Induced Changes in the Lipopolysaccharide of Yersinia pestis Affect Plasminogen Activation by the Pla Surface Protease.* Infection and Immunity, 2010. 78(6): p. 2644-2652.

187. Galvan, E.M., M.A.S. Lasaro, and D.M. Schifferli, *Capsular antigen fraction 1 and pla modulate the susceptibility of Yersinia pestis to pulmonary antimicrobial peptides such as cathelicidin.* Infection and Immunity, 2008. 76(4): p. 1456-1464.

188. Chen, T.H. and S.S. Elberg, *Scanning electron-microscopic study of virulent Yersinia-pestis and Yersinia-pseudotuberculosis type-i.* Infection and Immunity, 1977. 15(3): p. 972-977.

189. Runco, L.M., et al., *Biogenesis of the fraction 1 capsule and analysis of the ultrastructure of Yersinia pestis.* Journal of Bacteriology, 2008. 190(9): p. 3381-3385.

190. Cavanaugh, D.C. and R. Randall, *Role of multiplication of Pasteurella-pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague.* Journal of Immunology, 1959. 83(4): p. 348-363.

191. Zavialov, A.V., et al., *Structure and biogenesis of the capsular F1 antigen from Yersinia pestis: Preserved folding energy drives fiber formation.* Cell, 2003. 113(5): p. 587-596.

192. MacIntyre, S., et al., *An extended hydrophobic interactive surface of Yersinia pestis Caf1M chaperone is essential for subunit binding and F1 capsule assembly.* Molecular Microbiology, 2001. 39(1): p. 12-25.

193. Davis, K.J., et al., *Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative Yersinia pestis in African green monkeys (Cercopithecus aethiops).* Archives of Pathology & Laboratory Medicine, 1996. 120(2): p. 156-163.

194. Drozdov, I.G., et al., *virulent non-capsulate yYersinia-pestis variants constructed by insertion mutagenesis.* Journal of Medical Microbiology, 1995. 42(4): p. 264-268.

195. Chanteau, S., et al., *F1 antigenaemia in bubonic plague patients, a marker of gravity and efficacy of therapy.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1998. 92(5): p. 572-573.

196. Sebbane, F., et al., *Adaptive response of Yersinia pestis to extracellular effectors of innate immunity during bubonic plague.* Proceedings of the National Academy of Sciences of the United States of America, 2006. 103(31): p. 11766-11771.

197. Andrews, G.P., et al., *Protective efficacy of recombinant Yersinia outer proteins against bubonic plague caused by encapsulated and nonencapsulated Yersinia pestis.* Infection and Immunity, 1999. 67(3): p. 1533-1537.

198. Rocke, T.E., et al., *Recombinant F1-V fusion protein protects black-footed ferrets (Mustela nigripes) against virulent Yersinia pestis infection.* Journal of Zoo and Wildlife Medicine, 2004. 35(2): p. 142-146.

199. Jones, S.M., et al., *Protection conferred by a fully recombinant sub-unit vaccine against Yersinia pestis in male and female mice of four inbred strains.* Vaccine, 2000. 19(2-3): p. 358-366.

200. Osorio, J.E., et al., *Recombinant raccoon pox vaccine protects mice against lethal plague.* Vaccine, 2003. 21(11-12): p. 1232-1238.

201. Quenee, L.E., et al., *Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines.* Vaccine, 2011. 29(38): p. 6572-6583.

202. Erova, T.E., et al., *Evaluation of Protective Potential of Yersinia pestis Outer Membrane Protein Antigens as Possible Candidates for a New-Generation Recombinant Plague Vaccine.* Clinical and Vaccine Immunology, 2013. 20(2): p. 227-238.

203. Heath, D.G., et al., *Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine.* Vaccine, 1998. 16(11-12): p. 1131-1137.

204. Anisimov, A.P., et al., *Intraspecies and temperature-dependent variations in susceptibility of Yersinia pestis to the bactericidal action of serum and to polymyxin B.* Infection and Immunity, 2005. 73(11): p. 7324-7331.

205. Matsuura, M., et al., *Immunomodulatory Effects of Yersinia pestis Lipopolysaccharides on Human Macrophages.* Clinical and Vaccine Immunology, 2010. 17(1): p. 49-55.

206. Kawahara, K., et al., *Modification of the structure and activity of lipid A in Yersinia pestis lipopolysaccharide by growth temperature.* Infection and Immunity, 2002. 70(8): p. 4092-4098.

207. Telepnev, M.V., et al., *Tetraacylated Lipopolysaccharide of Yersinia pestis Can Inhibit Multiple Toll-Like Receptor-Mediated Signaling Pathways in Human Dendritic Cells.* Journal of Infectious Diseases, 2009. 200(11): p. 1694-1702.

208. Montminy, S.W., et al., *Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response.* Nature Immunology, 2006. 7(10): p. 1066-1073.

209. Knirel, Y.A., et al., *Temperature-dependent variations and intraspecies diversity of the structure of the lipopolysaccharide of Yersinia pestis.* Biochemistry, 2005. 44(5): p. 1731-1743.

210. Skurnik, M., A. Peippo, and E. Ervela, *Characterization of the O-antigen gene clusters of Yersinia pseudotuberculosis and the cryptic O-antigen gene cluster of Yersinia pestis shows that the plague bacillus is most closely related to and has evolved from Y-pseudotuberculosis serotype O : 1b.* Molecular Microbiology, 2000. 37(2): p. 316-330.

211. Erickson, D.L., et al., *Acute oral toxicity of Yersinia pseudotuberculosis to fleas: implications for the evolution of vector-borne transmission of plague.* Cellular Microbiology, 2007. 9(11): p. 2658-2666.

212. Erickson, D.L., et al., *Serotype differences and lack of biofilm formation characterize Yersinia pseudotuberculosis infection of the Xenopsylla cheopis flea vector of Yersinia pestis.* Journal of Bacteriology, 2006. 188(3): p. 1113-1119.

213. Spinner, J.L., et al., *Yersinia pestis insecticidal-like toxin complex (Tc) family proteins: characterization of expression, subcellular localization, and potential role in infection of the flea vector.* BMC Microbiol, 2012. 12: p. 296.

214. Lorange, E.A., et al., *Poor vector competence of fleas and the evolution of hypervirulence in Yersinia pestis.* J Infect Dis, 2005. 191(11): p. 1907-12.

215. Spinner, J.L. and B.J. Hinnebusch, *The Life Stage of Yersinia pestis in the Flea Vector Confers Increased Resistance to Phagocytosis and Killing by Murine Polymorphonuclear Leukocytes*, in *Advances in Yersinia Research*, A.M.P. DeAlmeida and N.C. Leal, Editors. 2012. p. 159-163.

216. Lowell, J.L., et al., *Identifying sources of human exposure to plague.* J Clin Microbiol, 2005. 43(2): p. 650-6.

217. Gabastou, J.M., et al., *An outbreak of plague including cases with probable pneumonic infection, Ecuador, 1998.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 2000. 94(4): p. 387-391.

218. Ruiz, A., *Plague in the Americas.* Emerging Infectious Diseases, 2001. 7(3): p. 539-540.

219. Tian, G., et al., *Histopathological observation of immunized rhesus macaques with plague vaccines after subcutaneous infection of Yersinia pestis.* PLoS One, 2011. 6(4): p. e19260.

220. Sebbane, F., et al., *Kinetics of disease progression and host response in a rat model of bubonic plague.* American Journal of Pathology, 2005. 166(5): p. 1427-1439.

221. Watson, R.P., et al., *Histopathology of experimental plague in cats.* Veterinary Pathology, 2001. 38(2): p. 165-172.

222. Doll, J.M., et al., *Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona.* Am J Trop Med Hyg, 1994. 51(1): p. 109-14.

223. Anderson, D.M., et al., *Pneumonic Plague Pathogenesis and Immunity in Brown Norway Rats.* American Journal of Pathology, 2009. 174(3): p. 910-921.

224. Finegold, M.J., *Pneumonic Plague in Monkeys - An Electron Microscopic Study.* American Journal of Pathology, 1969. 54(2): p. 167-&.

225. Du, Z. and X. Wang, *Pathology and Pathogenesis of Yersinia pestis.* Adv Exp Med Biol, 2016. 918: p. 193-222.

226. Laforce, F.M., et al., *Clinical and epidemiological observations on an outbreak of plague in nepal.* Bulletin of the World Health Organization, 1971. 45(6): p. 693-&.

227. Cowan, C., et al., *Invasion of epithelial cells by Yersinia pestis: Evidence for a Y pestis-specific invasin.* Infection and Immunity, 2000. 68(8): p. 4523-4530.

228. Gonzalez, R.J., et al., *Bioluminescence imaging to track bacterial dissemination of Yersinia pestis using different routes of infection in mice.* Bmc Microbiology, 2012. 12.

229. Lukaszewski, R.A., et al., *Pathogenesis of Yersinia pestis infection in BALB/c mice: Effects on host macrophages and neutrophils.* Infection and Immunity, 2005. 73(11): p. 7142-7150.

230. Rebeil, R., et al., *Variation in lipid A structure in the pathogenic yersiniae.* Molecular Microbiology, 2004. 52(5): p. 1363-1373.

231. Pujol, C. and J.B. Bliska, *The ability to replicate in macrophages is conserved between Yersinia pestis and Yersinia pseudotuberculosis.* Infection and Immunity, 2003. 71(10): p. 5892-5899.

232. Pujol, U. and J.B. Bliska, *Turning Yersinia pathogenesis outside in: subversion of macrophage function by intracellular yersiniae.* Clinical Immunology, 2005. 114(3): p. 216-226.

233. Plano, G.V. and K. Schesser, *The Yersinia pestis type III secretion system: expression, assembly and role in the evasion of host defenses.* Immunologic Research, 2013. 57(1-3): p. 237-245.

234. Bergsbaken, T. and B.T. Cookson, *Innate immune response during Yersinia infection: critical modulation of cell death mechanisms through phagocyte activation.* Journal of Leukocyte Biology, 2009. 86(5): p. 1153-1158.

235. Bergsbaken, T. and B.T. Cookson, *Macrophage activation redirects Yersinia-infected host cell death from apoptosis to caspase-1-dependent pyroptosis.* Plos Pathogens, 2007. 3(11): p. 1570-1582.

236. Brubaker, R.R., *Interleukin-10 and inhibition of innate immunity to yersiniae: Roles of Yops and LcrV (V antigen).* Infection and Immunity, 2003. 71(7): p. 3673-3681.

237. Zahorchak, R.J. and R.R. Brubaker, E*ffect of exogenous nucleotides on ca-2+ dependence and v-antigen synthesis in Yersinia-pestis.* Infection and Immunity, 1982. 38(3): p. 953-959.

238. Nakajima, R., V.L. Motin, and R.R. Brubaker, *Suppression of cytokines in mice by protein a-V antigen fusion peptide and restoration of synthesis by active immunization.* Infection and Immunity, 1995. 63(8): p. 3021-3029.

239. Nakajima, R. and R.R. Brubaker, *Association Between Virulence of Yersinia-pestis and Suppression of Gamma-Interferon and Tumor-Necrosis-Factor-Alpha.* Infection and Immunity, 1993. 61(1): p. 23-31.

240. Saikh, K.U., et al., *Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells.* Journal of Immunology, 2004. 173(12): p. 7426-7434.

241. Marketon, M.M., et al., *Plague bacteria target immune cells during infection.* Science, 2005. 309(5741): p. 1739-1741.

242. Robinson, R.T., et al., *Yersinia pestis evades TLR4-dependent induction of IL-12(p40)(2) by dendritic cells and subsequent cell migration.* Journal of Immunology, 2008. 181(8): p. 5560-5567.

243. Velan, B., et al., *Discordance in the effects of Yersinia pestis on the dendritic cell functions manifested by induction of maturation and paralysis of migration.* Infection and Immunity, 2006. 74(11): p. 6365-6376.

244. DePaolo, R.W., et al., *Toll-Like Receptor 6 Drives Differentiation of Tolerogenic Dendritic Cells and Contributes to LcrV-Mediated Plague Pathogenesis.* Cell Host & Microbe, 2008. 4(4): p. 350-361.

245. Ye, Z., et al., *Gr1(+) Cells Control Growth of YopM-Negative Yersinia pestis during Systemic Plague.* Infection and Immunity, 2009. 77(9): p. 3791-3806.

246. Kerschen, E.J., et al., *The plague virulence protein YopM targets the innate immune response by causing a global depletion of NK cells.* Infection and Immunity, 2004. 72(8): p. 4589-4602.

247. Parent, M.A., et al., *Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary Yersinia pestis infection.* Infection and Immunity, 2006. 74(6): p. 3381-3386.

248. Parent, M.A., et al., *Cell-mediated protection against pulmonary Yersinia pestis infection.* Infection and Immunity, 2005. 73(11): p. 7304-7310.

249. Kummer, L.W., et al., *Antibodies and cytokines independently protect against pneumonic plague.* Vaccine, 2008. 26(52): p. 6901-6907.

250. Bohn, E., et al., *Early Gamma-Interferon Messenger-Rna Expression is Associated With Resistance of Mice Against Yersinia-enterocolitica.* Infection and Immunity, 1994. 62(7): p. 3027-3032.

251. Autenrieth, I.B., et al., *Immune-Responses to Yersinia-enterocolitica in Susceptible Balb/C and Resistant C57bl/6 Mice - An Essential Role For Gamma-Interferon.* Infection and Immunity, 1994. 62(6): p. 2590-2599.

252. Elvin, S.J. and E.D. Williamson, *Stat 4 but not Stat 6 mediated immune mechanisms are essential in protection against plague.* Microbial Pathogenesis, 2004. 37(4): p. 177-184.

253. Meyer, K.F., et al., P*lague immunization .4. Clinical reactions and serologic response to inoculations of haffkine and freeze-dried plague vaccine.* Journal of Infectious Diseases, 1974. 129: p. S30-S36.

254. Meyer, K.F., *Effectiveness of live or killed plague vaccines in man.* Bulletin of the World Health Organization, 1970. 42(5): p. 653-&.

255. Marshall, J.D., et al., *Plague immunization .2. Relation of adverse clinical reactions to multiple immunizations with killed vaccine.* Journal of Infectious Diseases, 1974. 129: p. S19-S25.

256. Russell, P., et al., *A comparison of plague vaccine, usp and ev76 vaccine-induced protection against Yersinia-pestis in a murine model.* Vaccine, 1995. 13(16): p. 1551-1556.

257. Li, B., et al., *Antibody profiling in plague patients by protein microarray.* Microbes Infect, 2008. 10(1): p. 45-51.

258. Li, B., et al., *Protein microarray for profiling antibody responses to Yersinia pestis live vaccine.* Infect Immun, 2005. 73(6): p. 3734-9.

259. Lawson, J.N., C.R. Lyons, and S.A. Johnston, *Expression profiling of Yersinia pestis during mouse pulmonary infection.* DNA and Cell Biology, 2006. 25(11): p. 608-616.

260. Qi, Z., et al., *Comparison of mouse, guinea pig and rabbit models for evaluation of plague subunit vaccine F1+rV270.* Vaccine, 2010. 28(6): p. 1655-60.

261. Rocke, T.E., et al., *Consumption of baits containing raccoon pox-based plague vaccines protects black-tailed prairie dogs (Cynomys ludovicianus).* Vector Borne Zoonotic Dis, 2010. 10(1): p. 53-8.

262. Rocke, T.E., et al., *Further development of raccoon poxvirus-vectored vaccines against plague (Yersinia pestis).* Vaccine, 2009. 28(2): p. 338-44.

263. Lawrenz, M.B., *Model systems to study plague pathogenesis and develop new therapeutics.* Frontiers in Microbiology, 2010. 1.

264. Williamson, E.D., et al., *Human immune response to a plague vaccine comprising recombinant F1 and V antigens.* Infection and Immunity, 2005. 73(6): p. 3598-3608.

265. Williamson, E.D., et al., *A new improved subunit vaccine for plague - The basis of protection.* Fems Immunology and Medical Microbiology, 1995. 12(3-4): p. 223-230.

266. Wang, S.X., et al., *Relative immunogenicity and protection potential of candidate Yersinia Pestis antigens against lethal mucosal plague challenge in Balb/C mice.* Vaccine, 2008. 26(13): p. 1664-1674.

267. Benner, G.E., et al., *Immune response to Yersinia outer proteins and other Yersinia pestis antigens after experimental plague infection in mice.* Infection and Immunity, 1999. 67(4): p. 1922-1928.

268. Li, B., et al., *Humoral and Cellular Immune Responses to Yersinia pestis Infection in Long-Term Recovered Plague Patients.* Clinical and Vaccine Immunology, 2012. 19(2): p. 228-234.

269. Liu, H., et al., *Transcriptional profiling of a mice plague model: insights into interaction between Yersinia pestis and its host.* J Basic Microbiol, 2009. 49(1): p. 92-9.

270. Li, B., et al., *High-throughput identification of new protective antigens from a Yersinia pestis live vaccine by enzyme-linked immunospot assay.* Infect Immun, 2009. 77(10): p. 4356-61.

271. Das, R., et al., *Study of proinflammatory responses induced by Yersinia pestis in human monocytes using cDNA arrays.* Genes and Immunity, 2007. 8(4): p. 308-319.

272. Vadyvaloo, V., et al., *Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in Yersinia pestis.* PLoS Pathog, 2010. 6(2): p. e1000783.

273. Hinnebusch, B.J., F. Sebbane, and V. Vadyvaloo, *Transcriptional Profiling of the Yersinia pestis Life Cycle*. Yersinia: Systems Biology and Control, ed. E. Carniel and B.J. Hinnebusch. 2012. 1-18.

274. Comer, J.E., et al., *Transcriptomic and Innate Immune Responses to Yersinia pestis in the Lymph Node during Bubonic Plague.* Infection and Immunity, 2010. 78(12): p. 5086-5098.

275. Comer, J.E., E.A. Lorange, and B.J. Hinnebusch, *Examining the vector-host-pathogen interface with quantitative molecular tools.* Methods Mol Biol, 2008. 431: p. 123-31.

CHAPTER 2 - CLINICAL RESPONSE OF COYOTES (*CANIS LATRANS*) TO EXPERIMENTAL INOCULATION WITH *YERSINIA PESTIS*

SUMMARY

Multiple studies have utilized coyotes (*Canis latrans*) in animal-based surveillance efforts for the serological detection of *Yersinia pestis*. Coyotes are likely exposed to bacteria via flea bite or the consumption of infected mammals or their fleas and are presumed to be resistant to the development of clinical disease. Because the canid clinical response to *Y. pestis* has not been thoroughly characterized, we conducted an experimental inoculation of captive-reared, juvenile coyotes (n=12) with *Y. pestis* CO92 via oral or intradermal routes. No clinical signs of disease were observed, and minimal changes were noted in body temperature and white blood cell counts during the 7 days following exposure. Gross pathology was unremarkable, and minimal histopathological changes were noted at days 3 and 7 post-inoculation. Rechallenge at 14 weeks post-inoculation using the same dosage and exposure routes did not result in changes in clinical behavior, body temperature, or white blood cell counts. Information gathered from this experimental trial indicates that coyotes appear to be refractory to the development of clinical disease when exposed to *Yersinia pestis*.

INTRODUCTION

Plague is a flea-borne disease that continues to persist in endemic foci throughout the world. In the United States, human infection with this zoonotic pathogen is highly dependent upon peridomestic exposure to infected animals or the fleas that infest them [1-3]. Although *Y. pestis* is generally maintained in a sylvatic cycle between rodent species and their associated fleas, the organism can spill over and infect a variety of wild [1, 4-11] and domestic animals [12-14]. There are substantial differences among species in their susceptibility to disease manifestations of *Y. pestis* infection. For example, infection is highly lethal in prairie dogs

(*Cynomys* spp.) [7, 9] and black-footed ferrets (*Mustela nigripes*) [8] and moderately virulent in domestic cats (*Felis catus*) [13, 15], whereas canids (*Canis* spp.) are considered refractory to development of clinical disease [15], but can act as a source for disease transmission [3, 6, 16, 17].

In recent years, coyotes (*Canis latrans*) and red foxes (*Vulpes vulpes*) have increased in number in urban environments of North America [18-24]. These rodent hunters likely acquire *Y. pestis* infection through the bite of infected rodent fleas or through ingestion of infected rodents. Although there is no evidence of antemortem transmission from these canids, there are historic reports in which coyote carcasses have been linked to human plague cases [6, 17]. There is a clear potential for non-domestic canids to transfer infected fleas over significant distances [25, 26] as compared to peridomestic rodents. Additionally, the increase in urbanization of wildlife enhances the risk of disease transmission to domestic dogs (*Canis lupus familiaris*) and cats, which, in turn, could result in increased exposure and disease in human populations [2, 3, 27].

There has been an assumption that canids, including coyotes, usually survive infection with *Y. pestis* and produce detectable and specific antibody responses. These species have thus been considered useful sentinels of disease and many animal-based surveillance programs conducted over the past century have been based on serological data obtained from wild coyotes [28-35]. The mechanism of infection, frequency, and the timing of infection in these natural settings are difficult to determine. The present study was undertaken to better describe the clinical response of coyotes following controlled routes of oral and intradermal (ID) infection with *Y. pestis*.

MATERIALS AND METHODS

Bacteria: *Y. pestis* CO92 was received from the Centers for Disease Control and Prevention (CDC) and cultivated from frozen stock, grown in brain heart infusion (BHI) broth at 28˚C for 48 hours, and diluted in phosphate buffered saline to the desired concentrations. The dose

inoculated was determined by back titration of the inoculum plated in serially dilutions on BHI agar plates. Confirmation of pigmentation locus in *Y. pestis* was made by demonstration of pigmented growth on Congo Red agar [36].

Experimental animals: Juvenile captive-reared coyotes (n=12) (USDA-APHIS-Wildlife Services-Predator Field Station, Millville, Utah, USA) were obtained at approximately four months of age and housed in animal biosafety level 3 isolation at the Animal Disease Laboratory (Colorado State University, Fort Collins, Colorado, USA). Before transfer, coyotes were vaccinated with standard canine vaccines (Vanguard®Plus5/CV and Defensor®3; Pfizer Animal Health, New York, New York, USA). Control animals (n=2) were age-matched but maintained at the captive breeding facility in outdoor pens and euthanized at the termination of the study. Experimental animals were housed individually in standard elevated dog runs (1.2 m x 2.1 m; 2.6 m^2), four runs per room, each with a manufactured polyvinyl chloride (PVC) den box (53 cm diameter x 56 cm height) (Figure 2.1). Animals were provided water and commercial dry dog food *ad libitum* supplemented with dead mice, peanut butter, eggs, fruit, rawhide, long bones, or pig ears as daily environmental enrichment. Clinical evaluations were made twice daily on each animal throughout the study period to detect changes in behavior, food consumption, fecal output, and activity level. At approximately five months of age, animals were anesthetized for surgical implantation of temperature recorders (Thermochron iButton®, Maxim Integrated Products, Inc., Sunnyvale, California, USA) into the peritoneal cavity of all coyotes via a caudal left flank incision. All coyotes were treated with topical selamectin (Revolution®, Pfizer Animal Health, Madison, New Jersey, USA) at 6 mg/kg approximately four weeks prior to inoculation. Coyotes used in the inoculation trials were stratified by litter (three litters) and then randomized into two inoculation groups.

Yersinia pestis challenges: At approximately 6 months of age, six animals were inoculated by intradermal (ID) injection (50 µL) bilaterally in the pre-scapular region with a target dose of 10^3 colony forming units (cfu) *Y. pestis* CO92. The other six coyotes were offered two to three *Y. pestis*-infected mice for consumption. These 6-wk-old ICR mice had been inoculated subcutaneously (SC) with 10^2 cfu of *Y. pestis* CO92 (50% lethal dose {LD_{50}} of approximately 20 cfu) in a 0.1 mL volume and were euthanized by cervical dislocation once moribund (approximately 48 hours PI). One animal from each inoculation group was humanely euthanized on day 3 and day 7 postinoculation (PI) to assess gross and microscopic lesions during the course of early infection; all others were euthanized at the termination of the study. At 14 weeks PI, four coyotes from each inoculation group were re-challenged with *Y. pestis* using the same route and dosage as the original challenge.

Figure 2.1: The photograph depicts the experimental housing for animals used in this study. Coyotes were housed individually in standard elevated dog runs (1.2 m x 2.1 m; 2.6 m^2) that each contained a PVC den boxes (53 cm diameter x 56 cm height).

Diagnostic testing: The coyotes were manually or chemically restrained for serial venous blood sample collection in gel serum separation and ethylenediaminetetraacetic acid tubes (Vacutainer®, Becton Dickson, Franklin Lakes, New Jersey, USA) on days -1, 2,3 and 5 days postinoculation. Complete blood cell counts were performed with an automated hematology

analyzer (HemaTrue Hematology Analyzer, Heska, DesMoines, Iowa, USA). Whole blood and selected tissues collected during necropsy were cultured for *Y. pestis* using standard techniques [37]. Briefly, whole blood was plated directly or diluted 1:5, and tissues (organs, lymph nodes, 0.1 gm) were homogenized (Mixer Mill MM 400, Newton, Pennsylvania, USA) in 0.9 mL of BHI broth, followed by plating in serial dilutions to BHI agar. Feces were collected daily from each animal for the first 7 days PI and cultured for detection of *Y. pestis* after 24-hour enrichment in buffered peptone water. All enriched fecal samples were plated to the selective and differential media; cefsulodin-irgasan-novobiocin agar (Becton Dickinson) and incubated at 37°C with 5% CO_2 for at least 72 hours. Isolates were confirmed as *Y. pestis* based upon characteristic colony morphology and Gram stain; and biochemical confirmation was performed using Micro-ID® (Thermo Fisher Scientific, Lenexa, Kansas, USA).

Tissues collected during the postmortem examinations at days three and seven included all major internal organs (liver, lung, spleen, heart, kidney and adrenal glands) and lymphoid tissues, including palatine tonsil, retropharyngeal lymph node (RPLN), submandibular lymph node (SMLN), bronchiolar or tracheal lymph node, and mediastinal, prescapular, axillary, popliteal, and mesenteric lymph nodes. Tissues were fixed in 10% neutral buffered formalin or stored frozen at -80°C until further analysis. Formalin fixed tissues were processed by routine methods and embedded in paraffin. Blocks were sectioned at 5µm and stained with hematoxylin and eosin (HE). One section from each organ and two sections from each lymph node were submitted for histopathological examination [38]. Additional sections of paraffin embedded lung were deparaffinized and rehydrated. Slides were then processed for *Y. pestis*-specific immunohistochemistry (IHC) as previously described [38, 39].

Statistics analysis: Repeated measures analysis of variance was used to determine differences among and between groups by means of Proc Mixed procedures implemented in SAS 9.2. Unless noted P-values of <0.05 were considered statistically significant.

RESULTS

All animals used in this study were antibody negative (anti-F1 titers <1:4) before inoculation with *Y. pestis*. Following inoculation by either route, no mortality occurred and no appreciable signs of morbidity were noted in any animals during daily observations. Specifically, food consumption, fecal scores and activity levels of the coyotes did not change from baseline observations throughout the study.

Intra-abdominal body temperatures as measured by iButtons were recorded for each animal at 1-hour intervals for the animals that were used for pathological evaluation and at 3-hour intervals for the animals maintained for serological evaluation. Broad circadian variations were noted with pre-inoculation temperatures recorded between 36.1 – 39.9°C. High body temperatures were noted during high stress conditions such as manual restraint and husbandry manipulations. All these factors made the assessment of febrile conditions in the coyotes difficult. Daily maximum, mean, median, and minimum body temperatures for the infected groups over days 0-7 PI are depicted in Figure 2.2. For the orally infected group, evaluation of mean daily body temperatures before and after inoculation demonstrated a 0.6°C (range 0.3-0.9 °C) increase on day 1 and 0.3°C (range 0.1-0.6 °C) on day 2 PI (Fig 2.2.A). Although statistically significant (P = <0.0001 and 0.02, respectively), the clinical relevance is arguable. No statistical difference was noted in body temperatures taken from animals in the ID group between 1 and 7 days PI (Figure 2.2.B).

Complete blood cell counts were assessed at days -1, 2, 3 and 5 PI. All hematological values were normal except white blood cell (WBC) counts in the group inoculated orally. Values for WBC counts are delineated in Table 2.1. Increases in WBC counts were attributed to increases in absolute and percent granulocyte counts only. The WBC counts in conditioned, captive wild coyotes were previously reported in a range of 5.3-16.0 x 10^3/µL [40] and the reported normal range for domestic dogs for the instrumentation used was 6.0-17.0 x 10^3/µL.

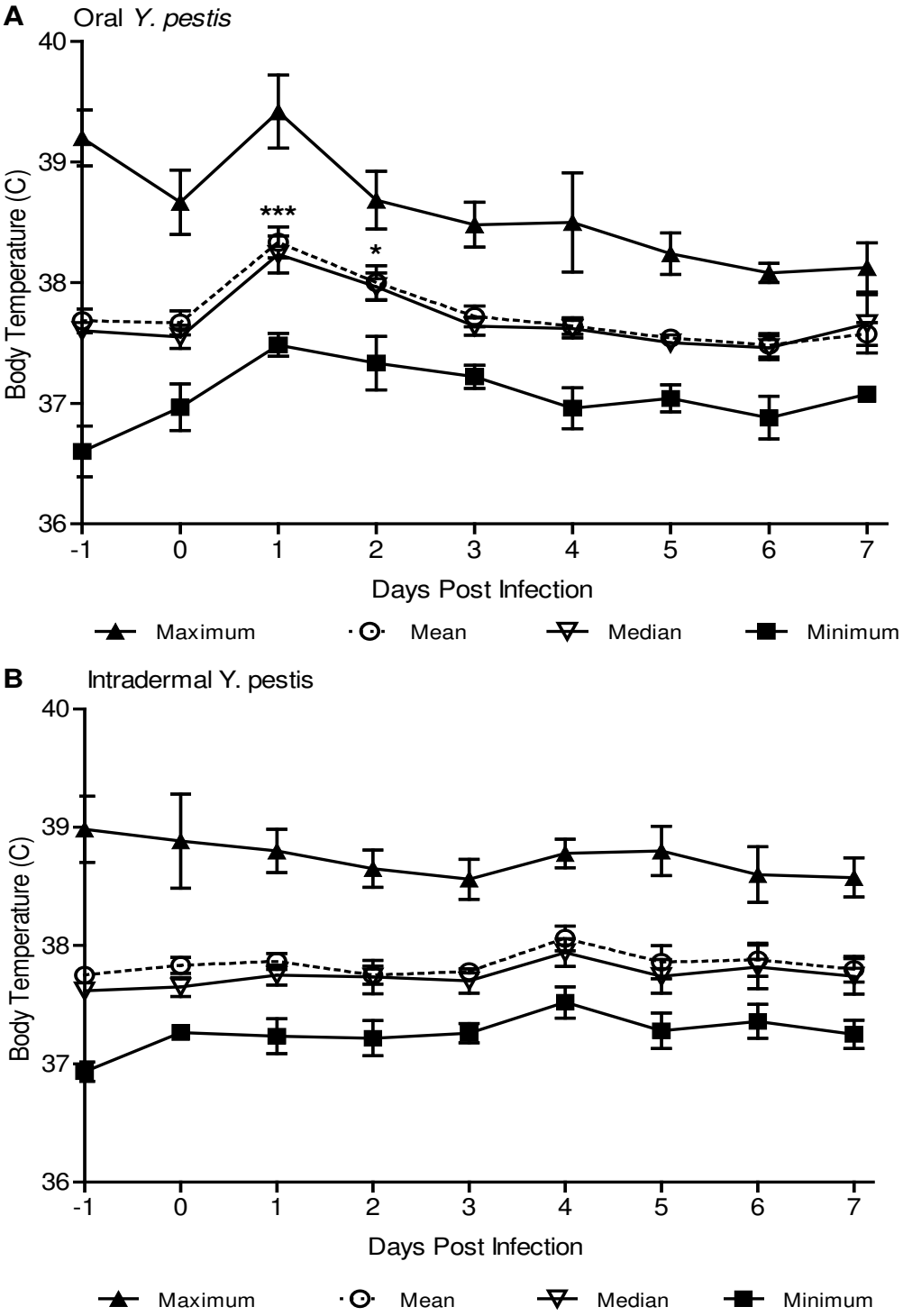


Figure 2.2: Body temperature (˚C) in coyotes (*Canis latrans*) experimentally infected with *Yersinia pestis*. A) Coyotes (n=6) infected by oral inoculation. B) Coyotes (n=6) infected by intradermal inoculation. Data represented as group maximum, mean, median and minimum body temperatures as measured by intra-abdominal thermo recorders; error bars represent standard deviation. *** *p* value = <0.0001, * *p value* = 0.020.

Table 2.1: White blood cell counts (10^3/µL) for coyotes (*Canis latrans*) experimentally infected with *Yersinia pestis*

	Days Postinfection			
Animal ID§	Day -1	Day 2	Day 3	Day 5
Or 1	6.9	15.9	14.0	8.0
Or 2	8.0	17.6	7.3	9.6
Or 3	10.2	22.2	13.7	14.9
Or 4	8.3	23.9	12.2	13.6
Or 5	6.0	16.4	7.0	NT
Or 6	7.2	30.2	15.2	19.9
Mean Oral	7.77	21.03***	11.57	13.20
SD^ Oral	1.45	5.54	3.55	4.69
Id 1	7.8	11.2	9.6	9.6
Id 2	9.2	9.7	11.2	12.8
Id 3	9.6	12.5	8.5	14.4
Id 4	6.9	15.9	14.0	8.0
Id 5	8.6	11.2	10.3	NT
Id 6	5.4	11.2	12.2	11.7
Mean Intradermal	7.92	11.95	10.97	11.30
SD^ Intradermal	1.57	2.13	1.96	2.54

§ Or, oral inoculation group; Id, intradermal inoculation group

^ SD, standard deviation

*** Statistically significant, *p* value = <0.0001

The WBC counts for the group inoculated ID remained within range considered to be normal for captive coyotes and/or domestic dogs during the first five days PI. While considered within the normal range, WBC counts increased above baseline values at 2, 3, and 5 days PI. However, these increases from baseline were not statistically significant in the ID group (*p* value > 0.05).

In the group of animals inoculated orally, WBC counts were within the normal range except for values obtained on day 2 PI. The mean WBC count on day 2 PI was 21.0 × 10^3/µL with a range of 15.9-30.2 × 10^3/µL for the group. The increase in WBC counts on day 2 PI was

considered significantly different from days 0, 3 and 5 PI (*p* value = <.0001). While the WBC count for animal Or1 on day 2 PI was considered within the normal range for captive coyotes, there was a greater than two-fold increase in WBCs from baseline.

Gross pathology of the animals from each group euthanized on days 3 and 7 PI (n=1 per day per group) was unremarkable. Lesions were not observed at ID inoculation sites and superficial lymph nodes were not palpable. There were no lesions noted in internal organs and *Y. pestis* was not isolated from homogenates of spleen, liver, heart, kidney, and adrenal glands. Of all of the lymphoid tissues examined, the palatine tonsils, SMLNs and RPLNs were slightly edematous on day 3 PI in the coyote that was inoculated by ingestion. However, *Y. pestis* was not isolated from any of the lymphoid tissues, including palatine tonsils, RPLNs, SMLNs, bronchiolar or tracheal, and mediastinal, pre-scapular, axillary, popliteal, and mesenteric lymph nodes. *Y. pestis* was not isolated from blood cultures or feces collected between days 0-7 PI.

Histologic abnormalities were noted in the spleen, liver, lung, and cervical lymph nodes in the animals inoculated by ingestion of infected mice. Mild lymphoid depletion was noted in the spleen of the day 3 animal inoculated by ingestion, and mild splenitis was noted in the day 7 animal. Mild hydropic degeneration of the central lobular regions of the liver was noted in the day 3 animal inoculated by ingestion. The lungs had small areas of focal, interstitial thickening in both animals (Figure 2.3.A). However, *Y. pestis* was not detected by IHC in the lung tissues. Mild lymphoid hyperplasia with suppurative tonsillitis (Figure 2.3.B) was noted in the palatine tonsil and mild suppurative lymphadenitis in the SMLN of the day 3 animal inoculated orally. Compression of the subcapsular sinusoids was noted in the RPLN and SMLN of the day 7, orally inoculated animal. The other lymph nodes were described as reactive or quiescently active (tracheal/bronchial, prescapular, axillary, popliteal, and mesenteric) and did not differ in presentation from that of the control animals.

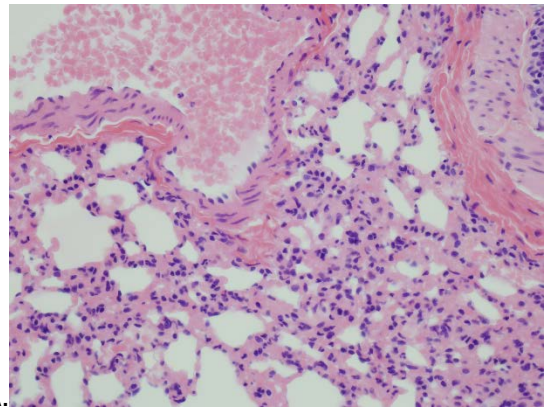
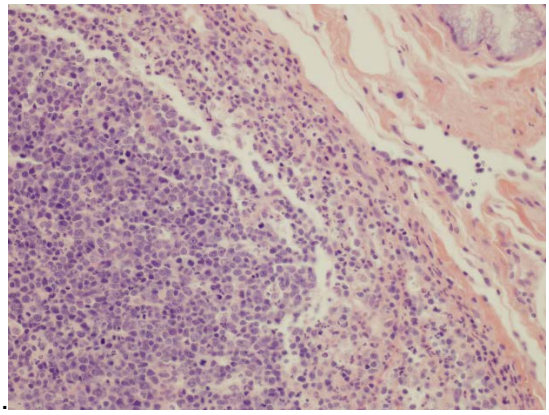
A.  B. 

Figure 2.3: Histological changes in tissues at day 3 postinoculation in animals inoculated by ingestion. A) Lung: focal, alveolar, interstitial thickening, H&E stain, 40x. B) Tonsil: mild lymphoid hyperplasia with suppurative tonsillitis, H&E stain, 40x.

Histological changes were less pronounced in the animals inoculated ID. A mild splenitis was noted in the animal euthanized on day 3 PI. Focal, alveolar, interstitial thickening was present in lung tissue from the animals euthanized on both day 3 and day 7 PI, but were negative for *Y. pestis* by IHC. The lymphoid tissue in the ID-inoculated animals were similar to those of controls, except for the SMLN of the day 3 animal, which had focal areas of compression of the subcapsular sinusoids, but without significant accumulation of neutrophils.

DISCUSSION

The results of the experimental infection described here are similar to those of historical reports of studies performed in coyotes. One study used captive bred coyotes (four adults and five juveniles) inoculated with varying doses of *Y. pestis* via oral or subcutaneous (SC) routes [41]. No mortality was observed in the nine animals inoculated, and no apparent clinical signs were described. However, *Y. pestis* was isolated from blood collected on day 5 PI from an adult exposed SC. Another study described a lack of morbidity and no change in body temperature in an undisclosed number of coyotes exposed by ingestion [42]. Cultures of blood and throat swabs were found to be negative for *Y. pestis* in all of the animals in this group. However, in a postmortem evaluation of a coyote carcass that was linked to a human clinical case, *Y. pestis*

was detected in bone marrow and spleen by florescent antibody staining. This finding implies that during natural exposure, systemic spread can occur [6].

Numerous historical descriptions of natural and experimental infection in domestic dogs have been published [15, 43-45]. The first experimental inoculations were described in the early 1700s. Routes of infection and bacterial sources varied considerably in these historic reports and usually involved inoculation with infected tissues from humans. Oral and SC exposures were the most commonly described. In general, mortality associated with infection in domestic dogs was considered to be low and associated with high dose or aerosol exposures. Clinical disease was generally mild with descriptions of fever, anorexia, and bubo formation. Gross lesions at inoculation sites, palatine tonsil, cervical lymphoid tissues, spleen, liver, and lung have been described.

More contemporary experimental infection trials in domestic dogs resulted in 100% morbidity with clinical descriptions of lethargy accompanied by transient increases in body temperature for approximately 72 hours [15]. No mortalities were noted in the experimentally infected dogs. While poorly described, these results are similar to clinical cases of plague described in domestic dogs with natural exposure to *Y. pestis* [12, 14]. These reports delineate a total of 65 cases of plague diagnosed in domestic dogs. The majority of these dogs were labeled as rabbit or rodent hunters living in New Mexico, USA. Common clinical signs in these three dogs included lethargy, transient fever, anorexia, and lymphadenopathy. Abnormal changes in WBCs were noted in 85% of these dogs. Two mortalities were noted in these clinical cases with one animal reported to have concurrent disease; however, most of the dogs were treated with antibiotics.

The clinical manifestation of infection described in these three groups of domestic dogs was markedly different from that noted in the coyotes used in the present study. The WBC counts

were found to be elevated above normal values in 29 of the domestic dog clinical cases, and degenerate neutrophils were noted in a second case. The elevation in body temperature in these domestic dogs was clinically significant compared to the minimal spike (0.5 – 1.0 °C) in temperature in the orally inoculated group of coyotes. Lymphadenopathy was noted in most of the domestic dogs but were not detected in the coyotes. In addition, *Y. pestis* was isolated from multiple tissues in the naturally and experimentally infected domestic dogs but not isolated from any tissues cultured from the coyotes at days 3 and 7 PI.

While the histological changes we observed in the coyote lymph nodes, spleen, liver, and lung suggest that mild systemic inflammation was present, *Y. pestis* was not recovered by culture or visualized in regional lymph nodes or organs at day 3 or day 7 PI. The minimal change in histology suggests that the ID inoculation used in this study resulted in a localized pro-inflammatory response and it is suspected that *Y. pestis* experienced rapid local clearance by phagocytes. It is hypothesized that isolation of organism might have been possible if earlier time points (0-72 hours) would have been evaluated as rodent models show that *Y. pestis* is cleared to regional lymph nodes within 6-24 hours [46].

In the present study, the ID route was chosen to mimic natural exposure via flea bite. The low dose of the organism was designed to approximate a dose delivered by a single flea bite. Previous experimental inoculation studies used the SC route rather than an ID route, and little is known about the difference in pathogenesis or antigen presentation between exposure routes. In a recent rodent model, progression of disease was found to be different between ID and SC routes of exposure [47]. The dose administered was similar to that reported in the literature for flea transmission [48, 49]; however, the inoculum did not contain components of flea saliva which may have immunomodulatory effects [50-53]. Recent microarray analyses compared the transcriptome of *Y. pestis* recovered from infected fleas with that of the organism grown in culture or recovered from infected rat lymph nodes. Differences in gene upregulation suggest

that bacterial growth within the flea midgut results in characteristics that are different than *in vitro* cultures and *in vivo* propagation [53, 54]. This is further characterized by the observation that a decrease in phagocytosis of *Y. pestis* by murine macrophages [53], murine neutrophils [52], and human neutrophils [49] was noted after passage through a flea. These findings suggest that the flea midgut environment may prepare *Y. pestis* for its exposure to, and evasion of, the mammalian innate immune response.

The small sample size per group used in this study is limiting in the overall assessment of *Y. pestis* pathogenesis in coyotes. The study included mostly young male coyotes, therefore, limiting the evaluation of age and sex differences in pathogenicity that have been noted in ground squirrels (*Spermophilis beecheyi*) [55]. The coyotes were also captive-reared and likely in better overall body condition than wild coyotes, which may have altered their immunological status and susceptibility to infection. Based upon the results in the study, further characterization of the potential effects of dose and flea-induced changes to *Y. pestis* on pathogenicity may be warranted.

LITERATURE CITED

1. Von reyn, c.f., et al., *bubonic plague from exposure to a rabbit: a documented case, and a review of rabbit-associated plague cases in the united states.* Am j epidemiol, 1976. 104(1): p. 81-7.

2. Gage, k.l., et al., *cases of cat-associated human plague in the western us, 1977-1998.* Clin infect dis, 2000. 30(6): p. 893-900.

3. Gould, l.h., et al., *dog-associated risk factors for human plague.* Zoonoses public health, 2008. 55(8-10): p. 448-54.

4. Jessup, d.a., et al., *ocular lesions of plague (yersinia-pestis) in a black-tailed deer (odocoileus-hemionus-columbianus).* Journal of zoo and wildlife medicine, 1989. 20(3): p. 360-363.

5. Wild, m.a., t.m. shenk, and t.r. spraker, *plague as a mortality factor in canada lynx (lynx canadensis) reintroduced to colorado.* J wildl dis, 2006. 42(3): p. 646-50.

6. Von reyn, c.f., et al., *bubonic plague from direct exposure to a naturally infected wild coyote.* Am j trop med hyg, 1976. 25(4): p. 626-9.

7. Ubico, s.r., et al., *a plague epizootic in the white-tailed prairie dogs (cynomys leucurus) of meeteetse, wyoming.* J wildl dis, 1988. 24(3): p. 399-406.

8. Williams, e.s., et al., *plague in a black-footed ferret (mustela nigripes).* Journal of wildlife diseases, 1994. 30(4): p. 581-585.

9. Pauli, j.n., et al., *a plague epizootic in the black-tailed prairie dog (cynomys ludovicianus).* J wildl dis, 2006. 42(1): p. 74-80.

10. Thorne, e.t., et al., *plague in a free-ranging mule deer from wyoming.* Journal of wildlife diseases, 1987. 23(1): p. 155-159.

11. Wong, d., et al., *primary pneumonic plague contracted from a mountain lion carcass.* Clin infect dis, 2009. 49(3): p. E33-8.

12. Orloski, k.a. and m. Eidson, *yersinia pestis infection in three dogs.* J am vet med assoc, 1995. 207(3): p. 316-8.

13. Eidson, m., j.p. thilsted, and o.j. rollag, *clinical, clinicopathologic, and pathologic features of plague in cats: 119 cases (1977-1988).* J am vet med assoc, 1991. 199(9): p. 1191-7.

14. Nichols, m.c., et al., *yersinia pestis infection in dogs: 62 cases (2003-2011).* Javma-journal of the american veterinary medical association, 2014. 244(10): p. 1176-1180.

15. Rust, j.h., et al., *the role of domestic animals in the epidemiology of plague. I. Experimental infection of dogs and cats.* J infect dis, 1971. 124(5): p. 522-6.

16. Wang, h., et al., *a dog-associated primary pneumonic plague in qinghai province, china.* Clin infect dis, 2011. 52(2): p. 185-90.

17. Poland, j.d., a.m. barnes, and j.j. herman, *human bubonic plague from exposure to a naturally infected wild carnivore.* Am j epidemiol, 1973. 97(5): p. 332-7.

18. Grinder, m.i. and p.r. krausman, *home range, habitat use, and nocturnal activity of coyotes in an urban environment.* Journal of wildlife management, 2001. 65(4): p. 887-898.

19. Quinn, t., *using public sighting information to investigate coyote use of urban habitat.* Journal of wildlife management, 1995. 59(2): p. 238-245.

20. Atwood, t.c., h.p. weeks, and t.m. gehring, *spatial ecology of coyotes along a suburban-to-rural gradient.* Journal of wildlife management, 2004. 68(4): p. 1000-1009.

21. Riley, s.p.d., et al., *effects of urbanization and habitat fragmentation on bobcats and coyotes in southern california.* Conservation biology, 2003. 17(2): p. 566-576.

22. Gehrt, s.d., c. Anchor, and l.a. white, *home range and landscape use of coyotes in a metropolitan landscape: conflict or coexistence?* Journal of mammalogy, 2009. 90(5): p. 1045-1057.

23. Adkins, c.a. and p. Stott, *home ranges, movements and habitat associations of red foxes vulpes vulpes in suburban toronto, ontario, canada.* Journal of zoology, 1998. 244: p. 335-346.

24. Rosatte, r.c., *long distance movement by a coyote, canis latrans, and red fox, vulpes vulpes, in ontario: implications for disease-spread.* Canadian field-naturalist, 2002. 116(1): p. 129-131.

25. Mcgee, b.k., et al., *possible vector dissemination by swift foxes following a plague epizootic in black-tailed prairie dogs in northwestern texas.* J wildl dis, 2006. 42(2): p. 415-20.

26. Boone, a., j.p. kraft, and p. Stapp, *scavenging by mammalian carnivores on prairie dog colonies: implications for the spread of plague.* Vector borne zoonotic dis, 2009. 9(2): p. 185-90.

27. Runfola, j.k., et al., *outbreak of human pneumonic plague with dog-to-human and possible human-to-human transmission--colorado, june-july 2014.* Mmwr morb mortal wkly rep, 2015. 64(16): p. 429-34.

28. Willeberg, p.w., et al., *epidemiologic survey of sylvatic plague by serotesting coyote sentinels with enzyme immunoassay.* Am j epidemiol, 1979. 110(3): p. 328-34.

29. Thomas, c.u. and p.e. hughes, *plague surveillance by serological testing of coyotes (canis latrans) in los angeles county, california.* J wildl dis, 1992. 28(4): p. 610-3.

30. Gese, e.m., et al., *serological survey for diseases in free-ranging coyotes (canis latrans) in yellowstone national park, wyoming.* J wildl dis, 1997. 33(1): p. 47-56.

31. Arjo, w.m., et al., *serologic survey for diseases in free-ranging coyotes (canis latrans) from two ecologically distinct areas of utah.* J wildl dis, 2003. 39(2): p. 449-55.

32. Gese, e.m., et al., *serologic survey for canine infectious diseases among sympatric swift foxes (vulpes velox) and coyotes (canis latrans) in southeastern colorado.* J wildl dis, 2004. 40(4): p. 741-8.

33. Salkeld, d.j. and p. Stapp, *seroprevalence rates and transmission of plague (yersinia pestis) in mammalian carnivores.* Vector borne zoonotic dis, 2006. 6(3): p. 231-9.

34. Brinkerhoff, r.j., et al., *are carnivores universally good sentinels of plague?* Vector-borne and zoonotic diseases, 2009. 9(5): p. 491-497.

35. Bevins, s.n., et al., *yersinia pestis: examining wildlife plague surveillance in china and the usa.* Integrative zoology, 2012. 7(1): p. 99-109.

36. Surgalla, m.j. and e.d. beesley, *congo red-agar plating medium for detecting pigmentation in pasteurella pestis.* Appl microbiol, 1969. 18(5): p. 834-7.

37. Chu, m.c., *laboratory manual of plague diagnostic tests*. 2000, atlanta, ga: centers for disease control and prevention.

38. Guarner, j. And s.r. zaki, *histopathology and immunohistochemistry in the diagnosis of bioterrorism agents.* J histochem cytochem, 2006. 54(1): p. 3-11.

39. Guarner, j., et al., *immunohistochemical detection of yersinia pestis in formalin-fixed, paraffin-embedded tissue.* Am j clin pathol, 2002. 117(2): p. 205-9.

40. Gates, n.l. and e.k. goering, *hematologic values of conditioned, captive wild coytoes.* Jounral of wildlife disease, 1976. 12(3): p. 402-404.

41. Marchette, n.j., et al., *studies on infectious diseases in wild animals in utah. Ii. Susceptibility of wild mammals to experimental plague.* Zoonoses res, 1962. 1: p. 225-49.

42. Barnes, a.m., *surveillance and control of bubonic plague in the united states*. Symposium zoological society of london. Vol. 50. 1982, london, england. 237-270.

43. De souza, a., j. Arruda, and m. Pinto, *report on experiments undertaken to discover whether the common domesticated animals of terceira island are affected by plague.* J hyg (lond), 1910. 10(2): p. 196-208.

44. Mullett, c.f., *the bubonic plague in england; a problem in public health.* Bull hist med, 1946. 20(2): p. 299-309.

45. Strong, r.p. and o. Teague, *studies on pneumonic plague and plague immunization.* Phillipine journal of science, 1912. 7(3): p. 223-28.

46. Sebbane, f., et al., *kinetics of disease progression and host response in a rat model of bubonic plague.* American journal of pathology, 2005. 166(5): p. 1427-1439.

47. Gonzalez, r.j., et al., *comparison of models for bubonic plague reveals unique pathogen adaptations to the dermis.* Infection and immunity, 2015. 83(7): p. 2855-2861.

48. Lorange, e.a., et al., *poor vector competence of fleas and the evolution of hypervirulence in yersinia pestis.* J infect dis, 2005. 191(11): p. 1907-12.

49. Jarrett, c.o., et al., *transmission of yersinia pestis from an infectious biofilm in the flea vector.* J infect dis, 2004. 190(4): p. 783-92.

50. Andersen, j.f., et al., *an insight into the sialome of the oriental rat flea, xenopsylla cheopis (rots).* Bmc genomics, 2007. 8.

51. Schoeler, g.b. and s.k. wikel, *modulation of host immunity by haematophagous arthropods.* Annals of tropical medicine and parasitology, 2001. 95(8): p. 755-771.

52. Spinner, j.l. and b.j. hinnebusch, *the life stage of yersinia pestis in the flea vector confers increased resistance to phagocytosis and killing by murine polymorphonuclear leukocytes.* Advances in yersinia research, 2012. 954: p. 159-163.

53. Vadyvaloo, v., et al., *transit through the flea vector induces a pretransmission innate immunity resistance phenotype in yersinia pestis.* Plos pathog, 2010. 6(2): p. E1000783.

54. Vadyvaloo, v., et al., *analysis of yersinia pestis gene expression in the flea vector.* Adv exp med biol, 2007. 603: p. 192-200.

55. Williams, j.e., m.a. moussa, and d.c. cavanaugh, *experimental plague in the California ground-squirrel.* Journal of Infectious Diseases, 1979. 140(4): p. 618-621.

CHAPTER 3 - IMMUNOLOGICAL RESPONSE OF COYOTES (*CANIS LATRANS*) TO EXPERIMENTAL *INOCULATION WITH YERSINIA PESTIS*

SUMMARY

Coyotes (*Canis latrans*) have historically been used in animal-based surveillance efforts for the detection of *Yersinia pestis*. Coyotes are likely exposed via flea bite and/or oral routes and appear to be resistant to the development of disease. Sampling of canids has been useful for the evaluation of the geographic distribution of *Yersinia pestis* in the landscape. As the canid immunological response to *Yersinia pestis* has not been thoroughly characterized, this study reports the experimental inoculation of captive-reared juvenile coyotes (n=8) with *Yersinia pestis* CO92 via oral and intradermal routes. The innate immune response was characterized by changes in acute phase proteins during the seven day period following exposure with little to no response noted in the intradermal exposed group. A brief peak in acute phase proteins between day two and five post inoculation was detected in the oral exposure group with values returning to normal levels by day 7. The humoral response to *Y. pestis* fraction 1 capsular protein (anti-F1) was measured with a significant difference noted between inoculation groups in magnitude and duration of antibody production. The anti-F1 titers as measured by passive hemagglutination assay in animals exposed by intradermal route peaked at day 10 post-inoculation (range 1:32 to 1:128) with titers remaining stable at 1:32 through week 12. In contrast, orally inoculated animals developed higher titers (range 1:256 to 1:1024) that remained stable at 1:256 to 1:512 through day 42. Re-challenge at day 98 post inoculation via similar dosage and routes resulted in marked differences in antibody response between groups. Animals in the orally-inoculated group produced a striking increase in anti-F1 titers (up to 1:4096) within three days (day 101), while there was minimal to no increase in antibody response noted in the intradermal group. Using western blot, all serum samples were screened

to profile additional antibodies against *Y. pestis* antigens. Antibodies against Yersinia outer proteins D and H (YopD, YopH), plasminogen activator (Pla) and low calcium response V (LcrV) were detected but no specific patterns were noted between inoculation routes. Information gathered from this experimental trial may provide additional insight into the evaluation of coyote plague serosurveillance data as well as pathogen-host responses that might contribute to apparent resistance in canids.

INTRODUCTION

Plague is a zoonotic disease caused by the bacterium *Yersinia pestis*. It has been determined that plague has a complex disease ecology involving transmission by a diverse number of flea species to a wide array of rodent species of varying susceptibilities. The pathogen has the capability of spilling over into a diverse range of mammalian species including humans [1-3]. In the United States, disease occurrence in humans is closely related to exposures to peridomestic rodents and domestic pets [4-8]. Increased anthropic changes due to urbanization are linked to increased risk factors for exposures in endemic areas of the western states [9, 10].

It has been suggested that climatic changes may contribute to geographic shifts in distribution of the *Y. pestis* and/or its flea and rodent reservoirs [10-12]. Therefore, diligence is still required in maintaining surveillance programs for this highly pathogenic organism. Traditional surveillance programs developed in the early 1900s after the emergence of plague in the United States involved the capture of rodents for serological screening and flea burden assessments. This was labor intensive and costly and it was found that rodent reservoirs varied by geographic regions within the United States making identification of specific foci difficult [3, 6].

In the 1900s, it was reported that canids often survive infection and produce detectable, *Y. pestis* specific antibody responses [6, 13, 14]. They were thus, considered useful sentinels and

canid sample collections were incorporated into animal-based surveillance programs [15-21]. The use of domestic dogs (*Canis lupus familiaris*) to monitor for plague on Native American reservations in New Mexico, Arizona and California was found to be useful if combined with enhanced rodent surveillance in hot spot regions [22, 23]. The use of wild canids in surveillance programs was implemented in the 1980s. In comparison of coyotes (*Canis latrans*), foxes (*Vulpes spp*) and wolves (*Canis spp*), it was determined that coyotes offered the most valuable data [15], due to their diet choice, large geographic range and common interactions with reservoir species and their fleas. Despite the 30+ years of canid surveillance data, their refractory response to infection, mechanism of resistance, as well as the frequency, timing and the duration of an antibody response in these natural settings are poorly understood. The study reported here seeks to better describe the immunologic responses of coyotes following controlled routes of oral and intradermal (ID) infection with *Y. pestis*. This data provides a baseline characterization of both the innate and adaptive immune response with which to better understand surveillance data and mechanisms of resistance in this species.

MATERIALS AND METHODS

Experimental animals: Juvenile captive-reared coyotes (n=8) (USDA-APHIS-Wildlife Services, Predator Field Station, Millville, Utah, USA) were obtained at approximately four months of age and housed in animal biosafety level 3 isolation at the Animal Disease Laboratory (Colorado State University, Fort Collins, Colorado, USA). Before transfer, coyotes were vaccinated with standard canine vaccines (Vanguard®Plus5/CV and Defensor®3; Pfizer Animal Health, New York, New York, USA). Animals were housed individually in standard elevated dog runs (1.2 m x 2.1 m; 2.6 m^2), four runs per room, each with a manufactured polyvinyl chloride (PVC) den box (53 cm diameter x 56 cm height). Animals were provided water and commercial dry dog food *ad libitum* supplemented with dead mice, peanut butter, eggs, fruit, rawhide, long bones, or pig ears as daily environmental enrichment. Clinical evaluations

were made twice daily on each animal throughout the study period to detect changes in behavior, food consumptions, fecal output, and activity level. All coyotes were treated with topical selamectin (Revolution®, Pfizer Animal Health, Madison, New Jersey, USA) at 6 mg/kg approximately four weeks prior to inoculation. Coyotes used in the inoculation trials were stratified by litter (three litters) and then randomized into two inoculation groups. One animal from each inoculation group was humanely euthanized at day 3 and day 7 post-inoculation. Control animals (n=2) were age-matched but maintained at the captive breeding facility in outdoor pens and euthanized at the termination of the study.

Bacteria: *Yersinia pestis* CO92 was obtained from the Centers for Disease Control and Prevention (CDC). Bacteria were cultivated from frozen stock, grown in brain heart infusion (BHI) broth at 28˚C for 48 hours, and diluted in phosphate buffered saline to the desired concentrations. The dose inoculated was determined by back titration of the inoculum by plating serial dilution on BHI agar plates. Confirmation of pigmentation locus in *Y. pestis* was made by demonstration of pigmented growth on Congo red agar [24].

Yersinia pestis challenges: At approximately 6 months of age, four animals were inoculated by intradermal (ID) injection (50 µL) bilaterally in the pre-scapular region with a target dose of 10^3 colony forming units (cfu) of *Y. pestis* CO92. The other four coyotes were offered one to three *Y. pestis*-infected mice for consumption. These 6-wk-old imprinting control region (ICR) mice were inoculated subcutaneously (SC) with 10^2 cfu of *Y. pestis* CO92 (50% lethal dose {LD_{50}} of approximately 20 cfu) in a 0.1 mL volume and mice were euthanized by cervical dislocation once moribund (approximately 48 hours PI). At 14 weeks PI, four coyotes from each inoculation group were re-challenged with *Y. pestis* CO92 using the same route and dosage as the original challenge. The coyotes were manually or chemically restrained for serial venous blood sample collection in gel serum separation tubes (Vacutainer®, Becton Dickson, Franklin

Lakes, New Jersey, USA) on days 0, 5, 7, 10, 14, 21, 28, 42, 56, 98, 101,105, 108, 112, 119 and 126 PI.

Serology: Whole blood was harvested at the time points indicated above. Serum was separated, and heat inactivated at 56°C for 30 minutes and stored at -80°C prior to analysis. Titers of antibody reactivity to *Y. pestis* fraction 1 capsular protein (anti-F1) were determined by passive hemagglutination (PHA) based upon World Health Organization procedures as previously described [25]. Briefly, serum or FP eluates were absorbed for 30 minutes at ambient temperature with sheep red blood cells (RBC). After incubation, samples were centrifuged at 23,000 x g for 5 minutes. The supernatants were transferred to a fresh tube for analysis. Using a starting dilution of 1:16, supernatants were added to hemagglutination diluent containing F1-antigen sensitized sheep RBC. Agglutination reactions were evaluated to determine serological titer. C-reactive protein (CRP) and serum amyloid A (SAA) values were determined by enzyme-linked immunoassay (ELISA) (PHASE™ Canine CRP assay and PHASE™ Serum Amyloid A multispecies assay, Tridelta Development Limited, Maynooth, Kildare, Ireland) according to manufacturer specifications.

Western Blot: The anti-plague antibody repertoire was screened using western blot techniques as previously described [26]. Plasmid encoded loci *lcrV*, *ypkA*, *yopD*, *pla* and pesticin (*pst*) were amplified by polymerase chain reaction (PCR) from *Y. pestis* CO92 plasmid DNA using gene-specific primers and Pfu Ultra Master Mix (Stratagene, La Jolla, California, USA). The PCR products were inserted into the pETA46 Ek/LIC system (EMD Biosciences, La Jolla California, USA), pET21 (*yopD),* or pET19 (*pla*). The ligated DNA was transformed into NovaBlue competent cells for plasmid amplification and clone selection. Recombinant DNA was purified using Small Plasmid Prep kit (Qiagen, Valencia, California, USA). Recombinant plasmids were then transformed into Rosetta 2 (DE3) competent cells (EMD Biosciences), and selected clones were induced to express recombinant protein with addition of 1 mM isopropyl β-

D-1-thiogalactopyranoside (IPTG). Expressed histidine-tagged proteins were confirmed by evaluation on SDS-Page and Western blot, using anti-histidine monoclonal antibody (EMD Biosciences). Recombinant strains correctly expressing the desired proteins were used for scale up production of proteins. Cultures were collected 6 hr postinduction with IPTG and centrifuged, and supernatant was discarded. The cell pellets were treated with Bugbuster HT (EMD Biosciences), and lysates were passed across HIS Gravi Trap columns (GE Healthcare, Piscataway, New Jersey, USA). to capture the histidine-tagged recombinant products. Nonrecombinant, cell-free F1 capsular antigen was isolated from overnight cultures of *Y. pestis* CO92 *p*Lcr$^-$ as previously described [27]

Serum samples were tested individually from coyotes in each inoculation group at all time points. Samples were probed against F1 and *Y. pestis* (recombinant) virulence proteins LcrV, Pla, Pst, YopD, YopH, and YpkA (Table 3.1) at 1:10 dilutions. After SDS-PAGE, the antigen proteins were transferred onto polyvinylidene fluoride (PVDF) transfer membranes (Pall Life Sciences, Ann Arbor, Michigan, USA) using a semidry electroblot apparatus (Owl Instruments, Rochester, New York, USA). Immuno-labelling of blotted, electrophoresed proteins were performed as previously described [28]. Briefly, membranes were washed in phosphate buffered saline (PBS) then incubated with a 1:5000 dilution of alkaline phosphatase-conjugated Protein G (Rockland, Gilbertsville, PA) in skim milk for 1 hour. Following secondary labeling, the membrane was washed with PBS and incubated for 5 min with stabilized substrate (Promega). After color development, the reaction was stopped by rinsing the membranes with deionized water for 5 min.

Table 3.1. *Yersinia pestis* immunogenic antigens evaluated by Western blot

Protein	Descriptor	Function	Plasmid
F1	Capsular antigen fraction 1	Gel-like capsule	pFra
LcrV	Low calcium response V antigen	*T3SS translocator	pCD1
Pla	Plasminogen activator	Protease, adhesin	pPla
Pst	Pesticin	Colicin-like protein	pPla
YopD	*Yersinia* outer protein D	T3SS translocator	pCD1
YopH	*Yersinia* outer protein H	T3SS effector protein	pCD1
YpkA	*Yersinia* protein kinase	T3SS effector protein	pCD1

* T3SS = type III secretory system

RESULTS

The acute phase proteins (APP), C-reactive protein (CRP) and serum amyloid A (SAA), were measured by ELISA at 0, 2, 3, 5 and 10 days PI using a 1:500 dilution of serum. Elevations in CRP and SAA values were noted in the group exposed by ingestion (Table 3.2). The highest CRP levels were above the assay detection limit (> 120 µg/ml) on days 2 and 3 PI, with a marked decline noted on day 5 and a return to within the normal reference range for domestic dogs (0-7 µg/mL) [29, 30] by day 10 PI. The SAA levels were greater than 160 µg/mL on days 2 and 3 PI but were below detectable limits (< 5µg/mL) on days 0, 5 and 10 PI. Slight increases in CRP above the normal reference range were detected in three of the six animals in the intradermal exposure group on days 2 or 3 PI. However, elevations in SAA were not detected in this group at those same time points (Table 3.3). One animal in the intradermal exposure group (Id4) did show a detectable elevation of CRP and SAA (80 and 145 µg/mL, respectively) at day 5 PI and the APP responses are likely due to trauma rather than a response to infection due to its fractious nature in captivity. No acute phase proteins were detected in the two control animals.

Table 3.2. C-reactive protein (µg/mL) production after experimental inoculation with *Y. pestis.*

DPI	Or1	Or2	Or3	Or4	Or5	Or6	Id1	Id2	Id3	Id4	Id5	Id6	C1	C2
0	nd	nd	nd	nd	nd	53	5	nd	nd	nd	8	nd	nd	nd
2	34	>120	>120	>120	>120	>120	nd	10	6	24	25	nd		
3	>120	>120	>120	>120	>120	>120	nd	18	6	10	18	nd		
5	26	30	60	45	>120	92	nd	13	7	80		nd		
10	7	10	nd	8										

Data are listed for individual animals as µg/mL as measured by PHASE™ Canine CRP assay, Tridelta Development Limited, Maynooth, Kildare, Ireland. DPI = days postinoculation; Or = oral, Id = intradermal; C = uninoculated control; nd = below detection limit (< 4 µg/mL); >120 = above detection limit (≥ 120 µg/mL).

Table 3.3. Serum amyloid A (µg/mL) production after experimental inoculation with *Y. pestis.*

DPI	Or1	Or2	Or3	Or4	Or5	Or6	Id1	Id2	Id3	Id4	Id5	Id6	C1	C2
0	nd	nd	nd	nd	nd	nd >16	nd	nd	nd	nd	nd	nd	nd	nd
2	nd >16	nd >16	>160	>160	>160	0 >16	nd	nd	nd	nd	nd	nd		
3	0	0	>160	>160	>160	0	nd	nd	nd	nd	nd	nd		
5	6	nd	nd	nd	nd	5	nd	nd	nd	145		nd		
10	nd	nd	nd	nd			nd	nd	nd	nd				

Data are listed for individual animals as µg/mL as measured by PHASE™ Serum Amyloid A multispecies assay, Tridelta Development Limited, Maynooth, Kildare, Ireland. DPI = days postinoculation; Or = oral, Id = intradermal; C = uninoculated control; nd = below detection limit (< 5 µg/mL); >160 = above detection limit (≥ 160 µg/mL).

Antibody titers as measured by passive hemagglutination assay (PHA) against capsular antigen fraction 1 (F1) are listed in Table 3.4. All animals used in this study were found to be seronegative (anti-F1 titers <1:4) prior to inoculation with *Y. pestis*. There were major differences in the magnitude of antibody responses between inoculation groups. Antibody was detected in the two animals euthanized at day 7 (Or6, Id6). Anti-F1 titers for animals in the ID exposure group had already reached their maximum levels by day 10 PI, ranging from 1:32 to 1:128 (Fig. 3.1). These antibody titers remained stable with a group median titer of 1:32 through day 98 PI. In contrast, the orally inoculated animals had higher anti-F1 titers (range 1:256 to 1:1,024) that remained stable at 1:256 or 1:512 through day 42. Subsequently there was a two-fold decline in titers between days 42 and 98, (Fig. 3.2).

Table 3.4. Antibody production against *Y. pestis* Fraction 1 capsular antigen after experimental inoculation of coyotes.

DPI	Or1	Or2	Or3	Or4	Id1	Id2	Id3	Id4
0*	0	0	0	0	0	0	0	0
10	256	256	1024	256	32	32	64	128
14	256	512	512	512	32	32	64	128
21	256	512	256	512	32	32	32	64
28	256	512	256	512	32	32	32	64
42	256	512	256	512	32	32	32	64
56	128	512	128	512	32	32	32	32
84	64	128	64	256	16	32	16	32
98	64	128	64	256	16	32	16	32

Data are listed for individual animals as reciprocal titer measured by PHA. DPI = days post inoculation; Or = oral, Id = intradermal; * seronegative equals titer of <1:4.

To assess the anamnestic response associated with re-exposures that may be occurring in the field, coyotes were re-challenged on day 98 by the same dose and route. Marked differences in antibody production were again noted between groups. Animals in the group exposed by ingestion produced a striking increase in anti-F1 titer of up to 1:4096 within 7 days after re-challenge (day 105), and those antibody titers remained elevated through day 126 (Fig. 3.2). In contrast, there was minimal to no increase in the antibody response noted over four weeks in the animals re-inoculated by the ID route (Fig. 3.1). To further investigate the lack of an anamnestic response, the animals in the ID group were re-challenged a second time (four weeks after initial re-challenge, day 126) and again, there was no change in anti-F1 titers over two additional weeks (data not shown). To characterize the anamnestic response further, the coyotes in the intradermal inoculation group were then divided into two groups and re-inoculated a third time (day 149). One group (n=2) received *Y. pestis* by oral route as previously described and one group (n=2) received 3.6×10^3 cfu by intradermal inoculation. At day 7 post challenge (day 156), antibody titers were dramatically increased in the individuals that received the oral dosing (1:8192) and moderately increased by the intradermal dosing (1:512 to 1:1024).

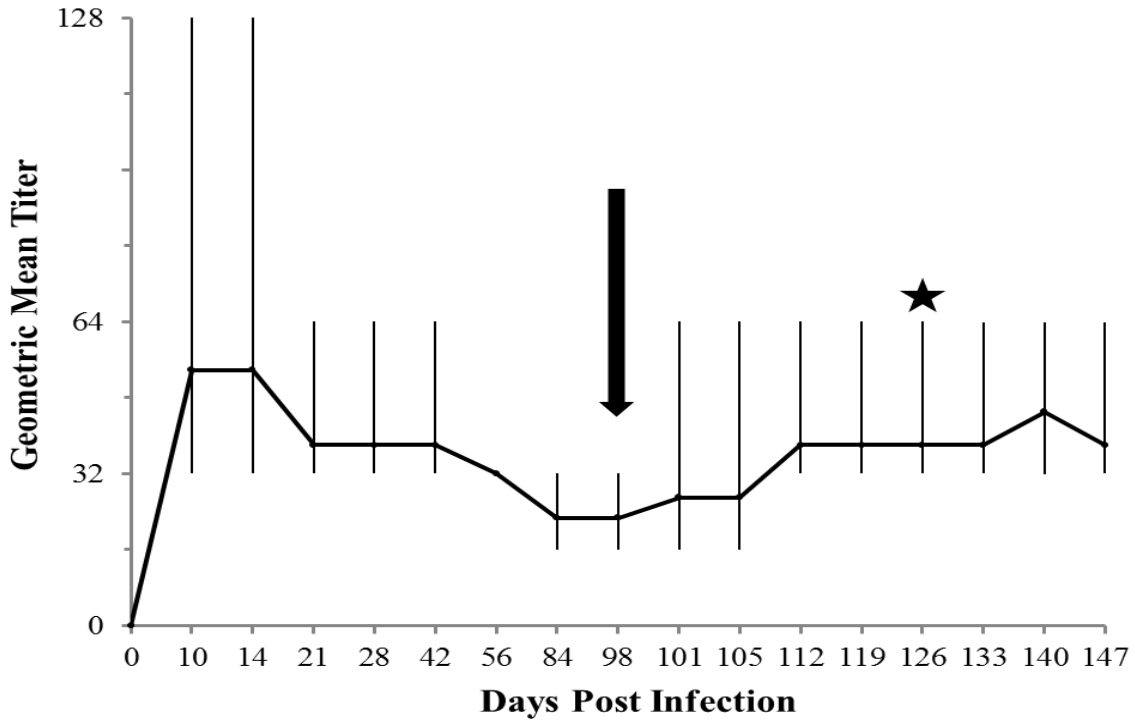


Figure 3.1. Geometric mean titers as measured by hemagglutination assay against *Yersinia pestis* F1 capsular antigen for the intradermal exposure group. Error bars represent the range of antibody titers for the coyotes (n=4) in this group. The arrow designates the re-challenge at 98 days post-infection. The star designates the second re-challenge at 126 days post-infection.

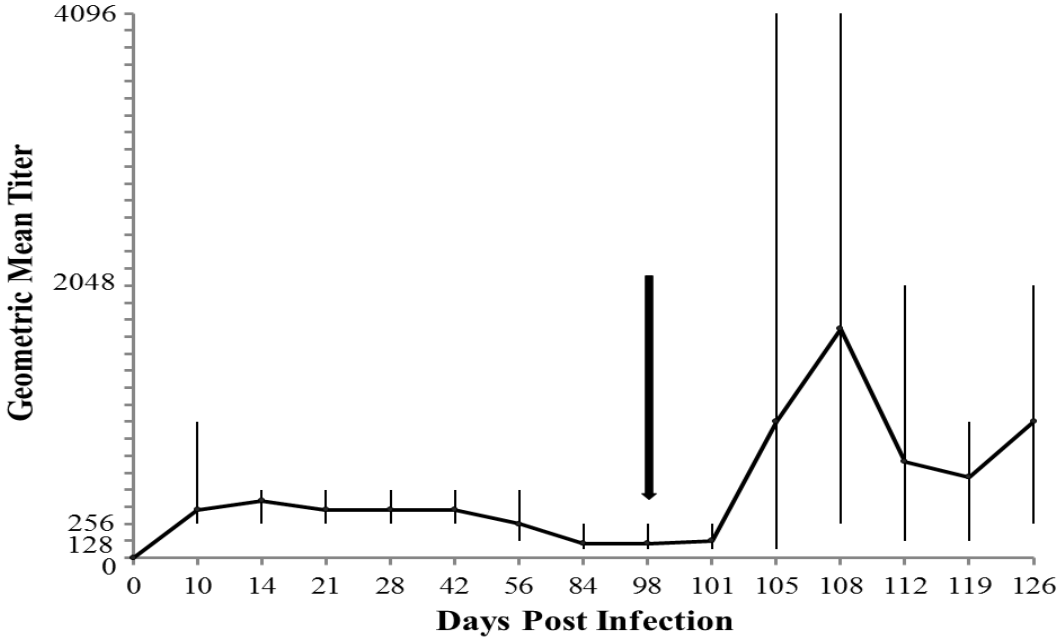


Figure 3.2. Geometric mean titers as measured by hemagglutination assay against *Yersinia pestis* F1 antigen for the oral exposure group. Error bars represent the range of antibody titers for the coyotes (n=4) in this group. The arrow designates the re-challenge at 98 days post-infection.

To investigate the antibody profile produced by coyotes in response to infection with *Y. pestis*, serum samples were evaluated by Western blot for the presence of antibody against known *Y. pestis* virulence proteins F1, LcrV, Pla, Pst, YopD, YopH and YpkA. Representative western blots are depicted in the Figure 3.3. In day 0 serum samples, antibodies were detected against YopD, YopH, and YpkA indicating probable prior exposure to enteropathogenic *Yersinia* species (Table 3.5). Similarly, antibody responses in the serum collected from the two control animals were detected against YopD, YopH, and LcrV. Post-infection, antibodies were detected against all six virulence proteins tested in at least one animal from each group (Table 3.5). Subtle differences were noted in intensity of reactions between groups but due to issues with variations in background staining between blots, quantitative assessment was not possible. Antibody intensity was generally similar across time and reactions appeared weaker for intradermal exposed individuals compared to those animals in the oral exposure group. Increases in intensity were usually noted in samples collected after re-challenge

.

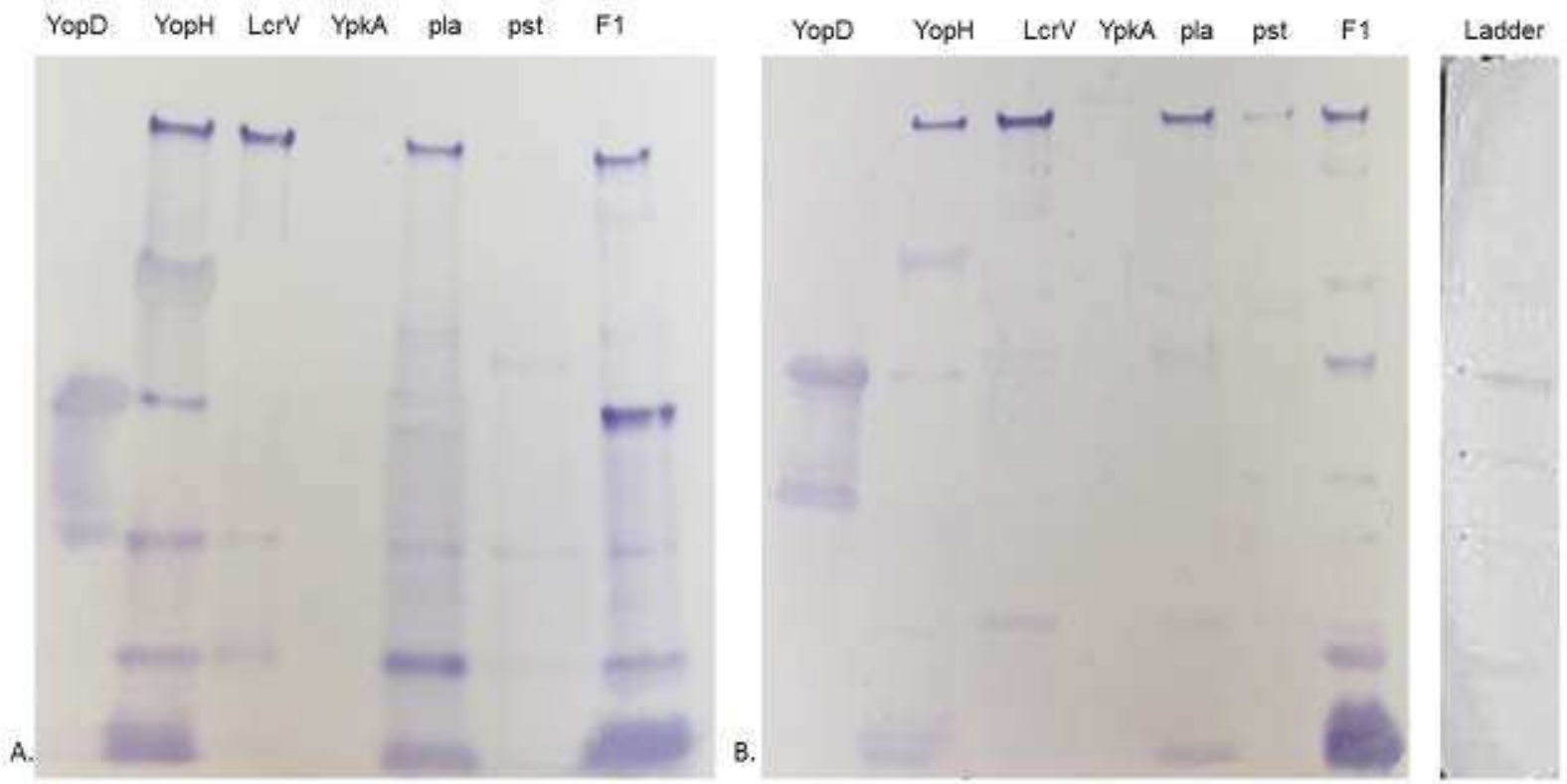


Figure 3.3. Western blot images showing antibody reactions against *Y. pestis* virulence proteins. Lanes from left to right show reactions to YopD, YopH, LcrV, YpkA, Pla, Pst, and F1.A) antibody responses at day 21 postinfection for coyote inoculated by the intradermal route, Id2; B) antibody response at day 21 postinfection for coyote inoculated by oral route, Or4.

Table 3.5 Antibodies detected by Western blot against immunogenic virulence proteins produced by coyotes after experimental infection with *Yersinia pestis*

Oral exposed group							
DPI	YopD	YopH	LcrV	YpkA	Pla	Pst	F1
0	2	4	0	2	0	1	0
14	3	4	3	2	4	2	4
28	4	3	2	2	2	1	4
42	4	4	2	2	3	3	4
56	4	4	2	1	3	4	4
84	4	4	2	1	3	1	4
98	4	4	3	2	4	4	4

Intradermal exposed group							
DPI	YopD	YopH	LcrV	YpkA	Pla	Pst	F1
0	3	4	0	3	0	3	0
14	3	4	3	3	4	4	4
28	4	4	2	2	4	4	4
42	4	4	3	0	4	4	4
56	4	4	1	1	3	3	4
84	4	4	1	2	3	3	4
98	4	4	0	0	4	4	4

Values are represented as the number of individuals that produced an antibody response for each of the *Y. pestis* virulence proteins (n=4 tested per inoculation group). Antibody detection was determined by western blot; DPI = days post infection.

DISCUSSION

In domestic dogs, CRP and SAA have been found to be major acute phase proteins (APP) [29]. Levels of these two blood proteins are commonly used to evaluate inflammation related to tissue injury, infection, stress, or neoplasia [30-33]. These proteins are produced by hepatocytes in response to pro-inflammatory signaling (IL1, IL6, TNF_{γ}) from activated macrophages and induce the acute phase response. CRP acts as an opsonin and modulates cytokine production and chemotaxis [34]. SAA is a chemotaxin and may play a role in downregulation of an inflammatory response [34]. These APPs are routinely produced within 4 hours of insult and have a short half-life (18 hours) making them useful markers of inflammation. The elevations in the CRP and SAA seen in this study support the findings that *Y. pestis* induces an acute phase/innate immune response in coyotes despite their apparent resistance to development of

disease. The response in CRP noted, although minimal, in the intradermal group supports our assumption that the ID *Y. pestis* inoculum was infective despite poor humoral responses.

The low dose of organism and ID inoculation route used in the present study was designed to mimic a natural exposure by delivery through a single flea bite. Due to the anatomy of the flea, *Y. pestis* would only be deposited in the dermal layer during natural flea exposure [35]. Assuming coyotes are incidental hosts [36, 37], minimal feeding by rodent fleas would be expected due to species-specific blood meal preferences [38]. As such, the low antibody titers in the intradermal group were to be expected. The amplitude of antibody production noted in these animals differed somewhat from previous canid studies. These differences were likely due to variations in the route of exposure and dose of inoculum. Two dogs inoculated with *Y. pestis* SC at 10^3 or 10^7 cfu had reported peak geometric mean titers (GMT) of 1:1024 that persisted at 1:128 thru day 330 [39]; further comparison to this study is limited since individual titers were not available.

Similar results have been described in domestic ferrets (*Mustela putorius furo*) inoculated SC with ≤10^3 organisms where individuals showed poor antibody responses to F1 [40]. Likewise, a raccoon inoculated ID with 10^4 organisms produced a peak antibody titer of only 1:16 [41] and multiple rat (*Rattus*) species inoculated SC with serial dilutions of *Y. pestis* (10^2 to 10^7 cfu) failed to produce a consistent antibody response at doses of <10^4 cfu. In addition, rats in the same study lacked an anamnestic response after low-dose re-challenge but mounted a significant antibody response with subsequent moderate-to high-dose re-challenges [42], which was similar to the subsequent re-challenge experiments described here for the ID exposure group.

Scientists are just beginning to unravel the role of fleas in the transmission of *Y. pestis* to mammalian host species. Transit through the flea gut appears to enhance the ability of *Y. pestis*

to evade portions of the host immune system [43-47], and it is speculated that flea saliva may play a role in transmission as noted in other insect vectors [38, 48]. Recent experiments in rodent models indicates that there are significant differences in the *Y. pestis* pathogenesis between routes of exposure [49], and mouse strains [49, 50]. As such, *Y. pestis* inoculated by naturally infected fleas in the dermis and its associated evasion of the innate immune response may result in the production of higher antibody titers in natural infection.

The serological response to F1 capsule antigen in coyotes exposed by ingestion was similar to that described in previous studies using canids. Domestic dogs (*Canis lupus familiaris*) offered *Y. pestis*-infected rat viscera showed peak geometric mean titers (GMT) of 1:2048 at day 10 which gradually declined to 1:256 by day 90 and subsequently remained stable through day 330 [39]. In another study, a number of wild carnivores, including coyotes, were orally inoculated with *Y. pestis*, but inoculation dose and antibody titers were not reported, making it difficult to compare to other studies. Nonetheless, the timing of the antibody response reported was similar, with the first detection at 8-14 days; and a peak in titers between days 20 and 30 PI that later declined to below detection levels by six to eight months [6].

Previously reported experimental trials using oral inoculation have demonstrated that the probable route of entry for *Y. pestis* is via palatine tonsil/pharyngeal exposure [51-53]. In this study, individual variation in consumption of infectious material was observed (Fig 3.4); some mice were fully consumed whereas others were partially ingested, and it is speculated that some of the variability in antibody titer and within the group may be due to differences in the dose and length of exposure to *Y. pestis* within the oral cavity. These results are similar to those presented for oral infection in swine (*Sus scrofa*) [54], mongoose (*Herpestes auropunctatus*) [55], Siberian pole cats (*Mustela eversmannii*) [56], and raccoons (*Procyon lotor*) [41]. This variable dose exposure may also explain the differences noted in antibody response between the coyotes in this study and the dogs infected via ingestion of infected rat viscera [39, 41].

Fig 3.4 Photograph depicts the variation in consumption of mice by individual coyotes during experimental infection trials (n=4 mice).

The substantial variation in antibody titers noted between oral and ID inoculation groups is not only a reflection of the route of exposure but differences in dose, and possibly *Y. pestis* characteristics related to cultivation (i.e. 28°C vs 37°C) [57, 58]. The inoculum used for ID exposure was grown at 28°C, and therefore, it is reasonable to assume that *Y. pestis* was presented to the immune system with hexa-acylated LPS. This form of LPS likely triggers the TLR4 response and induces a localized pro-inflammatory response, both of which might result in the rapid clearance of the organism by an efficient, localized, innate immune response. To investigate the extent to which the lack of response in the ID exposure group was dose related, animals in this group were re-challenged a third time (day 147); two animals were inoculated ID with 10^4 cfu and two animals were offered infected mice. Those animals receiving the fourth ID inoculation at $1x10^4$ cfu (approximating 10-20 flea bites), demonstrated a 10-20 fold titer increase, with peak titers reaching 1:512 and 1:1024 within 3-7 days (days 150-154). Even more pronounced, but in keeping with higher antibody responses to oral inoculation, the two animals receiving a final oral exposure reached titers of 1:8192 to 1:16384 within 3-7 days (days 150-

154). These findings do not specifically address the effects of inoculation route but strongly suggest a dose-dependent antibody response.

The antibody titer range found in the two groups of coyotes in the experimental inoculation trial (1:32–1:4096) are strikingly similar to that noted in historical surveillance records. Looking at historical coyote surveillance data (Figure 3.5), the majority of antibody titers detected (as measured by PHA) in free-ranging coyotes were less than 1:256. In comparison to the experimental data, these results would be consistent with low dose exposures and may be interpreted that animals with oral exposures to infected rodents during active plague periods would have titers ≥1:512. The lack of titers >1:4096 is interesting and suggest that limited re-exposures may be occurring in the wild. Alternatively, this surveillance data is based upon the collection of post mortem blood samples from coyotes stored on filter paper strips [15]. Methods of storage and duration between collection and testing has been shown to effect the antibody titers by PHA [59] which may also contribute to the lower titer range reported.

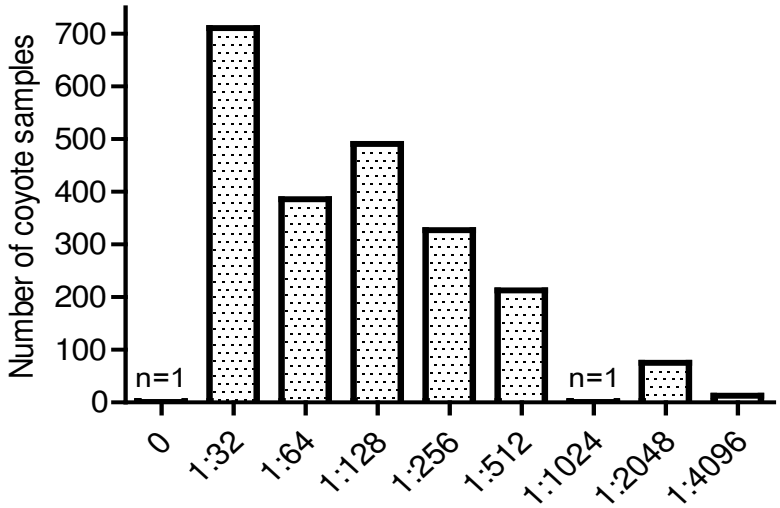


Figure 3.5. Historical plague serosurveillance data using free-ranging coyotes. Data presented as the number of coyote surveillance samples that were tested from plague endemic regions (1970-2006). Titers were determined by PHA on extractions from blood collected on filter paper strips (Nobuto strips). Data provided by S. Bevins and USDA-APHIS-WS-National Wildlife Disease Program.

The results of the antibody profiling conducted in this study were similar to antibody profiles observed in samples collected from free-ranging coyotes [26] as well as those found in dog and cat samples collected in plague endemic regions [60], experimentally infected rabbits [28], and human cases of plague [61]. The finding of antibody responses to *Y. pestis* virulence proteins (YopD, YopH, YpkA) at day 0 in this cohort as well as in PHA negative domestic dogs and free-ranging coyotes suggests that exposures to enteropathogenic *Yersinia* species are a common occurrence. As such, these anamnestic humoral responses to the virulence proteins of the T3SS may contribute to rapid clearance of *Y. pestis*. This hypothesis is in line with partial protection noted in experimental vaccine trials in rodents using antigenic components of the T3SS [62-66]. With the increase in availability of recombinant proteins and high throughput analyses, further assessment of responses to newly identified virulence proteins in the serum samples available from this study may shed light on the mechanisms of apparent resistance in canids.

Limitations of this study include a lack of data to assess the duration of antibody titers over multiple seasons. The assumption is that in plague endemic areas coyotes will have continued exposures throughout a season which is why emphasis was placed on re-challenge antibody titer measurements rather than the duration of antibody persistence. In addition, this study had a small sample size per group, which included mostly males. Previous serological assessments to *Y. pestis* in rodents and coyotes indicate that there may be differences in the level and duration of antibody production between sexes [20, 26, 67].

Based upon the results in this study, further characterization of the potential effects of dose and flea-induced changes to *Y. pestis* on antibody production may be warranted. Nonetheless, the data presented here provide a baseline from which to interpret serological titers collected from free-ranging coyotes. These data may be helpful in refining the current epidemiological models that use coyote serosurveillance data to predict human risk in plague-endemic areas

[19, 68]. High antibody titers should indicate that exposure to *Y. pestis* is likely recent and/or recurrent. It is possible that isotypic analysis of anti-*Y. pestis* antibodies (IgG, IgM) may enable better determinations of acute, convalescent and distant-past infections in sentinel coyotes and better assessment of disease occurrence and associated human risk. Further evaluation is necessary for the interpretation of low to moderate antibody titers to differentiate waning titers from low dose exposures. In addition, these results suggest that the use of a standard four fold or greater titer change between acute and convalescent titers to confirm canid *Y. pestis* infection may not be clinically sound in low dose exposures.

LITERATURE CITED

1. Poland, J.D. and A.M. Barnes, *Plague*, in *CRC Handbook Series in Zoonoses*, J.H. Steele, Editor. 1979, CRC Press: Boca Raton, FL. p. 515-597.

2. Pollitzer, R., *Plague*, W.H. Organization, Editor. 1954: Geneva. p. 698.

3. Perry, R.D. and J.D. Fetherston, *Yersinia pestis - Etiologic agent of plague.* Clinical Microbiology Reviews, 1997. 10(1): p. 35-+.

4. Gage, K.L., et al., *Cases of cat-associated human plague in the Western US, 1977-1998.* Clin Infect Dis, 2000. 30(6): p. 893-900.

5. Gould, L.H., et al., *Dog-associated risk factors for human plague.* Zoonoses Public Health, 2008. 55(8-10): p. 448-54.

6. Barnes, A.M., *Surveillance and Control of Bubonic Plague in the United States*. Symposium Zoological Society of London. Vol. 50. 1982, London, England. 237-270.

7. Craven, R.B., et al., *Reported cases of human plague infections in the United-States, 1970-1991.* Journal of Medical Entomology, 1993. 30(4): p. 758-761.

8. Brown, H.E., et al., *Climatic predictors of the intra- and inter-annual distributions of plague cases in New Mexico based on 29 years of animal-based surveillance data.* Am J Trop Med Hyg, 2010. 82(1): p. 95-102.

9. Bradley, C.A. and S. Altizer, *Urbanization and the ecology of wildlife diseases.* Trends in Ecology & Evolution, 2007. 22(2): p. 95-102.

10. Altizer, S., et al., *Climate Change and Infectious Diseases: From Evidence to a Predictive Framework.* Science, 2013. 341(6145): p. 514-519.

11. Holt, A.C., et al., *Spatial analysis of plague in California: niche modeling predictions of the current distribution and potential response to climate change.* Int J Health Geogr, 2009. 8: p. 38.

12. Enscore, R.E., et al., *Modeling relationships between climate and the frequency of human plague cases in the southwestern United States, 1960-1997.* Am J Trop Med Hyg, 2002. 66(2): p. 186-96.

13. De Souza, A., J. Arruda, and M. Pinto, *Report on Experiments undertaken to Discover whether the Common Domesticated Animals of Terceira Island are Affected by Plague.* J Hyg (Lond), 1910. 10(2): p. 196-208.

14. MARCHETTE, N.J., et al., *Studies on infectious diseases in wild animals in Utah. II. Susceptibility of wild mammals to experimental plague.* Zoonoses Res, 1962. 1: p. 225-49.

15. Bevins, S.N., et al., *Yersinia pestis: examining wildlife plague surveillance in China and the USA.* Integrative Zoology, 2012. 7(1): p. 99-109.

16. Gese, E.M., et al., *Serologic survey for canine infectious diseases among sympatric swift foxes (Vulpes velox) and coyotes (Canis latrans) in southeastern Colorado.* J Wildl Dis, 2004. 40(4): p. 741-8.

17. Gese, E.M., et al., *Serological survey for diseases in free-ranging coyotes (Canis latrans) in Yellowstone National Park, Wyoming.* J Wildl Dis, 1997. 33(1): p. 47-56.

18. Arjo, W.M., et al., *Serologic survey for diseases in free-ranging coyotes (Canis latrans) from two ecologically distinct areas of Utah.* J Wildl Dis, 2003. 39(2): p. 449-55.

19. Brown, H.E., et al., *Annual seroprevalence of Yersinia pestis in coyotes as predictors of interannual variation in reports of human plague cases in Arizona, United States.* Vector Borne Zoonotic Dis, 2011. 11(11): p. 1439-46.

20. Willeberg, P.W., et al., *Epidemiologic survey of sylvatic plague by serotesting coyote sentinels with enzyme immunoassay.* Am J Epidemiol, 1979. 110(3): p. 328-34.

21. Hopkins, D.D. and R.A. Gresbrink, *Surveillance of sylvatic plague in Oregon by serotesting carnivores.* American Journal of Public Health, 1982. 72(11): p. 1295-1297.

22. Thomas, C.U. and P.E. Hughes, *Plague surveillance by serological testing of coyotes (Canis latrans) in Los Angeles County, California.* J Wildl Dis, 1992. 28(4): p. 610-3.

23. Archibald, W.S. and S.J. Kunitz, *Detection of plague by testing serums of dogs on the Navajo Reservation.* HSMHA Health Records, 1971. 86(April 1971): p. 377-380.

24. Surgalla, M.J. and E.D. Beesley, *Congo red-agar plating medium for detecting pigmentation in Pasteurella pestis.* Appl Microbiol, 1969. 18(5): p. 834-7.

25. Chu, M.C., *Laboratory manual of plague diagnostic tests*. 2000, Atlanta, GA: Centers for Disease Control and Prevention.

26. Vernati, G., et al., *Antigenic profiling of yersinia pestis infection in the Wyoming coyote (Canis latrans).* J Wildl Dis, 2011. 47(1): p. 21-9.

27. Andrews, G.P., et al., *Fraction 1 capsular antigen (F1) purification from Yersinia pestis CO92 and from an Escherichia coli recombinant strain and efficacy against lethal plague challenge.* Infection and Immunity, 1996. 64(6): p. 2180-2187.

28. Andrews, G.P., et al., *Identification of in vivo-induced conserved sequences from Yersinia pestis during experimental plague infection in the rabbit.* Vector Borne Zoonotic Dis, 2010. 10(8): p. 749-56.

29. Ceron, J.J., P.D. Eckersall, and S. Martinez-Subiela, *Acute phase proteins in dogs and cats: current knowledge and future perspectives.* Veterinary Clinical Pathology, 2005. 34(2): p. 85-99.

30. Mansfield, C.S., F.E. James, and I.D. Robertson, *Development of a clinical severity index for dogs with acute pancreatitis.* Javma-Journal of the American Veterinary Medical Association, 2008. 233(6): p. 936-944.

31. Schmidt, E.M.D. and P.D. Eckersall, *Acute phase proteins as markers of infectious diseases in small animals.* Acta Veterinaria-Beograd, 2015. 65(2): p. 149-161.

32. Eckersall, P.D. and R. Bell, *Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine.* Veterinary Journal, 2010. 185(1): p. 23-27.

33. Jitpean, S., et al., *Increased concentrations of Serum amyloid A in dogs with sepsis caused by pyometra.* Bmc Veterinary Research, 2014. 10.

34. Cray, C., J. Zaias, and N.H. Altman, *Acute Phase Response in Animals: A Review.* Comparative Medicine, 2009. 59(6): p. 517-526.

35. Sebbane, F., et al., *Role of the Yersinia pestis plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague.* Proc Natl Acad Sci U S A, 2006. 103(14): p. 5526-30.

36. Gage, K.L., J.A. Montenieri, and R.E. Thomas, *The role of predators in the ecology, epidemiology, and surveillance of plague in the United-States.* Sixteenth Vertebrate Pest Conference, 1994: p. 200-206.

37. Boone, A., J.P. Kraft, and P. Stapp, *Scavenging by mammalian carnivores on prairie dog colonies: implications for the spread of plague.* Vector Borne Zoonotic Dis, 2009. 9(2): p. 185-90.

38. Marquardt, W.C., B.C. Kondratieff, and W.H. Marquardt, *Biology of Disease Vectors*. 2004, Burlington, United States: Elsevier Science & Technology.

39. Rust, J.H., et al., *The role of domestic animals in the epidemiology of plague. I. Experimental infection of dogs and cats.* J Infect Dis, 1971. 124(5): p. 522-6.

40. Williams, E.S., et al., *experimental-infection of domestic ferrets (mustela-putorius-furo) and Siberian polecats (Mustela-eversmanni) with Yersinia-pestis.* Journal of Wildlife Diseases, 1991. 27(3): p. 441-445.

41. Shepherd, G.G., *The response of raccoons (Procyon lotor) to experimental infection with Yersinia pestis*. 1973, Colorado State University.

42. Chen, T.H. and K.F. Meyer, *Susceptibility and antibody-response of Rattus species to experimental plague.* Journal of Infectious Diseases, 1974. 129: p. S62-S71.

43. Vadyvaloo, V., et al., *Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in Yersinia pestis.* PLoS Pathog, 2010. 6(2): p. e1000783.

44. Spinner, J.L. and B.J. Hinnebusch, *The Life Stage of Yersinia pestis in the Flea Vector Confers Increased Resistance to Phagocytosis and Killing by Murine Polymorphonuclear Leukocytes*, in *Advances in Yersinia Research*, A.M.P. DeAlmeida and N.C. Leal, Editors. 2012. p. 159-163.

45. Hinnebusch, B.J., C.O. Jarrett, and D.M. Bland, *"Fleaing" the Plague: Adaptations of Yersinia pestis to Its Insect Vector That Lead to Transmission.* Annu Rev Microbiol, 2017. 71: p. 215-232.

46. Hinnebusch, B.J. and D.L. Erickson, *Yersinia pestis biofilm in the flea vector and its role in the transmission of plague*, in *Bacterial Biofilms*, T. Romeo, Editor. 2008. p. 229-248.

47. Hinnebusch, B.J., *Biofilm-Dependent and Biofilm Independent Mechanisms of Transmission of Yersinia pestis by Fleas.* Advances in Yersinia Research, 2012. 954: p. 237-243.

48. Schoeler, G.B. and S.K. Wikel, *Modulation of host immunity by haematophagous arthropods.* Annals of Tropical Medicine and Parasitology, 2001. 95(8): p. 755-771.

49. Gonzalez, R.J., et al., *Comparison of Models for Bubonic Plague Reveals Unique Pathogen Adaptations to the Dermis.* Infection and Immunity, 2015. 83(7): p. 2855-2861.

50. Bosio, C.F., et al., *Kinetics of Innate Immune Response to Yersinia pestis after Intradermal Infection in a Mouse Model.* Infection and Immunity, 2012. 80(11): p. 4034-4045.

51. Rust, J.H., et al., *Susceptibility of rodents to oral plague infection: a mechanism for the persistence of plague in inter-epidemic periods.* J Wildl Dis, 1972. 8(2): p. 127-33.

52. Butler, T., et al., *Experimental Yersinia-pestis infection in rodents after intra-gastric inoculation and ingestion of bacteria.* Infection and Immunity, 1982. 36(3): p. 1160-1167.

53. Chen, T.H., S.S. Elberg, and D.M. Eisler, *Immunity in plague - protection induced in Cercopithecus-aethiops by oral-administration of live, attenuated Yersinia-pestis.* Journal of Infectious Diseases, 1976. 133(3): p. 302-309.

54. Marshall, J.D., et al., *Role of domestic-animals in epidemiology of plague .3. Experimental infection of swine.* Journal of Infectious Diseases, 1972. 125(5): p. 556-&.

55. Higa, H.H., W.T. Matsuura, and W.H. Watanabe, *Plague antibody response in the mongoose.* Hawaii Med J, 1971. 30(2): p. 92-4.

56. Castle, K.T., et al., *Susceptibility of the Siberian polecat to subcutaneous and oral Yersinia pestis exposure.* J Wildl Dis, 2001. 37(4): p. 746-54.

57. Gyles, C.L. and C. Ebooks, *Pathogenesis of bacterial infections in animals*. 4th ed.. ed. 2010, Ames, Iowa: Ames, Iowa : Wiley-Blackwell.

58. Straley, S.C. and R.D. Perry, *Environmental modulation of gene-expression and pathogenesis in Yersinia.* Trends in Microbiology, 1995. 3(8): p. 310-317.

59. Bevins, S., et al., *Effect of storage time and storage conditions on antibody detection in blood samples collected on filter paper.* J Wildl Dis, 2016. 52(3): p. 478-83.

60. Li, B., et al., *Serologic Survey of the Sentinel Animals for Plague Surveillance and Screening for Complementary Diagnostic Markers to F1 Antigen by Protein Microarray.* American Journal of Tropical Medicine and Hygiene, 2008. 79(5): p. 799-802.

61. Li, B., et al., *Antibody profiling in plague patients by protein microarray.* Microbes Infect, 2008. 10(1): p. 45-51.

62. Balada-Llasat, J.M., et al., *Oral inoculation with Type III secretion mutants of Yersinia pseudotuberculosis provides protection from oral, intraperitoneal, or intranasal challenge with virulent Yersinia.* Vaccine, 2007. 25(8): p. 1526-1533.

63. Andrews, G.P., et al., *Protective efficacy of recombinant Yersinia outer proteins against bubonic plague caused by encapsulated and nonencapsulated Yersinia pestis.* Infection and Immunity, 1999. 67(3): p. 1533-1537.

64. Erova, T.E., et al., *Evaluation of Protective Potential of Yersinia pestis Outer Membrane Protein Antigens as Possible Candidates for a New-Generation Recombinant Plague Vaccine.* Clinical and Vaccine Immunology, 2013. 20(2): p. 227-238.

65. Wang, S.X., et al., *Relative immunogenicity and protection potential of candidate Yersinia Pestis antigens against lethal mucosal plague challenge in Balb/C mice.* Vaccine, 2008. 26(13): p. 1664-1674.

66. Derbise, A., et al., *An Encapsulated Yersinia pseudotuberculosis Is a Highly Efficient Vaccine against Pneumonic Plague.* Plos Neglected Tropical Diseases, 2012. 6(2).

67. Jones, S.M., et al., *Protection conferred by a fully recombinant sub-unit vaccine against Yersinia pestis in male and female mice of four inbred strains.* Vaccine, 2000. 19(2-3): p. 358-366.

68. Lowell, J.L., et al., *Colorado animal-based plague surveillance systems: relationships between targeted animal species and prediction efficacy of areas at risk for humans.* J Vector Ecol, 2009. 34(1): p. 22-31.

CHAPTER 4 - INVESTIGATING SPECIES DIFFERENCES IN MACROPHAGE ACTIVITY AFTER EXPOSURE TO YERSINIA PESTIS

SUMMARY

Yersinia pestis, the causative agent of plague, is a zoonotic, vector-borne disease agent and there are considerable differences in specie's susceptibility to this pathogen and severity of ensuing disease. Although the pathogenesis of plague has been delineated in several mammalian hosts, defining the factors determining these inter-species differences in susceptibility have been elusive. Pathogenesis studies in rodent models imply that an early, effective innate immune response may protect against the development of plague. The severity of clinical disease appears to be related to the host's ability to mount an effective neutrophilic response and limit intracellular replication within macrophages. Here we evaluate the role of the macrophage response to *Y. pestis* in cells from mammalian species with different susceptibilities to development of clinical disease. Experimental infection of canine, feline and murine macrophages was performed to look for differences in efficiency of phagocytosis, intracellular survival, and replication of *Y. pestis*. Subtle differences were noted in phagocytic uptake and intracellular survival between species. Intracellular replication of *Y. pestis* was not detected in the cell culture systems utilized in this study. Macrophage-like cell lines infected in a similar manner to those of primary cell cultures showed similar bioparticle uptake and phagocytic responses to bacteria indicating their potential utility in future cell culture work.

INTRODUCTION

Yersinia pestis evolved from a primary enteric pathogen, *Y. pseudotuberculosis*, to become an obligate vector-borne pathogen by the gain of gene(s) enabling flea transmission and silencing of gene(s) facilitating enteric transmission [1-3]. Once established as a pathogen in

rodents, it appears to have subsequently acquired gene(s) that allowed for the dissemination and systemic spread within mammalian host tissues [4, 5]. The complexity of its disease ecology is mimicked in its adaptability to a biphasic lifestyle and varied expression of virulence factors under different environmental conditions [6-8].

The resiliency of *Y.* pestis is exemplified by its complex disease ecology. Primarily a disease of rodents and their fleas within enzootic cycles, incidental hosts including humans can develop severe disease. While the most common route of exposure is flea-borne transmission, infection can be established by parenteral, oral, and/or aerosol exposures. Dermal exposures result in trafficking to draining lymph nodes and development of necrosuppurative lymphadenopathy (bubonic plague). Primary pneumonic plague is the most severe form of the disease and oral exposures are most likely to occur in domestic and wild carnivores but human cases have been documented [9]. Systemic spread from severe or untreated infections is typical and can lead to endotoxic manifested disease and mortality [5, 10].

There is extensive variability among mammalian hosts in the severity of clinical disease that follows infection with *Y. pestis* [10, 11]. Through evolution from a pathogen of rats (*Rattus* spp) with the Oriental rat flea (*Xenopsylla cheopis*) as its primary vector, it has adapted to different flea-rodent life cycles worldwide despite variances in transmission efficiency across flea species [12] and varied pathogenesis within rodents [11]. Carnivores are thought to be fairly refractory to the development of clinical disease [11, 13, 14] yet variable morbidity and mortality has been observed following experimental infections of felids and mustelids [13, 15-20].

As depicted in various rodent pathogenesis models [7, 21-24], the variability in susceptibility to *Y. pestis* is likely linked to the mammalian host innate immune response during the first 48 hours of infection. Whether this is related to the robustness of the neutrophilic response,

complement-mediated lysis, intracellular survival in macrophages, or some other difference in the host acute phase or innate immune response is unclear.

In this study, I examined the response of macrophages during infection with *Y. pestis* and *Salmonella* to look for intrinsic differences in cellular function among mammalian host species. First, I used established macrophage-like cell lines from canine, feline, and murine lineages to evaluate the uptake of *Salmonella* and its intracellular survival. These assays were then used to compare results from exposure to *Y. pestis* in the same cell lines as well as bone marrow-derived mononuclear cells from dogs (*Canis lupus familiaris*), cats (*Felis catus*) and prairie dogs (*Cynomys ludovicianus*).

MATERIALS AND METHODS

Bacteria and Culture Conditions: Bacterial isolates of *Yersinia pestis* CO92 (CDC isolate), *Salmonella enterica* subspecies enterica serovar Typhimurium ATCC 14028s (*Salmonella*) and Escherichia coli (*E. coli*; ATTC 25922) were obtained from frozen stock and cultivated in brain heart infusion (BHI) or Luria-Bertani (LB) broth overnight at 37°C with 5% CO_2 unless otherwise stated. Sub-cultures grown on brain heart infusion (BHI) agar were diluted in physiological saline using Prompt™ inoculation system (Becton Dickinson and Company, Sparks, MD) prior to use for experimental infections to standardize bacterial colony forming units (CFU) between experiments [25].

Experimental Tissues: Dogs and cats – Femurs and blood were obtained from dogs (*Canis lupus familiaris*) and cats (*Felis catus*) euthanized for other research purposes. These animals were 1-3 years of age. Mice – Femurs and blood were obtained from CD1 mice (Jackson Laboratories, Bar Harbor, Maine, USA) euthanized for other research purposes. All mice were approximately 8 weeks of age. Prairie Dogs - Femurs and blood were obtained from wild-caught prairie dogs (*Cynomys ludovicianus*) euthanized for use as a food source for black-footed ferrets at the US-FWS Black-footed Ferret Conservation Center (Carr, Colorado, USA).

Tissue culture cell lines: Macrophage-like cell culture lines used in this study included murine (J774), feline (Fwcf-4), canine (DH82) and bovine (BoM, an uncharacterized source used for cultivation of *Toxoplasma gondii* [26]). Cell lines were obtained from frozen stocks and propagated in DMEM (D1152, Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 5-10% fetal bovine serum, 4mM/L glutamine and 1% penicillin-streptomycin at 37˚C with 5% CO_2 unless otherwise noted. Species-specific macrophage colony stimulating factor (M-CSF, 100 IU/mL; Sigma-Aldrich, St. Louis, Missouri, USA) was added to established cultures to activate cells prior to experimentation. At 18 hours prior to experimental plating, cells were maintained in fresh media without antibiotics or M-CSF.

Bone Marrow-Derived Macrophages*:* Bone marrow-derived macrophages (BMDM) were obtained from mouse, cat, dog, and prairie dog femurs. After appropriate euthanasia methods, femurs were dissected under sterile conditions. Bone marrow stroma was flushed from the femoral bone cavity using phosphate buffered saline pH 7.4 (PBS). Immediately after collection, cells were disrupted from the bone marrow matrix and allowed to stand for 2-3 minutes to allow settling of bone spicules and other debris. Cell suspensions were prepared under separation techniques as previously described [27]. Briefly, cell suspensions were washed via centrifugation (500 x g for 10 minutes) and resuspended in DMEM. BMDM were isolated by density gradient medium (Histopaque® 1083, Sigma-Aldrich, St. Louis, Missouri, USA) per manufacturer specifications. Mononuclear cells were recovered, washed by centrifugation and resuspended in cell culture media as described below. Cell suspensions were plated at approximately $1x10^7$ in 25 cm^2 cell culture flasks. Flasks were incubated for 72 hours and then gently rinsed with fresh media to remove non-adherent or dead cells. For initial differentiation of BMDM, cells were propagated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml species-specific macrophage colony stimulating factor (M-CSF, Sigma-Aldrich, St. Louis, Missouri, USA) and penicillin-streptomycin (100 IU/mL and 50 µg/mL, respectively). Unless

otherwise indicated, all cultures were incubated at 37°C with 5% CO_2. Media changes were completed every two days and cells were used for experimental purposes at 5-7 days. At 18 hours prior to experimental plating, cells were maintained in fresh media with 5% FBS, sans antibiotics or M-CSF to stop cell proliferation.

Peripheral Blood-Derived Macrophages*:* Peripheral blood-derived macrophages (PBDM) were isolated from whole blood collected from mice, cats and dogs. Whole blood was collected in EDTA from the jugular vein of cats and dogs being bled for other research purposes. Blood collected from mice was by cardiac puncture while under anesthesia. Buffy coats were collected after centrifugation of whole blood at 500 x g, 20˚C for 5 minutes and resuspended in PBS supplemented with 2% FBS for isolation of PBDM by density gradient medium (Histopaque® 1083, Sigma-Aldrich, St. Louis, Missouri, USA) as previously described [27]. Mononuclear cells were recovered, washed by centrifugation and resuspended in cell culture media. Cell suspensions were plated at approximately $1x10^7$ in 25 cm^2 cell culture flasks. Flasks were incubated for 72 hours and then gently flushed with fresh media to remove non-adherent or dead cells. For initial differentiation of PBDM, cells were propagated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml species-specific macrophage colony stimulating factor (M-CSF, Sigma-Aldrich, St. Louis, Missouri, USA) and 1% penicillin-streptomycin. Unless otherwise indicated, all cultures were incubated at 37°C with 5% CO_2. Media changes were completed every two days and cells were used for experimental purposes at 5-7 days. At 18 hours prior to experimental plating, cells were maintained in fresh media with 5% FBS sans antibiotics or M-CSF to stop cell proliferation.

Experimental Assays: Unless otherwise stated, BMDM were seeded in triplicate at 1×10^4 cells per well in 96 well cell culture plates or 8-well culture slides (BD Biocoat™, Bedford, MA) for experimental manipulations as described below. Each well was assessed separately and values for each run expressed as a mean of the triplicate wells.

Phagocytic Activity: The phagocytic activity of cell lines was assessed by particle internalization using pHrodo™ phagocytosis kit (Molecular Probes) per manufacturer instructions. Cells were plated in triplicate at 10^4 per well in a 100µl volume (8-well slide culture). BioParticles® (20µl) were incubated at 37˚C for 30 min after which cells were chilled at 4˚C to stop phagocytosis followed by immediate microscopic evaluation. The number of fluorescent particles (540 nm) per cell was estimated in each well.

Intracellular uptake of bacteria: Intracellular uptake and survival of *bacteria* by the three cell lines, BMDM and PBDM were determined after exposure to *Salmonella and Y. pestis* (grown at 28 and 37°C) at a multiplicity of infection of 10:1. After inoculation, plates were centrifuged at 600 x g, 18˚C for 4 minutes to synchronize infection followed by incubation at 37°C for 30 minutes. Media containing non-adherent bacteria was decanted, wells rinsed (PBS x2) and fresh media supplemented containing gentamicin (100 µg/mL) was added (Time 0). After 1 hour incubation at 37°C, plates were washed (PBS x3) and refilled with fresh media containing gentamicin (25 µg/mL). Infected cell lines or BMDM were harvested at various time points (1, 4 or 6, 12 and 24 hours) post infection for bacterial quantification. Cell lysates were prepared using 0.05% Tween-20 in PBS in a 2 minute incubation at 4˚C followed by a mechanical disruption step. To quantify intracellular bacteria in cell lysates, 10 fold serial dilutions were plated to LB agar and incubated for 72 hours at 28°C or 37°C. Data was recorded as colony forming units (CFU) per well and mean values of triplicate wells were reported.

Lipopolysaccharide Activation and Nitric Oxide Production: BMDM and cell lines were activated in the presence of LPS-EK (InvivoGen, San Diego, CA) in concentrations of 1, 10 and 100 *n*g/ml LPS [28]. Cell supernatants were collected at 1, 4, 12, and 24 hours post stimulation. To characterize the BMDM and cell lines for their activation response, the level of nitric oxide (NO) produced by LPS stimulated macrophages was determined using the Griess method (Griess Reagent System, Promega, Madison, WI) per manufacturer instructions. In brief, 100 µl

of supernatant was obtained from appropriate stimulation experiments and incubated with 100 µl NO working reagent (1% sulfanilimide, 0.1% napthylethylenediamide, 2.5% H_3PO_4). After a 10 minute incubation at room temperature in the dark, the optical density (OD) values were read at 540 nm and concentrations (µM) determined from the kit derived standard curve.

Statistical Analysis: Statistical analyses were performed using GraphPad PRISM (version 5). Due to limitations in cell numbers, only group means and standard deviations were calculated. Unless otherwise stated, p values < 0.05 were considered significant.

RESULTS

Macrophage-like cells (J774, DH82, BoMo, Fcwf) were propagated under similar culture conditions for several passages to allow acclimation to the same media conditions across cell lines. The J774, DH82, and BoMo cell lines produced confluent cell layers within 3-5 days under the established cell culture conditions. The feline cell line (Fcwf) failed to perform consistently under the described culture conditions and, therefore, its use was limited in assay evaluations.

Propagation of PBDM from canine, feline or mouse blood was attempted as described above. However, PBDM cells failed to proliferate under established cell culture conditions. Alternate growth conditions were investigated that included replacement of FBS with homologous serum; change in species source of M-CSF, and replacement of M-CSF with granulocyte-macrophage cells stimulating factor. Despite extensive troubleshooting efforts, there was a lack of proliferation of PBDM cells, therefore none of this cell type was available for experimental assays.

Propagation of BMDM from mouse and prairie dogs femurs was attempted as described above. These cells failed to proliferate under established cell culture conditions. Alternate growth conditions were investigated included replacement of FBS with homologous serum; change in species sources of M-CSF, and replacement of M-CSF with granulocyte-macrophage

cells stimulating factor. Due to the limited availability of animals, there were not enough BMDM from these two species for experimental assays.

The phagocytic activity of the various macrophage sources was compared to assess potential inter-species differences in particulate uptake. Using the pHrodo BioParticle kit, the ability to internalize particles was evaluated using the cell lines J774, DH82, Fcwf and BoMo, and two canine BMDM cultures. This kit utilizes E. coli BioParticles® that are labeled with pH-sensitive fluorogenic dye to help differentiate attachment from internalization of particles. The particles fluoresce at a pH ≤ 4.5 indicating internalization within host cell phagosome. Fluorescence was noted in all four cell lines confirming internalization of particles within one hour of exposure. Qualitative species differences were noted in uptake with an apparent higher number of particles (2-5 per cell) in DH82 cells (Figure 4.1) compared to the other three cell lines (1-2 per cell). Uptake was also noted in two canine BMDM sources using the same methods (Figure 4.2). Due to limitations in imaging equipment available, quantification of differences between species was not possible. Overall numbers of feline BMDM cells were limited therefore no particle internalization testing was performed.

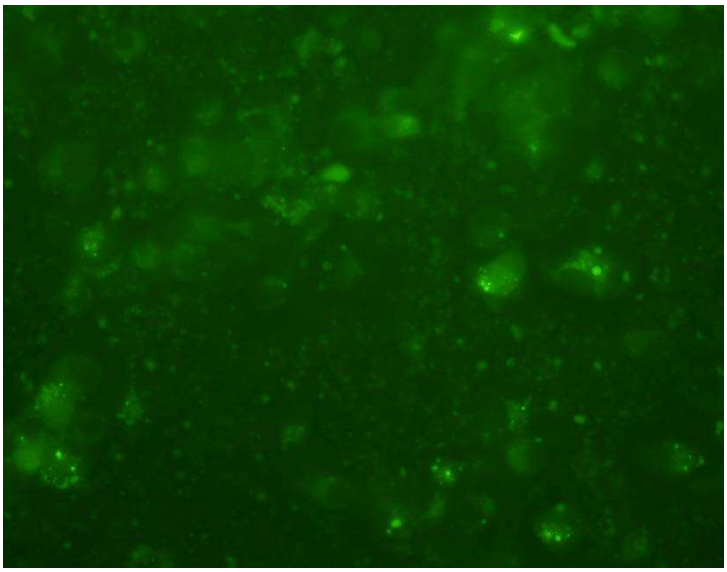


Figure 4.1 Representative image of pHrodo Green BioParticles® within canine macrophage-like cells (DH82) after one-hour incubation, observed at a wavelength of 540 nm, 40x.

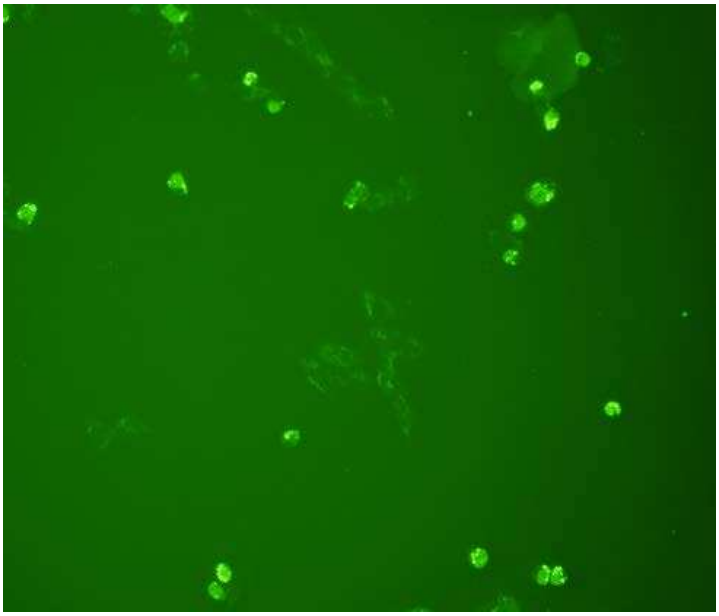


Figure 4.2. Representative image of pHrodo Green BioParticles® within canine BMDM after one-hour incubation, observed at a wavelength of 540nm, 40x.

Lipopolysaccharide stimulation using LPS-EK at 1, 10 and 100 ng/mL was performed on J774 and DH82 cells as well as BMDM cells obtained from three dogs and four cats. Multiple concentrations were tested due to known species differences in sensitivity to LPS. Supernatants collected at 1, 4, 12, and 24 hours from the stimulated cells were analyzed for detection of nitric acid production [28, 29]. Nitric acid was not detected in any of the supernatants tested (detection limit ≥ 3.13 µM). These results indicate that the cells tested were either not adequately stimulated by LPS; were not activated macrophages; did not possess appropriate receptors for activation; or incubation periods or collection times points were inppropriate for detection; or there were not enough cells to elicit a detectable response.

To determine the testing conditions for the bacterial intracellular survival experiments, multiple assays using J774 and BoMo cell lines were performed to determine optimal concentrations of gentamicin, cell lysing techniques and bacterial recovery times. Bacterial isolates were incubated in gentamicin at 25, 50,100 µg/mL for 30 and 60 minutes to determine optimal concentration and time required to effectively kill extracellular bacteria in the cell culture

systems. It was determined that 100 µg/mL for 60 minutes was adequate for killing *Salmonella* and *Y. pestis* when cell cultures were subsequently maintained with 25 µg/mL gentamicin. For the preparation of cell lysates, multiple solutions (distilled water, saponin, Tween-20 (.05%), and Triton X100 (1, 0.5, 0.1, 0.05, 0.03%) were evaluated for their efficiency in cell disruption and inhibition of bacterial recovery. Tween-20 at 0.05% in PBS was found to produce optimal cell lysates with the least inhibition of bacterial growth (data not shown).

Assay optimization was confirmed by means of exposing cultured macrophages to *Salmonella* (positive for intracellular growth) and *E. coli* (negative for intracellular growth). The *Salmonella enterica* subspecies enterica serovar Tyhimurium was chosen as the positive control because of its broad host specificity [30]. Preliminary experiments using J774, DH82 and BoMo confirmed uptake and intracellular survival of *Salmonella* under the proposed experimental conditions and no apparent uptake or intracellular survival was noted in cells exposed to *E. coli* (data not shown).

To ascertain if there may be any inter-species differences in intracellular survival of bacteria within macrophages, *Salmonella* was incubated with cultured macrophages (feline BMDM, DH82, canine BMDM, and J774) and cell lysates were harvested at set time points (1, 4, 12 and 24 hours post exposure) to assess the number of bacteria associated with each cell type. The number of intracellular bacteria varied between time points as well as between species (Figure 4.3, Table 4.1) Statistical evaluation beyond mean/standard deviation were limited due to disparities in group numbers. Bacterial counts were highest in the feline BMDM cells with mean uptake of $4.7x10^4 \pm 4.1x10^4$ CFU noted at 1 hour. Apparent intracellular replication was noted at 4 hours ($7.2x10^4 \pm 4.6x10^4$ CFU) followed by a slight decline at 12 hours ($6.6x10^4 \pm 4.2x10^4$ CFU). Viable bacteria were still present in feline BMDM cells at 24 hours ($5.1x10^3 \pm 4.0x10^4$ CFU) post exposure to *Salmonella*. Overall bacterial counts were found to be lower in DH82,

canine BMDM and J774 macrophages at all times points compared to the feline BMDM (Figure 4.3, Table 4.1). Probable intracellular replication was also noted in these cultured macrophages, which peaked at 12 hours in the canine macrophages and 4 hours in the murine macrophages. Statistical analyses beyond group means were not possible due to low sample size and high variances between assays.

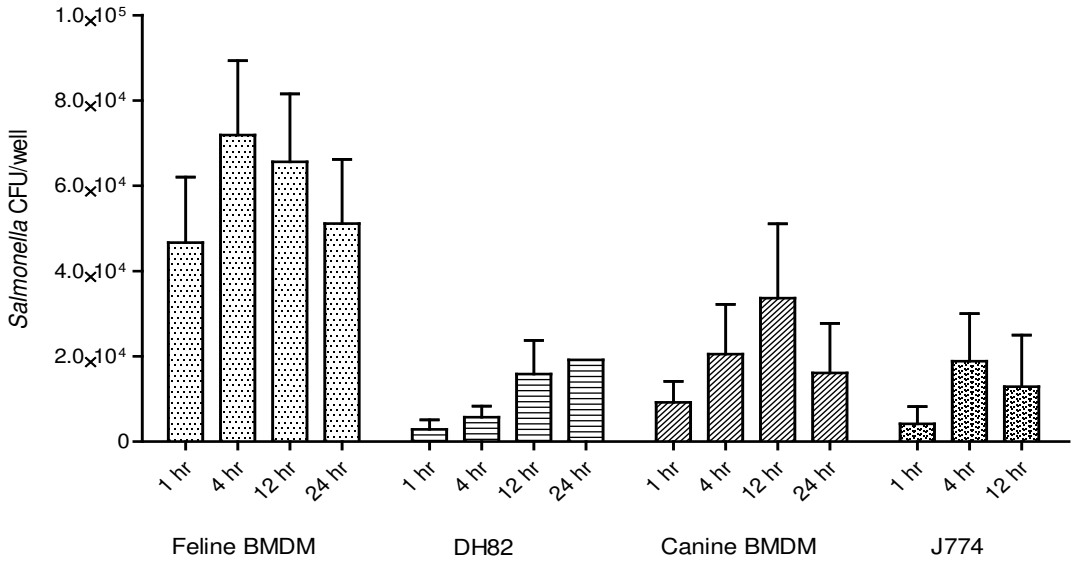


Figure 4.3. *Salmonella* detected in cultured macrophages. Bars represent bacterial colony forming units (CFU) per well (10^4 macrophages per well). Values are expressed as group means ± standard deviation of at least two experiments done in triplicate. Cultured macrophages included feline BMDM (n=7), DH82 (n=3), canine BMDM (n=4), and J774 (n=2).

Assays were repeated in cultured macrophages, replacing *Salmonella* with *Y. pestis* propagated at 28˚C and 37˚C. Differences in uptake and intracellular survival in cultured macrophages (canine BMDM, DH82, and J774) were noted between *Y. pestis* isolates in cell lysates harvested at set time points (1, 6, 12 and 24 hours post exposure). As noted in Table 4.1, the uptake of 28˚C cultivated *Y. pestis* was ten-fold higher in DH82 cells ($2.6x10^4 \pm 3.3x10^4$ CFU) compared to canine BMDM macrophages ($2.6x10^3 \pm 2.8x10^3$ CFU) and J774 cells ($2.1x10^3 \pm 1.5x10^3$ CFU). No apparent intracellular replication was noted as CFU values declined rapidly between 6-24 hours post-exposure in DH82 cells. In contrast, uptake was

dramatically reduced in DH82 cells exposed to 37˚C *Y. pestis* ($3.8x10^3 \pm 3.4x10^3$ CFU). This is consistent with the known phenotypic characteristics of *Y. pestis* where propagation at 28˚C has inhibitory effects on phagocytes *in vivo* [10, 31, 32] due to lack of capsule and altered LPS structure [33]. Statistical analyses beyond group means were not possible due to low sample size and high variances between assays.

Table 4.1. Survival of bacterial isolates within cultured macrophages.

	Feline BMDM	Canine BMDM	DH82	J774
Salmonella				
1 hr	$4.7x10^4 \pm 4.1x10^4$	$2.9x10^3 \pm 3.8x10^3$	$9.2x10^3 \pm 9.8x10^3$	$4.2x10^3 \pm 5.7x10^3$
4 hr	$7.2x10^4 \pm 4.6x10^4$	$5.7x10^3 \pm 4.6x10^3$	$1.9x10^4 \pm 2.0x10^4$	$2.1x10^4 \pm 1.6x10^4$
12 hr	$6.6x10^4 \pm 4.2x10^4$	$1.5x10^4 \pm 1.4x10^4$	$1.3x10^4 \pm 3.0x10^4$	$1.7x10^4 \pm 3.0x10^4$
24 hr	$5.1x10^4 \pm 4.0x10^4$	$1.9x10^4 \pm 0$	$1.6x10^4 \pm 1.6x10^4$	
Yersinia 28˚C				
1 hr		$2.6x10^3 \pm 2.8x10^3$	$2.7x10^4 \pm 3.3x10^4$	$2.1x10^3 \pm 1.5x10^3$
6 hr		$3.0x10^3 \pm 2.6x10^3$	$1.6x10^4 \pm 1.3x10^4$	$1.7x10^3 \pm 3.2x10^3$
12 hr		$1.4x10^3 \pm 1.5x10^3$	$7.2x10^3 \pm 6.6x10^3$	$1.2x10^2 \pm 1.1x10^2$
24 hr		$6.4x10^2 \pm 5.3x10^2$	$6.8x10^2 \pm 3.7x10^2$	
Yersinia 37˚C				
1 hr		$1.5x10^4 \pm 1.5x10^4$	$3.8x10^3 \pm 3.4x10^3$	$2.2x10^3 \pm 1.9x10^3$
6 hr			$3.0x10^3 \pm 2.9x10^3$	$1.3x10^3 \pm 1.8x10^3$
12 hr		$3.1x10^3 \pm 5.4x10^3$	$1.2x10^3 \pm 1.2x10^3$	6 ± 6
24 hr		$3.9x10^2 \pm 6.4x10^2$	$1.4x10^2 \pm 1.4x10^2$	0

Data are presented as bacterial colony forming units (CFU) per well (10^4 macrophages per well) in cultured macrophages. Values are expressed as group means ± standard deviation of at least two experiments per cell type with individual time points run in triplicate. Cultured macrophages included feline BMDM (n=7), canine BMDM (n=4), DH82 (n=3), and J774 (n=2) for *Salmonella* experiments and canine BMDM (n=3), DH82 (n=7), and J774 (n=6) for *Yersinia* experiments.

DISCUSSION

Even with years of research, little has been done to fully characterize species differences in susceptibility to *Y. pestis* infection. Most of the historical work described was limited in scope [34-43]. More recent experimental models attempting to characterize the kinetics of disease and host related responses have highlighted significant differences among rodent species, routes of bacterial exposure, and preparation of the bacterial isolates [7, 8, 24, 44-46]. Despite these variations, the general consensus is that the robustness of the innate immune response during the first 72 hours of infection is likely to determine the resulting disease progression. The key cells identified in this innate response are neutrophils and macrophages [24, 31, 47-54]. Bubonic plague kinetic models hypothesize three phases during this early infection period. Upon entry into the host, Y. pestis will remain at the inoculation site or traffic directly to draining lymph nodes depending upon the dose, route of exposure and bacterial phenotype. Phagocytosis of bacteria during this phase is predominately by neutrophils. However, intracellular bacteria are also noted in macrophages. It is suggested that the degree of intracellular replication of *Y. pestis* within macrophages may contribute to the progression and subsequent severity of disease in susceptible mammals [55]. It has been found that some of these mechanisms are conserved between pathogenic *Yersinia* species [56].

It is known that domestic cats and dogs differ in their oxidative stress responses [57] and glucuronidation capacity [58]. There have also been reports of subtle differences between species in activities of pulmonary macrophages [59], and antigen presenting cells [60, 61]. Therefore it is reasonable to hypothesize that there may be differences in macrophage function that may explain the differences in susceptibility to plague between species. The study described here was designed to evaluate whether differences in phagocytosis, survival and intracellular replication might exist among rodent, canine, and feline macrophages.

The methods for cell culture were derived to generate a uniform population of macrophages from bone marrow precursors in primary cell culture. BMDM are likely to be more representative of *in vivo* macrophages that are recruited from the peripheral blood to sites of inflammation in tissues [62]. In this study evaluation of intracellular survival of *Salmonella* was chosen to provide a subjective comparison to previous work done comparing bacterial survival and replication in human and murine macrophage cell lines. Schwan and colleagues showed that propagated cells lines (J774 and U937) were similar to primary cultivated macrophages (murine peritoneal macrophages and human PBDM) in uptake, replication and intracellular survival [63].

Three macrophage cell types have been described in the literature based upon their phenotypic characteristics (classical, alternative, and regulatory) [64]. In the experiments described here, we hoped to propagate the cells to enhance their classical activation characteristics [28, 29, 65]. Based upon the NO assays, it is unclear if the cultured macrophages were activated in these experiments. Other studies pursuing the comparison between species cell lines did not utilize the same activation mechanisms as described and were able to define subtle differences between murine and canine cells in intracellular survival of *Salmonella* [63] using autologous serum for activation (thus providing M-CSF) rather than priming with IFNɣ prior to LPS stimulations.

The reasons for the poor response to LPS stimulation by the cells used in this study are unclear. Apparent uptake of bacteria and evidence of intracellular survival and/or replication were noted in cells exposed to *Salmonella* and *Y. pestis*, therefore cultured macrophages appeared to have responded appropriately. In an attempt to evaluate alternate methods of cell activation, some canine cells were exposed to granulocyte macrophage-CSF in place of M-CSF but no differences were noted in bacterial uptake or intracellular survival. Others have noted a requirement for opsonization of bacterial isolates when assessing phagocytosis [49, 66]. To evaluate this potential effect, canine macrophages were incubated with coyote serum (naïve

and *Y. pestis* antibody positive) during the bacterial exposure but no differences were noted in uptake and intracellular survival of *Y. pestis* between treatments.

This study was significantly limited by a lack of technological resources due to requirements for working with a select agent in a biosafety level 3 environment. Based upon the bioparticle uptake results and phagocytic responses to bacteria in this experiment, the macrophage-like cell lines used in this study appear to be adequate representatives for future, more sophisticated assessments of species differences in macrophage responses to *Y. pestis*. The DH82 cell line originally developed from a canine malignant histiocytosis case had been characterized and found to possess Fcɣ receptors but not able to produce IL-1 (DH82- ATTC bulletin). They have been shown to phagocytize latex particles and functioned in a similar manner to canine BMDM macrophages in the experiments described here. The J774 cell line is derived from Balb/c mice and has been found to produce IL-1, lysozyme and have been shown to be active in antibody-dependent phagocytosis (J774- ATTC bulletin). Based upon the limited reactions observed with feline BMDM, further cell culture work using the Fcwf cell line is warranted. This cell line was originally developed from feline fetal tissue and was used to propagate feline coronavirus and has been found to possess characteristics of macrophages including non-specific esterases, phagocytic activities and Fc receptors (Fcwf- ATTC bulletin).

New modalities in the quest to define novel treatment and prevention measures for inflammation and infectious diseases are emerging. A translational systems approach has emerged that is attempting to model the complexity of the inflammatory response [67, 68]. The other emerging concept is the investigation of the role of resilience [69, 70], which is defined as the tolerance to pathogen loads by limiting the effects of inflammation. Linking these concepts, some recent studies have elucidated the potential protective effects that serum proteins might play in the innate immune response [71]. However, little has been done to investigate these factors in the context of susceptibility differences in *Yersinia* infections. These assessments

combined with technological advances in *vivo* imaging [72, 73], mass cytometry [74-76] and protein microarray derived antigen and antibody profiling [6, 33, 77-83] could unlock clues to the complexity of the inter-species differences noted in the disease ecology of *Y. pestis*.

LITERATURE CITED

1. Achtman, M., et al., *Microevolution and history of the plague bacillus, Yersinia pestis.* Proceedings of the National Academy of Sciences of the United States of America, 2004. 101(51): p. 17837-17842.

2. Achtman, M., et al., *Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis.* Proceedings of the National Academy of Sciences of the United States of America, 1999. 96(24): p. 14043-14048.

3. Vadyvaloo, V., et al., *Analysis of Yersinia pestis gene expression in the flea vector.* Adv Exp Med Biol, 2007. 603: p. 192-200.

4. Sebbane, F., et al., *Role of the Yersinia pestis plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague.* Proc Natl Acad Sci U S A, 2006. 103(14): p. 5526-30.

5. Oyston, P.C.F. and K.E. Isherwood, *The many and varied niches occupied by Yersinia pestis as an arthropod-vectored zoonotic pathogen.* Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 2005. 87(3): p. 171-177.

6. Sebbane, F., et al., *Adaptive response of Yersinia pestis to extracellular effectors of innate immunity during bubonic plague.* Proceedings of the National Academy of Sciences of the United States of America, 2006. 103(31): p. 11766-11771.

7. Sebbane, F., et al., *Kinetics of disease progression and host response in a rat model of bubonic plague.* American Journal of Pathology, 2005. 166(5): p. 1427-1439.

8. Guinet, F. and E. Carniel, *A technique of intradermal injection of Yersinia to study Y-pestis physiopathology*, in *Genus Yersinia: Entering the Functional Genomic Era*, M. Skurnik, J.A. Bengoechea, and K. Granfors, Editors. 2003. p. 73-78.

9. Christie, A.B., T.H. Chen, and S.S. Elberg, *Plague in camels and goats: their role in human epidemics.* J Infect Dis, 1980. 141(6): p. 724-6.

10. Perry, R.D. and J.D. Fetherston, *Yersinia pestis - Etiologic agent of plague.* Clinical Microbiology Reviews, 1997. 10(1): p. 35-+.

11. Barnes, A.M., *Surveillance and Control of Bubonic Plague in the United States*. Symposium Zoological Society of London. Vol. 50. 1982, London, England. 237-270.

12. Eisen, R.J., L. Eisen, and K.L. Gage, *Studies of vector competency and efficiency of North American fleas for Yersinia pestis: state of the field and future research needs.* J Med Entomol, 2009. 46(4): p. 737-44.

13. Rust, J.H., et al., *The role of domestic animals in the epidemiology of plague. I. Experimental infection of dogs and cats.* J Infect Dis, 1971. 124(5): p. 522-6.

14. Baeten, L.A., et al., *Immunological and clinical response of coyotes (Canis latrans) to experimental inoculation with Yersinia pestis.* J Wildl Dis, 2013. 49(4): p. 932-9.

15. Williams, S.K., et al., *Effects of low-temperature flea maintenance on the transmission of Yersinia pestis by Oropsylla montana.* Vector Borne Zoonotic Dis, 2013. 13(7): p. 468-78.

16. Williams, E.S., et al., *Plague in a black-footed ferret (Mustela nigripes).* Journal of Wildlife Diseases, 1994. 30(4): p. 581-585.

17. Williams, E.S., et al., *Experimental-infection of domestic ferrets (Mustela-putorius-furo) and siberian polecats (Mustela-eversmanni) with Yersinia-pestis.* Journal of Wildlife Diseases, 1991. 27(3): p. 441-445.

18. Castle, K.T., et al., *Susceptibility of the Siberian polecat to subcutaneous and oral Yersinia pestis exposure.* J Wildl Dis, 2001. 37(4): p. 746-54.

19. Wild, M.A., T.M. Shenk, and T.R. Spraker, *Plague as a mortality factor in Canada lynx (Lynx canadensis) reintroduced to Colorado.* J Wildl Dis, 2006. 42(3): p. 646-50.

20. Wong, D., et al., *Primary pneumonic plague contracted from a mountain lion carcass.* Clin Infect Dis, 2009. 49(3): p. e33-8.

21. Hancock, G.E., R.W. Schaedler, and T.T. Macdonald, *Yersinia-enterocolitica infection in resistant and susceptible strains of mice.* Infection and Immunity, 1986. 53(1): p. 26-31.

22. Autenrieth, I.B., et al., *Immune-responses to Yersinia-enterocolitica in susceptible Balb/c and resistant C57bl/6 mice - an essential role for gamma-interferon.* Infection and Immunity, 1994. 62(6): p. 2590-2599.

23. Autenrieth, I.B., et al., *The cellular immune-response against yersinia-enterocolitica in different inbred strains of mice - evidence for an important role of t-lymphocytes.* Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases, 1993. 278(2-3): p. 383-395.

24. Lukaszewski, R.A., et al., *Pathogenesis of Yersinia pestis infection in BALB/c mice: Effects on host macrophages and neutrophils.* Infection and Immunity, 2005. 73(11): p. 7142-7150.

25. Lund, M.E. and R.W. Hawkinson, *Evaluation of the prompt inoculation system for preparation of standardized bacterial inocula.* Journal of Clinical Microbiology, 1983. 18(1): p. 84-91.

26. Dubey, J.P. and G. Desmonts, *Serological responses of equids fed Toxoplasma-gondii oocysts.* Equine Veterinary Journal, 1987. 19(4): p. 337-339.

27. Paulnock, D.M., *Macrophages : a practical approach*. 2000, Oxford ; New York: Oxford ; New York : Oxford University Press.

28. Reiner, N.E., *Macrophages and Dendritic Cells Methods and Protocols*. 2009, Totowa, NJ, New York, N.Y.: Humana Press.

29. Dietert, R.R., *Immunotoxicity Testing Methods and Protocols*. 1.. ed. 2010, Totowa, NJ, New York, N.Y.: Humana Press.

30. Gyles, C.L. and C. Ebooks, *Pathogenesis of bacterial infections in animals*. 4th ed.. ed. 2010, Ames, Iowa: Ames, Iowa : Wiley-Blackwell.

31. Bergsbaken, T. and B.T. Cookson, *Innate immune response during Yersinia infection: critical modulation of cell death mechanisms through phagocyte activation.* Journal of Leukocyte Biology, 2009. 86(5): p. 1153-1158.

32. Ke, Y.C., Z.L. Chen, and R.F. Yang, *Yersinia pestis: mechanisms of entry into and resistance to the host cell.* Frontiers in Cellular and Infection Microbiology, 2013. 3.

33. Li, B., et al., *Use of protein microarray to identify gene expression changes of Yersinia pestis at different temperatures.* Can J Microbiol, 2011. 57(4): p. 287-94.

34. Rust, J.H., et al., *Susceptibility of rodents to oral plague infection: a mechanism for the persistence of plague in inter-epidemic periods.* J Wildl Dis, 1972. 8(2): p. 127-33.

35. Butler, T., et al., *Experimental Yersinia-pestis infection in rodents after intra-gastric inoculation and ingestion of bacteria.* Infection and Immunity, 1982. 36(3): p. 1160-1167.

36. Butler, T., et al., *Yersinia pestis infections of rodents by the oral route.* Clinical Research, 1979. 27(2): p. A341-A341.

37. Thomas, R.E., et al., *Susceptibility to Yersinia-pestis in the Northern grasshopper mouse (Onychomys-leucogaster).* Journal of Wildlife Diseases, 1988. 24(2): p. 327-333.

38. Hudson, B.W., et al., *Serological and bacteriological investigations of an outbreak of plague in an urban tree squirrel population.* Am J Trop Med Hyg, 1971. 20(2): p. 255-63.

39. Chen, T.H. and K.F. Meyer, *Susceptibility and immune-response to experimental plague in 2 species of langurs and in African-green (Grivet) monkeys.* Journal of Infectious Diseases, 1974. 129: p. S46-S52.

40. Williams, J.E. and D.C. Cavanaugh, *Differential signs of plague in young and old California ground-squirrels (Spermophilus-beecheyi).* Journal of Wildlife Diseases, 1983. 19(2): p. 154-155.

41. Quan, T.J., et al., *Experimental plague in rock squirrels, Spermophilus-variegatus (erxleben).* Journal of Wildlife Diseases, 1985. 21(3): p. 205-210.

42. Shepherd, A.J., P.A. Leman, and D.E. Hummitzsch, *Experimental plague infection in South-African wild rodents.* Journal of Hygiene, 1986. 96(2): p. 171-183.

43. Thomas, R.E., et al., *Experimentally induced plague infection in the Northern grasshopper mouse (Onychomys-leucogaster) acquired by consumption of infected prey.* Journal of Wildlife Diseases, 1989. 25(4): p. 477-480.

44. Bosio, C.F., et al., *Kinetics of Innate Immune Response to Yersinia pestis after Intradermal Infection in a Mouse Model.* Infection and Immunity, 2012. 80(11): p. 4034-4045.

45. Demeure, C.E., et al., *Early Systemic Bacterial Dissemination and a Rapid Innate Immune Response Characterize Genetic Resistance to Plague of SEG Mice.* Journal of Infectious Diseases, 2012. 205(1): p. 134-143.

46. Weening, E.H., et al., *The Dependence of the Yersinia pestis Capsule on Pathogenesis Is Influenced by the Mouse Background.* Infection and Immunity, 2011. 79(2): p. 644-652.

47. Janssen, W.A. and M.J. Surgalla, *Plague bacillus: survival within host phagocytes.* Science, 1969. 163(3870): p. 950-2.

48. Cavanaugh, D.C. and R. Randall, *Role of multiplication of Pasteurella-pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague.* Journal of Immunology, 1959. 83(4): p. 348-363.

49. Hamrick, T.S., et al., *Host and bacterial factors involved in the innate ability of mouse macrophages to eliminate internalized unopsonized Escherichia coli.* Infection and Immunity, 2000. 68(1): p. 125-132.

50. Pujol, C. and J.B. Bliska, *The ability to replicate in macrophages is conserved between Yersinia pestis and Yersinia pseudotuberculosis.* Infection and Immunity, 2003. 71(10): p. 5892-5899.

51. Bergsbaken, T. and B.T. Cookson, *Macrophage activation redirects Yersinia-infected host cell death from apoptosis to caspase-1-dependent pyroptosis.* Plos Pathogens, 2007. 3(11): p. 1570-1582.

52. Pujol, C., et al., *Yersinia pestis Can Reside in Autophagosomes and Avoid Xenophagy in Murine Macrophages by Preventing Vacuole Acidification.* Infection and Immunity, 2009. 77(6): p. 2251-2261.

53. Webster, S.J., et al., *Distinct Cell Death Programs in Monocytes Regulate Innate Responses Following Challenge with Common Causes of Invasive Bacterial Disease.* Journal of Immunology, 2010. 185(5): p. 2968-2979.

54. Isin, Z.M. and B.M. Suleimenov, *Comparative investigation of PMN leucocyte antimicrobial potential in animals of different species susceptibility to the plague agent.* Journal of Hygiene Epidemiology, Microbiology, Immunology, 1987. 31(3): p. 307-312.

55. Guinet, F., et al., *Defective Innate Cell Response and Lymph Node Infiltration Specify Yersinia pestis Infection.* Plos One, 2008. 3(2).

56. Pujol, U. and J.B. Bliska, *Turning Yersinia pathogenesis outside in: subversion of macrophage function by intracellular yersiniae.* Clinical Immunology, 2005. 114(3): p. 216-226.

57. McMichael, M.A., *Oxidative stress, antioxidants, and assessment of oxidative stress in dogs and cats.* Javma-Journal of the American Veterinary Medical Association, 2007. 231(5): p. 714-+.

58. van Beusekom, C.D., J. Fink-Gremmels, and J.A. Schrickx, *Comparing the glucuronidation capacity of the feline liver with substrate-specific glucuronidation in dogs.* Journal of Veterinary Pharmacology and Therapeutics, 2014. 37(1): p. 18-24.

59. Dillon, A.R., et al., *Activity of pulmonary intravascular macrophages in cats and dogs with and without adult Dirofilaria immitis.* Veterinary Parasitology, 2008. 158(3): p. 171-176.

60. Haverson, K. and S. Riffault, *Antigen presenting cells in mucosal sites of veterinary species.* Veterinary Research, 2006. 37(3): p. 339-358.

61. Alvarez, B., et al., *Antigen targeting to APC: From mice to veterinary species.* Developmental and Comparative Immunology, 2013. 41(2): p. 153-163.

62. Zhang, X. and D.M. Mosser, *Macrophage activation by endogenous danger signals.* Journal of Pathology, 2008. 214(2): p. 161-178.

63. Schwan, W.R., et al., *Differential bacterial survival, replication, and apoptosis-inducing ability of Salmonella serovars within human and murine macrophages.* Infection and Immunity, 2000. 68(3): p. 1005-1013.

64. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nature Reviews Immunology, 2008. 8(12): p. 958-969.

65. Mosser, D.M. and X. Zhang, *Activation of murine macrophages.* Curr Protoc Immunol, 2008. Chapter 14: p. Unit 14.2.

66. Uppington, H., et al., *Effect of immune serum and role of individual Fc gamma receptors on the intracellular distribution and survival of Salmonella enterica serovar Typhimurium in murine macrophages.* Immunology, 2006. 119(2): p. 147-158.

67. Vodovotz, Y., *Translational systems biology of inflammation and healing.* Wound Repair and Regeneration, 2010. 18(1): p. 3-7.

68. Vodovotz, Y., et al., *Translational Systems Approaches to the Biology of Inflammation and Healing.* Immunopharmacology and Immunotoxicology, 2010. 32(2): p. 181-195.

69. Schneider, D.S. and J.S. Ayres, *Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases.* Nature Reviews Immunology, 2008. 8(11): p. 889-895.

70. Ayres, J.S. and D.S. Schneider, *Tolerance of Infections.* Annual Review of Immunology, Vol 30, 2012. 30: p. 271-294.

71. Warren, H.S., et al., *Resilience to Bacterial Infection: Difference between Species Could Be Due to Proteins in Serum.* Journal of Infectious Diseases, 2010. 201(2): p. 223-232.

72. Nham, T., et al., *Imaging of Bubonic Plague Dynamics by In Vivo Tracking of Bioluminescent Yersinia pestis.* Plos One, 2012. 7(4).

73. Gonzalez, R.J., et al., *Bioluminescence imaging to track bacterial dissemination of Yersinia pestis using different routes of infection in mice.* Bmc Microbiology, 2012. 12.

74. Janes, K.A. and C.C. Wang, *Bringing systems biology to cancer, immunology and infectious disease.* Genome Biology, 2014. 15(7).

75. Candia, J., et al., *From Cellular Characteristics to Disease Diagnosis: Uncovering Phenotypes with Supercells.* Plos Computational Biology, 2013. 9(9).

76. Herderschee, J., et al., *Emerging single-cell technologies in immunology.* Journal of Leukocyte Biology, 2015. 98(1): p. 23-32.

77. Li, B., et al., *Protein microarray for profiling antibody responses to Yersinia pestis live vaccine.* Infect Immun, 2005. 73(6): p. 3734-9.

78. Lawson, J.N., C.R. Lyons, and S.A. Johnston, *Expression profiling of Yersinia pestis during mouse pulmonary infection.* DNA and Cell Biology, 2006. 25(11): p. 608-616.

79. Das, R., et al., *Study of proinflammatory responses induced by Yersinia pestis in human monocytes using cDNA arrays.* Genes and Immunity, 2007. 8(4): p. 308-319.

80. Li, B., et al., *Antibody profiling in plague patients by protein microarray.* Microbes Infect, 2008. 10(1): p. 45-51.

81. Liu, H., et al., *Transcriptional profiling of a mice plague model: insights into interaction between Yersinia pestis and its host.* J Basic Microbiol, 2009. 49(1): p. 92-9.

82. Li, B., et al., *High-Throughput Identification of New Protective Antigens from a Yersinia pestis Live Vaccine by Enzyme-Linked Immunospot Assay.* Infection and Immunity, 2009. 77(10): p. 4356-4361.

83. Comer, J.E., et al., *Transcriptomic and Innate Immune Responses to Yersinia pestis in the Lymph Node during Bubonic Plague.* Infection and Immunity, 2010. 78(12): p. 5086-5098.

CHAPTER 5 - DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF ANTIBODIES AGAINST YERSINIA PESTIS IN WILD MAMMALS

SUMMARY

Plague remains endemic in the western United States resulting in approximately seven human cases a year. The causative agent, *Yersinia pestis*, is maintained in flea-rodent cycles that vary by geographic regions. The distribution of plague has routinely been monitored by regional serosurveillance of rodents and carnivores. Serological testing has historically been restricted to a small number of laboratories due to the limited availability of *Yersinia* specific reagents. Here we described the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies using recombinant proteins of known immunogenic antigens of *Yersinia pestis*. The assay was first developed to characterize isotypic immunoglobulin responses after experimental infection of coyotes with *Y. pestis*. The isotypic antibody detected using rF1-V antigen showed peak IgM production at day 10 postinfection with a duration of 42 days. In contrast, IgG production peaked at day 28 and persisted for greater than 84 days. The ELISA was then optimized for use with samples collected on nobuto filter paper strips, which are commonly used for serosurveillance of wildlife samples. Using recombinant F1-V, F1 and LcrV antigens, ELISA values were compared to passive hemagglutination (PHA) and sensitivity and specificity was determined to be 100% when testing serum from experimentally infected coyotes. However, sensitivity and specificity dropped significantly when the ELISA was applied to field samples. The addition of conjugated protein A/G to the ELISA platform proved useful in the development of a reliable multi-species serological assay for detection of anti-*Y. pestis* antibodies.

INTRODUCTION

Plague is a vector-borne disease that circulates worldwide in sylvatic cycles between rodents and their fleas. Zoonotic transmission in the United States is linked to epizootic outbreaks and peri-domestic exposures [1]. Approximately seven human cases are reported per year with a mortality rate of 14-16% [2, 3]. Canids and rodents have long been considered useful sentinels for the detection of endemic foci of plague [4-6]. Multiple animal-based plague surveillance programs conducted over the last century have been well documented [7-11]. These surveillance data has been useful in developing predictive models that mitigate impacts on human health [4]. Several climatic factors appear to be involved that can affect densities of the flea and rodent reservoirs [12-15]. Availability of serological testing has historically been limited to a few laboratories due to limited resources for the production of appropriate reagents and the classification of the causative agent, *Yersinia pestis*, as a category A pathogen and a federal select agent [16].

The current gold standard for serological evaluation of exposure to plague is the passive hemagglutination assay (PHA) [17]. This assay is designed to detect antibody produced against the major immunogenic antigen of *Y.pestis*, the fraction 1 capsular protein (F1). The assay utilizes a whole cell lysate or semi-purified F1 antigen preparation [18, 19] to sensitize sheep red blood cells, and the degree of agglutination of the red blood cells is used to quantify the amount of anti-F1 antibody that is present in samples being tested. The preparation of the F1 antigen has traditionally required the production of *Y. pestis* in large batch cultures. Variations in quantity and quality of this critical test reagent require extensive validation efforts for each batch and inhibit the ability to standardize the assay between laboratories. Upscaling for production of large quantities of antigen is difficult and limited to only a few laboratories due to the organism's federal select agent status. Large scale production of live organism also poses a significant risk of exposure to laboratory personnel generating these products.

There is a significant need for readily available methods for plague serological testing, specifically in wildlife samples collected for disease surveillance programs, vaccination field studies, and endangered species conservation projects. Multiple enzyme-linked immunosorbent assays (ELISA) have been described for use in human medicine [17, 20, 21] and wildlife surveillance programs [22] but have been limited by the availability of antigenic reagents. In general, most ELISA platforms have been found to improve assay sensitivity compared to agglutination tests such as PHA. With the recent development of recombinant protein technologies, ELISA development has become more commonplace for select agent assay development.

In addition, new conjugates have been developed. Protein A and protein G can be extracted from the cell walls of *Staphylococcus aureus* and *Streptococcus* group G, respectively. These proteins have been found to be Fc receptors for mammalian IgG. Recent technologies have resulted in the ability to produce recombinant proteins in the chimeric form, protein A/G. The high binding affinity of this chimera to the Fc region of IgG from numerous mammalian species without interfering with antigen specific binding sites has made it a remarkable immunochemical reagent [23].

The ELISA described here was evaluated using multiple antigens for the detection of antibodies to *Y. pestis* in experimentally infected coyotes (*Canis latrans*) and was shown to have high sensitivity and specificity. Recombinant proteins (rF1-V, rF1, and rLcrV) were used in an ELISA format modeled after existing platforms described in the literature [20, 24]. We also compared the use of protein A/G and anti-canine secondary antibodies to determine the utility of this assay in the screening of wildlife field samples.

MATERIALS AND METHODS

Experimental animals: Juvenile captive-reared coyotes (n=8) (USDA-APHIS-Wildlife Services, Predator Field Station, Millville, Utah, USA) were obtained at approximately four months of age and housed in animal biosafety level 3 isolation at the Animal Disease Laboratory (Colorado State University, Fort Collins, Colorado, USA). Before transfer, coyotes were vaccinated with standard canine vaccines (Vanguard®Plus5/CV and Defensor®3; Pfizer Animal Health, New York, New York, USA). Animals were housed individually in standard elevated dog runs (1.2 m x 2.1 m; 2.6 m^2), four runs per room, each with a manufactured polyvinyl chloride (PVC) den box (53 cm diameter x 56 cm height). Animals were provided water and commercial dry dog food *ad libitum* supplemented with dead mice, peanut butter, eggs, fruit, rawhide, long bones, or pig ears as daily environmental enrichment. Clinical evaluations were made twice daily on each animal throughout the study period to detect changes in behavior, food consumptions, fecal output, and activity level. All coyotes were treated with topical selamectin (Revolution®, Pfizer Animal Health, Madison, New Jersey, USA) at 6 mg/kg approximately four weeks prior to inoculation. Coyotes used in the inoculation trials were stratified by litter (three litters) and then randomized into two inoculation groups. One animal from each inoculation group was humanely euthanized at day 3 and day 7 post-inoculation. Control animals (n=2) were age-matched but maintained at the captive breeding facility in outdoor pens and euthanized at the termination of the study.

Bacteria: *Yersinia pestis* CO92 was obtained from the Centers for Disease Control and Prevention (CDC). Bacteria were cultivated from frozen stock, grown in brain heart infusion (BHI) broth at 28˚C for 48 hours, and diluted in phosphate buffered saline to the desired concentrations. The dose inoculated was determined by back titration of the inoculum by plating serial dilution on BHI agar plates. Confirmation of pigmentation locus in *Y. pestis* was made by demonstration of pigmented growth on Congo red agar [25].

Yersinia pestis challenges: At approximately 6 months of age, four animals were inoculated by intradermal (ID) injection (50 µL) bilaterally in the pre-scapular region with a target dose of 10^3 colony forming units (cfu) of *Y. pestis* CO92. The other four coyotes were offered one to three *Y. pestis*-infected mice for consumption. These 6-wk-old imprinting control region (ICR) mice were inoculated subcutaneously (SC) with 10^2 cfu of *Y. pestis* CO92 (50% lethal dose {LD_{50}} of approximately 20 cfu) in a 0.1 mL volume and mice were euthanized by cervical dislocation once moribund (approximately 48 hours PI). At 14 weeks PI, four coyotes from each inoculation group were re-challenged with *Y. pestis* CO92 using the same route and dosage as the original challenge. The coyotes were manually or chemically restrained for serial venous blood sample collection in gel serum separation tubes (Vacutainer®, Becton Dickson, Franklin Lakes, New Jersey, USA) on days 0, 5, 7, 10, 14, 21, 28, 42, 56, 98, 101,105, 108, 112, 119 and 126 PI. Whole blood samples (EDTA) were collected at termination of the study and 100 µL applied to saturate Nobuto filter strips (FP). FP were stored at -20˚C until analysis.

Free ranging wildlife samples: Archived samples from free-ranging coyotes were obtained from the USDA-APHIS-WS-National Wildlife Disease Program archives. During standard wildlife damage management operations, blood samples were collected on Nobuto filter paper strips (FP), dried and stored at -80ºC prior to analysis [7]. The FP are designed to collect 100 µL of whole blood yielding approximately 50 µL of serum when eluted. The elution of serum from FP was performed per manufacturer instructions. Briefly, the blood saturated portion of the FP was cut into 5-7 pieces and vortexed with 1.25 mL of phosphate buffered saline pH 7.4, containing 2.5 mg/mL casein (Sigma, St. Louis, Missouri, USA) (DBc). Following incubation for one hour at 37˚C, the eluates were mixed by vortexing and centrifuged at 5,000 x g for 5 minutes to remove particulate matter. Supernatants were transferred to a fresh tube for analysis. Serum samples from wild-caught swift fox (*Vulpes velox*) collected for research purposes were obtained from North Dakota State University. Serum samples from black-tailed prairie dogs (*Cynomys*

ludovicianus) collected for research purposes were obtained from Colorado Parks and Wildlife. Serum samples from black-footed ferrets (*Mustela nigripes*) were obtained from the US-FWS Black-footed Ferret Conservation Center. An additional 12 domestic ferret archived serum samples previously collected for research purposes were made available for evaluation as surrogate plague negative samples.

Antigens and antibodies: *Y. pestis* antigens used in this study were recombinant proteins purified from *Escherichia coli* expression. These included the F1-V fusion protein (rF1-V), F1 capsular antigen (rF1) and low calcium residue V antigen (rLcrV) (BEI Resources, Manassas, Virginia, USA). Horseradish peroxidase-conjugated antibodies used in this study were sheep anti-dog IgG heavy chain, goat anti-dog IgM, goat anti-dog IgA (Bethyl Laboratories, Montgomery, Texas, USA), and Protein A/G (ThermoFisher, Rockford, IL). Polyclonal antibodies against *Y. pestis* F1 (goat anti-*Y. pestis* F1) and LcrV (goat anti-*Y. pestis* LcrV) were used for positive controls (BEI Resources, Manassas, Virginia, USA).

Passive Hemagglutination: Titers of antibody reactivity to *Y. pestis* fraction 1 capsular protein (anti-F1) were determined by passive hemagglutination (PHA) based upon World Health Organization procedures as previously described [17]. Briefly, serum or FP eluates were absorbed for 30 minutes at ambient temperature with sheep red blood cells (RBC). After incubation, samples were centrifuged at 10,000 x g for 5 minutes and the supernatants were transferred to a fresh tube for analysis. Using a starting dilution of 1:16, supernatants were added to hemagglutination diluent containing F1-antigen sensitized sheep RBC. Agglutination reactions were evaluated to determine serological titer after overnight incubation at 4˚C.

ELISA - Isotypic antibodies: Checkerboard titration assays [26], were performed to optimize the parameters of the ELISA platform, including antigen concentrations, coating, blocking, dilution and wash buffers, secondary antibody concentrations, incubation times and

temperatures (Table 5.1). Optimal signal to noise ratios were obtained at 1:1,000 for anti-F1 and 1:2,000 for anti-LcrV against 2 µg/mL rF1-V antigen. The isotypic antibody profile was evaluated using an indirect ELISA modified from previously published methods [17, 20, 24]. Wash steps were performed in triplicate between each assay step detailed below using 300 µL volumes of phosphate buffered saline pH 7.4, containing 0.5% Tween 20 (PBSt) and a Nunc Immuno Washer (Thermo Scientific, Rockford, Illinois, USA). Antigen (rF1-V) was coated on flat bottom, 96 well polystyrene plates (Polysorp Nunc-Immuno plates, Nalgene Nunc International, Penfield, New York, USA) at 2 µg/mL in 0.1M carbonate buffer (pH 9.6) for 16-18 hours at 4ºC in 50 µl volume (wash). Serum samples were prepared by dilution in phosphate buffered saline pH 7.4, containing 2.5 mg/mL casein (Sigma, St. Louis, Missouri, USA) (DBc). Unless otherwise noted, 50 µL of serum samples diluted 1:1000 were assayed in triplicate and incubated for two hours at 37ºC in antigen coated wells. After washing, 50 µL volumes horseradish peroxidase-conjugated anti-dog IgG, anti-dog IgM or anti-dog IgA diluted in DBc at 1:1,000 (IgG), or 1:500 (IgM, IgA) were added to wells, incubated at 37ºC for one hour, and plates were washed again. Peroxidase substrate (ABTS® Peroxidase substrate, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Maryland, USA), was added (50 µL) and incubated at room temperature for 10 minutes followed by addition of 50 µL of stop solution (ABTS® peroxidase stop solution, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Maryland, USA). Optical density was measured at 410 nm (OD_{410}) using a DynaTech 5000 plate reader (DynaTech Laboratories, Chantilly, Virginia, USA). A positive antibody response was defined as an OD_{410} greater than the mean pre-infection value plus 3 standard deviations.

Table 5.1. ELISA Optimization - checkerboard titrations

ELISA buffer			
Wash	Phosphate base	Tris base	
Surfactant	± Tween-20	± Triton X100	
Coating	0.1 M Carbonate pH 9.6	0.05M Carbonate, pH 8.5	
	4˚C vs 25˚C	4˚C vs 25˚C	
Blocking	5% FBS	5% Milk	5% BSA
Dilution	PO_4 base w/ 2.5% casein	PO_4 base	Tris base + Triton
Antigen	F1-V	F1	LcrV
	0-20 µg/ml	0-10 µg/ml	0-10 µg/ml
Antibody	sheep anti-canine IgG	goat anti-canine IgM	Protein A/G
	1:100 to 1:2,000	1:500 to 1:2,000	1:1000 to 1:20,000
Plates	Polysorp[1]	Immulon[2]	CoStar[3]
Incubation	37˚C	25˚C	4˚C
	1-2 hours	1-2 hours	16-18 hours
Elutions	37˚C	25˚C	4˚C
	1-2 hours	16-18 hours	
	PO_4 buffer w/ 2.5% casein	PO_4 buffer	

[1] Nunc-Immuno plates, [2] Immulon 2HB, [3] Corning, untreated. FBS = fetal bovine serum, BSA = bovine serum albumin., PO_4 = Phosphate

ELISA – rF1-V, rF1, and rLcrV antigens: Optimization of ELISA reagents was repeated due to a change in laboratory and equipment, addition of Nobuto filter paper samples and a desire to conserve antigenic reagents. Antigens (rF1-V, rF1,and rLcrV) were coated on flat bottom, 96 well polystyrene plates (Polysorp Nunc-Immuno plates, Nalgene Nunc International, Penfield, New York, USA) at concentrations of 2 µg/mL, 4 µg/mL, 2 µg/mL, respectively in 0.1M carbonate buffer (pH 9.6) for 16-18 hours at 4ºC in 100µl volumes followed by a wash step. Serum or FP eluate samples (100µl volumes) were serially diluted in DBc (1:50 to 1:400) and incubated for 1 hour at 37ºC in antigen-coated wells followed by a wash step. Horseradish peroxidase-conjugated antibodies were diluted in DBc at 1:10,000 (protein A/G), 1:1,000 (anti-dog IgG) or 1:500 (anti-dog IgM) with 100µL volumes incubated at 37ºC for one hour followed by a wash step. Peroxidase substrate (ABTS® Peroxidase substrate, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Maryland, USA), was added at 50 µL volumes and incubated at room temperature for 10 minutes followed by 50 µl of stop solution (ABTS® peroxidase stop

solution, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Maryland, USA). Optical density was measured at 405 nm (OD_{405}) using a BioTek ELX800 plate reader (BioTek Instruments Inc, Winooski, Vermont, USA). Wash steps were performed in triplicate between each assay step detailed above (wash) using 300 µL volumes of PBSt and a BioTek ELX50 strip washer (BioTek Instruments Inc, Winooski, Vermont, USA).

The assay cut-off values for each antigen ELISA were calculated by using a population of negative control sera (8 pre-inoculation and 2 age-matched naïve coyotes) tested at least in triplicate over 10 runs. An additional 71 FP samples collected from free-ranging coyotes captured in geographic regions without plague were assayed for comparison. The frequency of the OD_{405} values for these two negative populations was normally distributed. Therefore the cut-off values for each antigen were calculated as the mean plus 3 standard deviations.

Statistical analysis: Statistical analysis was performed using GraphPad PRISM version 5. Means, and standard deviation were calculated and unless noted, *p* values of <0.05 were considered statistically significant.

RESULTS

ELISA Optimization: The first task in development of the ELISA was to confirm the binding capacity of anti-*Y. pestis* antibodies to rF1-V antigen. This was characterized by using serial dilutions of polyclonal goat anti-*Y. pestis* F1 and polyclonal goat anti-*Y. pestis* LcrV antibodies with protein A/G as the conjugate (Figure 5.1). The binding specificity of rF1 and rLcrV was also confirmed by western blot as previously described [27]. Optimal antigen coating concentrations varied between products and final antigen concentrations were based upon the OD_{410} values obtained with serum samples from experimentally infected coyotes in the optimal detection range (slope) and the least signal to noise ratio in blank wells and negative control samples. Of note, the addition of casein to the dilution buffer as previously described [20] was sufficient in decreasing non-specific binding, thus eliminating the need for a blocking step.

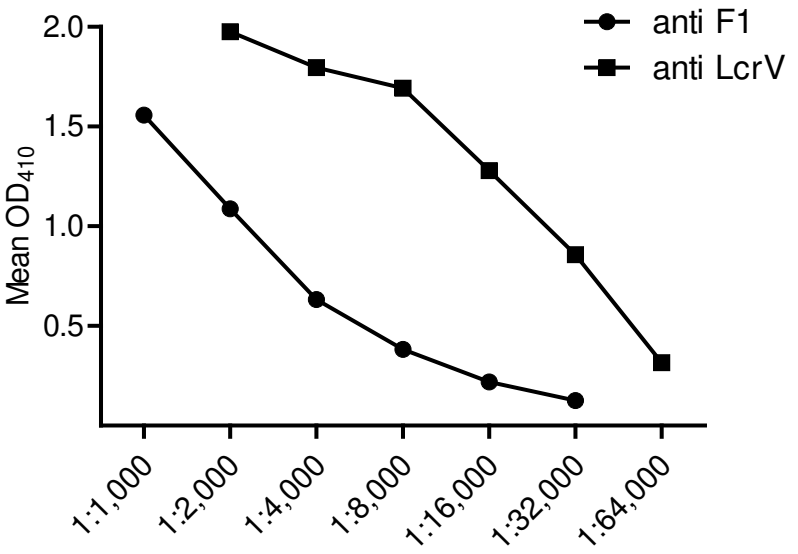


Figure 5.1 Characterization of polyclonal antibody responses to rF1-V antigen by ELISA. Values are expressed as the mean optical density reading 410 nm (OD_{410}) of serial dilutions run in duplicate for goat anti-*Y. pestis* F1 and goat anti-Y. pestis LcrV binding to rF1-V as detected by conjugated protein A/G.

Isotypic Antibody Response: The isotypic anti-*Y. pestis* immunoglobulin response in serum collected from experimentally infected coyotes (n=8) was measured by an indirect ELISA using rF1-V as the detection antigen (Figure 5.2). Assessment of IgA production was not successful due to high signal-to-noise ratios (measured as a comparison of OD_{410} of the positive samples to the OD_{410} for the negative samples). Increases in mean OD_{410} for IgM in the oral inoculation group were detected at day 5 PI (0.198 ± 0.010), peaked at day 14 PI (0.443 ± .186) followed by a return to baseline by day 56 PI (0.193 ± .061). There was no significant increase in IgM detected in the ID inoculation group (Figure 5.2.A). The earliest detection of IgG was noted at day 7 PI (0.212 ± 0.043) in the oral inoculation group, with peak levels noted at day 28 PI (0.800 ± 0.221) and a slight decline through day 84 PI (0.516 ± .243). The peak detection of IgG in the intradermal exposure group was noted at day 21PI (0.191 ± .09) and values returned to baseline levels by day 84 PI (0.146 ± 0.013) (Figure 5.2.B.).

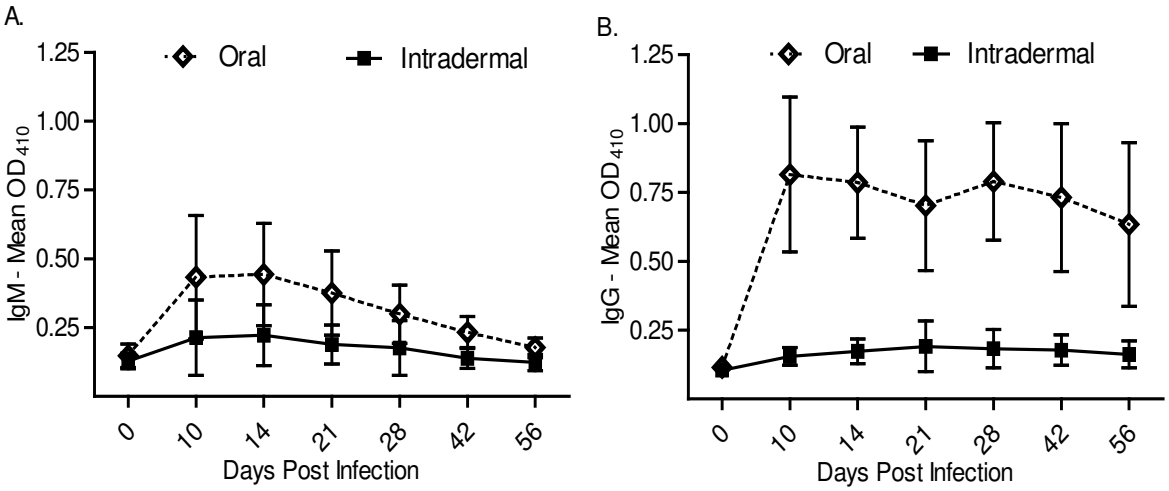


Figure 5.2. Isotypic immunoglobulin levels as measured by indirect ELISA against *Yersinia pestis* rF1-V antigen in serum collected from coyotes experimentally infected by oral and intradermal routes from days 0-56 post infection. Values for IgM (A) and IgG (B) are represented as the group mean optical density at 410 nm (OD_{410}) of samples run in duplicate. Error bars represent one standard deviation of values for the coyotes in each inoculation group (n=4 per group).

Assay Comparison: Anti-*Y. pestis* antibody titers were evaluated in serum samples from experimentally infected coyotes by PHA and ELISA (Figure 5.3). ELISA results were based upon antibody binding to rF1-V or rF1 antigens, with detection by conjugated protein A/G. The cut-off values were calculated as the mean OD_{405} value + 3 SD (0.207) using pre-infection (n=8) and naïve control (n=2) serum samples. ELISA titers were determined as the highest dilution with OD_{405} found to be above the cut-off value. All animals used in this study were found to be seronegative by both PHA and ELISA prior to inoculation with *Y. pestis*. All post-inoculation samples were found to be positive by both PHA and ELISA, thus these assays were found to be of equivalent sensitivity. However, there were differences in the amplitude and duration of antibody responses when comparing PHA to ELISA titers.

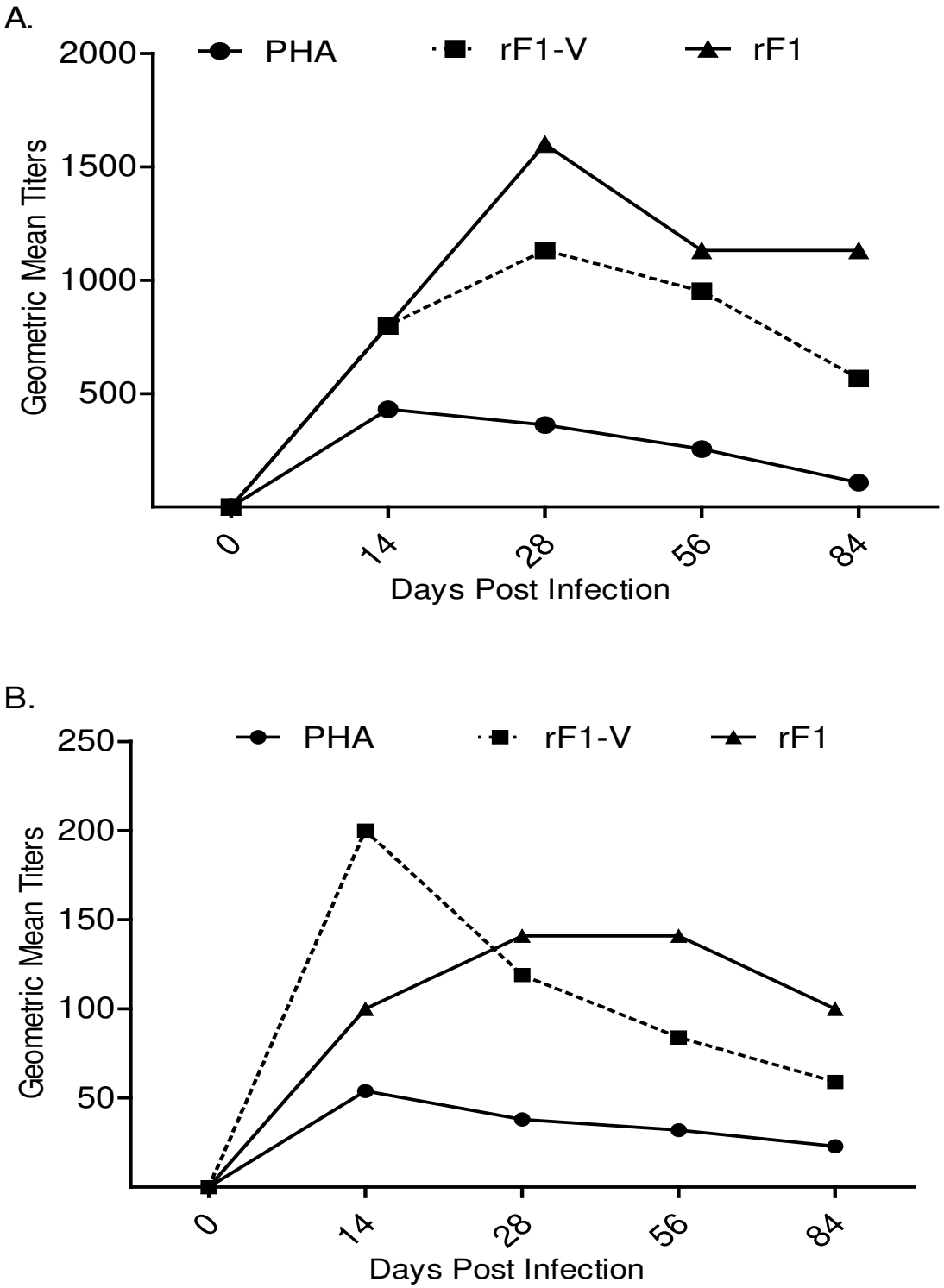


Figure 5.3. Geometric mean reciprocal anti-*Y.pestis* antibody titers as measured by passive hemagglutination assay (PHA) or ELISA (using rF1-V or rF1) for the coyotes experimentally infected with *Y.pestis* by oral (A) or intradermal (B) inoculation (n=4). Positive cutoff values are ≥ 1:16 for PHA and ≥ 1:50 for ELISA.

Free-ranging Coyote Samples: The assay was optimized for use with FP samples by evaluation of elution buffer solutions, incubation times and starting dilutions as noted in the methods section. Each sample was assayed in a row with 4 doubling dilutions (1:50-1:400) for each antigen (rF1-V, rF1, rLcrV). The cut-off values were calculated as the mean OD_{405} value + 3SD (0.207) using pre-infection (n=8) and naïve control (n=2) serum samples (Table 5.2). The ranges and cut-off values were found to be fairly similar between antigens. However, the OD_{405} values using FP samples were found to be higher than values obtained using serum samples from the same animals.

Table 5.2 Optical density (410 nm) values for negative control filter paper samples as detected by indirect enzyme-linked immunosorbent assay using recombinant *Y. pestis* antigens and detection by conjugated protein A/G.

Y. pestis Antigen	Min	Max	Mean	SD	Cut-off*
rF1-V	0.096	0.380	0.201	0.081	0.443
rF1	0.078	0.420	0.168	0.079	0.409
rLcrV	0.089	0.407	0.174	0.080	0.413

Samples run at 1:50 dilution, *Calculated as mean negative OD value + 3 SD for each antigen

Cross-reactivity was assessed using FP samples collected in geographically plague-negative areas. A total of 71 FP samples collected from free-ranging coyotes in West Virginia (68) and Kentucky (3) were assayed. All 71 samples were determined to be ELISA negative when tested against rF1. A total of four samples were found to be positive when tested against rF1-V with an OD_{405} range of 0.457-1.145. These same four samples were also found to be positive against LcrV antigen, suggesting possible exposure to enteric *Yersinia*. The mean OD_{405} for these probable negative field samples were found to be lower than the FP samples collected from experimental coyote samples (rF1-V 0.180 ± 0.161; rF1 0.088 ± 0.029; rLcrV 0.120 ± 0.63). Due to limited access to PHA testing of non-human samples, results were not available for statistical comparison to PHA for this group of samples.

An additional 198 FP samples that were collected from free-ranging coyotes in Colorado (22), Kansas (10), Montana (9), and New Mexico (157) were tested by PHA and ELISA. Of the 198 samples tested, 72 were determined to be negative and 126 were determined to be positive by PHA (≥1:16). The distribution of results for rF1-V and rF1 ELISAs are presented in Table 5.3. Of the 126 PHA positive samples, 82 were found to be positive by rF1-V ELISA and 44 were negative. Of the 72 PHA negative samples, 15 were rF1-V ELISA positive and 57 were negative. Of the 15 PHA negative/rF1-V positive samples, five were found to be rLcrV positive. These results indicate a sensitivity of 65% and specificity of 79% for the rF1-V ELISA when compared to the PHA results.

Table 5.3 Comparison of anti-*Y. pestis* antibodies detected in coyote Nobuto filter paper samples by passive hemagglutination assay (PHA) and enzyme-linked immunosorbent assay (ELISA) using rF1-V or rF1 antigens.

		PHA					PHA		
		positive	negative	totals			positive	negative	totals
ELISA rF1-V	positive	82	15	97	ELISA rF1	positive	64	11	75
	negative	44	57	101		negative	62	61	123
	totals	126	72	198		totals	126	72	198

Of the 126 PHA positive samples, 64 were found to be positive by rF1 ELISA and 62 were negative. Of the 72 PHA negative samples, 11 were rF1 ELISA positive and 61 were negative. These results indicate a sensitivity of 51% and specificity of 85% for the rF1 ELISA when compared PHA.

Species Range of Protein A/G: To evaluate the utility of conjugate protein A/G in a non-species-specific ELISA, samples were obtained from free-ranging swift fox (n=29), black-tailed prairie dogs (n=36) and captive-reared black-footed ferrets (n= 37). Unless otherwise noted, cut-off values were arbitrarily set at OD_{405} of 0.200 based upon data generated from the experimental coyote samples (See Chapter 3). Of 29 serum samples collected from free-ranging swift fox serum samples (NW South Dakota), one sample tested positive by ELISA-rF1. These results were subsequently confirmed by F1-Luminex plague assay [27]. The 36 serum

samples collected from prairie dogs were part of a plague vaccination trial in Colorado [28]. Of the 12 animals sampled in vaccine-baited colonies, 12 tested positive by ELISA-rF1 and ELISA-rLcrV. Of the 24 samples collected in placebo-baited colonies, 2 tested positive by ELISA-rF1 and ELISA-rLcrV. The 37 black-footed ferret serum samples were collected from captive-bred animals that were vaccinated with F1-V vaccine [24]. All 4 of the pre-vaccination samples tested negative for ELISA-rF1 and ELISA-rLcrV and the 33 post-vaccination samples tested positive by both assays. Cut-off values for these samples were determined based upon mean OD_{405} values +3SD calculated from a run of 12 domestic ferret serum samples run in duplicate (rF1 0.157; rLcrV 0.180).

DISCUSSION

Serological methods for plague diagnostics have historically been limited due to *Y. pestis*' federal select agent status. The PHA has remained the gold standard for serosurveillance of wildlife due to the lack of readily available or standardized reagents for ELISA [17]. In addition, agglutination assays continue to be a desirable format for serological testing for wildlife as commercially available species-specific reagents are often not available.

The ELISA described here was demonstrated to be an acceptable tool for the serological assessment of coyotes experimentally infected with *Y. pestis* and with sera collected at defined times post-infection. The rF1-V antigen, originally developed as a fusion protein for application in vaccine development [29], was found to be a satisfactory alternative for the detection of anti-*Y. pestis* antibodies showing comparable results to rF1 antigen in the same ELISA platform.

Results reported here for field samples were similar to previous analytical comparisons of PHA and ELISA [30, 31] where significant differences were noted in sensitivity between assays. Based upon these comparisons using samples with well characterized samples of known immunological responses (Figure 5.3), it does appear that PHA may be biased in the detection

of IgM [22, 32]. Further analysis of anti-*Y. pestis* isotypic immunoglobulins are therefore warranted to help characterize temporal evaluations in wildlife surveillance data.

Although the sensitivity of the ELISA was found to be low when applied to FP field samples, I suspect that immunological characterization of the discrepant samples would show that ELISA is more sensitive in the detection of IgG. In subsequent testing of free-ranging coyote FP samples using the F1 Luminex plague assay (LPA) and immunoblotting, the 16 PHA negative:LPA positive samples were subsequently confirmed as positive [27]. Other factors such as variations in saturation of FP strips during field collections, storage conditions and time to testing may have contributed to the discrepancies between assays noted here [33].

The recent development of the immunochemical reagent protein A/G has expanded the capabilities for the development of multi-species assay development. Per manufacturer instructions, protein A/G has been determined to have an affinity to detect IgG in human, mouse, rat, goat, rabbit, and sheep serum. In the present study, we established the utility of protein A/G for the detection of anti-*Y. pestis* antibodies in coyotes. While animal numbers are small and designated control samples were not readily available, we determined that protein A/G does bind to IgG from prairie dogs, ferrets, and fox. These results are similar to those found in the recently developed lateral flow assay used for the detection of anti-plague antibodies in the field [34]. There are additional descriptions of protein A/G use in serological assays for brucellosis (domestic and wild ungulates, swine, marine mammals, and polar bears) [35, 36], toxoplasmosis (ungulates, dogs, swine, felids, mice and marine mammals) [37, 38] and Lyme disease (ungulates, domestic dogs) [39]. Another study reported the ability to detect antibody responses to 160 species commonly found in zoos using protein A and protein G in separate Lyme disease assays [40] emphasizing the breadth of utility for these reagents.

Further evaluation of this ELISA format was curtailed due to limited availability of rF1 antigen and access to PHA comparisons. Efforts were diverted to the development of a high throughput assay where a larger number of samples could be tested with smaller amounts of antigen, as well as decreased handling times. The samples from the experimentally infected coyotes played a pivotal part in a successful comparison of PHA to the LPA [27]. The ultimate goal will be to have an assay developed that could detect IgM and IgG production against multiple pathogens of concern in the landscape where wildlife management efforts are occurring. Availability of these datasets will enable better determinations of temporal and spatial assessment of disease occurrence in wildlife and direct models use to evaluate human risk.

LITERATURE CITED

1. Gage, K.L., R.S. Ostfeld, and J.G. Olson, *Nonviral vector-borne zoonoses associated with mammals in the United-States.* Journal of Mammalogy, 1995. 76(3): p. 695-715.

2. Craven, R.B., et al., *Reported cases of human plague infections in the United-States, 1970-1991.* Journal of Medical Entomology, 1993. 30(4): p. 758-761.

3. Prevention, C.f.D.C.a., *Human plague - four states, 2006: Morbidity and Mortality Weekly Report*. 2006.

4. Gage, K.L., J.A. Montenieri, and R.E. Thomas, *The role of predators in the ecology, epidemiology, and surveillance of plague in the United-States.* Sixteenth Vertebrate Pest Conference, 1994: p. 200-206.

5. Barnes, A.M., *Surveillance and Control of Bubonic Plague in the United States*. Symposium Zoological Society of London. Vol. 50. 1982, London, England. 237-270.

6. Williams, J.E., *Use of ELISA to reveal rodent infections in plague surveillance and control programs.* Bulletin of the World Health Organization, 1990. 68(3): p. 341-345.

7. Bevins, S.N., et al., *Yersinia pestis: examining wildlife plague surveillance in China and the USA.* Integrative Zoology, 2012. 7(1): p. 99-109.

8. Brown, H.E., et al., *Annual seroprevalence of Yersinia pestis in coyotes as predictors of interannual variation in reports of human plague cases in Arizona, United States.* Vector Borne Zoonotic Dis, 2011. 11(11): p. 1439-46.

9. Lowell, J.L., et al., *Colorado animal-based plague surveillance systems: relationships between targeted animal species and prediction efficacy of areas at risk for humans.* J Vector Ecol, 2009. 34(1): p. 22-31.

10. Chomel, B.B., et al., *Serological surveillance of plague in dogs and cats,California, 1979-1991.* Comparative Immunology Microbiology and Infectious Diseases, 1994. 17(2): p. 111-123.

11. Archibald, W.S. and S.J. Kunitz, *Detection of plague by testing serums of dogs on the Navajo Reservation.* HSMHA Health Records, 1971. 86(April 1971): p. 377-380.

12. Nakazawa, Y., et al., *Climate change effects on plague and tularemia in the United States.* Vector Borne Zoonotic Dis, 2007. 7(4): p. 529-40.

13. Hall, R.J., L.M. Brown, and S. Altizer, *Modeling Vector-Borne Disease Risk in Migratory Animals Under Climate Change.* Integrative and Comparative Biology, 2016. 56(2): p. 353-364.

14. Enscore, R.E., et al., *Modeling relationships between climate and the frequency of human plague cases in the southwestern United States, 1960-1997.* Am J Trop Med Hyg, 2002. 66(2): p. 186-96.

15. Parmenter, R.R., et al., *Incidence of plague associated with increased winter-spring precipitation in New Mexico.* Am J Trop Med Hyg, 1999. 61(5): p. 814-21.

16. Oyston, P.C. and C. Davies, *Q fever: the neglected biothreat agent.* J Med Microbiol, 2011. 60(Pt 1): p. 9-21.

17. Chu, M.C., *Laboratory manual of plague diagnostic tests*. 2000, Atlanta, GA: Centers for Disease Control and Prevention.

18. Chen, T.H. and K.F. Meyer, *An evaluation of Pasteurella pestis fraction-1-specific antibody for confirmation of plague infections.* Bulletin of the World Health Organization, 1966. 34(6): p. 911-&.

19. Baker, E.E., et al., *Studies on immunization against plague .1. The isolation and characterization of the soluble antigen of Pasteurella-pestis.* Journal of Immunology, 1952. 68(2): p. 131-145.

20. Lindsey, C.Y., et al., *Validation of quantitative elisas for measuring anti-Yersinia pestis F1 and V antibody concentrations in nonhuman primate sera.* Journal of Immunoassay & Immunochemistry, 2012. 33(1): p. 91-113.

21. Neubauer, H., et al., *Serodiagnosis of human plague by an anti-F1 capsular antigen specific IgG/IgM ELISA and immunoblot.* Epidemiology and Infection, 2000. 125(3): p. 593-597.

22. Willeberg, P.W., et al., *Epidemiologic survey of sylvatic plague by serotesting coyote sentinels with enzyme immunoassay.* Am J Epidemiol, 1979. 110(3): p. 328-34.

23. Eliasson, M., et al., *Chimeric IgG-binding receptors engineered from Staphylococcal protein-a and Streptococcal protein-G.* Journal of Biological Chemistry, 1988. 263(9): p. 4323-4327.

24. Rocke, T.E., et al., *Recombinant F1-V fusion protein protects black-footed ferrets (Mustela nigripes) against virulent Yersinia pestis infection.* Journal of Zoo and Wildlife Medicine, 2004. 35(2): p. 142-146.

25. Surgalla, M.J. and E.D. Beesley, *Congo red-agar plating medium for detecting pigmentation in Pasteurella pestis.* Appl Microbiol, 1969. 18(5): p. 834-7.

26. Crowther, J.R. and J.R. Crowther, *The ELISA Guidebook*. 2.. ed. 2009, Totowa, NJ,New York, NY.: Humana Press.

27. Chandler, J.C., et al., *A Bead-Based Flow Cytometric Assay for Monitoring Yersinia pestis Exposure in Wildlife.* J Clin Microbiol, 2018. 56(7).

28. Rocke, T.E., et al., *Sylvatic Plague Vaccine Partially Protects Prairie Dogs (Cynomys spp.) in Field Trials.* Ecohealth, 2017. 14(3): p. 438-450.

29. Heath, D.G., et al., *Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine.* Vaccine, 1998. 16(11-12): p. 1131-1137.

30. Williams, J.E., et al., *Comparison of passive hemagglutination and enzyme-linked immunosorbent-assay for serodiagnosis of plague.* Bulletin of the World Health Organization, 1982. 60(5): p. 777-781.

31. Shepherd, A.J., et al., *A comparison of serological techniques for plague surveillance.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1984. 78(6): p. 771-773.

32. Greenbury, C.L., D.H. Moore, and L.A.C. Nunn, *Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells.* Immunology, 1963. 6(5): p. 421-&.

33. Bevins, S., et al., *Effect of storage time and storage conditions on antibody detection in blood samples collected on filter paper.* J Wildl Dis, 2016. 52(3): p. 478-83.

34. Abbott, R.C., et al., *A rapid field test for sylvatic plague exposure in wild animals.* J Wildl Dis, 2014. 50(2): p. 384-8.

35. Nielsen, K., et al., *Validation of a second generation competitive enzyme immunoassay (CELISA) for the diagnosis of brucellosis in various species of domestic animals.* Veterinary Immunology and Immunopathology, 2008. 125(3-4): p. 246-250.

36. Nymo, I.H., et al., *A protein A/G indirect enzyme-linked immunosorbent assay for the detection of anti-Brucella antibodies in Arctic wildlife.* Journal of Veterinary Diagnostic Investigation, 2013. 25(3): p. 369-375.

37. Schaefer, J.J., et al., *Chimeric protein A/G conjugate for detection of anti-Toxoplasma gondii immunoglobulin G in multiple animal species.* Journal of Veterinary Diagnostic Investigation, 2012. 24(3): p. 572-575.

38. Al-Adhami, B.H. and A.A. Gajadhar, *A new multi-host species indirect ELISA using protein A/G conjugate for detection of anti-Toxoplasma gondii IgG antibodies with comparison to ELISA-IgG, agglutination assay and Western blot.* Veterinary Parasitology, 2014. 200(1-2): p. 66-73.

39. Bhide, M.R., et al., *Protein A/G dependent ELISA a promising diagnostic tool in Lyme disease seroprevalence in game animals and hunting dogs.* Comparative Immunology Microbiology and Infectious Diseases, 2004. 27(3): p. 191-199.

40. Stobel, K., A. Schonberg, and C. Staak, *A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s. l. in zoo animals.* International Journal of Medical Microbiology, 2002. 291: p. 88-99.

CHAPTER 6: SUMMARY AND CONCLUSIONS

SUMMARY OF WORK

The primary goal of the work described here was to characterize the serological response of coyotes through the course of a well characterized experimental infection with *Y. pestis* in order to inform serosurveillance efforts. Detection of antibodies against *Y. pestis* has been a staple of plague surveillance programs since the 1920s. The data obtained from the experimental infection trial were deemed necessary for the temporal and spatial evaluation of serosurveillance data collected from free-ranging coyotes by the USDA-APHIS-WS-National Wildlife Disease Program. The key component of these testing methods has been targeting antibody production against the capsular antigen F1 by passive hemagglutination assay (PHA).

As variability of susceptibility in canid species has been reported but poorly characterized, a secondary goat, as described in Chapter 2, was to evaluate the clinical and microbiological responses of juvenile captive-reared coyotes after inoculation of *Y. pestis* by both oral and intradermal routes of exposure. Little to no clinical responses were noted and histological evaluation of tissues collected at day 3 post-inoculation showed minimal inflammation indicating rapid clearance of organism and lack of establishment of characteristic lymphotropic changes noted in other mammalian species. As described in Chapter 3, I also characterized the immunological responses of these same animals using assays to evaluate the acute phase response of the inflammatory response as well as the antibody profile produced by the humoral immune response to inoculation with *Y. pestis*. Acute phase protein (CRP and SAA) responses were significant but short-lived, again indicating rapid clearance and a limited inflammatory response to infection. Pre-infection antibodies against the *Yersinia* T3SS components were detected and may indicate a cross-protection mechanism with an anamnestic response presumably due to previous exposure to enteric *Yersinia* species. Further evaluation of this

possible mechanism of resistance to development of clinical disease is warranted as similar antibody profiles have been detected in free-ranging coyotes with uncharacterized exposures to *Y. pestis* [1].

Plague serological testing has been limited to a few laboratories due to the microbiological techniques and biosafety requirements necessary to create *Y. pestis*-specific reagents. Due to these limitations, there is an ongoing and significant need for serological testing for samples collected from wildlife species. As such, Chapter 5 describes the development of an ELISA using recombinant *Y. pestis* immunogenic proteins (rF1, rF1-V, rLcrV) to characterize and quantify antibody titers for in-house testing of wildlife samples. A comparison of IgG and IgM antibody levels indicates that PHA may be biased in the detection of IgM compared to ELISA. The ELISA was further modified to create a multi-species assay using the chimeric conjugate protein A/G. The addition of this conjugate will expand the utility of this assay to cover testing of most wildlife species involved in the ecological niche of plague.

In my early review of the pathobiology of *Y. pestis*, I had identified several mechanisms where species differences in biological function might be contributing to the differences in severity of disease between species. Highest on my list was to determine if there were innate differences in the host's ability to subvert Y. pestis' capability to control host macrophage functions allowing intracellular survival and replication within macrophages. Preliminary experiments were performed to see if there were differences in macrophage phagocytic uptake of *Y. pestis* and intracellular survival and replication between species in cell culture. Unfortunately, the experimental design was limited by the availability of equipment in the BSL3 laboratory resulting in limited critical troubleshooting mechanisms. The study was also hindered in its assessment of the various cells in culture due to poor cell proliferation. However, based upon the results outlined in chapter 4 and the work of others [2, 3], the use of macrophage-like cell lines holds promise for further work. I recommend that similar assays be conducted with

PBDM, BMDM and splenic macrophages collected from infected animals during the first 72 hours of infection. I suspect these cells would likely yield some insightful results in evaluation of the differences among species in susceptibility to *Y. pestis*.

FUTURE DIRECTIONS

Based upon the identification of potential cross-protective antibodies in our experimental animals, it would be important to investigate their role in the natural infection of coyotes. To determine the role that these antibodies pay in the possible acquired resistance, experimental inoculations should be repeated using naïve coyotes. These experiments should be proceeded by screening free-ranging coyotes for antibodies against all known *Y. pestis* virulence effectors in both plague-free and plague-endemic areas. This effort could easily be accomplished using existing surveillance samples.

As highlighted in various rodent models, the pathobiology in response to *Y. pestis* exposure appears to be determined by the immunological events that occur during the first 48 hours post-exposure [4, 5]. My study, as well as the experimental infection of dogs [6], missed that window for evaluation as the first animal was euthanized at 72 hours post exposure. As such, it would be informative to repeat experimental infection trials where serial evaluation of the *in vivo* responses at the inoculation site, draining lymph nodes and lymphotropic organs such as spleen and liver were conducted. Sampling should consist of blood components, lymph nodes and organ suspensions to detail specific reactions and time points in species with low (canid), moderate (felid) and high susceptibility (prairie dog) to development of bubonic plague. Using current technologies in imaging, mass cytometry, cytokine and chemokine production, and antigen profiling on multiple species of varying susceptibility, we can expand our knowledge of this organism's manifestation of virulence factors to generate a spectrum of clinical disease. These data would be informative in the development of novel therapeutic modalities as well as vaccine development.

LITERATURE CITED

1. Vernati, G., et al., *Antigenic profiling of yersinia pestis infection in the Wyoming coyote (Canis latrans).* J Wildl Dis, 2011. **47**(1): p. 21-9.

2. Ponnusamy, D. and K.D. Clinkenbeard, *Yersinia pestis Intracellular Parasitism of Macrophages from Hosts Exhibiting High and Low Severity of Plague.* Plos One, 2012. **7**(7).

3. Bashaw, J., et al., *Development of in vitro correlate assays of immunity to infection with Yersinia pestis.* Clinical and Vaccine Immunology, 2007. **14**(5): p. 605-616.

4. Sebbane, F., et al., *Kinetics of disease progression and host response in a rat model of bubonic plague.* American Journal of Pathology, 2005. **166**(5): p. 1427-1439.

5. Bosio, C.F., et al., *Kinetics of Innate Immune Response to Yersinia pestis after Intradermal Infection in a Mouse Model.* Infection and Immunity, 2012. **80**(11): p. 4034-4045.

6. Rust, J.H., et al., *The role of domestic animals in the epidemiology of plague. I. Experimental infection of dogs and cats.* J Infect Dis, 1971. **124**(5): p. 522-6.

CHAPTER 7: COLLABORATIVE WORK

There is limited availability of serological testing methods for detection of *Y. pestis* from serum samples routinely collected from various wildlife species. As such, generation of the a series of diagnostic samples from representative animals exposed to organism under controlled experimental conditions proved to be an important endeavor for development of future testing modalities. The references below outline the additional work that was done by myself and others using serum samples generated from the experimental trails in coyotes outlined in Chapter 2 or the ELISA developed in Chapter 5.

COLLABORATIVE STUDIES - *YERSINIA*

Title: A rapid field test for sylvatic plague exposure in wild animals.

Abstract: Plague surveillance is routinely conducted to predict future epizootics in wildlife and exposure risk for humans. The common surveillance method for sylvatic plague is detection of antibodies to *Yersinia pestis* F1 capsular antigen in sentinel animals, such as coyotes (*Canis latrans*). Current serologic tests for *Y. pestis*, hemagglutination (HA) test and enzyme-linked immunosorbent assay (ELISA), are expensive and labor intensive. To address this need, we developed a complete lateral flow device for the detection of specific antibodies to *Y. pestis* F1 and V antigens. Our test detected anti-F1 and anti-V antibodies in serum and Nobuto filter paper samples from coyotes, and in serum samples from prairie dogs (*Cynomys ludovicianus*), lynx (*Lynx canadensis*), and black-footed ferrets (*Mustela nigripes*). Comparison of cassette results for anti-F1 and anti-V antibodies with results of ELISA and HA tests showed correlations ranging from 0.68 to 0.98. This device provides an affordable, user-friendly tool that may be useful in plague surveillance programs and as a research tool.

Contributions: My main contribution to this study was the creation of the coyote serum samples used in validation of the assay. I performed off site test assay performance and assisted with test signal evaluations, and manuscript preparation and review.

Reference: Abbot RC, Hudak R, Mondesire R, Baeten, LA, Russell RE, Rocke TE. A rapid field test for sylvatic plague exposure in wild animals. Journal of Wildlife Diseases. 2014: Apr; 50(2): 384-388.

Title: Effect of storage time and storage conditions on antibody detection in blood samples collected on filter paper.

Abstract: Using filter paper to collect blood from wildlife for antibody analysis can be a powerful technique to simplify the collection, transport, and storage of blood samples. Despite these advantages, there are limited data that detail how long these samples can be stored and how storage conditions affect antibody longevity. We used blood samples collected on filter paper from coyotes experimentally infected with *Yersinia pestis* to determine optimum sample storage conditions over time. Blood samples collected on filter paper were stored for 454 d or more in four groups: 1) at ambient temperature and at ambient relative humidity, 2) at ambient temperature with desiccant, 3) at 4˚C with desiccant, and 4) at -20˚C with desiccant. Samples stored at 4˚C or -20˚C with desiccant had detectable antibody for a longer period of time than the samples stored at room temperature.

Contributions: My main contribution to this study was the creation of the Nobuto filter paper samples used in validation of the assay. Whole blood samples from coyotes at multiple time points post-infection were created and transferred to the collaborators for storage and testing. In addition, I used stored Nobuto filter paper strips from NWDP surveillance efforts in the ELISA development project which offered insight into the quality of the field samples over time. I assisted with the manuscript preparation and review.

Reference: Bevins SN, Pappert R, Young J, Schmit B, Kohler D, Baeten LA. Effect of storage time and storage conditions on antibody detection in blood samples collected on filter paper. Journal of Wildlife Diseases. 2016. Jul;52(3);478-483.

Title: A bead-based flow cytometric assay for monitoring Yersinia pestis exposure in wildlife.

Abstract: *Yersinia pestis* is the causative agent of plague and is considered a category A priority pathogen due to its potential for high transmissibility and the significant morbidity and mortality is causes in humans. *Y. pestis* is endemic to the western United States and much of the world, necessitating programs to monitor for this pathogen on the landscape. Elevated human risk of plague infection has been spatially correlated with spikes in seropositive wildlife numbers, particularly rodent-eating carnivores, which are frequently in contact with the enzootic hosts and the associated arthropod vectors of *Y. pestis*. In this this study, we describe a semiautomated bead-based flow cytometric assay developed for plague monitoring in wildlife called the F1 Luminex plague assay (F1-LPA). Based up upon Luminex/Bio-Plex technology, the F1-LPA targets serological responses to the F1 capsular antigen of *Y.pestis* and was optimized to analyze antibodies eluted from wildlife blood samples preserved on Nobuto filter paper strips. In comparative evaluations with passive hemagglutination, the gold standard tool for wildlife plague serodiagnosis, the F1-LPA demonstrated as much as 64x improvement in analytical sensitivity for F1-specific IgG detection and allowed for unambiguous classification of IgG status. The functionality of the F1-LPA was demonstrated for coyotes and other canids, which are the primary sentinels in wildlife plague monitoring, as well as felids and raccoons. Additionally, assay formats that do not require species-specific immunological reagents, which are not routinely available for several wildlife species used in plague monitoring, were determined to be functional in the F1-LPA.

Contribution: My main contribution to this study was the creation of the serum and some of the Nobuto filter paper samples used in development and validation of the F1-LPA assay. I provided

consultation and initial reagents used to develop the assay. I provided data used for the validation of the assay (PHA and ELISA). I also helped with interpretation of validation data, manuscript preparation and review.

Reference: Chandler JC, Baeten LA, Griffin DL, Gidlewski T, DeLiberto TJ, Petersen JM, Pappert R, Young JW, Bevins, SN. A bead-based flow cytometric assay for monitoring Yersinia pestis exposure in wildlife. Journal of Clinical Microbiology. 2018: 56(7).

RAPIDD workshop – Translating serology data to disease dynamics

I was a presenter and participant in the workshop. Data from the experimental infection of coyotes with *Yersinia pestis* is being used in modeling exercises that have come out of the workshop. Proposed publication: Inferring disease prevalence from wildlife serosurveillance data.

Serological support for plague related projects

I contributed to the master's dissertations for two students at the University of South Dakota by providing the serological data for swift fox studies in the Dakotas. Both projects, I ran the ELISA that I developed (see chapter 5) to detect antibodies against *Yersinia pestis* in swift fox samples. I also provided consultation and assisted with the diagnostic testing for distemper and parvovirus in one of the studies.

Mitchell, E. (2018). Distribution, ecology, disease risk, and genetic diversity of swift fox (Vulpes velox) in the Dakotas. MS Dissertation University of South Dakota

Nevins, S. (2019). MS Dissertation University of South Dakota (title not available)

I contributed to the master's dissertations for one student at Colorado State University. I was responsible for training the student to develop an ELISA for detecting antibodies against plague in the student's vaccine development project for black-footed ferrets. In addition, I ran plague

serology on wild prairie dogs samples for related studies at the Colorado Parks & Wildlife and black-footed ferret samples for the USFWS Black-footed Ferret Conservation Center.

McCuen, D.G. (2019) MS Dissertation Colorado State University (title not available)

COLLABORATIVE STUDIES – *COXIELLA*

Due to logistical issues in between *Yersinia* experiments, I became involved with a colleague studying the detection of *Coxiella* in marine mammals. These efforts subsequently led to my involvement in additional research efforts in our lab associated with the a DTRA funded grant, in collaboration with the Vaccine and Immunotherapy Center at Massachusetts General Hospital (MGH) and collaborating institutions including EpiVax, Innatoss, Harvard University, and Oxford University. The following is a summary of my contributions to these research projects. These efforts as they contributed significantly to the development of my scholarly knowledge and immunological expertise.

Characterization of non-pregnant ruminant model for *Coxiella burnetii.*

IDSC grant from Colorado State University: June 2013 - September 2014.
Contributions: I was lead investigator, in collaboration with Dr. Colleen Duncan on this project to determine if non-pregnant ruminants would be an effective model for the study of *Coxiella* pathogenesis. Grant summary and manuscript prepared but manuscript not submitted due to the need for experimental follow-up.

Q-VaxCelerate: Development of a T-cell based vaccine for Q-fever

Contributions: I was the lead investigator for all of the mouse and guinea pig experiments done in the BSL3 laboratories at Colorado State University (CSU) used in the identification of peptide epitopes that could be used in the development of the T-cell based vaccine. This involved experimental design, CyTOF assay development, diagnostic testing, and data analysis experiments performed in mice and guinea pigs infected with *Coxiella burnetti*.

Four publications have been developed covering the activities to date. The first publication highlights the Q-VaxCelerate working group approach to the development of the *Coxiella* vaccine candidates. The three other publications review the animal model development and epitope selection process. The grant will be completed with validation of Adeno-vector vaccine candidates in mouse and guinea pig models to be completed at CSU and a non-human primate immunogenicity trial at MGH in the Spring of 2019.

Title: Q-VaxCelerate: A distributed development approach for a new *Coxiella burnetii* vaccine.

Abstract: Development of vaccines that are both safe and effective remains a costly and time-consuming challenge. To accelerate the pace of development and improve the efficacy and safety of candidate vaccines for both existing and emerging infectious agents, we have used a distributed development approach. This features the managed integration of individual expert groups having the requisite vaccine platforms, pre-clinical models, assays, skills and knowledge pertinent to a specific pathogen into a single, end-to-end development team capable of producing a new vaccine tailored to that particular agent. Distributed development focuses on integrating existing effort across multiple institutions rather than developing new capabilities or consolidating resources within an individual organization. Previously we have used the distributed development strategy to generate vaccine candidates for emerging viral diseases. *Coxiella burnetii* is a highly infectious and resilient bacterium and the causative agent of Q fever. Treatment for Q fever can require months of antibiotics. The current vaccine for Q-fever is only approved in Australia and requires prescreening due to the potential for severe reactogenicity in previously exposed individuals. Here we discuss Q-VaxCelerate, a distributed development consortium for the development of a new vaccine to prevent Q fever.

Reference: Reeves PM[1], Paul SR[1], Sluder AE[1], Brauns TA[1], Poznansky MC[1]. Hum Vaccin Immunother. 2017 Dec 2;13(12):2977-2981. doi: 10.1080/21645515.2017.1371377. Epub 2017 Sep 21

Title: Standardized guinea pig model for Q fever vaccine reactogenicity.

Abstract: Historically, vaccination with *Coxiella burnetii* whole cell vaccines has induced hypersensitivity reactions in humans and animals that have had prior exposure to the pathogen as a result of infection or vaccination. Intradermal skin testing is routinely used to evaluate exposure in humans, and guinea pig hypersensitivity models have been developed to characterize the potential for reactogenicity in vaccine candidates. Here we describe a refinement of the guinea pig model using an alternate vaccine for positive controls. An initial comparative study used viable *C. burnetii* to compare the routes of sensitizing exposure of guinea pigs (intranasal vs intraperitoneal), evaluation of two time points for antigen challenge (21 and 42 days) and an assessment of two routes (intradermal and subcutaneous) of challenge using the ruminant vaccine Coxevac as the antigenic control. Animals sensitized by intraperitoneal exposure exhibited slightly larger gross reactions than did those sensitized by intranasal exposure, and reactions were more pronounced when skin challenge was performed at 42 days compared to 21 days post-sensitization. The intradermal route proved to be the optimal route of reactogenicity challenge. Histopathological changes at injection sites were similar to those previously reported and a scoring system was developed to compare reactions between groups receiving vaccine by intradermal versus subcutaneous routes. Based on the comparative study, a standardized protocol for assessment of vaccine reactogenicity in intranasally-sensitized animals was tested in a larger confirmatory study. Results suggest that screens utilizing a group size of n = 3 would achieve 90% power for detecting exposure-related reactogenic responses of the magnitude induced by Coxevac using either of two outcome measures.

Reference: Baeten LA, Podell BK, Sluder AE, Garritsen A, Bowen RA, Poznansky MC. PLoS One. 2018 Oct 12;13(10):e0205882. doi: 10.1371/journal.pone.0205882. eCollection 2018.

Title: Promiscuous *Coxiella burnetii* CD4 Epitope Clusters Associated with Human Recall Responses Are Candidates for a Novel T-cell Targeted Multi-Epitope Q-fever Vaccine

Abstract: *Coxiella burnetii*, the causative agent of Q fever, is a Gram-negative intracellular bacterium transmitted via aerosol. Regulatory approval of the Australian whole-cell vaccine Q-VAX® in the US and Europe is hindered by reactogenicity in previously exposed individuals. The aim of this study was to identify and rationally select *C. burnetii* epitopes for design of a safe, effective and less reactogenic T-cell targeted human Q fever vaccine. Immunoinformatic methods were used to predict 65 HLA class I epitopes and 50 promiscuous HLA class II *C. burnetii* epitope clusters, which are conserved across strains of *C. burnetii*. HLA binding assays confirmed 89% of class I and 75% of class II predictions, and 11 HLA class II epitopes elicited IFNγ responses following heterologous DNA/DNA/peptide/peptide prime-boost immunizations of HLA-DR3 transgenic mice. Human immune responses to the predicted epitopes were characterized in individuals naturally exposed to *C. burnetii* during the 2007-2010 Dutch Q fever outbreak. Subjects were divided into three groups: controls with no immunological evidence of previous infection and individuals with responses to heat-killed *C. burnetii* in a whole blood IFNγ release assay (IGRA) who remained asymptomatic or who experienced clinical Q-fever during the outbreak. Recall responses to *C. burnetii* epitopes were assessed by cultured IFNγ ELISpot. While HLA class I epitope responses were sparse in this cohort, we identified 21 HLA class II epitopes that recalled T-cell IFNγ responses in 10-28% of IGRA+ subjects. IGRA+ individuals with past asymptomatic and symptomatic *C. burnetii* infection showed a comparable response pattern and cumulative peptide response which correlated with IGRA responses. None of the peptides elicited reactogenicity in a *C. burnetii* exposure-primed guinea pig model. These data demonstrate that a substantial proportion of immunoinformatically identified HLA class II epitopes show long-lived immunoreactivity in naturally infected individuals, making them desirable candidates for a novel human multi-epitope Q fever vaccine.

Reference: Anja Scholzen, Guilhem Richard, Leonard Moise, Laurie A Baeten, Patrick M Reeves, William D Martin, Timothy A Brauns, Christine M Boyle, Susan Raju Paul, Richard Bucala, Richard A Bowen, Anja Garritsen, Anne Searls De Groot, Ann E Sluder, Mark C Poznansky. Front. Immunol. 15 February 2019 | https://doi.org/10.3389/fimmu.2019.00207.

Title: Immune correlates of *Coxiella burnetii* vaccination and challenge identified by longitudinal deep profiling.

Abstract: Background: *Coxiella burnetii (Cb)* is a highly infectious and resilient intracellular bacterium that causes Q fever and is classified as a Category B pathogen. The current *Cb* vaccine is approved in Australia alone and has exhibited significant reactogenicity in some individuals who have been previously exposed to the bacterium. As part of the Q-VaxCelerate consortium project to develop a new effective and less reactogenic Q fever vaccine, we interrogated the breadth of the inflammatory and immune response to *Cb* in murine models of infection and vaccination. We used mass cytometry in conjunction with computational approaches to identify novel immune correlates of vaccination and challenge in this context.

Methods: To assess the immune response to *Cb* in transgenic mice expressing human HLA-DR3, blood samples were collected longitudinally in a study of vaccination and challenge with *Cb*. Samples from two independent replicate studies were analyzed by CyTOF along with an assessment of splenomegaly, splenic bacterial load and organ pathology at sacrifice 10 days post challenge. Computational tools were used to define and identify immune cell populations conserved between two independent replicate experiments were generated for each time point and replicate experiment independently. For each time point, immune populations conserved between replicate experiments were identified, manually validated, and assessed for their differential abundance and correlation to quantitative phenotypic measures.

Results: Vaccination with inactivated whole-cell *Cb* induced antibody production, and cellular responses in NK, T-cell, and B-cell populations. Following challenge, vaccinated mice exhibit

reduced inflammatory responses in both innate and adaptive immune cell populations as compared to unvaccinated mice. Vaccinated mice were further distinguished by reduced splenic bacterial burden and splenomegaly, as well as enhanced expression of Ly6C, CD73, and Tbet in multiple cell populations and expanded B-cell diversity following challenge.

Conclusion: Analysis of murine data has provided insights into the broad scope of the immune response to *Cb* vaccination and challenge. Here we provide a detailed description of the immune response to *Cb* vaccination and infection. We demonstrate a novel method for identification of shared immune populations in replicate time course studies. Analysis of conserved immune populations can discriminate between treatment groups following vaccination and challenge, which in turn define immunologic signatures of vaccination and resistance to infection. Insights gained from these studies will inform the design and assessment of candidate vaccines for *Cb.*

Reference: Patrick M Reeves, Susan Raju Paul, Laurie A Baeten, Guilhem Richard, Leonard Moise, Richard A Bowen, Anja Scholzen, Anja Garritsen, Anne Searls De Groot, Ann E Sluder, Mark C Poznansky In submission April 2019 PLoS