DISSERTATION

ENVIRONMENTAL MAINTENANCE AND TRANSMISSION OF *FRANCISELLA TULARENSIS* IN COTTONTAIL RABBITS, PRAIRIE VOLES, AND AMOEBAE

Submitted by

Vienna Rae Brown

Department of Microbiology, Immunology, and Pathology

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Doctoral Committee:

Advisor: Richard Bowen

Annette Bachand
Kristy Pabilonia
Jeannine Petersen

ABSTRACT

ENVIRONMENTAL MAINTENANCE AND TRANSMISSION OF *FRANCISELLA TULARENSIS* IN COTTONTAIL RABBITS, PRAIRIE VOLES, AND AMOEBAE

Tularemia is a zoonotic disease that is endemic in much of the Northern Hemisphere, capable of causing severe disease in a wide range of hosts. This disease is caused by the gram-negative bacterium *Francisella tularensis* and most human cases are caused by either subsp. *tularensis* (type A) or *holarctica* (type B). Genetic clustering has led to further differentiation within type A and type B strains; type A strains are currently classified as A1a, A1b, and A2. Due to the high virulence and low infectious dose of this pathogen, naïve immune status of the public, and previous weaponization, *F. tularensis* has been classified as a Tier 1 Select Agent by the Centers for Disease Control and Prevention. Although the *Francisella* bacterium was discovered over a century ago, understanding of ecological factors that contribute to environmental maintenance and transmission remains enigmatic. Extensive research has been performed in a variety of laboratory animal models to evaluate factors related to disease progression and vaccine and therapeutic options; however, very little is known about reservoir and/or amplification hosts in a natural setting.

Reported here are a series of experimental studies performed in cottontail rabbits and voles as well as *in vitro* infections of amoebae with multiple strains of *F. tularensis.* The objectives of the *in vivo* studies were to characterize clinical disease, tissue dissemination and organ burden, and morbidity and mortality in a species believed to play an important role in naturally acquired infections. Rabbits were inoculated using a strain and dose of organism as well as a route of infection in accordance with what would be expected in nature.

The initial experimental infections of cottontail rabbits involved intradermal inoculation with one of several strains of *F. tularensis* which resulted in varied patterns of clinical disease, gross pathology,

and histopathology. Each of the type A strains was highly virulent, with rabbits requiring euthanasia or succumbing to infection 3-13 days post-infection. Gross lesions observed in infected rabbits included numerous microabscesses in the livers and spleens, suggesting high bacterial organ burdens. In contrast, most rabbits infected with type B strains developed a mild fever and became lethargic, but the disease was infrequently lethal. Those rabbits infected with type B strains that survived longer than 14 days post-infection developed a robust humoral immune response, and *F. tularensis* was not isolated from liver, spleen, or lungs of those animals. These findings depict a clear difference in virulence and immune kinetics between type A and B strains of *F. tularensis* in cottontail rabbits.

Based on findings from the original study with cottontail rabbits, I evaluated the protection afforded against infection with a type A strain of *F. tularensis* by prior inoculation with a type B strain. Previous infection with a type B strain of the organism was found to lengthen survival time and, in some cases, prevented death following inoculation with a type A2 strain of *F. tularensis.* In contrast, inoculation of a type A1b strain was uniformly lethal in cottontail rabbits irrespective of a prior type B inoculation. These findings provide important insight about the role cottontail rabbits may play in environmental maintenance and transmission of this organism.

Prairie voles are believed to acquire a natural infection with *F. tularensis* from contact with infected waterways or cannibalism of another vole that died from a tularemia infection. To evaluate such infection experimentally, I inoculated prairie voles orally with 10^7 organisms of type B *F. tularensis* and serially euthanized them to characterize organ burdens and pathology. The inoculated voles failed to show any clinical signs of disease and upon necropsy did not present with any gross lesions. Furthermore, organisms were not recovered from the liver and spleen, and antibodies were not detected, despite evaluation >14 days post-infection. Eight voles were then challenged intranasally with 350-650 organisms of one of two strains of *F. tularensis*. Infection with one strain (OR96-0246) resulted in all the animals succumbing to death or euthanasia between 6 and 7 days post-infection, whereas voles infected with the

other strain (KY99-3387) survived to the end of the study period (10 days post-infection), with the exception of one vole which succumbed to infection. These findings were surprising and require further investigation to understand how voles become infected in nature and what role they may play in *F. tularensis* persistence and transmission.

Free-living amoebae are capable of harboring pathogens and have been implicated in various disease outbreaks. I evaluated 3 strains of *Acanthamoebae* and 1 strain of *Hartmannella* as hosts for three bacterial pathogens. All strains of amoebae were propagated in culture with virulent strains of *F. tularensis*, *Burkholderia pseudomallei* and methicillin-resistant *Staphylococcus aureus,* with the aim of elucidating both general principles and pathogen-specific mechanisms associated with bacteria-amoebae interactions. *F. tularensis* and *B. pseudomallei* were recoverable from the lysate for all four strains of amoebae at both 4 and 24 hours post-inoculation, whereas MRSA was recoverable from the lysate of all four strains at the 4 hour time point and from only two of the strains at the 24 hour time point. Confocal microscopy allowed for the visualization of labeled bacteria of each strain and differentiation of amoebae morphology was possible. These findings provide intriguing evidence that amoebae are capable of phagocytosing pathogenic bacteria and that protozoa may play a role in environmental maintenance and persistence.

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1.1 Introduction:

1.1.1 History

Francisella tularensis was first isolated in 1911 in Tulare County, California from ground squirrels suffering from what appeared to be a 'plague-like disease' (McCoy and Chapin, 1912). The bacterium was later isolated by George McCoy in 1912; however, reports of a disease that closely resembled tularemia were reported as early as 1653 in lemmings in Norway (Sjöstedt, 2007). The first confirmed human case was identified in 1913 in a 21 year old meat cutter in Ohio, who developed conjunctivitis in association with cervical lymphadenitis (Wherry and Lamb, 1914). The organism was serially passaged through guinea pigs until it was finally isolated using coagulated egg-yolk.

1.1.2 Taxonomy and Geography

Originally named *Bacterium tularense*, referring to the county in which it was first recognized, *F. tularensis*, was subsequently designated as *Pasteurella tularensis* in the 1920s based on serological analysis (Sjöstedt, 2007). Using DNA hybridization technology in the 1960s, it was determined that *F. tularensis* was distinctly different from *Pasteurella* (Ritter and Gerloff, 1966). Analysis of 16S rDNA sequences indicated that *Francisella* was dissimilar from all other classified genera and that taxonomically it belonged to the γ-subclass of *Proteobacteria* (Forsman *et al.*, 1994).

F. tularensis is now further classified into three distinct subspecies based on differences in virulence, geographical distribution, and biochemical properties (Petersen and Molins, 2010). Early pioneering studies found that the LD_{50} (concentration administered that results in 50% of the animals infected succumbing to a lethal infection) for *F. tularensis* subsp. *tularensis* was 1-10 bacterial cells in mice, guinea pigs, and rabbits whereas the LD_{50} for *F. tularensis* subsp. *holarctica* was 1 and 10 bacterial cells in mice and guinea pigs, respectively; however, domestic rabbits tolerated a much higher inoculum, 10^8 bacterial cells (Olsufjev and Meshcheryakova, 1982). *F. tularensis* subsp. *tularensis* (type A) is

found exclusively in North America and is highly virulent in both humans and many other species (Keim *et al.*, 2007). *F. tularensis* subsp. *holarctica* (type B), a less virulent strain, has been found in many parts of the Northern Hemisphere, including the United States and Canada, as well as much of Europe (Petersen *et al.,* 2009). Type A and B organisms are responsible for the vast majority of human and animal infections worldwide. *F. tularensis* subsp. *mediasiatica* has been detected in Central Asia (Kazakhstan and Turkmenistan) and Russia and was designated according to its origin and biological components (Keim, *et al.*, 2007). *F. novicida* was first isolated in Utah in the early 1950s and much later was designated as a subspecies of *F. tularensis* (Sjostedt, 2007). This strain is considerably less virulent than either type A or type B strains and was originally included as a subspecies of *F. tularensis* despite vast differences in culture requirements and metabolic processes. Recently calls have been made to suggest that *F. tularensis* and *F. novicida* be maintained as separate species due to vast differences in phenotypic and genomic characteristics (Kingry and Petersen, 2014).

Type A tularemia is further differentiated into two subpopulations: A1 and A2. A1 has been found to be distinctly more virulent than A2 and it often referred to as A-east due to geographic preferences; whereas A2 is referred to as A-west (Kugeler *et al.,* 2009). Furthermore, A1 strains have been further classified as A1a and A1b due to immense discrepancies in infection kinetics and mortality in humans and animals.

1.1.3 Ecology

Tularemia has a vast host range as infection has been described in over 250 species (both vertebrate and invertebrate) (Parola and Raoult, 2001). The primary species associated with *F. tularensis* infections include beavers, muskrats, voles, lagomorphs, and ground squirrels and, while not considered important for pathogen maintenance or persistence, non-human primates and humans are particularly susceptible to infection (Wobeser *et al.,* 2009). Both an aquatic disease cycle and a terrestrial disease cycle have been described: terrestrial cycles involve rabbits and hares as amplifying hosts with ticks and

deerflies as arthropod vectors; while the aquatic cycle involves the shedding of *F. tularensis* into the environment by muskrats, beavers, and voles (Nigrovic and Wingerter, 2008), and mosquitoes have been implicated in mechanical transmission of type B strains exclusively.

Infection of beavers with *F. tularensis* has been recorded in both Canada and the United States. In the late fall of 1939 to the late spring of 1940, the beaver population of Little Big Horn River in Montana and nearby water bodies was decimated (Jellison *et al.,* 1942). In an area where at one time there were greater than 150 beavers, the population was reduced to zero. Three beaver carcasses were in sufficient condition to be tested and all three were confirmed positive for *F. tularensis*; suggesting that a tularemia infection was responsible for the massive beaver population die-off. *F. tularensis* was found to persist in the body of water for thirty-three days without the presence of beavers; this indicates that perhaps water is a media for terrestrial species to pass the organism along to aquatic species, and then the aquatic species perpetuate the disease through shedding in the water (Jellison *et al.,* 1942). Sero-surveillance data from Eastern Europe and Sweden further demonstrated the capacity of beavers to become infected with *F. tularensis* as the prevalence of bacterium-specific serum antibodies was greater than 57% (Tarnvik and Berglund, 2003). This large proportion suggests that beavers are heavily exposed in their natural habitat and survive infection to a greater extent than do other wild mammals.

A large muskrat die-off occurred in Ontario, Canada in October of 1955 and salvageable carcasses were necropsied and gross pathological findings included splenomegaly with yellowish-white foci of necrosis in the spleen (Fyvie *et al.,* 1959). Guinea pigs inoculated with tissue homogenates collected from the muskrats or water samples from their habitat resulted in death of all the inoculated guinea pigs and biochemical analysis in the laboratory confirmed *F. tularensis.* This study suggests that *F. tularensis* can replicate in water under normal conditions – if the contamination of the water was due strictly to a carcass it seems logical that the organism would scatter into the water and become nearly impossible to detect; although that is not the case, thus suggesting bacterial replication. Furthermore, a

large outbreak of human tularemia occurred in Vermont in 1969 amongst individuals who had close contact with infected muskrats (Young *et al.,* 1969). An overwhelming number of the associated human cases were serologically confirmed and all had recent contact with muskrats, although the number of muskrats handled did not seem to have an impact on incidence of disease. Individuals who wore gloves did not become infected, nor did those who handled dry pelts only; illness seemed to be associated with direct cutaneous contact with moist muskrat carcasses, indicating that the bacterium can penetrate unbroken human skin. *F. tularensis* was isolated from the water of a highly contaminated area and the organism was cultured directly from the walls of an abandoned muskrat house.

A 1982 publication describes that of the seventy-three human tularemia illnesses across Canada in which the source of infection was known, contact with rabbits was involved in 29 (40%) and muskrats in 22 (30%) (Martin *et al.,* 1982). Almost all disease in the 1930s and 1940s was a result of contact with rabbits, but in the last three decades muskrats have become the primary source of infection in Canada. A 1995 study of trappers in Quebec, Canada compared *F. tularensis* seropositivity amongst muskrat trappers and the general population (Levesque *et al.,* 1995). Twenty-seven percent of trappers who caught 100 or more muskrats during the past season had antibodies against *F. tularensis* whereas antibodies for *F. tularensis* were not detected in trappers who captured fewer than 100 muskrats the previous season. The finding that the number of muskrats handled affected the likelihood of seropositivity is incongruent with another study (Young *et al.,* 1969) and differences observed are believed to be due to variations in sample handling, the type of antibody assay utilized, and/or real changes in risk during the 10 years between the studies.

A study of terrestrial tularemia in North America indicated that voles both carry *F. tularensis* and are susceptible to infection from the bacteria which they often acquire via cannibalism, a common practice both in wild and captive voles (Bell and Stewart, 1975). Additionally, voles have been shown to shed the bacterium in their urine which may subsequently infect aquatic environments through leaching

and run-off. Voles typically succumb to infection rather quickly; however, it has been shown that some voles develop nephritis with bacteriuria which can persist for up to two weeks – providing an opportunity for cross contamination between terrestrial and aquatic environments and animals (Bell and Stewart, 1975; Bell and Stewart, 1983). A Swedish study from 1978 asserted that voles (specifically, the bank vole, grey-sided vole, and field vole) are susceptible to tularemia and that the disease was likely a contributing factor for decreases in the vole population (Hornfeldt, 1978). In 1981, 400 laboratory confirmed human cases of tularemia were reported in Sweden, which was believed to be connected to the large number of dead and dying voles observed during the winter (Christenson, 1984). Infected voles shed the bacterium in their urine and feces, which may then be picked up by larval mosquitoes as they filter particulate matter in their aquatic environment. Furthermore, an extensive outbreak in 1966-1967 was reported in which tularemia occurred as a result of inhalation of dust from hay contaminated with vole feces containing *F. tularensis* (Dahlstrand *et al.,* 1971). Voles are distinctly indicated as reservoir species and are able to spread the pathogen around the environment, infecting both aquatic and terrestrial species as well as humans (Dahlstrand *et al.,* 1971; Bell and Stewart, 1975; Bell and Stewart, 1983; Rossow *et al.,* 2014a; Rossow *et al.,* 2014b).

Early in the course of recognizing and characterizing the disease, rabbits and other lagomorphs were considered to be the primary species associated with tularemia; as many as 90% of all human cases were attributed to contact with rabbits (McKeever *et al.,* 1958). While it has become widely known that many other species serve as reservoir and amplifying hosts for *F. tularensis*, rabbits are still implicated in numerous outbreaks and play an important role in disease persistence and transmission. An outbreak of tularemia, caused by organisms from both clade A1 and A2 organisms, was reported in humans in Utah, USA in 2007 (Petersen *et al.,* 2008). *F. tularensis* was detected in lagomorph carcasses found in the same vicinity using a multi-targeted real-time PCR assay, with rabbits demonstrating infection with clade A1, clade A2, or type B bacteria. These findings clearly indicate that multiple strains of the pathogen may be responsible for a single disease outbreak, rabbits are acutely susceptible hosts for *F. tularensis,* and that

the health of the local rabbit population can have significant impacts on the human population. Bacterium-contaminated aerosolized rabbit feces has been implicated in multiple cases of pneumonic tularemia (Thomas and Schaffner, 2010). Additionally, in Europe, lagomorphs are an important species for tularemia persistence; the European Brown hare may serve as a reservoir for human disease (Gyuranecz *et al.*, 2010). Evaluation of European Brown hares shot by hunters in Hungary between 2007 and 2009, detected high levels of seropositivity (71%) and gross lesions in a large proportion of seropositive hares. Despite these findings, the majority of the hares had body condition scores listed as moderate or above, suggesting that they are capable of surviving and thriving despite a tularemia infection; thus they may serve as an important reservoir for *F. tularensis* subsp. *holarctica* in Europe. A 142 person outbreak of tularemia in Northwestern Spain found that 97.2% of cases reported previous contact with hares, 83.8% of patients had prepared hare carcasses, and 13.3% had handled hare meat; in only 2.8% of cases there was no animal contact reported (Pérez-Castrillón *et al.*, 2001). An overwhelming majority of infected persons had contact with hares prior to infection, suggesting that contact with hares was associated with illness. In a recapture study from southern Illinois, antibodies to *F. tularensis* in cottontail rabbits were evaluated over a period of 2.5 years, and it was found that antibody presence varied dramatically between the rabbits (n=79) with some being seronegative over the course of the project, others converting from seronegative to seropositive for IgM but never IgG, others started with IgM seropositivity and developing IgG antibodies, and still others who started with both IgM and IgG antibodies that either remained the same or fell over the course of the analysis (Shoemaker *et al.*, 1997). Moreover, 44% of the rabbits sampled from a state park in Illinois were found to be seropositive for IgM (n=722) and 23% were found to be seropositive for IgG (n=805). These findings indicate that cottontail rabbits are capable of developing a robust antibody response that allows them to survive an infection with *F. tularensis*, although most likely these rabbits were exposed to type B strains as opposed to type A as it is probable that infection with a type A strain would result in a fatal infection. Furthermore, lagomorphs are often implicated in spillover events resulting in human cases, thus suggesting their importance in transmission.

While ground squirrels are not considered a primary reservoir for *F. tularensis*, they are linked to the discovery of the bacterium and have been implicated in several cases. Between the years of 1931 and 1944, there was a 40 person outbreak of tularemia in Alberta, Canada and two of the cases were traced back to contact with infected ground squirrels (Bow and Brown, 1946). In Washington, 29 Western and Eastern gray squirrels were submitted to the Washington Animal Disease Diagnostic Laboratory for tissue screening of *F. tularensis;* 52% were PCR positive for the type B biovar (Nelson *et al.,* 2014). 53% of the PCR-positive squirrels were found to be seropositive by a direct fluorescence antibody test and one-third of the squirrels were positive by culture from tissues. Of the PCR-positive squirrels, gross lesions were not identified in the majority of them and histopathologic lesions were not detected in one-third, suggesting that tularemia may present typically or atypically.

While beavers, muskrats, and voles are the most important hosts for type B tularemia and small rodents and lagomorphs are the most important terrestrial hosts, many other species have been implicated as hosts for *F. tularensis.* In the United States, tularemia has been reported in opossums, badgers, porcupines, mink, coyotes, and raccoons (McKeever *et al.*, 1958). Between 2002 and 2003, 60 coyotes and 60 raccoons in Nebraska were tested for antibodies to *F. tularensis* and 32% of coyotes and 38% of raccoons were found to be seropositive (Bischof and Rogers, 2005). Additionally, it appears that any actively infected animal can transmit the disease directly through contact or indirectly through arthropod vectors to humans (McKeever *et al.,* 1958). Between 1994 and 2004, blood was collected from white-footed mice, skunks, raccoons, squirrels, rabbits, rats, deer, and dogs on Martha's Vineyard to analyze antibody titers to *F. tularensis* via a micro-agglutination assay (Berrada *et al.,* 2006). None of the mice, squirrels, or rabbits were found to be seropositive, samples from a few of the dogs, deer, and rats were positive, and half of the samples collected for skunks and raccoons were positive. Both skunks and raccoons are definitive hosts for *Dermacentor variabilis*, which are highly competent vectors of *F.*

tularensis. Based on these findings, skunks and raccoons may be useful in sero-surveillance studies that will likely provide valuable information with regard to transmission risk.

In addition to natural mammalian hosts, captive monkeys appear to be highly susceptible to tularemia. In 2002, 2004, and 2005 blood was collected from thirty-five cynomolgus monkeys at the German Primate Center and analyzed for the presence of antibodies to *F. tularensis* (Matz-Rensing *et al.*, 2007). Tularemia was diagnosed in 18 of the 35 monkeys; while 6 animals died suddenly with unspecified clinical symptoms and 12 others seroconverted but remained asymptomatic. The six monkeys that died unexpectedly had necropsy findings similar to those found in human infections and PCR and immunohistochemistry were used to confirm widespread dissemination of *F. tularensis.* A second incident that suggests the acute susceptibility of monkeys occurred at the Assiniboine Park Zoo in Winnipeg during the summer of 1978 (Preiksaitis *et al.*, 1979). Four monkeys (3 tamarin and 1 talapoin) died suddenly and at necropsy *F. tularensis* was isolated from each monkey. Ground squirrels were frequently found around the monkey cages, yet the mesh around the cages was too small to permit entry to the cage; two squirrels were captured and necropsies yielded *F. tularensis* from both the squirrels and the fleas found on them.

Using a competitive ELISA, 632 wild animals were sero-screened for antibodies against *F. tularensis* in Japan including 150 Japanese black bears, 142 Japanese hares, 120 small rodents (mice and voles), 97 rats, 53 raptors, 26 Japanese monkeys, 21 Japanese raccoon dogs, 20 masked palm civets, and 3 Japanese red foxes (Sharma *et al.*, 2014). Twenty three and 18 black bears, 2 and 1 small rodents, and 3 and 2 Japanese raccoon dogs were found to be antibody positive using the cELISA and micro-agglutination assays, respectively. All of the samples positive by the micro-agglutination assay were found to be positive by the cELISA and 6 of the 7 cELISA-only positives were also found to be positive using a confirmatory Western Blot assay. The vast global distribution of this pathogen, capacity to infect

innumerable mammalian and non-mammalian hosts, and associated virulence, provides a strong impetus to sero-survey wild-trapped animals.

The idea of an environmental niche for *F. tularensis* has been suggested and the limited studies of this hypothesis seem to support this possibility. An infected carcass was found to contaminate water for 10 days and contaminated water stored in a refrigerated environment was found to infect animals for up to two weeks (Telford and Goethert, 2011). Furthermore, water that was naturally contaminated was found to maintain infectivity for up to 10 weeks and *F. tularensis* in silt could infect animals up to two months. A study in which rodents were immersed in contaminated water found that one-half of immersed rodents became infected after exposure to $1x10^2$ to $1x10^3$ organisms (Pavlovsky, 1966). Furthermore, water invertebrates such as shrimp and snails have been found to retain viable organisms for 20 days.

1.1.4 Amoebae as a Potential Reservoir for *F. tularensis*

The acute nature of infection, high mortality induced, and the vast reduction in fitness of infected ticks, suggests that a mammalian or vector species reservoir for *F. tularensis* is unlikely, especially for the highly virulent, type A strains. Free-living amoebae (FLA) are single-celled organisms that exist ubiquitously within the environment. They primarily consume bacteria and have been found to harbor pathogens, especially intracellular organisms; as such, they have been implicated in environmental maintenance and transmission. Amoebae have two distinct life stages: trophozoite, which is a vegetative, motile form, capable of feeding and dividing, and a cyst, which is a dormant, spore-like form, capable of persisting in sub-optimal conditions. It is thought that amoebae-resistant pathogens (including *F. tularensis*) escape amoebal digestion which allows the amoebae to serve as an important reservoir, by providing protection from adverse environmental conditions and nutrient availability, as well as facilitating pathogen entry and disguise within a mammalian host during transmission (Greub and Raoult, 2004). Furthermore, ingestion by amoebae and subsequent escape by the pathogen has been implicated in the selection of virulence traits for pathogenic bacteria as well as aiding microbes in adaptation to the

intracellular environment within the host, especially macrophages (Greub and Raoult, 2004; Molmeret *et al.*, 2005; Berdal *et al.*, 1996).

Amoebae appear to be a possible reservoir that could enhance persistence and amplification of *F. tularensis*. Several *in vitro* experiments have been conducted which support the hypothesis that amoebae may ingest and harbor this microbe. *Acanthamoeba castellanii* are free-living amoebae that have been associated with several other intracellular pathogens including *Legionella pneumophila* and *Mycobacterium avium,* and interestingly, *F. tularensis* organisms that have been associated with amoebae have an enhanced ability to survive and replicate in host macrophages (El-Etr *et al.,* 2009). Experimental inoculation of *A. castellanii* with the live vaccine strain (LVS) strain of *F. tularensis* (type B) showed an increase in the number of intracellular bacteria overtime, with many *F. tularensis* cells located within vacuoles, which appeared to attract amoebae cell organelles such as the mitochondria and rough endoplasmic reticulum (Abd *et al.,* 2003). Furthermore, the numbers of *F. tularensis* LVS co-cultured with *A. castellanii* increased overtime while the numbers of *F. tularensis* LVS cultured alone decreased over the same period. This inverse relationship is likely because *F. tularensis* LVS uses CO_2 produced by live amoebae and derives nutrients from dead amoebae (Abd *et al.,* 2003). Interestingly, the numbers of amoebae are reduced when co-cultured with *F. tularensis* LVS, suggesting that infection with this organism has deleterious effects on amoebae survival.

Using *F. tularensis* LVS, *F. novicida* (strain U112), SchuS4, and 10 other type A field isolates (labeled Ft-1 through Ft-10), El-Etr and colleagues (2009) demonstrated that infection of *A.castellanii* with multiple strains of *F. tularensis* showed substantial variations in efficiency for both bacterial entry into amoebae as well as intracellular replication. *F. novicida* U112, SchuS4, and Ft-1 infection resulted in the development of spacious vacuoles within 30 minutes of infection, while *F. tularensis* LVS infection resulted in tight vacuoles that were later determined to be lysosomal in nature. Moreover, viable organisms of *F. novicida* U112, Ft-1, and Ft-7 were recovered from amoebal cysts up to 3 weeks post-

infection. This rapid encystment phenotype was induced by all type A strains of *F. tularensis* as well as *F. novicida* U112; however, the 5 type A strains responsible for the highest levels of encystment were those associated with the highest rates of attachment, entry, and survival. This finding is not surprising as amoebae encystment is induced under stressful or sub-optimal environmental conditions; rapid entry and proliferation by an organism would undoubtedly induce amoebal stress leading to encystment. Furthermore, using a transwell culture system, it was determined that a soluble proteinaceous factor released by the pathogen is responsible for the induction of encystment. Most probably, *F. tularensis* promotes amoebal encystment as a means of survival, the cyst provides a nearly impenetrable fortress that facilitates bacterial survival in the environment which promotes microbial persistence and transmission.

1.1.5 Animal Models

A wide variety of animal models have been used to evaluate a variety of research questions related to *F. tularensis*, including determination of the lethal dose via numerous routes, characterization of transmission and pathogenesis, and evaluation of vaccine candidates and therapeutics. When determining which type of animal model is best suited for the specific research question, it is essential that use of the model is capable of differentiating between two or more potential outcomes to either validate or disprove a hypothesis (Lyons and Wu, 2007). Furthermore, the model should simulate human infection as closely as possible if the desired outcome is ultimately therapeutic treatment or vaccination options for humans.

Human trials using *F. tularensis* were conducted in the 1950s and 60s to evaluate vaccine candidates, antimicrobial treatments, and metabolic changes, specifically the kinetics of insulin (Shambaugh and Beisel, 1967), alkaline phosphatase (Beisel, 1967), amino acid composition (Feigin and Dangerfield, 1967), and iron metabolism (Pekarek *et al.*, 1969); as well as cognitive function (Alluisi *et al.*, 1973) following aerosol exposure to *F. tularensis.* A series of vaccine trials were performed, mostly using prisoner ‘volunteers’, to evaluate the efficacy of various vaccines delivered via one of several

routes. These trials typically involved vaccination followed by experimental infection with SchuS4 in order to assess the protection afforded to a highly virulent strain (McCrumb, 1961; Hornick and Eigelsbach, 1966; Saslaw *et al.*, 1961a; Saslaw *et al.*, 1961b).

Non-human primates (NHPs) became the animal model of choice for tularemia research in the 1970s as human trials became increasingly unfavorable and of questionable morality. NHPs develop a similar clinical course of disease, analogous lesions at both the macro- and microscopic levels, and appear to have similar susceptibility in terms of infectious dose and strain, as well as serving as a representative model for vaccine and antimicrobial efficacy. Studies were undertaken to evaluate the kinetics of infection following vaccination with either a live or dead strain of LVS (Eigelsbach *et al.*, 1962), to compare efficacy of vaccination route followed by inoculation with a virulent strain of *F. tularensis* (Tulis *et al.*, 1970), and to analyze clinical parameters following experimental inoculation (Hambleton *et al.*, 1978). Furthermore, a series of experiments were performed which provided descriptive data with respect to organismal persistence within tissues, the course of inflammation, antibody kinetics, and details on gross and histological presentation following inoculation with SchuS4 (White *et al.*, 1964; Baskerville *et al.*, 1978; McGavran *et al.*, 1962).

Mouse models have been used extensively to evaluate various aspects of *F. tularensis* infection. Killed vaccines were found to control infection against strains of lower virulence (Bell *et al.*, 1952) but only provided slight protection against virulent *F. tularensis* challenge by prolonging survival (Ruchman and Foshay, 1949). However, lowly-virulent strains of *F. tularensis* can induce a more protective immunity (Downs and Woodward, 1949). The kinetics and specificity of protection following LVS immunization were identified and *F. tularensis* subsp. *novicida* infection was determined to be ineffective in protecting against virulent strains of *F. tularensis* (Elkins *et al.*, 1992) despite having LPS with greater immunobiological activity as compared to LPS derived from *F. tularensis* (Kieffer *et al.*, 2003). Susceptibility to LVS was assessed in varying strains of mice via a variety of inoculation routes (Fortier

et al., 1991) and adoptive transfer of spleen cells from vaccinated mice was found to provide protection (Eigelsbach *et al.*, 1975). Furthermore, microbiological and histological descriptions were provided for mice inoculated via an aerosol route (Wu *et al.*, 2005; Conlan *et al.*, 2003).

A recent study performed by the Centers for Disease Control and Prevention entailed intradermal inoculation of mice with 10-16 organisms in order to evaluate differences amongst types A1a, A1b, A2, and B strains of *F. tularensis* (Molins *et al.*, 2010). Using several parameters, including time to death and variation of survival amongst mice within the same group, type A1b was found to be the most virulent strain. The bacterial load in the blood, spleen, and liver were found to be highest in the mice infected with type B strains followed closely by those infected with A2, whereas the bacterial burden found in the lungs was found to be highest in the mice infected A2 strains followed closely by type B strains. The bacterial burden was lower in the mice that died sooner (A1b and A1a) indicating that higher bacterial burdens are required in order for type A2 and B strains to be lethal to mice. During necropsy, splenomegaly was observed in mice that had been challenged with type B strains whereas mice exposed to type A1a, A1b, and A2 strains were found to have necrosis and granulomas present in the spleen. Prior to this study, mice were not considered a particularly useful animal model for evaluating variations in virulence due to their acute susceptibility; however, using an intradermal route and a very low inoculating dose, mice can be used to differentiate between strains of *F. tularensis.*

This study was also used to evaluate a method for standardizing the progression of *F. tularensis* by monitoring body temperature (Molins *et al.*, 2012). The researchers used a subcutaneous implant to monitor temperature as a non-subjective measurement of animal health. Mice infected with *F. tularensis* demonstrated a reproducible pattern, irrespective of the strain of organism used in the inoculation, which included sequential phases of normal, elevated, and finally lower than normal temperature. The point at which the mouse transitioned from pyrexia to hypothermia (the drop point) could be readily used as a

surrogate endpoint; the survival curves observed for the infected mice and those euthanized at the drop point were nearly identical and all differences observed were found to be non-significant.

Moreover, mice have been used extensively in determining the mechanism of *F. tularensis* infection as well as virulence factors associated with cellular adherence, entry, replication, and dissemination; this is reviewed in the 'virulence factors' section of this literature review (section 1.4.5).

Rats have also proven to be a useful model to evaluate vaccine candidates as well as providing descriptive insight into immunological and histological parameters. Vaccinated rats were found to have a less complete protection against virulent SchuS4 challenge as compared to rats who had been previously infected and recovered (Downs *et al.*, 1949), likely due to a limited replicative capacity within the spleen leading to lengthened survival following inoculation (Buchele and Downs, 1949). Fischer 344 rats have been found to be a particularly good model for studying pneumonic tularemia and evaluating vaccines (Jemski, 1981; Wu *et al.,* 2009) as well as characterizing histopathological changes following infection in either vaccinated or naïve rats (Moe *et al.,* 1975). Experimental infection of rats led to the postulation that cellular resistance to *F. tularensis* is primarily in response to non-specific inflammatory cells, especially macrophages and sensitized lymphocytes (Kostiala *et al.,* 1975). This concept was evaluated further through an experiment in which it was determined that in rats co-infected with *F. tularensis* LVS and *F. novicida* the number of viable *F. tularensis* cells in the spleen was suppressed up to 100-fold when compared to rats infected only with *F. tularensis* LVS (Cowley *et al.,* 1997). Due to the rapid replication of *F. novicida*, the immune system was likely alerted to the infection of *F. tularensis* LVS more quickly than in animals infected with *F. tularensis* LVS only, allowing the host to elicit a robust immune response more rapidly, leading to fewer viable cells. Metabolic changes were also evaluated, specifically serum and liver zinc concentration, amino acid uptake by the liver, seromucoid content, and α_2-macrofetoprotein production, and no metabolic sequelae was observed until liver damage was initiated, determined by the presence of pyogranulomatous lesions (Powanda *et al.,* 1975).

Rabbits are an alternative small animal model that has been used for *F. tularensis* research, however not nearly to the extent as rats or mice. Vaccine candidates have been evaluated using rabbit models to determine the best route, dose, and frequency of administration and subsequent lesion development (Downs, 1932; Nutter, 1969). Furthermore, descriptive studies have been conducted to determine gross and histopathological changes (Baskerville and Hambleton, 1976) as well as changes in blood analytes associated with SchuS4 infection in rabbits (Hambleton *et al.*, 1977). These studies, in conjunction with an experimental inoculation study by Reed and colleagues (2011), indicate that rabbits develop acute tularemia in a manner very similar to humans, suggesting that rabbits are a relevant model for studying human tularemia (Reed *et al.*, 2011).

1.2 Clinical Symptoms and Transmission:

Tularemia is a multi-systemic disease and has been differentiated into diverse types reflective of the point of bacterial entry into the body, specifically in human cases, and both type A and type B strains can be acquired through any of these routes. Infection acquired through the skin, typically via the bite of an infected vector or through direct contact with an infected animal, is the most common route and results in the ulceroglandular form (Foley and Nieto, 2010; Oyston, 2008). The ulcer develops at the site of exposure and is then surrounded by a zone of inflammation and organism can often be cultured from this type of lesion. Glandular tularemia is acquired in the same manner but lacks the ulcer at the site of exposure (Oyston, 2008). Both ulceroglandular and glandular tularemia cause fever, chills, malaise, headaches, sore throat, and local lymphadenopathy. Oculoglandular tularemia results from direct contamination of the eye by *F. tularensis* resulting in conjunctivitis, swelling of the lids, purulent secretion, and cervical lymphadenopathy. Oropharyngeal and gastrointestinal forms of tularemia arise following the consumption of contaminated food or water and result in tonsillitis, pharyngitis, and a sore throat as well as a range of other symptoms from mild but persistent diarrhea to a fatal disease that is characterized by extensive ulceration of the bowel. Pneumonic tularemia results from inhalation of the bacterium and manifests as a dry cough, dyspnea, chest pain, patchy infiltrates, lobar pneumonia or

bloody pleural effusion, often accompanied with a high fever, malaise, chills, delirium, pulse-temperature dissociation, and hilar lymphadenopathy (Foley and Nieto, 2010; Oyston, 2008). Typhoidal is the most lethal of all the forms and typically occurs as a secondary infection to pneumonic or ulceroglandular tularemia (Foley and Nieto, 2010). Regardless of the route of infection and the initial characterization of disease, the pathogen is capable of disseminating into the blood stream resulting in a systemic infection and septicemia.

There are well characterized differences in virulence to humans among strains of *F. tularensis*. Infection with type A1 strains was found to result in 14% mortality in humans as compared to 0% mortality in A2; however, marked differences were observed between A1a and A1b with 4% and 24% fatality, respectively (Kugeler *et al.,* 2009). Type A1 *F. tularensis* was much more likely to be recovered from the blood or lungs of an infected patient in comparison to type A2, which was typically recovered from a regional lymph node (Staples *et al.,* 2006). Based on the acute pathogenesis of type A1 tularemia, it was unsurprising that this organism was often isolated from a systemic location as opposed to type A2 which was often focal.

1.2.1 Vector Transmission

F. tularensis is capable of being transmitted via infected ticks as the bacterium penetrates the midgut and migrates to the salivary glands where it capable of inoculating its host during blood feeding. Alternatively, during feeding, ticks often defecate near the feeding site which can cause contamination of the wound leading to an ulceroglandular or glandular infection (Foley and Nieto, 2010).

Vector species play an important role in the ecology and transmission of pathogens; many microbes undergo developmental cycles within the vector and the saliva of the vector is an important immune modulator such that transmission is facilitated (Wikel, 1999). Tick saliva has been shown to include anti-coagulants, inhibitors of platelet aggregation, vasodilators, and immuno-suppressants. Tick-

mediated host immunosuppression includes inhibiting the activity of complement components, production of compounds that will counter the stimulation of the host itch response, disabling the function of natural killer cells, diminishing antibody production, reducing the proliferative response of T lymphocytes to mitogens, and down-regulating pro-inflammatory cytokines and responses. All of these immuno-suppressive activities are designed to promote tick engorgement from a mammalian host; however, they also facilitate pathogen establishment within the host. It has been shown that rabbits that have developed an acquired resistance to tick infestation are less susceptible to tick-transmitted infection with *F. tularensis* as compared to tick susceptible controls (Wikel, 1999). A similar finding has been reported for mice, bank voles, and guinea pigs for *Borrelia burgdorferi.*

1.2.1.1 Tick Transmission

F. tularensis is endemic in Arkansas and Missouri and despite great reductions in the number of cases across the U.S. over the last century, this region has not seen the dramatic decline afforded to the rest of the states. Prior to 1951, other states (California, Illinois, Indiana, Kansas, Louisiana, and Wisconsin) reported lagomorphs to be associated with >70% of the cases; although Arkansas and Missouri reported 56-76% of cases to be tick-borne (Eisen, 2007). Between 1978 and the present, the probable exposure of humans to *F. tularensis* in the south-central U.S. is due to ticks; temporal evidence is the greatest proponent of this theory as the highest number of tularemia infections perfectly coincides with the peak activity period of two suspected tick vectors - *Amblyomma americanum* and *Dermacentor variabilis*. Tick transmission is the suspected culprit as to why Arkansas and Missouri have had a much less pronounced decline in tularemia as compared to the other states after a general decline in human exposure to *F. tularensis*-infected lagomorphs (Eisen, 2007). Interestingly, despite the annual number of tularemia cases, the natural infection rates in ticks are surprisingly low, ranging from 0-3% with most areas having <1% of infected ticks.

A series of laboratory infections have been performed to evaluate the capacity of various species of ticks to transmit *F. tularensis. D. variabilis* nymphs were fed on mice infected with A1b (MA00-2987), A2 (WY96-2418), and B (KY99-3387) and nymphal attachment was timed such that their peak engorgement would coincide with maximal bacteremia (Reese *et al.,* 2011). The mice used to infect the nymphs with the A1b, A2, and B strains had a maximum average bacteremia of 9.11 $\log_{10}$ cfu/mL, 9.57 $\log_{10}$ cfu/mL, and 10.11 $\log_{10}$ cfu/mL, respectively; resulting in 100% infection prevalence in the fed nymphs. It was also found that *F. tularensis* had minimal impact on survivorship. There was no statistically significant difference in the number of ticks that survived to day 65 post-infection for either the A2 or B strains; however, the ticks infected with A1b had a statistically significant reduction in the number of ticks that survived to day 65 post-infection, although the number surviving still exceeded 80%. The proportion of ticks attached was the same for infected and uninfected ticks of the A1b group; however, ticks infected with the A2 and B strains had lower attachment rates as compared to their uninfected counterparts. Efficient trans-stadial transmission occurred for each of the three strains: 71%, 91%, and 88% for types A1b, A2, and B, respectively. Feeding of the infected adult ticks upon naive mice resulted in transmission rates of 67%, 89%, and 58%, respectively for the three stains (A1b, A2, and B). This study suggests that *D. variabilis* are very competent vectors of *F. tularensis* as the bacteria colonize nymphs easily, they are able to maintain infection during molting, and they can successfully transmit the bacterium to a naive mammal.

Interestingly, just one year before the above study (Reese *et al.,* 2011) was published, an experiment was reported by the same group demonstrating that *D. variabilis* larvae infected with *F. tularensis* resulted in high fitness costs and low transmission rates (Reese *et al.,* 2010). Mice were infected with one of the three strains of *F. tularensis* from the above study and infested with larvae such that the period of rapid engorgement would coincide with peak bacteremia; all of the larvae acquired the bacterium. Furthermore, the infected larvae were able to maintain infection after molting between the larval and nymphal stages. Infection with A2 and B strains caused statistically significant reductions in

survivorship between infected and uninfected nymphs; in the A2 group there was 88% mortality at day 65 post-infection. The attachment rates did not differ between the uninfected nymphs as compared to those infected with A1b, A2, and B. The length of nymphal feed was similar in all groups; however, A1b nymphs fed to repletion much slower than the other groups. The proportion of nymphs feeding to repletion was statistically significantly different between uninfected and A2 infected groups, 55% and 3.7%, respectively. Moreover, A1b nymphs that had fed to repletion were very small as compared to uninfected nymphs. All of the infected nymph groups had much higher levels of feeding induced mortality as compared to uninfected; A1b (75%), A2 (94%), B (19%), as compared to 2.5% for uninfected nymphs. The overall transmission rate of infected nymphs to naive mice was 0%, 8%, and 13.5% for nymphs infected by A1b, A2, and B strains, respectively. Based on the 2010 and 2011 studies published by this group, it appears that *D. variabilis* infected as larvae are subject to a very high fitness costs that results in low levels of transmission; however, infected as nymphs they are much more competent as vectors (Reese *et al.*, 2010).

The finding that infection with *F. tularensis* species has a dramatic negative impact on tick longevity suggests that tick-borne transmission may not be a major mechanism for transmission. A further study assessed tick longevity between *F. tularensis* infected and uninfected field-derived ticks on Martha's Vineyard (Goethert and Telford, 2011). Host-seeking *D. variabilis* ticks were collected and infection status determined via PCR by testing hemolymph. By September, 44% of the PCR positive ticks had died as compared to 24% of non-infected ticks ($p<0.05$); by December 80% of the remaining PCR positive ticks had died and only 32% of the remaining PCR negative ticks had died ($p<0.001$). By the end of the observation period, 89% of the PCR positive ticks had died as compared to 48% of the PCR negative ticks ($p<0.001$); thus, clearly suggesting that natural infection of *F. tularensis* contributes to decreased longevity in *D. variabilis*. The authors then sought to determine whether bacterial burden was found to be associated with mortality; it was not. However, the genotype of the bacteria was an excellent determinant of tick mortality. The 'common genotype' was found in 69% of the infected ticks, whereas the

'uncommon genotype' comprised of twelve genotypes and was found in the remaining 21% of the infected ticks. At the September observation point, 31% of the common genotype ticks had died whereas 61% of the uncommon genotype ticks had died ($p<0.05$); however, at the December observation point, the tick longevity was found to be similar between the two groups of genotypes. It was further found that there was no difference in longevity between uninfected ticks and ticks infected with the common genotype; thus, indicating that uncommon genotypes contribute to overall differences found in mortality (Goethert and Telford, 2011). These findings suggest that the common genotype has evolved with the tick vector and thus has modulated its effect on tick fitness.

Martha's Vineyard, Massachusetts has an interesting history with *F. tularensis*. In the 1930s tularemia endemic cottontail rabbits from Arkansas and Missouri were introduced to Cape Cod and Martha's Vineyard by game clubs for sport hunting; the first cases of tularemia were reported in this area shortly thereafter (Feldman *et al.,* 2001). The vast majority of human tularemia cases in the United States are of the ulceroglandular form; however, between 2000 and 2008 there were >70 cases of pneumonic tularemia (Goethert and Telford, 2009). The only other cases of pneumonic tularemia ever recorded in the U.S. were in 1978 on Martha's Vineyard, although how the pathogen was maintained between epidemics is unknown. The prevalence of *F. tularensis* DNA within American dog ticks (*D. variabilis*) on Martha's Vineyard ranges between 1-5% and using variable number tandem repeat (VNTR) analyses it was determined that the diversity of the bacterium in dog ticks from Martha's Vineyard is as great as that measured for all existing *F. tularensis* isolates from across North America (Goethert and Telford, 2009). This finding suggests that the bacteria have been endemic since its introduction in the 1930s. Host-seeking dog ticks were collected and evaluated by PCR for the presence of *F. tularensis* and all positives were further differentiated using VNTR analysis to evaluate the presence of specific haplotypes. One specific site was found to have a disproportionately high number of ticks that were *F. tularensis* positive and they were much more likely to be infected with an uncommon haplotype, suggesting that a microfocus may be responsible for the generation of infected ticks. VNTR haplotypes occur in a slip-

strand mispairing of the tandem repeats; the frequency with which this occurs depends on the number of repeats, in general the more repeats there are the more likely mispairing occurs. These mutations depend on the number of replication cycles the microbe undergoes as each replication cycle provides an opportunity for mispairing to occur. By mapping where ticks containing *F. tularensis* DNA with uncommon VNTR haplotypes are found, it is possible to determine where increased replication (more intense transmission) is occurring. The microfocus appeared to have equal rodent and small mammal activity as surrounding sites so the increased transmission is likely due to microhabitat-related factors such as temperature, humidity, soil composition or chemistry, protozoal fauna, and/or long-standing fomites (Goethert and Telford, 2009).

One particular field side on Martha's Vineyard had the highest prevalence of *F. tularensis* positive ticks and also had the most diversity; further indication that there is a long-standing enzootic transmission cycle as opposed to a point source or recent introduction (Goethert *et al.,* 2004). This finding was further substantiated by evaluating *F. tularensis* haplotypes within questing *D. variabilis* sampled at two distinct locations on Martha's Vineyard. Squibnocket (an area with known high incidence of *F. tularensis*) and Katama (a minimal number of tularemia cases despite a large number of dog ticks) were compared and results yielded a largely clonal population in Squibnocket as compared to a much more diverse population at Katama, likely derived from multiple founders (Goethert *et al.,* 2009).

1.2.1.2 Deerfly Transmission

Deerflies are capable of infecting humans with *F. tularensis* via mechanical transmission of contaminated mouthparts; this route of transmission has been demonstrated for both deer flies, *Chrysops discalis* (in the U.S.) and *Chrysops relictus* (in Russia) and horse flies, *Haematopota pluvialis* (in Russia) (Petersen *et al.,* 2009). Biting flies deliver a painful bite, and as such, often have interrupted feedings as they are forced by host-defense behavior to leave before repletion; once interrupted they actively and

persistently seek at the nearest available host to continue feeding. This propensity to feed on multiple hosts in a short window of time has been implicated in acute outbreaks of tularemia.

In the early summer of 1971 in Utah, there was an epizootic outbreak of tularemia in rabbits, followed soon thereafter by an outbreak in humans (Klock *et al.,* 1973). Over the course of 4 months there were 39 cases of tularemia reported, 28 cases had strong evidence of having been transmitted via the bite of an infected deerfly and 7 cases were suspected of having been the result of being bitten by an infected mosquito or biting gnat. In late July of the year of the outbreak, *F. tularensis* was isolated from one pool of 43 trapped deerflies and later isolated from two groups of 105 deerflies trapped at a site where a patient had been bitten by a deerfly 8 days previously. Having collected *F. tularensis* positive deerflies in conjunction with human cases both in terms of temporality and geography, in addition to patient reports of having recently been bitten by deerflies, strongly suggests that deerflies are capable of transmitting this organism.

1.2.1.3 Mosquito Transmission

Mosquitoes have long been implicated in transmitting *F. tularensis* subsp. *holarctica* in Scandinavia (especially Sweden) and Russia, although the evidence has been largely circumstantial. Furthermore, mosquitoes have been linked to some of the largest epidemics of tularemia ever reported (>400 cases) (Petersen *et al.,* 2009). An epidemiologic study conducted in Sweden determined that being bitten by a mosquito was a statistically significant risk factor for a tularemia diagnosis, further supported by the development of ulceroglandular tularemia which makes mosquito transmission plausible (Eliasson *et al.,* 2002).

Experimental infections in a laboratory setting have been able to provide information about how competent mosquitoes are as vectors of *F. tularensis.* Larvae samples collected from a tularemia endemic area of Sweden in August 2008 were laboratory reared to adults in their original pond water (Lundstrom

et al., 2011). The adult mosquitoes were killed and both pond water samples and mosquitoes were tested for DNA, via real-time PCR, to *F. tularensis;* 14 out of 48 pools (29%) of mosquitoes were positive for *F. tularensis lpnA* sequences. Furthermore, *F. tularensis* subsp. *holarctica* strains are able to survive in association with protozoa, indicating that aquatic protozoa may play an important role as a reservoir for this bacterium. This paper concludes that outbreaks of tularemia originate in the pond habitat of mosquito larvae, implicating that larvae are exposed via the pond environment and natural trans-stadial transmission occurs.

Wild-caught Alaskan mosquitoes were collected and sampled and 30% of the pooled samples were positive for the *fopA* gene via real-time qPCR (Triebenbach *et al.*, 2010). Trans-stadial transmission was evaluated by exposing *Aedes aegypti* and *Anopheles gambiae* larvae to *F. tularensis* subsp. *novicida* strain U112 ($2 \times 10^4 - 6 \times 10^{11}$ cfu) with and without green fluorescent protein (GFP). After several washings to remove extracellular bacteria, the larvae were assessed for fluorescence; exposed larvae exhibited fluorescence whereas non-exposed larvae did not. This finding indicates that the larvae pick up the bacteria as they strain their surroundings for food; no difference was observed in survival indicating that this pathogen is not lethal to larvae. Furthermore, adult mosquitoes were fed an artificial blood meal that was spiked with 1×10^8 cfu/mL and 72 hours post-feeding these mosquitoes were fed on anesthetized mice to determine if they were able to transmit the bacterium to naive animals. These mosquitoes were positive for the organism at all time points during the first 72 hours but the bacterial load fell ~4 orders of magnitude between 48 and 72 hours. None of the mice fed upon by the mosquitoes had any indication of infection. These findings implicate that the mosquito is not an efficient vector with which the organism can multiply, perpetuating the hypothesis that mosquito-borne transmission is a result of mechanical transmission, not biological. It has been previously reported that *A. aegypti* fed on moribund animals were able to transmit *Bacterium tularense* to healthy animals in a laboratory setting (Philip *et al.*, 1932). These contradictory results suggest that mosquito transmission of tularemia is dependent upon

several important factors, including strain of mosquito, strain of organism, concentration of organism, and interval between blood meals (the shorter the interval the more likely the organism is to transmit).

After feeding mosquitoes *F. tularensis* subsp. *holarctica* spiked blood meal, organism could be detected for more than 2 weeks. Furthermore, the feeding of 12 mosquitoes on infected mice with a bacteremia of 4+/- 0.5 x 10^5 cfu/mL resulted in 7 mosquitoes picking up the bacteria (58%) (Thelaus *et al.*, 2014). These results differ from the aforementioned study (Triebenbach *et al.*, 2010) using *F. novicida* indicating that strain of organism significantly impacts the competence of mosquitoes as vectors for tularemia. Moreover, the strains of mosquitoes used in the studies (*Aedes aegypti* and *Anopholes gambiae*) do not live in the northern part of the Northern Hemisphere and thus, are not a biologically viable vector species.

A predictive model for tularemia was created in Sweden in an attempt to predict human tularemia cases based on mosquito abundance in conjunction with hydrological and meteorological data (Ryden *et al.*, 2011). The presumed location of disease transmission was available for 332 of the cases and geographical clustering was clearly evident; also, there was a temporal correlation between mosquito abundance and cases of tularemia. The geographical clustering of the cases indicate a focus area in which the bacteria is environmentally stable and exists in some sort of 'nest'. Of the 20 environmental variables tested in the model, the most significant was the predicted mosquito abundance; thus, providing evidence for an important role of mosquitoes in transmission of tularemia to humans.

1.3 Pathogenesis:

1.3.1 Limitations of the Current Animal Models

As described in detail in the above ‘Animal Models’ section (1.1.5), a plethora of animal models have been used in a laboratory setting to assess a wide variety of questions associated with *F. tularensis* transmission, maintenance, and virulence, as well as for vaccine and therapeutic trials. While this work

has been instrumental in advancing our understanding of acute pathogenesis, susceptible species, and virulence factors, basic questions regarding environmental maintenance and persistence remain largely unanswered. Furthermore, the roles of mammalian and/or protozoan reservoir or amplification hosts are largely uncharacterized.

1.3.2 *Francisella* Entry and Intracellular Trafficking

Infection by *F. tularensis* begins when the organism enters the body through a cut in the skin, the alimentary tract, the conjunctiva, or via inhalation. The infectious dose for this pathogen is very small; 10-50 aerosolized organisms reliably caused disease in human subjects in trials conducted during the 1950s (Titball and Sjostedt, 2003). Despite the route of entry, the organism is capable of inducing a systemic infection resulting in multi-focal disease and a septicemia that is rapidly lethal if left untreated.

F. tularensis is a facultative intracellular pathogen that is capable of infecting a wide variety of cell types; however, *in vivo* it preferentially infects macrophages (Oyston *et al.*, 2004). TUL4 and FTT1103 are two lipoproteins that interact with TLR-2 and that may be responsible for pro-inflammatory cytokine induction during infection (Oyston, 2008). This induces macrophages to produce asymmetric spacious pseudopod loops which require complement activity in order to allow uptake. The organism enters the macrophage without eliciting the respiratory burst and once inside the cell is contained within a phagosome (Oyston *et al.*, 2004). Maturation of phagosomes can be determined via the assessment of lysosome-associated membrane glycoproteins (LAMPs) – LAMP1 and CD63 – as well as the acid hydrolase cathepsin D. Within 2-4 hours of infection, *Francisella*-containing vacuoles were found to stain abundantly for LAMPs, but very little for cathepsin D (Clemens *et al.*, 2004; Celli and Zahrt, 2013). Furthermore, evaluation of the pH of infected phagosomes demonstrated little acidification despite the presence of live *F. tularensis.* Within 8 hours of infection, the membrane of the non-acidified phagosome becomes morphologically disrupted allowing for bacterial entry into the cytoplasm and subsequent replication. It is believed that the lack of natural progression of the phagosome to a phagolysosome, and

ultimately to a lysosome results in membrane disruption that allows the pathogen to gain access to the cytosol. At the onset of replication, the numbers of bacteria rise slowly; however, after the first 12 hours, there is a rapid increase in proliferation (Oyston *et al.*, 2004). Within 24 hours of macrophage infection, apoptosis begins by what resembles the intrinsic pathway of cell death. Enormous numbers of bacteria are released from the dying cell, giving way to infection of new cells. The virulence associated with *F. tularensis* is not due to exotoxin production but rather to its ability to proliferate to large numbers within tissues and organs, disrupting normal function and inducing a robust inflammatory response (Oyston, 2008).

1.3.3 Dissemination and Localization of *Francisella* in Host Tissue and Associated Pathophysiology

F. tularensis specifically targets the lymph nodes, lungs, spleen, liver, and kidney in all mammalian species that have been evaluated (Dennis *et al.*, 2001). This is likely due to its preferential replication within macrophages which results in lysis and a transient extracellular period before it is uptaken by the next macrophage target. All of these tissues filter large quantities of blood and lymph daily making them ideal targets.

Host trafficking, organ involvement, and micro- and macroscopic changes associated with an *F. tularensis* infection are similar irrespective of the inoculating strain (either subsp. *tularensis* or *holarctica*), route, and dose (Ojeda *et al.*, 2008). As expected, larger challenge doses and more virulent strains result in, a) faster development of lesions, b) larger and more diffusely affected regions, and c) more extensive involvement of tissues. However, overall the organs implicated in infection remain fundamentally unchanged. Liver and spleen are the primary sites of dissemination for *F. tularensis*, and infection results in pyogranulomatous inflammation and necrosis characterized by cellular debris in conjunction with fibrin, live and dead neutrophils, and macrophages (Moe *et al.*, 1975; Baskerville *et al.*, 1978; Dennis *et al.*, 2001; Ferrecchia *et al.*, 2012; Fortier *et al.*, 1991; Guarner *et al.*, 1999; Twenhafel *et al.*, 2009; KuoLee *et al.*, 2007). Macroscopically microfocal abscesses that are widely and randomly

distributed throughout the tissue are visualized; these focal lesions are typically well demarcated, round, and whitish in color with smooth borders (Gyuranecz *et al.,* 2010). Similar lesions are often observed in kidneys and renal involvement reflects the possibility for contaminated urine to transmit infection (Bell and Stewart, 1983; Olsufjev *et al.,* 1984; Rossow *et al.,* 2014b). In some cases, voles infected with type B strains of *F. tularensis* become chronically infected and have been shown to shed organism in urine for up to 80 days following infection and are capable of transmitting *F. tularensis* via this route (Olsufjev *et al.,* 1984; Bell and Stewart, 1983).

The lungs are another primary target organ of *F. tularensis* and may be infected either in a direct capacity (aerosol, intranasal, or intratracheal inoculation) resulting in a primary pneumonic tularemia or via hematogenous spread of the organism as a result of an alternative route of infection (intraperitoneal, intradermal, subcutaneous, or oral). Pulmonary involvement typically manifests as bronchial, bronchiolar, and alveolar epithelium necrosis, which promptly develops into necrotizing pneumonia (Twenhafel *et al.,* 2009; Baskerville and Hambleton, 1976; Reed *et al.,* 2011) or bronchopneumonia and hemorrhage (Downs, 1932). The extent of tissue damage depends upon the route of infection, infecting strain, and dose; however, the liver, spleen, lungs, and regional lymph nodes will indubitably be involved.

1.3.4 Virulence Factors

The size of the *F. tularensis* genome is nearly 2.0 Mb and despite distinct differences in virulence and biochemical properties, type A and type B sub-species have been found to have nearly homologous genomes (Larsson *et al.,* 2005). The overall gene content of the subspecies *tularensis* and *holarctica* is highly conserved yet the level of genomic rearrangement that has occurred is striking and likely plays an important role in observed biological differences (Petrosino *et al.,* 2006).

The recently reported whole genome sequence performed on *F. tularensis* suggested a number of notable virulence factors encoded, including type IV pili, type II secretion system, a surface

polysaccharide, a putative poly-D-glutamic acid capsule, an iron-acquisition system, and >10% of genes which contained mutations – insertion/deletion or substitution (Sjostedt, 2006). Interestingly, a number of the disrupted genes encoded metabolic pathways, which is reflected by the fastidious growth requirements of this pathogen. A large proportion of these virulence genes are encoded by the *Francisella*-pathogenicity island (FPI), a 30 kb segment of genome which has a much lower G + C content as compared to the rest of the *F. tularensis* genome (Nano *et al.*, 2004). The FPI is a cluster of 16-19 genes that are found to be duplicated in virulent strains, *F. tularensis* subsp. *tularensis* and subsp. *holarctica*, while less virulent strains, *F. novicida*, are found to contain only 1 copy (Barker *et al.*, 2009). Sixteen of the genes are highly conserved, while 2-3 putative genes are often interrupted by stop codons or altogether absent depending on the strain (Nano and Schmerk, 2007). Significant attenuation of virulence in conjunction with reduced intracellular replication were found in strains that underwent experimental induction of mutations within the FPI.

The transcription of the FPI genes are under positive control by the global virulence regulator, MglA (Barker *et al.*, 2009; Lauriano *et al.*, 2004). MglA appears to regulate the expression of nearly 100 genes, many of which are involved in cellular metabolism as well as environmental and genetic information processing (Brotcke *et al.*, 2006). Through the use of MglA mutants, it was determined that MglA regulated genes are involved in macrophage cytotoxicity as well as both positive and negative regulation of intra-macrophage replication, which is likely crucial for *in vivo* survival as over-replication alerts the host immune system to the presence of an invader. *iglA* and *iglC*, two genes found on the FPI, have been found to be necessary for intra-macrophage growth (Nano and Schmerk, 2007). Moreover, IglC protein, which has no known homologs (Telepnev *et al.*, 2003), is robustly upregulated during intra-macrophage growth (Golovliov *et al.*, 1997) and is required for phagosome escape and inhibiting phago-lysosomal fusion (Santic *et al.*, 2006; Lindgren *et al.*, 2004). Also, IglC appears to modulate the host immune response via the down-regulation of Toll-like receptor signaling and *Francisella*-infection induced apoptosis is dependent upon action by this protein (Telepnev *et al.*, 2003).

Further evaluation of the FPI gene cluster resulted in the discovery of a sequence that shares homology with the type VI secretion system (Oyston, 2008; Barker *et al.*, 2009) found in *Pseudomonas aeruginosa* (Mongous *et al.*, 2006) and *Vibrio cholerae* (Pukalzki *et al.*, 2006). While the effector proteins remain largely uncharacterized (Oyston, 2008), a VgrG homologue was found to secrete products into host macrophages which are necessary for phagosomal escape, intracellular replication, and subsequently, virulence (Barker *et al.*, 2009).

In addition to virulence factors found on the FPI, several other mechanisms are utilized by *F. tularensis* to avoid immune detection and enhance virulence. For example, *F. tularensis* lipopolysaccharide (LPS) does not induce a typical immune reaction in response to the presence of endotoxin, suggesting that it is unable to interact with traditional LPS receptors (ex: TLR-4) (Ellis *et al.*, 2002). Due to variation in the O-antigen and lipid A moity, *F. tularensis* is capable of undergoing phase variation which affects antigenicity as well as the nitric oxide response by macrophages. As another example, citrulline ureidase is a carbon nitrogen hydrolase that is encoded by the *ctu* gene (FTT0435) and activity is only found in type A strains of *F. tularensis* (Mahawar *et al.*, 2009). Citrulline is recycled within the macrophage to facilitate the constant production and supply of nitric oxide. Citrulline ureidase functions to degrade citrulline into ornithine, carbon dioxide, and ammonia and is crucial for efficient intra-macrophage growth and inhibition of nitric oxide production, further contributing to the delay of the host immune response facilitating bacterial amplification and spread. The *cfu* gene in type B strains is interrupted by stop codons and a series of amino acid substitutions resulting in a truncated product.

1.4 Host Immune Response:

1.4.1 Innate Immunity

The innate immune system is characterized by a number of barriers that serve to protect the host from any number of pathogens that may enter and create an infection. The skin acts as the first line of defense and, if intact, provides a formidable obstacle for pathogens. Beyond the integumentary system,

bodily secretions (mucus, sweat, and urine), peristalsis, commensal bacteria, and the mucociliary elevator provide additional means to exterminate invading pathogens. A multitude of specific proteins are also involved in eradicating invaders and/or announcing their presence, such as defensins, complement, chemokines, and cytokines. Furthermore, many host cells contain receptors that recognize broad classes of microbial structures such as lipoproteins, LPS, flagellin, and non-self DNA, which are agonists for TLR-2, TLR-4, TLR-5, and TLR-9, respectively. Activation of these pattern recognition receptors (TLRs) induces a pro-inflammatory state in which macrophages/monocytes, neutrophils, and natural killer cells, amongst others, migrate to the site of infection and become activated in order to abolish the pathogen.

The complement system response involves a series of proteins that work in conjunction to target invading cells through the construction of the membrane attack complex (MAC) which causes the formation of transmembrane channels that destroy the pathogen. *F. tularensis* is highly resistant to the assembly of MAC as it binds Factor H which results in the cleavage of C3b (an essential complement protein) to its inactive form iC3b (Ben Nasr and Klimpel, 2008). Furthermore, pathogens opsonized by iC3b are targeted by macrophages for phagocytosis (Plow and Zhang, 1997); thus, *F. tularensis* facilitates its own survival by evading complement killing and harnessing this host system to increase cell entry and survival (Ben Nasr *et al.*, 2006).

Despite being a highly virulent pathogen that is capable of inducing acute disease with a very small infectious dose, *F. tularensis* is a highly stealthy pathogen, equipped with a series of mechanisms to evade immune cells and dampen the pro-inflammatory response when detected. *F. tularensis* targets macrophages and enters cells primarily via the mannose receptor (MR), non-opsonized entry, (Schulert and Allen, 2006) and the complement receptor CR3 (when opsonized by iC3b), none of which induces a strong pro-inflammatory signaling cascade (Aderem and Underhill, 1999; Zhang *et al.*, 2005; Bosio, 2011). TUL4, a surface lipoprotein, induces TLR-2 signaling; however, the observed result is not robust (Chase and Bosio, 2010). This is likely due to the absence of the co-receptor, CD14, on target cells, an

idea that was reinforced during a laboratory experiment that supplied soluble CD14 to macrophages and found that the cells induced an early, strong pro-inflammatory response that was able to elicit host control of the bacterial infection. In addition to minimal TLR-2 activation, *F. tularensis* lipopolysaccharide is a weak TLR-4 agonist due to the presence of only 4 acyl moieties (Dueñas *et al.,* 2006); optimal TLR-4 signaling involves a minimum of 6 acyl chains (Park *et al.,* 2009).

Immune modulation is another technique utilized by *F. tularensis* to subvert a robust, specific immune response. *F. tularensis* has been shown to induce alternative macrophage activation which results in dampened oxidative species generation (Shirey *et al.,* 2008). *F. tularensis* is susceptible to reactive oxygen and nitrogen species; thus, once inside the cell, the pathogen must prevent host cell generation of oxidative species or neutralize their function (Fortier *et al.,* 1992; Bosio and Elkins, 2001; Ireland *et al.,* 2010). Disruption of the assembly of complexes responsible for reactive species generation has been shown to occur via several routes, including interference of phosphorylation of the p47 subunit, inhibition of the accumulation of $gp91^{phox}/gp22^{phox}$ heterodimers in neutrophils (Allen and McCaffrey, 2007), and suppression of NADPH oxidase assembly in human neutrophils following exposure to unrelated stimuli (McCaffrey *et al.,* 2010); in addition to neutralization of oxidative species via catalase and superoxide dismutase (Lindgren *et al.,* 2007). Superoxide scavenging systems encoded as part of the pathogen genome further hobble the capacity of the host cell to develop a robust oxidative burst (Melillo *et al.,* 2010). Moreover, pro-inflammatory cytokine production is significantly abrogated, which results in a delayed reaction to the presence of *F. tularensis* allowing for undisturbed intracellular replication, promoting virulence (Chase *et al.,* 2009; Telepnev *et al.,* 2003; Bosio *et al.,* 2007; Greisman *et al.,* 1963).

Despite a delay in the immune response following infection with *F. tularensis*, pro-inflammatory T_H1 cytokines, particularly TNF-α, IFN-γ, and IL-12 begin to be produced 72-96 or 24-48 hours following infection with type A or type B strains, respectively (Oyston *et al.,* 2004; Kirimanjeswara *et al.,* 2008; Elkins *et al.,* 2007). AIM2 is an intracellular pathogen recognition receptor (also known as a Nod-

like receptor, NLR) that broadly detects cytosolic dsDNA and is activated by the presence of *F. tularensis* (Fernandes-Alnemri *et al.,* 2010). Following intracellular bacterial detection, an inflammasome, a multi-protein complex that contains at least one NLR and regulates caspase-1 activity, is then assembled. The inflammasome recruits and oligomerizes caspase-1, a cysteine protease, by activating the adaptor protein ASC (Weiss *et al.,* 2007). Caspase-1 becomes activated and is responsible for the production of mature pro-inflammatory cytokines, IL-1β (establishing fever) and IL-18 (upregulating IFN-γ) (Fernandes-Alnemri *et al.,* 2010; Rathinam *et al.,* 2010). Furthermore, caspase-1 induces cell death via pyroptosis, a form of apoptosis associated with an inflammatory response that leads to further immune activation and cellular trafficking to the site of infection (Fernandes-Alnemri *et al.,* 2010). The presence of numerous dead and dying macrophages, circulating pro-inflammatory cytokines, and a massive number of disseminating bacteria results in a robust inflammatory response via the innate immune system in the early stages, soon giving rise to a highly specific, adaptive response.

1.4.2 Adaptive Immunity

Due to the intracellular nature of *F. tularensis* replication, the cell-mediated immune response has been found to be much more efficacious in response to infection as compared to pathogen specific antibodies. However, *F. tularensis*-specific antibodies have been shown to develop following infection, with reported isotypes of IgG, IgM, and IgA (Elkins *et al.,* 2007). These antibodies typically appear 2 weeks after infection and a large proportion are targeted against the LPS (Cole *et al.,* 2009) and to a lesser extent the TUL4 lipoprotein (Savitt *et al.,* 2009), the outer membrane proteins FopA and FopB, and the O-antigen capsule (Apicella *et al.,* 2010). Anti-*Francisella* specific antibodies may play an important role during the extracellular period in which bacteria disseminate throughout the body. Prior exposure to heat-killed or fixed antigen as part of a vaccine trial, passive antibody transfer, or exposure to a less virulent strain of *F. tularensis* followed by challenge with a highly virulent strain of the organism has been met with mixed results (Stenmark *et al.,* 2003). The protection afforded by *F. tularensis* specific antibodies appears to be highly dependent on the subsequent challenge strain, dose, and concentration

(Fulop *et al.*, 2001; Cole *et al.*, 2009; Brown *et al.*, 2015). Furthermore, it is difficult to determine if the humoral immune response is responsible for the protection afforded, the T-cell response, or the interaction of B and T lymphocytes (Rhinehart-Jones *et al.*, 1994).

In addition to all of the modulatory mechanisms utilized for the innate immune response, *F. tularensis* alters the cell-mediated response by blocking T-cell proliferation (Woolard *et al.*, 2007). *In vitro* experiments demonstrate that the supernatant of macrophages infected with *F. tularensis* contains prostaglandin E_2 which is a potent inhibitor of IFN-γ production, skewing the immune response toward the production of IL-4 and IL-5 which promote T_H2 activation. T_H1 activation is crucial for enhancing cell-mediated immunity and phagocyte-dependent inflammation, whereas T_H2 activates a strong humoral immune response and has been found to inhibit some of the functions of phagocytic cells (Romagnani, 2000).

Membrane polypeptides, likely under the bacterial surface of *F. tularensis*, serve as the epitopes for T lymphocytes (Tarnvik, 1989). Both $CD4^+$ and $CD8^+$ cells play an important role in optimal protective immunity, responsible for producing pro-inflammatory cytokines such as IFN-γ and TNF-α (Elkins *et al.*, 2007). Interestingly, however, athymic mice (depleted of $CD4^+$ and $CD8^+$ cells) are still able to manage a primary, sub-lethal inoculation of *F. tularensis* LVS, but fail to completely resolve the infection (Conlan *et al.*, 1994). Following infection with *F. tularensis*, a robust, collaborative, multi-faceted immune response involving both innate and adaptive immunity is essential for managing, sequestering, and clearing the organism.

1.5 Diagnostics:

Aerobic culture is considered to be the gold standard of confirming an *F. tularensis* infection; however, a breadth of other laboratory assays can be used in conjunction and often provide useful information with regard to exposure status and other epidemiological factors (OIE, 2008). *F. tularensis*

must be grown in media supplemented with cysteine and is often cultured on Modified Mueller Hinton, Thayer-Martin, or chocolate agar supplemented with hemoglobin or defibrinated sheep blood (Baker *et al,* 1985; Dennis *et al.,* 2001). Indirect and direct fluorescent antibody assays and immunohistochemistry can be used to detect antigen disseminated throughout bodily tissue and provides a high level of specificity (OIE, 2008). Furthermore, PCR can be used to test tissue, swabs, and whole blood and has successfully been used to detect DNA directly from the site of an ulcer on a tularemia patient and allows for rapid diagnosis with a high level of sensitivity (Tarnvik and Chu, 2007). PCR protocols have been designed that are specific to either the genera or species level and are highly useful as a clinical diagnostic for human patients or to evaluate animal carcasses, vector species, or environmental samples. Long and colleagues (1993) describe a protocol using a primer sequence derived from TUL4, a T-cell epitope found on the surface of *F. tularensis.* This protocol is specific to the level of the genera. If sub-species differentiation is necessary, Johannson *et al,* describe a protocol using the C1 and C4 primers that results in an amplicon size that is differentiatable between type A and type B strains of *F. tularensis.*

Serological assays are often employed as a means of further confirmation or to evaluate a historical infection. Using an enzyme-linked immunosorbent assay (ELISA) as a screening tool and a Western blot as a confirmatory test has been found to be an extremely sensitive and efficacious tool for antibody detection (Porsch-Ozcurumez *et al.,* 2004, Schmitt *et al.,* 2005). An immunochromatographic test (a lateral flow assay) that detects *F. tularensis*-specific antibodies has also been developed to allow for rapid testing and has been shown to have both sensitivity and specificity >98% (Splettstoesser *et al.,* 2010).

1.6 Vaccination and Therapeutics:

A safe, efficacious vaccine for humans against tularemia has been under investigation since the 1930s and is of high importance due to concern over this pathogen as an agent of bioterrorism (Barry *et al.,* 2009). Vaccination is believed to be possible due to the highly specific immune response generated

and the subsequent protection afforded by a natural *F. tularensis* infection. This optimism has been supported by experimental trials indicating efficacy against challenge with wild-type SchuS4 following vaccination with the live vaccine strain of *F. tularensis* subsp. *holarctica* (LVS). The Foshay vaccine was the original candidate and involved a killed and formalinized preparation of whole *F. tularensis* cells (Foshay *et al.,* 1949). Vaccination with this candidate allowed for the generation of limited protection against challenge with a type A strain in human and animal models and often resulted in severe local and systemic side effects (Kadull *et al.,* 1950; Ruchman and Foshay, 1942). LVS was generated in the Soviet Union in 1946 as a vaccine candidate and originated from an attenuated type B strain (Tigertt, 1962). A sub-culture was transferred to the U.S. in 1956 and co-cultures from the LVS ampoules demonstrated the presence of two distinct colony types, a white/blue colony type that is immunogenic and a grey colony type that is not immunogenic. Vaccination followed by challenge with a virulent strain of *F. tularensis* in humans demonstrated that LVS delivered via an aerosol route provided increased protection; however, at a sufficient dose LVS induced a mild typhoidal tularemia that necessitated antibiotic treatment in several vaccinees (Hornick and Eigelsbach, 1966). The LVS vaccine is the current gold standard with which all new vaccine candidates are compared; however, it lacks federal approval as the mechanism of attenuation is largely unknown (Rohmer *et al.,* 2006).

Streptomycin was the first antibiotic found to be efficacious against tularemia; however, due to issues with oto- and nephrotoxicity as well as the need for parenteral administration, alternative antibiotic options were essential (Sawyer *et al.,* 1966; Enderlin *et al.,* 1994). Due to favorable tissue penetration, streptomycin is reserved for rare, severe cases of tularemia where the central nervous system is involved (Tarnvik and Chu, 2007). Gentamicin is an alternative aminoglycoside that has been found to have higher rates of relapse but is preferred over streptomycin as it is less toxic (Mason *et al.,* 1980; Dembek, 2011). In comparison to aminoglycosides, which are bactericidal for *F. tularensis*, tetracyclines are bacteriostatic, which results in a higher incidence of relapse, necessitating longer periods for treatment. However, the capacity of tetracyclines for oral administration in conjunction with much lower toxicity

makes them a strong candidate for first line treatment (Tarnvik and Chu, 2007). Doxycycline has been found to be readily bioavailable and minimum inhibitory concentrations can develop and be maintained in blood (Welling *et al.*, 1997). Quinolone antibiotics have also been found to be bactericidal and are far less toxic as compared to aminoglycosides, making them a treatment option for type B tularemia (Tarnvik and Chu, 2007). Practice guidelines published by the Infectious Disease Society of America indicates that severe cases of tularemia should be treated with streptomycin or gentamicin and mild cases should be treated with tetracycline or doxycycline (Stevens *et al.*, 2014). Most strains of *F. tularensis* are β-lactamase positive, which is believed to be largely responsible for the lack of efficacy of β-lactam antibiotics to treat a tularemia infection (Bina *et al.*, 2006).

1.7 Rationale for the Current Study:

The overall aim of the research described in the next four chapters of this dissertation was to study *Francisella* infection in species that have been implicated in environmental maintenance and transmission of this organism. Several aspects of *F. tularensis* pathogenesis, virulence, transmission, and therapeutic options have been extensively evaluated, but very few studies have been performed using species that are believed to play an important role in naturally acquired infections. Similarly, elucidating how *F. tularensis* is capable of surviving in the environment between outbreaks has been a subject of much research, but the resolution of these questions has been elusive. This organism is quite fastidious and it is unlikely to survive alone in the environment, yet it has been shown to cause severe reductions in tick fitness and is highly virulent (especially type A strains) in mammalian species making a true reservoir species unlikely. Amoebal reservoirs have been implicated as an alternative to vector or vertebrate hosts and examples of other bacterium that exploit protozoa, such as *Mycobacterium avium* and *Legionella pneumophila*, further the legitimacy of this possibility (Brieland *et al.*, 1997; Cirillo *et al.*, 1997).

Cottontail rabbits are distinctly implicated in the ecology of type A tularemia and, prior to the modern era, the vast majority of humans infected with tularemia reported handling cottontail rabbits, their

carcasses, or consuming their meat, leading to the moniker "rabbit fever". While previous work has been done to evaluate rabbits and their response to a *F. tularensis* infection, evaluating wild-caught cottontail rabbits is novel and significant as these wild lagomorphs are believed to play an important role in environmental maintenance and transmission. Exposure to various pathogens and parasites as well as the potential for sub-clinical infections, which is certainly common in wild-caught species, allows for a more representative evaluation of the potential role that cottontail rabbits may play in the ecology of the pathogen. Chapters 2 and 3 of this dissertation describe experimental infections performed using cottontail rabbits. A series of parameters were evaluated in this work, specific to immune kinetics, morbidity and mortality, gross and histopathology, and organism dissemination and tissue burden. Furthermore, cross-protection afforded for a type A infection following a type B infection was evaluated.

Subsequently, we sought to assess similar clinical and immunological factors using prairie voles, which are often suggested to play an important role in type B tularemia. Voles were inoculated orally and intranasally to best emulate the route of infection that is believed to be responsible for *F. tularensis* transmission. The findings of this work are described in chapter 4.

In vitro characterization of multiple strains of *F. tularensis* interacting with various genera and strains of amoebae is the clear first step in beginning to unravel the relationship between bacterium and protozoa. The evaluation of two biological systems interacting requires a significant amount of foundational work, the results of which are described in chapter 5.

CHAPTER 2: PATHOGENESIS AND IMMUNE RESPONSES OF *FRANCISELLA TULARENSIS* STRAINS IN WILD-CAUGHT COTTONTAIL RABBITS

2.1 Introduction:

Francisella tularensis is an intracellular, Gram negative zoonotic bacterium that causes significant disease in humans and domestic and wild animals and is also of concern as an organism for bioterrorism (Tarnvik and Berglund, 2003). Tularemia is characterized by multi-systemic disease and has been classified into six diverse forms that reflect the point of entry of the organism into the body (Foley and Nieto, 2010). Tularemia is transmitted by ticks and flies, water exposure, contaminated food, and aerosol dispersion and is endemic in Europe, North America, and Asia (Foley and Nieto, 2010; Oyston *et al.*, 2004; Petersen *et al.*, 2009). There are two main types of *F. tularensis*: subspecies *tularensis* and subspecies *holarctica*, also referred to as type A and type B, respectively (Kugeler *et al.*, 2009; Nakazawa *et al.*, 2010). Type A is endemic only in North America and is transmitted to humans primarily by ticks and biting flies, purportedly from a wildlife amplifying host such as rabbits or through direct contact with infected animals (Petersen *et al.*, 2009; Kugeler *et al.*, 2009). Bacteria of this subspecies are highly virulent, and depending on the route of administration, the LD_{50} in mice may be as low as 1 colony-forming unit (cfu) (Oyston *et al.*, 2004). The type A subspecies is further classified into two subpopulations: A1, which is predominant in the central United States, and A2, which is more common in the western United States (Petersen *et al.*, 2008; Farlow *et al.*, 2005). Type B *F. tularensis* is endemic in the Northern Hemisphere, and is commonly isolated in European countries. Furthermore, type B strains are typically associated with an aquatic lifecycle and have been linked to mosquito transmission (Oyston *et al.*, 2004; Thelaus *et al.*, 2014; Triebenbach *et al.*, 2010).

Previous experimental infections have described clinical presentation and histopathology associated with aerosol infection with *F. tularensis* and determined that rabbits are a potential model for human tularemia; however, neither study has evaluated this organism in a species implicated in natural infections, cottontail rabbits (Baskerville and Hambleton, 1976; Reed *et al.*, 2011). Despite the extensive

literature asserting rabbits as a primary species associated with *F. tularensis* infection, there is a significant gap in understanding with respect to transmission patterns, immune response, and the potential for environmental maintenance of *F. tularensis.* Three cottontail rabbit species are found throughout the majority of the United States and in many cases have overlapping geographical distribution. Desert and mountain cottontail rabbits were used in these experiments and are found to range through the center states (Texas, Oklahoma, Kansas and the Dakotas) and in the southern portion of California, Arizona, Utah and Nevada; and all Western states, respectively. The majority of the strains of *F. tularensis* used in these experiments were isolated from geographically relevant locations in comparison to the regions in which the cottontail rabbit species used in these experiments inhabit, less the MA00-2987. Eastern cottontail rabbits were trapped in Arkansas and Missouri and released in Martha's Vineyard in the late 1930s by game clubs and soon thereafter the first cases of tularemia were identified in the region and are believed to be involved in the ecology of *F. tularensis* in the northeast (Feldman *et al.*, 2001).The objective of this study was to provide an initial characterization of clinical disease, bacteremia, pathology, organ burden, and antibody kinetics of North American cottontail rabbits (*Sylvilagus spp*) experimentally infected with five strains of *F. tularensis*, including 3 type A and 2 type B strains. Additionally, we characterized the long-term humoral immune response and ability to clear infection in cottontail rabbits infected with two of the type B strains.

2.2 Materials and Methods:

2.2.1 Experimental Design and Animals

Two studies were conducted using a total of 46 cottontail rabbits, referred to as acute-phase versus long-term. In the acute-phase study, we infected a total of 20 cottontail rabbits with one of five strains of *F. tularensis* (4 rabbits per strain) to evaluate morbidity, mortality, gross and histopathology, and organ burden. The long-term study utilized an additional 20 cottontail rabbits, 10 of which were challenged with each of two type B strains, and euthanized between 2 and 12 weeks post-infection to assess humoral immune responses over time as well as ability to clear infection. In each phase, two or

three additional rabbits were sham inoculated, housed in the same room as inoculated rabbits, and served as handling controls to evaluate the potential for airborne transmission of *F. tularensis* which has previously been suspected in field studies with European Brown hares (Gyuranecz *et al.*, 2010).

Male and female cottontail rabbits were wild-trapped along the front range of Colorado. The rabbits were housed individually in standard stainless steel rabbit cages within an ABSL-3 containment facility approved for use of Select Agents. Rabbits were provided *ad libitum* access to alfalfa pellets, alfalfa hay, and water, and acclimatized to the laboratory setting for 3-4 weeks prior to infection during which time they were treated for fleas and ticks, and received a subcutaneous IPTT300 temperature transponder (BioMedic Data Systems, Inc., Seaford, DE). Ticks were not observed on any of the rabbits. PCR and DNA sequencing were used to determine rabbit species as previously described by Berkman and colleagues (Berkman *et al.*, 2009). The sequences of amplified products were compared to representative sequences from Genbank to determine species identity. All 22 rabbits used in the acute-phase study and 21 of 23 for the long-term study were found to be desert cottontails *(Sylvilagus audobonii),* with the remaining two being mountain cottontails *(Sylvilagus nuttallii).*

Rabbits were inoculated with *F. tularensis* intradermally on the right hip with 50 µL containing between 25 and 125 organisms; control animals were inoculated with 50 µL of sterile PBS. Prior to and after inoculation, body weight, temperature, and appetite of each rabbit was evaluated. Weight was measured using a Pesola scale with the rabbit wrapped tightly in a towel and placed in a cloth bag. Once daily the rabbits were provided a sweet treat of peaches, pears, or pineapple, and their enthusiasm for those items proved to be an effective means of assessing subtle changes in clinical presentation.

This work was approved by the Animal Care and Use Committee at Colorado State University (approval #13-4209A) and conducted in strict accordance with the NIH Guide for the Care and Use of

Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals).

2.2.2 Bacterial Strains and Culture Methods

Five strains of *F. tularensis* were used in these experiments; for clarity, each strain name is abbreviated to include its clade **(Table 2.1)**. All five strains of *F. tularensis were* provided by Dr. Jeannine Petersen at unknown passage history, and passaged in our laboratory one time. Stocks of strains Schu-A1a, MA-A1b, KY-B, and OR-B were prepared from cultures grown 24 to 36 hours in Modified-Mueller Hinton (MMH) broth at 37°C with 5% CO_2, and frozen in 15% glycerol (Baker *et al.*, 1985). The WY-A2 strain was grown on cysteine heart agar with 9% chocolatized sheep blood (CHAB), due to difficulty culturing this organism in MMH broth, for 48 hours at 37°C with 5% CO_2 after which the agar plate was flooded with MMH broth and the colonies collected; glycerol was added to the broth to achieve a final concentration of 15%.

Table 2.1: Strains of *F. tularensis* used in this study.

Project	Strain Name	Clade	Abbreviation	Number of rabbits
Acute	SchuS4	A1a	Schu-A1a	4
	MA00-2987	A1b	MA-A1b	4
	WY96-2418	A2	WY-A2	4
	KY99-3387	B	KY-B	4
	OR96-0246	B	OR-B	4
	PBS			2
Long-term	KY99-3387	B	KY-B	10
	OR96-0246	B	OR-B	10
	PBS			3

2.2.3 Bacteremia Evaluation

To assess bacteremia, 50 µL of blood collected from the marginal ear vein was immediately diluted with 450 µL of PBS. Serial ten-fold dilutions of this mixture were plated on Modified-Mueller

Hinton agar, except for samples from rabbits infected with the WY-A2 strain, which were plated on CHAB agar. Plates were incubated at 37°C with 5% CO_2 for 24-72 hours at which time they were counted and recorded. Colonies derived from whole blood samples were not individually confirmed using PCR; rather, if the number of colonies recorded was consistent with 10-fold serial dilutions and organisms were found disseminated in the liver, spleen, and/or lungs, and subsequently confirmed by PCR (protocol described below)the animal was considered to have had a bacteremia.

2.2.4 Euthanasia, Necropsy, Histopathology, and Organ Burden

Rabbits were euthanized at the end of the study or as necessary due to a moribund condition, by an overdose of pentobarbital administered intravenously. All survivors of the acute-phase study were euthanized at day 14, whereas rabbits used in the long-term experiment were euthanized at intervals between 2 and 12 weeks post-infection in order to assess organ burden at various time-points after inoculation. Necropsies were performed on all animals and gross lesions recorded, including microabscess formation, pulmonary consolidation, and splenomegaly. Splenomegaly was assessed qualitatively rather than by weight because control spleen weights for wild-caught cottontail rabbits were not available. The liver, spleen, and lungs were the primary organs evaluated for gross pathology lesions as *F. tularensis* preferentially traffics to these sites (Dennis *et al.*, 2001; Lamps *et al.*, 2004). The following tissues were collected and placed in 10% buffered formalin: liver, spleen, lungs, heart, duodenum, bladder, and kidneys. Samples were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for histological assessment. Sections were examined on a Nikon Eclipse 51E microscope and digital micrographs were taken with a Nikon DS-Fi1 camera with a DS-U2 unit and NIS elements F software. Images are reproduced without manipulations other than cropping and adjustment of light intensity. Severity of lesions was scored on a scale of 0 to 6 with zero denoting histologically normal appearance and 6 indicating the most severe lesions.

Samples (100 mg) of lung, liver, spleen, and kidney were collected in a vial with 0.9mL of Mueller-Hinton broth containing 15% glycerol and 2 stainless steel BBs, immediately homogenized in a mixer mill, and frozen at -80°C. Samples were thawed and ten-fold serial dilutions were made from 10^{-1} to 10^{-3}. Samples (100 µL) from each of the three dilutions were plated on MMH agar plates (except for rabbits infected with the WY-A2 strain which were plated on CHAB agar). The plates were incubated at 37°C with 5% CO_2 for 24-48 hours and colony counts recorded. For each rabbit with a positive culture, DNA was extracted from one bacterial colony derived from the spleen and its identity as *F. tularensis* was confirmed by PCR using a protocol described by Long and coworkers (Long *et al.*, 1993).

2.2.5 Serology

An ELISA was developed to detect antibodies to *F. tularensis* in serum. This assay followed the procedures outlined in the WHO Guidelines on Tularaemia (Tarnvik, 2007), with exceptions described below. Briefly, Nunc polysorp 96-well plates (Thermo Scientific, Rochester, NY) were coated overnight at room temperature with 100 µL of coating buffer containing 3 µg/mL of *F. tularensis* LPS obtained from BEI Resources (Manassas, Virginia, USA), blocked with 5% non-fat dry milk for 30 minutes and rinsed 5x with 300 µL of washing buffer. Serum samples were heat treated for 30 minutes at 56°C to ensure inactivation of any residual organisms, diluted 1:1000 in incubation buffer, and duplicate wells loaded with 100 µL. Positive and negative rabbit sera were used as controls in each assay. After a 1 hour incubation, the plate was emptied and rinsed 5x with 300 µL of washing buffer. Goat-anti-rabbit horseradish-peroxidase conjugate (Jackson ImmunoResearch, West Grove, Pennsylvania) was added to each well and incubated for 1 hour. The plate was again emptied, rinsed 5x with washing buffer, and substrate applied (TMB Peroxidase Substrate, KPL, Gaithersburg, MD). The reaction was allowed to proceed for 15-20 minutes, stopped via the addition of 50 µL of 1N hydrochloric acid, and optical density determined using a plate reader with a 450 nm filter (BioRad Model 680 plate reader, Hercules, California). The cutoff for determining a positive sample was set as 3 standard deviations above the mean of the values from negative control sera.

2.2.6 Statistical and Survival Analyses

STATA software (Stata, Statistical Software: Release 11.2, College Station, Texas) was used for descriptive statistics and survival analysis. Median survival times (in days) and 95% confidence intervals were calculated using a Kaplan Meier survival function. Univariate non-parametric analysis was conducted using the log rank test to compare the survival function (risk of death) among cottontail rabbits infected with different strains. Fisher's exact test was used to compare histopathological findings, frequency of dissemination, and organ burdens in the acute-phase study.

2.3 Results:

2.3.1 Acute-Phase Experiment

All of the rabbits inoculated with type A strains became ill, lost roughly 10% of their body weight, and either died or required euthanasia prior to 14 days post-infection (dpi) **(Table 2.2)**. Fever was observed in all rabbits starting at 2 dpi and persisted through the time of death in all but two rabbits. Baseline body temperature before infection ranged from 101 to 104^{o}F, and peak body temperature after challenge ranged from 107 to 109^{o}F. At necropsy on 5 dpi, rabbit 1 (Schu-A1a) was found to have a large intrathoracic abscess, likely as a preexisting infection; *F. tularensis* was not confirmed by PCR in samples from this rabbit and it was removed from the study. Bacteremia was detected on a single day in two of the rabbits inoculated with either the MA-A1b or Schu-A1a strains, but not in rabbits inoculated with the WY-A2 strain. Despite the low rate of bacteremia detected by once daily sampling, the type A organisms were widely disseminated in all but one rabbit, indicating hematogenous spread throughout the body. Gross lesions were detected in all 11 rabbits inoculated with type A strains of *F. tularensis* **(Figure 2.1A)**. The most common gross lesions were micro-abscesses randomly scattered throughout the liver and/or spleen and splenomegaly **(Table 2.2)**. Histopathologic lesions observed in rabbit #4 in liver, spleen, and lung 13 dpi with Schu-A1a are depicted in **Figure 2.2**. These lesions are representative of severely infected animals, as summarized in **Table 2.3**.

Table 2.2: Acute-phase experiment – Summary of clinical response, pathology, and microbiology.

						Organ burden*		
Strain	**Rabbit**	**DPI euthanized**	**Microabscesses?**	**Splenomegaly?**	**Bacteremia**	**Liver**	**Spleen**	**Lung**
Schu-A1a	2	5	Yes	No	No	(-)	+	+
	3	7	Yes	Yes	Yes (5 dpi)	+++	+++	+++
	4	13	Yes	Yes	Yes (8 dpi)	+++	+++	+++
MA-A1b	5	5	Yes	No	Yes (5 dpi)	+++	+++	+++
	6	4	Yes	No	No	+++	+++	+++
	7	4	Yes	No	Yes (3 dpi)	+++	+++	+++
	8	3	Yes	No	No	+++	+++	++
WY-A2	9	7	No	Yes	No	+++	+++	+++
	10	8	Yes	Yes	No	+++	+++	++
	11	3	Yes	No	No	+++	++	++
	12	7	No	Yes	No	+++	+++	+++
KY-B	13	14	No	No	No	(-)	+	+
	14	14	No	No	No	(-)	+	+++
	15	14	No	No	No	(-)	(-)	(-)
	16	14	Yes	Yes	No	(-)	+	(-)
OR-B	17	14	No	No	No	+	+	++
	18	14	No	No	No	+	+	+
	19	14	No	Yes	No	+	++	+
	20	14	Yes	Yes	No	+	(-)	+

*The number of (+) signs is associated with the number of organisms found in the first plated dilution (10^{-1}): (+) = 1-<10^4 cfu/gram; (++) = 10^4-10^5 cfu/gram; (+++) = >10^5 cfu/gram. Tissues that were not found to have any organism are denoted with a (-) sign.

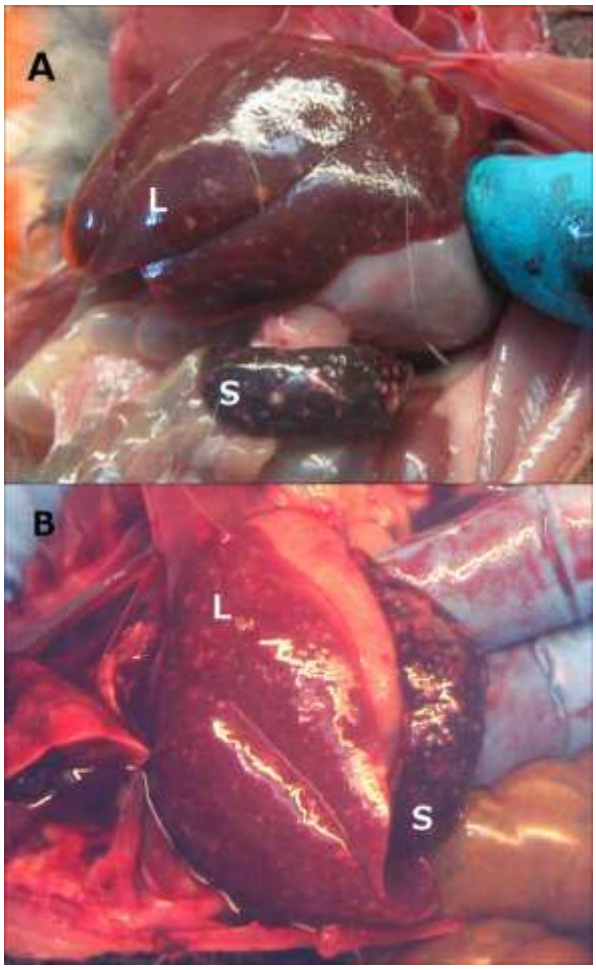


Figure 2.1: Presence of microabscesses resulting from *F. tularensis* infection. A) Liver and spleen from rabbit #3, 7 days following challenge with Schu-A1a; B) Liver and spleen from rabbit #20, 14 days following challenge with OR-B.

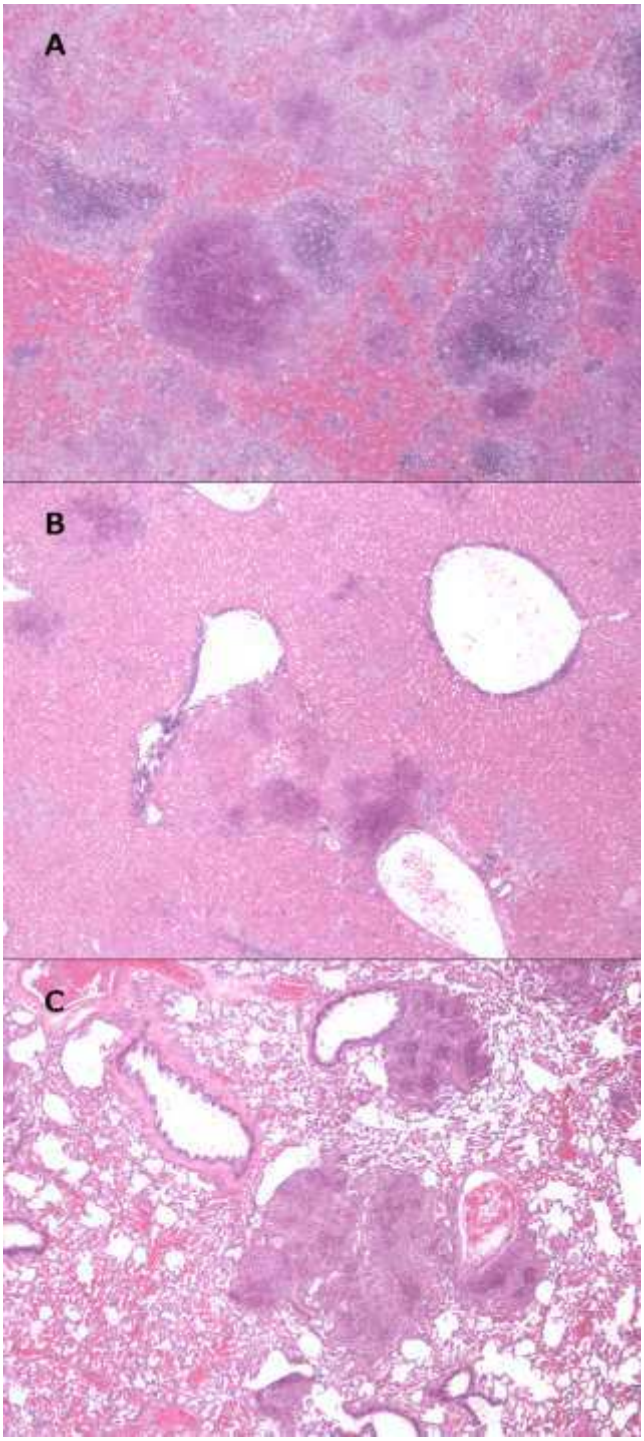


Figure 2.2: Histopathology associated with Schu-A1a infection. Micrographs of A) Spleen, B) Liver, C) Lung at 40x from rabbit #4, 13 days following challenge.

Table 2.3: Acute-phase experiment – Histopathology.

Strain	Total	Tissue	0*	1-2	3-4	5-6
Schu4 (A1a)		Liver	-	1	2	1
	4	Spleen	-	2	-	2
		Lung	1	1	1	1
MA00 (A1b)		Liver	-	1	1	2
	4	Spleen	-	-	4	-
		Lung	3	1	-	-
WY96 (A2)		Liver	-	2	2	-
	4	Spleen	-	4	-	-
		Lung	2	2	-	-
KY99 (B)		Liver	2	2	-	-
	4	Spleen	2	1	1	-
		Lung	2	1	1	-
OR96 (B)		Liver	-	3	1	-
	4	Spleen	-	2	2	-
		Lung	-	2	2	-

*0=normal, 1-2=minimal to mild changes, 3-4=moderate to focally marked, 5-6=severe, widespread, diffuse.

All three animals infected with Schu-A1a had severe focal (#2) or multifocal to coalescing, necrotizing splenitis. Two of the animals also had moderate, necrotizing hepatitis, while only one animal (#4) had evidence of pneumonia, characterized by multifocal to coalescing infiltration of macrophages, heterophils and fewer lymphocytes and marked pulmonary vasculitis. All four animals infected with strain MA-A1b had moderate to severe, necrotizing hepatitis and moderate splenitis. Only one of the animals (#5) had apparent pneumonia, presenting as focal, necrotizing lesions, while another (#7) had very severe lung edema; presumably an agonal change. The WY-A2 infected animals all had multifocal, mild to moderate, necrotizing hepatitis and mild splenitis. In three of the animals there was mild

leukocytosis in the pulmonary vasculature, but no frank parenchymal inflammation. Out of the four animals infected with OR-B, and terminated on 14 dpi, two had focal (#17) or multifocal (#20) severe, necrotizing pneumonia. All four animals had multifocal, mild to moderate splenitis and hepatitis, either necrotizing or granulomatous in character. In contrast, two of the animals infected with KY-B appeared histologically unremarkable (#13 & 14), whereas #15 and #16 had minimal to mild hepatitis, splenitis, and alveolitis.

In contrast to the response to each of the type A strains, none of 8 rabbits inoculated with the two type B strains manifested severe, overt clinical disease, nor did they succumb to their infection within 14 days **(Figure 2.3; Table 2.4)**; however, gross lesions were detected on 3 out of the 8 rabbits **(Figure 2.1B)**. Elevated body temperature was observed starting at 2 dpi and persisted until 8 dpi in all of the rabbits inoculated with either strain of type B *F. tularensis* (data not shown). Bacteremia was not detected in any of these animals, and the risk of gross lesions was limited in comparison to rabbits inoculated with type A strains **(Table 2.2)**. The frequency of dissemination in rabbits inoculated with type B strains was not different from those inoculated with type A strains (Fisher's exact test, $p=0.65$), but the magnitude of organ burdens in lung, liver, and spleen was significantly lower in rabbits infected with type B strains when comparing tissues with $>10^5$ cfu/gram to all others (Fisher's exact test, $p<0.0001$). When comparing histopathologic lesions between rabbits inoculated with type A versus type B strains, we compared rabbits with 'severe, widespread, diffuse' changes in liver, spleen, and/or lung to those with 'normal, minimal, mild, or moderate' changes in the same tissues and detected 18% and 0%, respectively (Fisher's exact test, $p=0.07$).

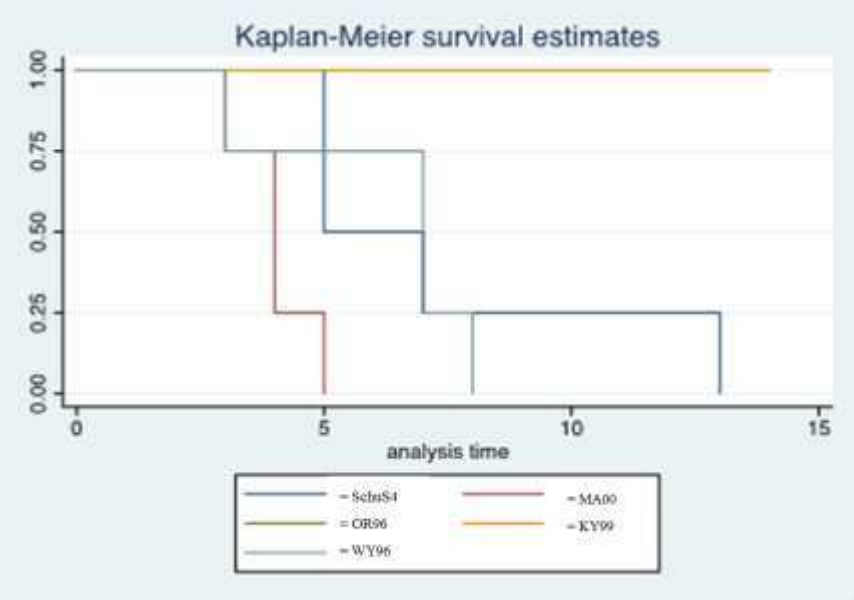


Figure 2.3: Kaplan-Meier survival function. Comparison for rabbits intradermally inoculated with one of five strains of *F. tularensis*, 3 type A strains and 2 type B strains.

Table 2.4: Median survival time (in days) following infection with one of five strains of *F. tularensis.*

Strains	Median	Survival Range
Schu-A1a	6	5-13
MA00-A1b	4	3-5
WY-A2	7	3-8
KY-B	14	14
OR-B	14	14

The type A strains of *F. tularensis* were considerably more virulent in cottontail rabbits than type B strains, as none of the rabbits infected with type A strains (n=11) survived to day 14 whereas all of those inoculated with type B strains (n=8) survived to day 14 **(Figure 2.3; Table 2.4)**. There were significant differences in the risk of mortality for rabbits inoculated with these two types of *F. tularensis* (log-rank test, $p<0.0001$) **(Table 2.2)**. Rabbits infected with type A strains were 14 times as likely to develop microabscesses in the liver and/or spleen compared to rabbits infected with type B strains (Fisher's exact test OR 95% CI = 1.6 – 111.8, p=0.0237). However, there was no difference in the

likelihood of splenomegaly between rabbits challenged with type A and type B strains of *F. tularensis* (Fisher's exact test OR 95% CI = 0.2 – 8.2, p=1.0).

Uninfected control rabbits remained healthy and active over the course of the experiment and did not exhibit elevation in body temperature or a decline in body weight.

2.3.2 Long-Term Experiment

Clinical and microbiologic responses of rabbits inoculated with KY-B or OR-B are summarized in **Table 2.5**. Three of the 10 rabbits inoculated with KY-B were euthanized due to clinical disease between 8 and 10 dpi; however, *F. tularensis* was detected post-mortem only in two of these animals. The third rabbit (#20) was euthanized due to a peri-ocular abscess determined (via a Gram stain) to be unrelated to infection with *F. tularensis.* The remaining seven rabbits inoculated with KY-B developed moderate fever for several days, but did not manifest overt disease. Mild, transient fever was also observed in all of the rabbits inoculated with OR-B, and two of those ten animals were euthanized during the course of the experiment due to conditions not related to the tularemia infection. Only one rabbit of the 20 inoculated with KY-B or OR-B was found to be bacteremic. That event was detected on 5 dpi, and the animal (rabbit #16, KY-B) was found moribund on 10 dpi and euthanized.

Table 2.5: Long-term experiment – Summary of clinical response, pathology, and microbiology.

							Organ burden		
Rabbit	**Strain**	**DPI euthanized**	**Microabscesses?**	**Splenomegaly?**	**Lung consolidation?**	**Bacteremia**	**Liver**	**Spleen**	**Lung**
1	OR-B	84	No	No	No	No	(-)	(-)	(-)
2		56	No	No	Yes	No	(-)	(-)	(-)
3		28	No	No	No	No	(-)	(-)	(-)
4		56	No	Yes	Yes	No	(-)	(-)	(-)
5		14	No	No	No	No	(-)	(-)	(-)
6		84	No	No	No	No	(-)	(-)	(-)
7		14	No	No	No	No	(-)	(-)	(-)
8		36**	No	No	Yes	No	(-)	(-)	(-)
9		28	Yes	No	No	No	(-)	(-)	(-)
10		17**	No	No	No	No	(-)	(-)	(-)
11	KY-B	28	No	No	No	No	(-)	(-)	(-)
12		84	No	No	No	No	(-)	(-)	(-)
13		84	No	No	No	No	(-)	(-)	(-)
14		8*	Yes	Yes	No	No	+++	+++	+++
15		56	No	Yes	Yes	No	(-)	(-)	(-)
16		10*	Yes	Yes	No	Yes	+++	+++	+++
17		56	No	No	No	No	(-)	(-)	(-)
18		14	Yes	Yes	No	No	(-)	(-)	(-)
19		28	No	No	No	No	(-)	(-)	(-)
20		9**	No	No	No	No	(-)	(-)	(-)

*Rabbits euthanized due to a moribund condition associated with *F. tularensis* infection.
**Rabbits euthanized due to a health condition unassociated with the experimental infection.

Of the ten animals infected with OR-B, three were terminated 14-17 dpi. Two of these had multifocal, subacute, necrotizing pneumonia, while the third had granulomatous splenitis. Of the remaining seven animals, terminated at dpi 28-84, all but one had mild to severe hepatitis, in two cases accompanied by amyloid depositions and marked Kupffer cell hypertrophy. The same six animals also had multifocal, moderate to severe, mostly necrotizing pneumonia. Of the ten animals infected with KY-

B, four animals were terminated 8-14 dpi. Three of these had necrotizing splenitis, varying from mild (#20) to very severe (#14). Two animals (#16 & 18) had multifocal, mild to moderate, subacute, necrotizing pneumonia, while one animal (#14) had multifocal to coalescing, necrotizing hepatitis. Of the remaining six animals in this group, terminated 28-84 dpi, only two had histopathological evidence of severe pneumonia. Other mild lesions observed in a couple of animals included interstitial nephritis, most likely a lesion unrelated to the experimental infection, and mild hepatitis.

No histopathologic comparisons were made among or within groups at the varying time points as the sample sizes were not sufficient to allow for comparisons. The histopathologic findings are summarized in **Tables 2.6** and **2.7**.

Table 2.6: Long-term experiment – Histopathology (KY-B).

		Liver				Spleen				Lung			
Necropsy day	Total	0	1-2	3-4	5-6	0	1-2	3-4	5-6	0	1-2	3-4	5-6
≤D14	4	-	2	1	1	1	-	1	2	2	1	1	-
D15 – 28	2	1	1	-	-	2	-	-	-	-	1	-	1
D29 – 56	2	-	1	1	-	1	1	-	-	1	-	-	1
D57-84	2	2	-	-	-	2	-	-	-	1	1	-	-

*0=normal, 1-2=minimal to mild changes, 3-4=moderate to focally marked, 5-6=severe, widespread, diffuse.

Table 2.7: Long-term experiment – Histopathology (OR-B).

		Liver				Spleen				Lung			
Necropsy day	Total	0	1-2	3-4	5-6	0	1-2	3-4	5-6	0	1-2	3-4	5-6
≤D14	2	-	2	-	-	1	1	-	-	1	-	1	-
D15 – 28	3	-	2	1	-	2	1	-	-	-	1	1	1
D29 – 56	3	-	1	2	-	2	-	-	1	-	-	-	3
D57-84	2	1	1	-	-	2	-	-	-	1	1	-	-

*0=normal, 1-2=minimal to mild changes, 3-4=moderate to focally marked, 5-6=severe, widespread, diffuse.

Humoral immune response was evaluated only in the long-term experiment and it was found that all of the rabbits surviving past 14 dpi developed an antibody response. Irrespective of the infecting strain, the peak antibody production (with an OD value between 2.0 and 2.5) occurred between days 14 and 21 post-infection, then typically declining slightly before leveling out and remaining stable for the during of the experiment **(Figure 2.4)**.

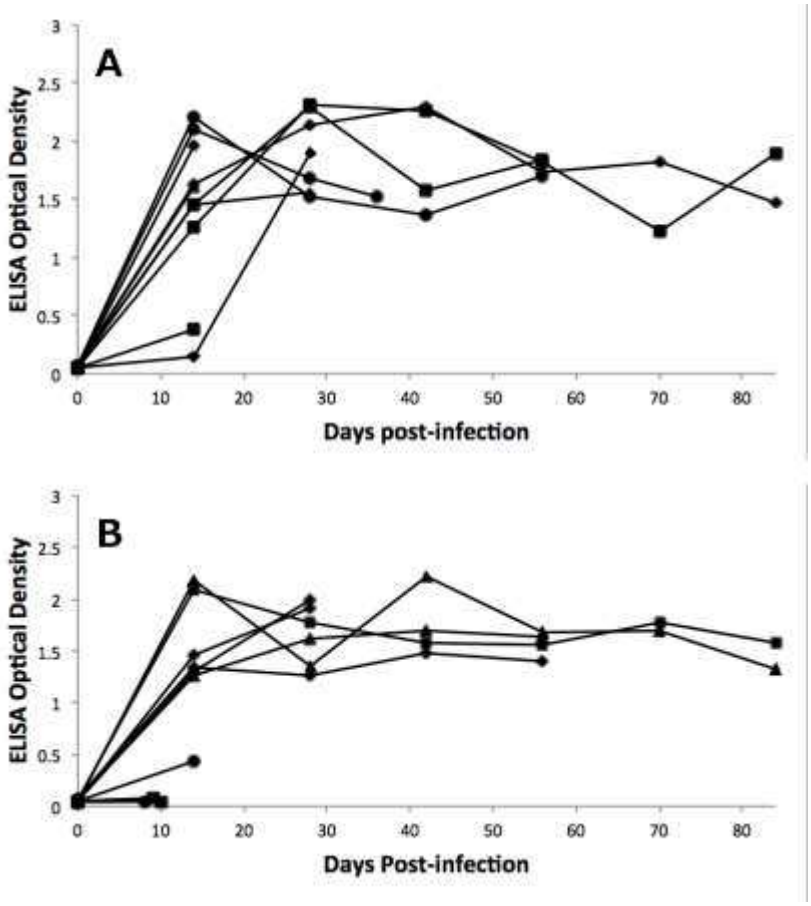


Figure 2.4: ELISA antibody responses of rabbits infected with *F. tularensis.* Panels depict: A) OR-B and B) KY-B between 8 and 84 days post-infection.

2.3.3 Control Rabbits

Control rabbits had no detectable antibodies to *F. tularensis*, nor was any organism detected when tissues were homogenized and plated after necropsy. Due to the territorial nature of cottontail rabbits, it is unfeasible to co-house these animals which may be necessary in order to evaluate transmission among rabbits. Two of the three mock-infected control animals, terminated on day 84, had a large hepatic granuloma, in one case with caseation and mineralization of the central core. The most likely cause of this type of lesion is aberrant parasite migration, as often observed in wild-caught cottontail rabbits (HBO, unpublished observations).

2.4 Discussion:

This study provides an initial assessment of the pathogenesis of infection in cottontail rabbits experimentally infected with several field isolates of *F. tularensis.* Understanding the dynamics of organism dissemination, gross and histopathology, organ burden, and mortality, as well as humoral immune response and ability to clear infection is a crucial start when attempting to determine the role of cottontail rabbits in maintenance and spread *F. tularensis*.

The acute-phase experiment identified the MA-A1b strain to be the most virulent of those tested in cottontail rabbits (all four infected rabbits succumbed to disease by day 5), followed successively by WY-A2 and Schu-A1a **(Table 2.2)**. As anticipated, the type B strains (OR-B and KY-B) were distinctly less virulent than type A strains, although capable of causing mortality in some instances (KY-B). These findings support what has been found in laboratory mice, but contrast the findings in humans, as the type B strains typically result in higher mortality as compared to A2 strains (Molins *et al.*, 2010; Reese *et al.*, 2011). Among human cases of tularemia reported in the U.S., the A1b strain resulted in 24% mortality as compared to 4% with A1a strains, 0% with A2 strains, and 7% with type B strains (Reese *et al.*, 2010; Staples *et al.*, 2006). However, type B strains appear to be fatality biased by age in humans which makes our findings in cottontail rabbits congruent with the findings in humans (Kugeler *et al.*, 2009). Increases in body temperature in our study were observed more rapidly following inoculation and were of much higher magnitude as compared to those reported by Reed and colleagues (Reed *et al.*, 2011).

The long-term experiment demonstrated that cottontail rabbits are capable of developing a robust humoral immune response following an intradermal challenge with *F. tularensis.* The level of protection afforded by this antibody response against a virulent challenge with *F. tularensis* has yet to be determined, although the intracellular nature of this pathogen may render antibodies insufficient (Celli and Zahrt, 2013).

Bacteremia was difficult to detect in both experiments, despite 90% of rabbits having organism in liver, spleen, and/or lungs, which is suggestive of hematogenous spread. In order to evaluate the presence of organisms in the bloodstream, rabbits were bled once daily (on the aforementioned days) in the morning. This suggests that the bacteremia was transient in nature and thus, not readily detected with only one bleed per day. We suspect that the organism was sequestered in micro-abscesses, specifically on the liver and spleen, and at various points these abscesses would rupture resulting in a 'bacterial seeding event' that facilitated organism dissemination. Alternatively, the numbers of organism in the bloodstream may have been below our limit of detection (100 cfu/mL). Samples were obtained in an aseptic manner however, it is a possibility that contaminating bacteria prohibited the growth of *F. tularensis* on the plate in some instances, which has previously been shown to occur (Petersen *et al.*, 2004).

This study was subject to several potential limitations, perhaps most importantly that it utilized wild-caught animals that were undoubtedly stressed by being held in captivity. We attempted to mitigate this problem by acclimatizing them for several weeks prior to challenge and handling as gently and infrequently as possible. While the laboratory is certainly an environment they were not familiar with, it is arguably less stressful than their natural environment. Additionally, our goal was to study the infection in a natural host, and the animals we used had been exposed to and perhaps harbored a variety of other pathogens. Finally, the inoculating dose and route were not necessarily representative of those experienced in a natural setting. There is no precise estimate from the literature indicating the amount of *F. tularensis* that would be transferred from an arthropod vector to a mammalian host, so we utilized a low inoculation dose administered intradermally in an attempt to mimic what a tick might deliver. Despite these challenges, we believe our findings to be novel and reliable as we pursue further understanding of *F. tularensis* infection in cottontail rabbits.

The primary *F. tularensis* strains in the U.S. are type A which have been associated with cottontail rabbits as the primary amplifying species; transmission to humans thought to result from

interaction with or ingestion of contaminated rabbit carcasses or an arthropod vector, primarily ticks and biting flies (Petersen *et al.*, 2009; Reese *et al.*, 2011; Eisen, 2007). Despite this longstanding notion, our understanding of cottontail rabbits and their role in *F. tularensis* transmission and maintenance is inadequate (Foley and Nieto, 2010; Farlow *et al.*, 2005; Telford and Goethert, 2011). Our findings show that rabbits infected with field isolates of type A strains, MA-A1b and WY-A2, all rapidly succumbed to infection (maximum survival of 8 days). This level of virulence is not typical in reservoir hosts, which must survive the infection in order to maintain the infective agent in nature. Furthermore, bacteremia was detected in only two of the 16 rabbits challenged with field isolates which further complicates the current perception that the bacterium is transmitted by arthropods after blood feeding on *F. tularensis* infected lagomorphs. These characteristics perhaps indicate that cottontail rabbits are an incidental host that are extremely susceptible to disease and may contribute to disease ecology by amplifying the bacteria. Recent publications articulate a similar message, "although the rabbit is commonly cited as the reservoir of tularemia, it is more likely that the actual reservoir is either an environmental fomite or the arthropod vector itself. Nevertheless, the rabbit is often associated with human exposure risk and may be more appropriately termed an amplification host rather than a reservoir" (Foley and Nieto, 2010). In many instances, large rabbit die-offs are indicative of the presence of *F. tularensis* which often spills over into the human population, "rabbits and hares may only be the epidemiological bridge and are not necessarily an element of natural focality" (Telford and Goethert, 2011). It may be that rabbits could be used as a sentinel species for tularemia as prairie dogs are used for plague, *Yersinia pestis*, in the United States (Cully *et al.*, 2000; Lowell *et al.*, 2009). Further research is needed to elucidate the role cottontail rabbits play in the maintenance and transmission of *F. tularensis* among wildlife populations and to humans.

CHAPTER 3: PRIOR INOCULATION WITH TYPE B STRAINS OF *FRANCISELLA TULARENSIS* PROVIDES PARTIAL PROTECTION AGAINST VIRULENT TYPE A STRAINS IN COTTONTAIL RABBITS

3.1 Introduction:

Francisella tularensis (*F. tularensis*) is an intracellular, zoonotic bacterium, and infection with this organism causes tularemia (Sjostedt, 2006). This organism is capable of causing severe disease in a wide variety of species and, due to its low infectious dose and high virulence (the LD_{50} for some type A strains of *F. tularensis* has been found to be as low as one colony-forming unit (cfu) in mice), is classified as a Tier 1 Select Agent by the Centers for Disease Control and Prevention (CDC) (Dennis *et al.*, 2001; Oyston *et al.*, 2004). *F. tularensis* is classified into two subspecies: *tularensis* and *holarctica* which are referred to as type A and B respectively, and are responsible for the vast majority of human tularemia cases (Kugeler *et al.*, 2009; Molins *et al.*, 2010). Despite a largely homologous genome, type A and type B can be readily distinguished due to large differences in virulence. Additionally, these two strains differ in global geographical distribution (Petersen and Molins, 2010). Type A is endemic in North America and transmission is primarily via bites from infected vectors (ticks and biting flies) or direct contact with amplifying species, such as cottontail rabbits (Nakazawa *et al.*, 2010; Akimana and Kwaik, 2011; Reese *et al.*, 2011). Based on genetic clustering, type A strains can be further differentiated into two subpopulations: A1, primarily found in the central United States and on both coasts, and A2, which is predominantly found in the western United States (Kugeler *et al.*, 2009; Nakazawa *et al.*, 2010). Furthermore, in cases of human tularemia, A1a has been associated with 4% mortality compared with 24% for A1b and 0% for A2 (Staples *et al.*, 2006; Reese *et al.*, 2010). Type B has been found in North America and is the only species endemic in Europe; this organism is associated with mosquito-borne transmission and an aquatic cycle, primarily involving beavers, muskrats, and voles (Keim *et al.*, 2007). These reservoir species become infected and contaminate waterways via their carcasses and urine which then serve as a route of infection for mosquito larvae, other aquatic mammals, and humans (Bell and

Stewart, 1975; Rossow *et al.*, 2014b). Type B strains of *F. tularensis* cause mortality in 7% of human cases (Staples *et al.*, 2006; Reese *et al.*, 2010).

Tularemia has been recognized for over a century and has long been associated with cottontail rabbits primarily due to rabbit die-offs or contact with a rabbit preceding human cases of tularemia (Foley and Nieto, 2010; Telford and Goethert, 2011). Our laboratory has previously demonstrated that type A strains are highly virulent in cottontail rabbits and challenge with 50-100 organisms results in 100% fatality within 13 days of inoculation (Brown *et al.*, 2015a). Inoculation of type B strains rarely resulted in mortality in cottontail rabbits, and challenged rabbits elicited a robust humoral immune response through 12 weeks post-infection. Importantly rabbits challenged with type B strains appeared capable of clearing the organism (Brown *et al.*, 2015a). The objective of this study was to determine if a prior infection with a type B strain would provide cross-protection against subsequent challenge with a type A organism in North American cottontail rabbits (*Sylvilagus spp*).

3.2 Materials and Methods:

3.2.1 Ethics Statement

All aspects of this work, including experimental manipulations and sampling were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Colorado State University (approval #13-4209A). Rabbits were trapped on public lands using Havahart traps baited with grain following approval from the Colorado Department of Natural Resources, Parks and Wildlife; cottontail rabbits are not endangered or protected. At the end of the study period, rabbits were euthanized by intravenous overdose of pentobarbital.

3.2.2 Bacterial Strains and Culture Methods

All four strains of *F. tularensis* used in this study were provided by the CDC and were subsequently maintained in our laboratory; passage number is unknown. For simplicity, the strains are abbreviated to include the U.S. state in which they were originally isolated and their clade distinction **(Table 3.1)**. MA-A1a, KY-B, and OR-B were prepared from cultures grown in Modified-Mueller Hinton (MMH) broth at 37°C with 5% CO_2 and frozen in 15% glycerol (Baker *et al.*, 1985). Due to difficulty culturing the WY-A2 strain in MMH broth, cysteine heart agar with 9% chocolatized sheep blood (CHAB) was used under identical incubation settings as the strains above. Following 48 hours of growth, the agar plate was flooded with MMH broth and colonies were collected and frozen with 15% glycerol.

Table 3.1: Strains of *F. tularensis* used in this study.

Strain Name	Clade	Abbreviation
MA00-2987	A1b	MA-A1b
WY96-2418	A2	WY-A2
KY99-3387	B	KY-B
OR96-0246	B	OR-B

3.2.3 Experimental Design and Animals

Thirty-five cottontail rabbits (16 males and 19 females) were wild-trapped along the front range of Colorado. Rabbits were transported to an ABSL-3 facility at Colorado State University and acclimatized for 2-3 weeks prior to infection. During that time they were treated for ectoparasites, a IPTT-300 temperature transponder (BioMedic Data Systems, Inc., Seaford, DE) was implanted subcutaneously under lidocaine anesthesia, and an ELISA performed on pre-inoculation serum to provide some assurance that the rabbits were naïve. The rabbits were individually housed in standard size, stainless steel rabbit cages and provided free access to alfalfa hay, commercial rabbit pellets, and water.

Rabbits were inoculated intradermally on the right hip with 50 µL of inoculum that was confirmed by backtitration to contain between 40-80 cfu of one of two strains of *F. tularensis* on day 0 and one of four strains on day 28 **(Table 3.1)**. On the first inoculation day (day 0), fifteen rabbits were inoculated with OR-B, fifteen rabbits were inoculated with KY-B, and five rabbits were sham inoculated with sterile phosphate buffered saline (PBS). Four weeks following this initial inoculation (day 28), six rabbits from each of the type B groups (OR-B and the KY-B) were inoculated with a type A strain, either MA-A1a or WY-A2 **(Table 3.2)**. Three rabbits from each group were re-challenged with the same organism as that used for the day 0 inoculations, OR-B or KY-B. Of the five rabbits sham inoculated on day 0, two were inoculated on day 28 with MA-A1a and three were inoculated with WY-A2, to serve as positive controls. The days post-infection following the initial challenge with a type B strain are referred to as 'dpi-1' while days post-infection following the challenge with a type A strain are referred to as 'dpi-2'.

Table 3.2: Challenge days, strains, and number of rabbits used.

Challenge 1 (Day 0)	Challenge 2 (Day 28)	Abbreviation	Number of rabbits
KY-B	MA-A1b	KY-B/MA-A1b	6
KY-B	WY-A2	KY-B/WY-A2	6
KY-B	KY-B	KY-B/KY-B	3
OR-B	MA-A1b	OR-B/MA-A1b	6
OR-B	WY-A2	OR-B/WY-A2	6
OR-B	OR-B	OR-B/OR-B	3
PBS	MA-A1b	PBS/MA-A1b	2
PBS	WY-A2	PBS/WY-A2	3

Body weight, temperature, and appetite of each rabbit were evaluated prior to and daily following inoculation. Weight was determined using a Pesola scale in which the rabbits were tightly wrapped in a towel and placed in a cloth bag before being suspended from the scale. Each morning the rabbits were provided a treat of peaches, pears, or pineapple and consumption was recorded. Rabbit enthusiasm for the treat proved to be an efficacious method for evaluating small changes in clinical presentation.

3.2.4 Euthanasia, Necropsy, Histopathology, and Organ Burden

All rabbits were euthanized at 14 dpi-2, or earlier as necessary due to a moribund condition, which included extreme lethargy, poor appetite, hypo-responsivity, or a recumbent position. Rabbits were monitored every 12 hours for signs of progressing disease; however, despite this frequency several rabbits succumbed to death naturally due to tularemia. Gross lesions, specifically the detection of microabscesses, pulmonary consolidation, and splenomegaly were recorded for each rabbit at the time of necropsy. Due to the unavailability of control spleen weights for cottontail rabbits, splenomegaly was evaluated qualitatively based on visual appearance.

Organ burden was evaluated by collecting 100 mg samples of liver, spleen, lung, and kidney in a vial with 0.9 mL of MMH broth containing 15% glycerol and 2 stainless steel BBs; these samples were immediately homogenized in a mixer mill and frozen at -80°C. Serial ten-fold dilutions were made from 10^{-1} to 10^{-3} for the liver and spleen of rabbits challenged with the type B strains (either KY-B or OR-B) at both time points in order to quantify the organ burden. Duplicate samples (100 µL) from each of the three dilutions were plated on MMH or CHAB agar plates, incubated at 37°C with 5% CO_2 for 24-48 hours, and colony counts recorded. For each rabbit with a positive culture, DNA was extracted from a bacterial colony and PCR was used to confirm its identity as *F. tularensis* using a protocol described by Long and colleagues (Long *et al.*, 1993). For rabbits sequentially inoculated with type B followed by type A strains, tissues were processed similarly, but a subspecies-specific PCR (Johansson *et al.*, 2000) was used to confirm the identity of the recovered organism.

3.2.5 Serology

Rabbits were manually restrained and bled from the jugular vein prior to infection and on 14, 28, and 42 dpi-1, or upon euthanasia due to a moribund condition. In rare instances when rabbits died prior to the observation of a moribund condition (hunched/recumbent position or hypo-responsive), blood was not collected. Humoral antibody response was evaluated using an ELISA developed in our laboratory based

on the World Health Organization Guidelines on Tularaemia (Tarnvik, 2007) and described in detail by Brown and colleagues (Brown *et al.*, 2015a). Briefly, 96-well plates were coated overnight at room temperature with 3 µg/mL of *F. tularensis* LPS obtained from BEI Resources (Manasas, Virginia, USA), rinsed, and blocked with 5% non-fat dry milk. Serum samples were diluted 1:1,000 in incubation buffer and loaded in duplicate wells. Following a 1-hour incubation, the plate was emptied and rinsed. Goat anti-rabbit horseradish-peroxidase conjugate (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) was used as the secondary antibody and incubated for 1-hour. The plate was again emptied and rinsed prior to the application of substrate (TMB Peroxidase Substrate, KPL, Gaithersburg, Maryland, USA) and proceeded for 15-20 minutes before the reaction was stopped via the addition of 1N hydrochloric acid. Pooled serum from laboratory rabbits was used as a negative control.

3.2.6 Statistical and Survival Analyses

Descriptive statistics and survival analysis were performed using STATA software (Stata, Statistical Software: Release 11.2, College Station, Texas). Time to death was measured in days from the time of inoculation with different strains of *F. tularensis.* Median survival time (in days) and 95% confidence intervals were calculated using a Kaplan Meier survival function and univariate non-parametric analyses were conducted using the log rank test to compare the survival function among cottontail rabbits infected with a combination of *Francisella* strains. Results were considered statistically significant with p-values <0.05.

3.3 Results:

Baseline body temperature was found to be between 38.3 and 39.4°C for all rabbits. Initial inoculation with either type B strain (KY-B or OR-B) resulted in elevated body temperatures starting 2-3 dpi-1 and peaking between 40.6 and 41.7°C. Overt clinical disease was not observed with the exception of one rabbit (#19) that became moribund following the initial inoculation with OR-B, and was euthanized at 8 dpi-1.

Body temperature following MA-A1b inoculation at 0 dpi-2 appeared to have been influenced by which type B strain the rabbit had originally received. Rabbits inoculated with OR-B at the first time point were found to develop a fever 3 to 5 dpi-2, whereas rabbits that received KY-B at the first time point developed a fever 5 to 7 dpi-2. Fever associated with inoculation with WY-A2 was found to be the same for both groups and was observed 4 to 6 dpi-2. The peak body temperature for rabbits inoculated with MA-A1b and WY-A2, irrespective of which type B strain had been administered previously, was found to be between 40.6 and 41.7°C (data not shown).

The median time to euthanasia (survival time) and range following inoculation with a type A strain is summarized in **Table 3.3**. Rabbits challenged with either KY-B or OR-B at the first time point followed by MA-A1b at the second time point survived for an increased length of time **(Figure 3.1)** as compared to rabbits challenged only with MA-A1b (log-rank test, p=0.0082 and p=0.0143, respectively). Similarly, rabbits challenged with either type B (KY-B or OR-B) strain at the first time point followed by WY-A2 at the second time point survived for an increased length of time as compared to rabbits challenged only with WY-A2 (log-rank test, p=0.0391 and p=0.0388, respectively).

Table 3.3: Median survival time following inoculation with a virulent type A strain (dpi-2) after a previous inoculation with a type B strain or a sham inoculation with PBS.

Strains	Median	Survival range
KY-B/MA-A1b	7.5	7-13
KY-B/WY-A2	10	5-10
OR-B/MA-A1b*	7	5-8
OR-B/WY-A2	14	7-14
PBS/MA-A1b	4	4
PBS/WY-A2	7	5-7

*Rabbit #19 is not included in this table; thus, the median and range for the OR/MA group is based only on 5 rabbits.

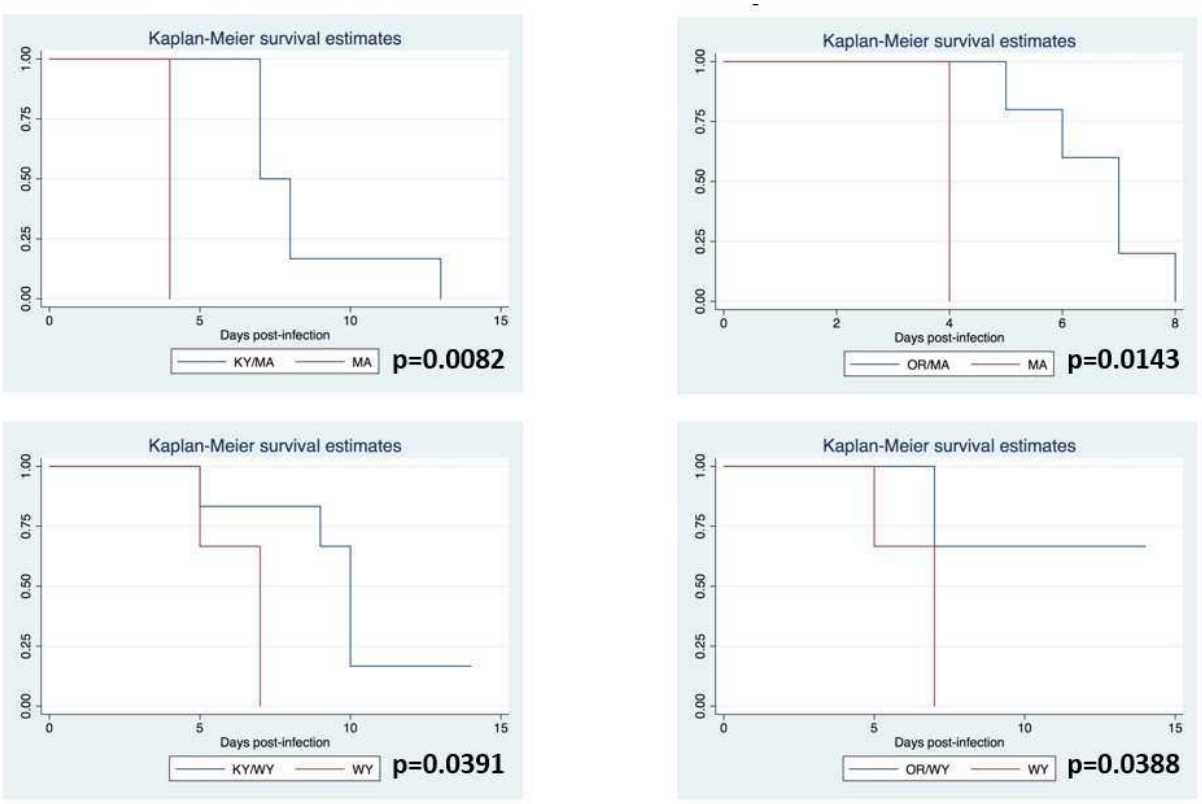


Figure 3.1: Kaplan-Meier survival functions. Comparisons for rabbits inoculated with type B strains followed by type A strains of *F. tularensis.*

All rabbits inoculated with a type B strain followed by challenge with a type A strain at day 28 were found to have type A organism in the spleen upon necropsy **(Table 3.4)**. Urine was collected from two rabbits (#4 and #26) due to the observed presence of microabscesses on the kidneys at necropsy. *F. tularensis* was cultured in the urine from each rabbit, type A and type B, respectively. Rabbits challenged with type B at both time points were found to have cleared the organism from the liver and spleen upon euthanasia at the end of the study period, with an exception for one rabbit (#13) that was found to have 700 organisms/gram in the spleen.

Table 3.4: Summary of clinical response, pathology, and organism isolated following inoculation with a type A strain.

Strains	Rabbit	DPI-2 Euthanized	Splenomegaly?	Microabscesses?	Organism Type Isolated
KY-B/MA-A1b	1	8	-	+	A
	2	7	-	+	A
	3	7	-	+	A
	4	13	+	+	A
	5	8	-	+	A
	6	7	-	+	A
KY-B/WY-A2	7	10	-	-	A
	8	14	-	-	A
	9	10	-	-	A
	10	5	+	-	A
	11	9	-	-	A
	12	10	+	+	A
KY-B/KY-B	13	14	-	-	B
	14	14	-	-	-
	15	14	-	-	-
OR-B/OR-B	16	14	-	-	-
	17	14	+	-	-
	18	14	-	-	-
OR-B/MA-A1b	19	8*	+	-	N/A
	20	7	+	+	A
	21	5	+	+	A
	22	8	-	+	A
	23	6	+	+	A
	24	7	-	+	A
OR-B/WY-A2	25	14	-	-	A
	26	7	+	+	A
	27	7	+	+	A
	28	14	-	-	A
	29	14	-	+	A
	30	14	+	-	A
PBS/WY-A2	31	5	+	-	A
	32	7	+	-	A
	33	7	-	-	A
PBS/MA-A1b	34	4	-	+	A
	35	4	-	+	A

*The dpi euthanized for this rabbit are associated with days following the first inoculation (dpi-1), as this rabbit succumbed to infection following the initial inoculation with the type B strain (OR-B).

The primary gross lesions observed were microabscesses on the liver or spleen and splenomegaly **(Table 3.4)**. Rabbits inoculated with MA-A1b at either time point were found to have a much higher incidence of microabscesses upon necropsy than rabbits challenged with WY-A2 (OR = 35.8, 95% CI = 3.47 – 368.8, p = 0.0027). Splenomegaly occurred at the same frequency in rabbits, irrespective of inoculation with MA-A1b or WY-A2 (OR = 0.635, 95% CI = 0.143 – 2.82, p = 0.5505). Consistent with previous work completed in our laboratory, lung consolidation was observed in two of the rabbits inoculated with OR-B at both time points (data not shown).

With a few exceptions, rabbits developed a robust antibody response characterized by a rise in antibodies by 14 dpi-1 which peaked at 28 dpi-1 and remained stable until euthanasia at 42 dpi-1 or earlier **(Figure 3.2)**. Serologic responses from several rabbits appeared aberrant; for example, antibodies were not detected for rabbit #10 at any time point following inoculation and rabbit #11 was found to have an antibody response at 14 dpi-1 but not at at 28 dpi-1. We re-tested all of these samples, obtained the same result, and were unable to explain these apparent discrepancies. Rabbit #13 was not found to have antibodies until 42 dpi-1; however, the magnitude of the response at that time point was similar to other rabbits of the same group. Rabbit #21 did not have detectable antibodies at 14 dpi-1 but was found to have response equivalent to others in its group by 28 dpi-1. Rabbit #23 seroconverted by 14 dpi-1 but antibodies were not detected at 28 dpi-1; however, upon euthanasia at 34 dpi-1 the antibody response was found to be comparable to the other rabbits in the group.

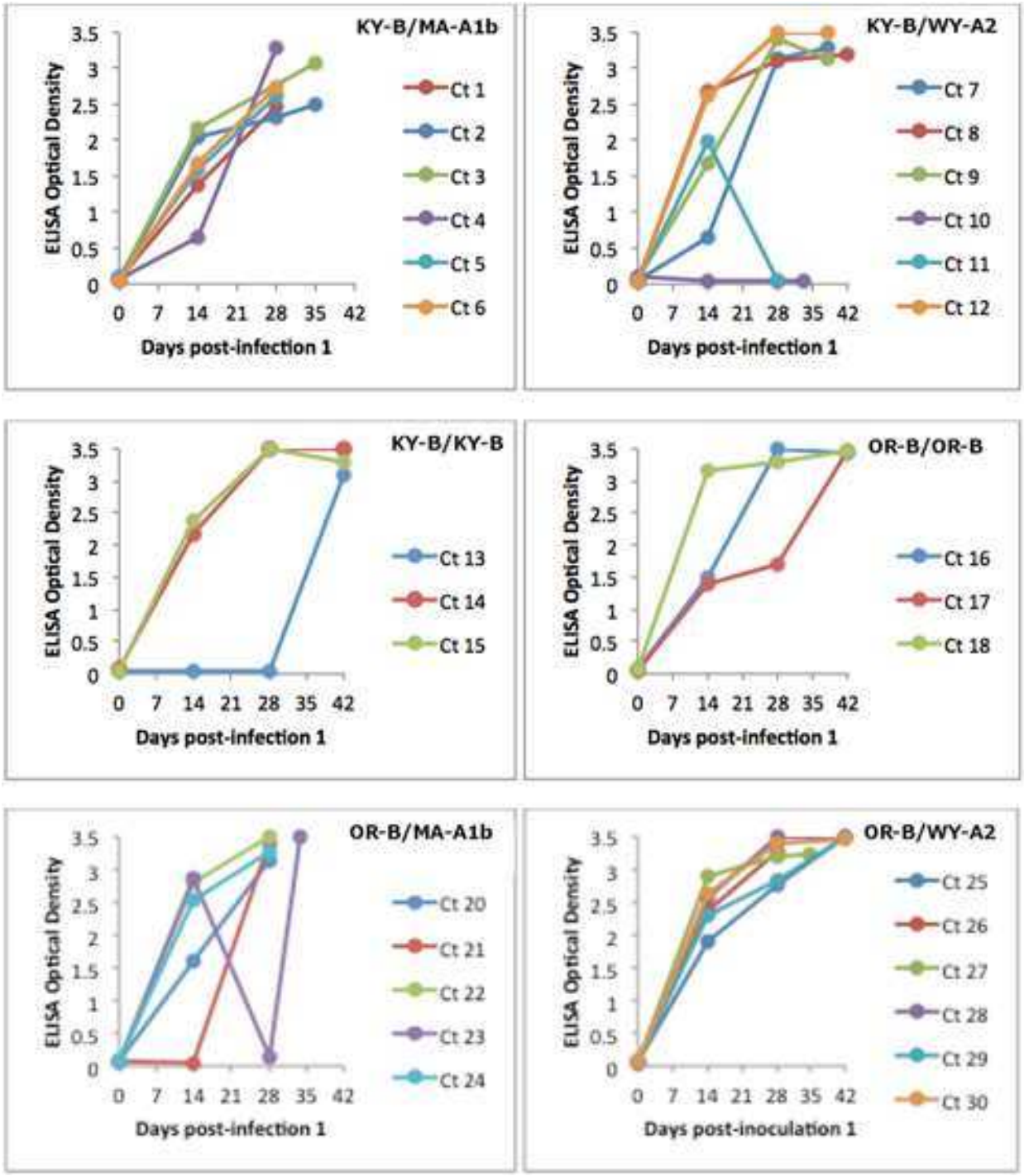


Figure 3.2: ELISA antibody responses of rabbits infected with a combination of *F. tularensis* strains. Rabbits were inoculated with *F. tularensis* on days 0 and 28.

3.4 Discussion:

This study provided an initial characterization in cottontail rabbits of the effect of a prior type B infection on the outcome of subsequent inoculation with a virulent type A strain of *F. tularensis.* Infection of laboratory mice with both type A and B strains results in uniform mortality (Molins *et al.*, 2010) and it was of interest to evaluate sequential infections in a natural host species. Understanding how a prior inoculation with a less virulent strain of this organism (type B), resulting in a robust humoral immune response, impacts host survival following exposure to a highly virulent strain of *F. tularensis* has important implications for further understanding the role of cottontail rabbits in the maintenance and transmission of *F. tularensis.* Clearly, humoral immunity *per se* likely does not mediate protective immunity to an intracellular pathogen like *F. tularensis*, but was used in this study as an index of immune response to infection.

Our study establishes that a robust immune response, as assessed by antibody production, initiated by challenge with a type B strain of *F. tularensis* is, in some cases, sufficient to lengthen the survival time following infection with a virulent type A strain. Inoculation with MA-A1b was uniformly lethal prior to 14 dpi regardless of whether or not the rabbit was previously inoculated with a type B strain; however, rabbits that were previously exposed were found to survive for a longer duration as compared to those that received PBS at the first time point. Inoculation with WY-A2 alone was found previously (Brown *et al.*, 2015a) to cause uniform mortality in cottontail rabbits; however, when WY-A2 was delivered subsequent to a challenge with either KY-B or OR-B, we observed lengthened survival periods in some rabbits and complete protection from mortality in others (n=4) during the first 14 days following inoculation. This finding is not altogether surprising as A1b and A2 have very different mortality ratios in humans, 24% and 0%, respectively; which is suggestive of differences in ability to colonize the host, capability to evade the immune system, or both (Kugeler *et al.*, 2009).

Interestingly, the two rabbits (#26 and #27) that were euthanized from the OR/WY group prior to 14 dpi-2) were found to have antibody levels similar to the rabbits that survived which, as has been previously observed, provides an additional indication that antibody production is not the major influence on host outcome following exposure to *F. tularensis*. This observed difference indicates that a robust antibody response is associated with partially protecting rabbits from inoculation with a virulent strain of *F. tularensis* (WY-A2), although is insufficient to afford protection against highly virulent strains (MA-A1b).

The pattern and magnitude of the antibody response proved to be fairly uniform irrespective of the inoculating strain and length of survival following inoculation. Sequential exposure to a type B strain at days 0 and 28 was found to result in a humoral immune response of a similar magnitude to those that received only one inoculation with a type B strain. Additionally, fever was observed between 3 and 7 dpi in the majority of rabbits regardless of the inoculating strain. This febrile response was similar to that described following aerosol exposure of New Zealand White rabbits with the Schu S4 strain of *F. tularensis* (Reed *et al.*, 2011).

Surprisingly, all rabbits inoculated with type A strains were found to have spleens that were culture positive for this organism (either MA-A1b or WY-A2) at the time of necropsy, even in instances where the rabbit survived until 14 dpi-2. Because both type A strains have been found to be 100% fatal when administered solely, the finding of culture positive tissues in apparently healthy animals is suggestive of an important role for the adaptive immune response.

Furthermore, culturing *F. tularensis* from the urine of two cottontail rabbits inoculated with a type A strain followed by a type B strain was a highly significant finding and the first time bacteriuria has been reported in cottontail rabbits infected with this pathogen. The urine of these rabbits was evaluated due to the observance of microabscesses present on the kidney and much further work is needed to

determine if this is a common manifestation of a tularemia infection in cottontail rabbits. Excreta containing *F. tularensis* from tularemia infected cottontail rabbits may serve as a source for environmental contamination and could provide a route of exposure for amoebae or other protozoa.

This study was subject to some limitations. First, the cottontail rabbits utilized in this study were wild-caught and thus, were undoubtedly harboring various organisms that could influence the immune responses observed in our study. However, our intent was to study *F. tularensis* in its natural host and thus, we believe our findings to be representative of a natural setting. Secondly, the likelihood in a natural setting of a sequential infection in cottontail rabbits of a type B strain of *F. tularensis* followed by a type A strain is unknown. These strains do overlap geographically and thus, it is certainly a possibility that a single animal could be infected with several strains of the organism. Next, captivity for cottontail rabbits is indisputably a stressful environment. We attempted to alleviate the stress of the laboratory setting by handling the rabbits gently and infrequently. Finally, chocolate agar plates were used only for the WY-A2 strain due to growth requirements which may have confounded the culture results.

Our results strongly suggest that although a previous exposure to a type B strain of *F. tularensis* does not provide full protection against challenge with a virulent type A strain, it does lengthen the survival period for rabbits inoculated with either KY-B or OR-B followed by WY-A2 and in some cases, rabbits survive infection altogether. These findings are important and help to shape our understanding of the role that cottontail rabbits may play in the maintenance and transmission of *F. tularensis* amongst humans and other animals.

CHAPTER 4: COMPARISON OF ORAL AND INTRANASAL INOCULATION ROUTES OF *FRANCISELLA TULARENSIS* SUBSP. *HOLARACTICA* IN PRAIRIE VOLES (*MICROTUS OCHROGASTER*)

4.1 Introduction:

Tularemia, caused by *Francisella tularensis*, is a severe disease in a wide variety of species (Dennis *et al.*, 2001). Two distinct strains of *F. tularensis* are responsible for the vast majority of disease: *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B). Both strains are naturally found in the Northern Hemisphere, however, type A is found only in North America (Kugeler *et al.*, 2009). This organism is classified as a Tier 1 Select Agent due to its high virulence and low infectious dose and obtaining a clear understanding of propagation of this agent in nature is of high importance (Oyston *et al.*, 2004). Cottontail rabbits, amongst other lagomorphs, are considered to be an important species for type A maintenance and transmission, and aquatic mammals, including beavers, muskrats, and voles, are implicated as reservoirs in type B ecology (Nakazawa *et al.*, 2010; Keim *et al.*, 2007). Voles, in particular, are thought to acquire infection via cannibalization of animals that died from tularemia (Bell and Stewart, 1975).

Previous experiments with meadow voles (*Microtus pennsylvanicus*) demonstrated the acute susceptibility of the majority of infected voles to oral inoculation (via contaminated drinking water) with *F. tularensis* subsp. *holarctica*, with 100% mortality following an inoculation of $1x10^7$ organisms (Bell and Stewart, 1983). However, a population of voles in the same study group was found to have order of magnitude differences in susceptibility to type B *F. tularensis* following oral inoculation. In a prior study, a subset of more resistant voles were found to develop nephritis with bacteriuria, which allowed them to shed organism via infected urine (Bell and Stewart, 1975). Furthermore, experimental infections using bank and field voles supported a role for voles as amplification hosts (Rossow *et al.*, 2014b). These findings are suggestive that voles may play an important role in fueling an outbreak as a result of heavy environmental contamination via excreta and carcass leaching.

Prairie voles inhabit a large portion of North America, ranging as far east as West Virginia, with their habitat dipping south into Kentucky and Tennessee, and finally spreading west into parts of Colorado, Wyoming, and Montana as well as the southern provinces of Canada. Infection kinetics were evaluated in two distinct strains of *F. tularensis,* isolated in Oregon (OR96-0246) and Kentucky (KY99-3387), with the KY99-3387 strain being the only strain with geographical overlap with prairie voles. The objective of the current study was to evaluate the susceptibility of prairie voles (*Microtus orchogaster*) to an oral or intranasal inoculation of two strains of *F. tularensis* subsp. *holarctica.* A vole cannibalizing a fellow vole that died of a tularemia infection would certainly be exposed to organism via the alimentary tract; however, it is likely that they would also inhale the organism while eating. In this study we sought to determine the infection kinetics of these two inoculation routes via the evaluation of gross pathology, serology, organ burden, and morbidity and mortality.

4.2 Materials and Methods:

4.2.1 Experimental Design and Animals

Captive bred prairie voles were graciously provided by colleagues at the University of Texas, El Paso. Same sex sibling pairs (9-11 weeks of age) were co-housed in standard rat cages within an ABSL-3 containment facility approved for use of Select Agents. Voles were provided *ad libitum* access to alfalfa blocks, hay, and water and were provided a small piece of apple daily. They were acclimatized to our facility for 2-3 weeks prior to infection.

Twenty-three voles were orally infected with $1x10^7$ cfu of OR96-0246, a type B strain isolated in Oregon and previously characterized in experimental inoculations using cottontail rabbits (Brown *et al.,* 2015a; Brown *et al.,* 2015b). A small pilot study preceded this experiment and found voles to respond equally following oral challenge to both OR96-0246 and KY99-3387, an alternative type B strain isolated from a human; thus, we inoculated the voles only with the OR96-0246 strain in order to increase our

animal numbers and subsequently, the power of the study. The oral inoculum was prepared in 100 µL of PBS and delivered via a pipette tip into the oral cavity in manually restrained, un-anesthetized animals.

In a subsequent experiment, 8 voles were inoculated intranasally with 350-650 colony forming units (cfu) in 25 µL of media. Four voles were inoculated with KY99-3387 and the other 4 with OR96-0246. Voles were anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively) during this challenge.

All of this work was conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals) and was approved by the Animal Care and Use Committee at Colorado State University (approval #15-5813A).

4.2.2 Bacterial Strains and Culture Methods

Both strains of *F. tularensis* used in this study were kindly provided by Dr. Jeannine Petersen (CDC) and passaged one time in our laboratory. Cultures were grown for 24 to 36 hours in Modified-Mueller Hinton (MMH) broth at 37°C with 5% CO_2, and glycerol was added to the broth to achieve a final concentration of 15% prior to freezing (Baker *et al.,* 1985). The inoculum was prepared immediately prior to infection and a back titration confirmed the target dose was achieved in both experiments.

4.2.3 Euthanasia, Necropsy, Gross Pathology, and Organ Burden

Voles were euthanized by an overdose of pentobarbital administered intraperitoneally at the end of each study or as necessary due to a moribund condition, characterized by >25% weight loss, piloerection, and/or a lack of responsiveness. In the oral inoculation experiment, voles were serially euthanized following inoculation in order to characterize organ burden, gross pathology, and humoral

immune response at various intervals following challenge **(Table 4.1)**. The intranasal inoculation experiment was designed to evaluate acute disease and thus, all survivors were euthanized at 10 dpi.

Table 4.1: Number of voles euthanized at each time point following oral infection.

Number of voles	Euthanasia: Days post-infection
4	3
4	7
8	14
7	28

Necropsies were performed on all animals and observations of gross lesions were recorded. The liver and spleen were the organs of peak interest for gross pathology and organ burden due to the preferential tissue tropism of *F. tularensis* (Dennis *et al.,* 2001). The presence or absence of splenomegaly was evaluated qualitatively due to the lack of availability of the weight for a control vole spleen.

During necropsy, samples of liver, spleen, and kidney were collected in a vial with Mueller-Hinton broth with 15% glycerol and 2 stainless steel BBs. A mixer mill was used to homogenize the tissues and the samples were immediately plated on Modified-Mueller Hinton agar plates at the 10^{-1} dilution. Colony counts were recorded after a 24-hour incubation at 37°C in 5% CO_2 in air. DNA was extracted from a single cell colony pluck from the spleen homogenate and PCR was used to confirm an *F. tularensis* identity for any voles with a positive culture (Long *et al.,* 1993).

4.2.4 Serology

Voles were manually restrained and bled via the facial vein to confirm baseline sero-negativity and a cardiac puncture was performed upon necropsy. The World Health Organization Guidelines on Tularemia (Tarnvik, 2007) was used to develop an ELISA for the evaluation of an *F. tularensis*-specific antibody response; the details of this assay can be found in an alternative manuscript (Brown *et al.,* 2015a). Concisely, 3 µg/mL of *F. tularensis* LPS, obtained from BEI resources (Manasas, Virginia,

USA), was used to coat 96-well plates. Plates were rinsed and blocked with 5% non-fat dry milk and serum samples were diluted 1:1,000 in incubation buffer and loaded in duplicate. The plate was emptied and rinsed following a 1-hour incubation and a goat anti-mouse horseradish-peroxidase conjugate (Jackson ImmunoReserach, West Grove, Pennsylvania, USA) was incubated for 1-hour. Following another rinse, substrate (TMB Peroxidase Substrate, KPL, Gaithersburg, Maryland, USA) was applied and allowed to react for 15-20 minutes before the addition of 1N hydrochloric acid. Due to the unavailability of known vole serum positive or negative for *F. tularensis*-specific antibodies, mouse samples from prior experiments were used as the controls. The threshold for a positive sample was 3 standard deviations above the mean of the negative controls.

4.2.5 Survival Analysis

Survival analysis was performed using STATA software (Stata, Statistical Software: Release 11.2, College Station, Texas) and time to death was measured in days post-infection. Univariate non-parametric analysis was conducted using the log rank test to compare the survival function (risk of death) among voles infected intranasally with OR96-0246 or KY99-3387.

4.3 Results:

No gross lesions were observed on any voles inoculated via the oral route; however, all 4 voles inoculated intranasally with OR96-0246 developed a robust splenomegaly (5-6x normal size) with detectable microabscesses. Liver and spleen homogenates were found to contain bacteria and PCR confirmed its identity as *F. tularensis.* Interestingly, voles inoculated with KY99-3387 developed a slight splenomegaly (1-2x normal size), however, the tissues plated were culture negative.

None of the voles, irrespective of inoculating dose, strain, route, or day of euthanasia were found to have *F. tularensis*-specific antibodies.

The OR96-0246 strain of *F. tularensis* was considerably more virulent in voles as compared to KY99-3387, as 3 of the 4 voles inoculated with KY99-3387 survived to the end of the study period (10 dpi) whereas none of the voles inoculated with OR96-0246 survived. There was a significant difference in the risk of mortality for voles inoculated with these two strains of type B *F. tularensis* (log-rank test, p=0.01) **(Figure 4.1).**

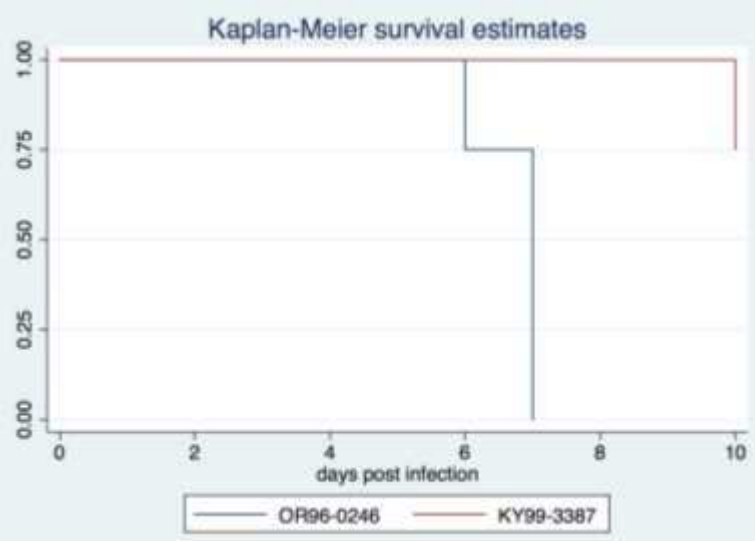


Figure 4.1: Kaplan-Meier survival function. Comparison for voles intranasally inoculated with OR96-0246 or KY99-3387 (p=0.01).

4.4 Discussion:

This pilot study sought to evaluate the infection kinetics and susceptibility of prairie voles to oral and intranasal inoculation with type B strains of *F. tularensis.* Understanding the similarity and differences in response to infection between prairie and meadow voles is important, as these species have been implicated in environmental maintenance and transmission of *F. tularensis* (Bell and Stewart, 1975; Bell and Stewart, 1983; Rossow *et al.,* 2014a; Rossow *et al.*, 2014b).

Surprisingly, neither gross pathological lesions nor an antibody response was observed in voles orally inoculated with OR96-0246, despite a serial euthanasia scheme designed to capture acute and subacute changes after inoculation with what was considered a very high dose. Furthermore, *F. tularensis* was not detectable from either the liver or spleen in any of the voles. The lack of antibodies at 28 dpi (the longest time point following challenge) suggests that the bacteria did not replicate to any significant effect in the animals. Although the challenge appeared seamless and the back titration values indicated the target inoculation was indeed delivered, it appeared that these voles never became infected. These findings suggest large differences in susceptibility between meadow and prairie voles as $1x10^7$ cfu delivered orally was found to be 100% fatal in meadow voles (Bell and Stewart, 1983).

In contrast to results obtained using oral inoculation, voles inoculated intranasally were found to have gross lesions irrespective of infecting strain, albeit that OR96-0246 resulted in more severe changes. None of these animals were found to have *F. tularensis*-specific antibodies, however the study ended 10 dpi, which is likely too soon to have a detectable humoral immune response. *F. tularensis* was confirmed by culture from the liver and spleen of all voles inoculated with OR96-0246, whereas it was not detected in tissues from voles inoculated with KY99-3387. As it is likely that an earlier evaluation would detect bacteria within the tissues, it seems that 10 dpi allowed for the clearance of the KY99-3387 infection. Furthermore, all of the voles challenged intranasally with OR96-0246 succumbed to infection while 3 of the 4 voles inoculated with KY99-3387 survived to the end of the study period. This too is a surprising finding as these two strains were found to be similarly virulent in experiments performed in our laboratory on cottontail rabbits (Brown *et al.,* 2015a; Brown *et al.,* 2015b).

Further work is certainly necessary to more fully elucidate the role of voles in the ecology of *F. tularensis* using a larger sample size and comparing outcomes amongst various vole species using multiple strains of organism. However, the acute susceptibility of prairie voles to an intranasal inoculation

of OR96-0246 suggests that perhaps voles become infected naturally due to inhalation of the bacterium during cannibalistic activities. Increased knowledge of the role of amplification or reservoir hosts for *F. tularensis* is crucial to understanding the ecology of natural disease and for the prevention and mitigation of epizootic events.

CHAPTER 5: EVALUATION OF FREE-LIVING AMOEBAE AS A POTENTIAL RESERVOIR FOR *FRANCISELLA TULARENSIS*

5.1 Introduction:

The acute virulence of *F. tularensis*, especially type A strains, in conjunction with the distinct lack of a convincing reservoir species, mammalian or vector, makes the possibility of an environmental niche of utmost interest. Free-living amoebae (FLA) are single-celled organisms that are often considered ancestral macrophages and exist nearly universally within the environment (Siddiqui and Khan, 2012; Barker and Brown, 1994). Amoebae have at least two developmental stages: trophozoites, which are the metabolically active form capable of feeding and dividing, and cysts, which are a dormant, inactive form (Greub and Raoult, 2004). Trophozoites encyst when environmental conditions are suboptimal and are adept at withstanding a variety of insults in this form. When conditions improve, they are capable of excysting and resuming metabolic functions (Aguilar-Diaz *et al.*, 2011). Bacteria compose the primary diet of amoebae and several pathogens have been shown to escape digestion and thus be harbored within the amoebae. Examples of pathogens that have been shown in the laboratory to be taken up and resist digestion by amoeba include *Vibrio cholerae* (Abd *et al.*, 2005), *Legionella pneumophila* (Brieland *et al.*, 1997; Molmeret *et al.*, 2005; Swanson and Hammer, 2000), *Mycobacterium avium* (Cirillo *et al.*, 1997); *Burkholderia cepacia* (Marolda *et al.*, 1999), *Mycobacterium bovis* (Taylor *et al.*, 2003), *Listeria monocytogenes* (Zhou *et al.*, 2007), and *Mycobacterium leprae* (Wheat *et al.*, 2014). Two previously published manuscripts demonstrate the tolerance of a variety of *F. tularensis* strains to uptake by *Acanthamoebae castellanii* (Abd *et al.*, 2003; El-Etr *et al.*, 2009). These findings support the idea that amoebae may play a crucial role in environmental maintenance and persistence of this pathogen by providing a robust citadel that affords nutrition and protection until a transmission event. Infected mammalian species are certainly capable of contaminating the environment through their carcasses and/or bacteria-laden excreta providing an opportunity for amoebal species to come into contact with *F. tularensis* in a natural setting and phagocytose the bacterium (Brown *et al.*, 2015a; Brown *et al.*, 2015b; Rossow *et al.*, 2014a; Rossow *et al.*, 2014b).

A series of pilot experiments were performed to elucidate foundational information crucial for further understanding the role amoebae may play in maintenance of *F. tularensis.* This work consisted of *in vitro* infections of 4 strains of amoebae with SchuS4, a highly virulent strain of *F. tularensis,* to assess bacterial uptake by amoebae, rates of encystment following infection, and pathogen replicative capacity within amoebae. We conducted these same experiments with *Burkholderia pseudomallei*, another gram-negative organism, and an isolate of methicillin-resistant *Staphylococcus aereus* (MRSA), a gram-positive bacteria, to compare amoebal response and bacterial replicative capacity. *B. pseudomallei* is an environmental pathogen that causes melioidosis which causes mortality in 20-50% of human cases and is endemic in Asia and northern Australia (Cheng and Currie, 2005). MRSA is of serious concern especially in hospital settings which allows for ready transmission of antibiotic resistance genes between various bacterial species and to patients who are often immunocompromised. The sewage system provides an excellent habitat for gene transfers between microorganisms and exposure to sub-therapeutic levels of clinically important antibiotics as well as serving as a superb environment for amoebae; thus, we desired to begin to evaluate these organisms to compare and contrast between *F. tularensis* as well as providing critical information for other pathogens of high priority (Grabow and Prozesky, 1973; Scheikl *et al.,* 2014).

5.2 Materials and Methods:

5.2.1 General Methods

5.2.1.1 Bacterial Culture and Staining Protocol

F. tularensis SchuS4 was grown in our laboratory in Modified-Mueller Hinton (MMH) broth at 37°C with 5% CO_2 for 24-36 hours or until the broth was turbid (Baker *et al.,* 1985). Before freezing, glycerol was added to the broth to obtain a final concentration of 15%. In all instances where *F. tularensis* was cultured, it was grown on MMH agar plates at 37°C with 5% CO_2 for 48 hours. *B. pseudomallei* strain 1026b and a local hospital isolate of MRSA were both grown overnight in Brain-Heart Infusion (BHI) broth on a shaking incubator at 37ºC and glycerol was added to media before freezing (Lee *et al.,*

2011; Vitko and Richardson, 2013). Direct culture assays were performed on BHI agar plates which were incubated at 37°C with 5% CO_2 overnight.

PKH26 is a red fluorescent cell membrane dye (Sigma-Aldrich, St. Louis, Missouri, USA) that was used to label all bacterial strains used in these experiments, allowing visualization with confocal microscopy. To stain the bacteria, a vial of each pathogen was thawed and pelleted via centrifugation at 10,000 rpm for 10 minutes. The supernatant was removed, the pellet re-suspended in 100 µL of Diluent C and 100 µL of 0.4% dye, and incubated at room temperature for 3-5 minutes. The reaction was then stopped with the addition of 200 µL of fetal bovine serum and centrifuged at 10,000 rpm for 10 minutes. The pellet was washed 1-2 times with PBS and used to infect the amoebae cultures.

5.2.1.2 Amoebae Culture and Media

Stocks of axenic *Acanthamoebae castellanii* (ATCC 30232), *Acanthamoebae lenticulata* (ATCC 30841), *Acanthamoebae polyphaga* (CCAP 1501/18), and *Hartmannella vermiformis* (ATCC 50237) were provided by Dr. William Wheat and derived from subculture. Cultures were maintained in our laboratory in 75 cm^2 flasks at 28°C and cells were passaged once weekly. The medium used for culture of *Acanthamoebae* trophozoites as 1X PYG medium, which is comprised of Page's amoebae saline (PAS) [60mg NaCl, 2mg MgSO4·7H2O, 68mg KH_2PO_4, 71mg $NaHPO_4$ and 2 mg $CaCl_2$ in 500 ml dH_2O (pH= 6.9)] and 10X PYG solution [50 g proteose peptone (Difco); 5 g yeast extract (Difco); 2.45 g $MgSO_4·7H_2O$; 2.5 g sodium citrate·$2H_2O$; 0.05 g ammonium iron sulfate $(NH_4)2Fe(SO_4)2·6H_2O$; 0.85 g KH_2PO_4; 0.89 g $Na_2HPO_4·7H_2O$; 22.5 g a-D-glucose; 0.295 g $CaCl_2$ in 250ml dH_2O], diluted 10-fold with PAS. *Hartmannella* trophozoites were maintained in Modified PYNFH media [5 g Proteose Peptone (Difco); 5 g yeast extract (Difco); 0.5 g yeast nucleic acid; 7.5 mg folic acid; 0.5 mg hemin in 440 ml dH_2O with 10 mL buffer solution (9.05 g KH_2PO_4 and 12.5g NA_2HPO_4 per 500 mL dH_2O) and 50 mL heat-inactivated fetal bovine serum added after the autoclave cycle]. Encystment buffer [0.1 M KCl; 0.02

M Tris (pH=8); 8 mM $MgSO_4$; 0.4 mM $CaCl_2$; 1 mM $NaHCO_3$ in dH_2O] was used to induce cyst formation.

5.2.2 Pilot Experiments

A series of experiments were performed to evaluate various components that were crucial for more complex studies, including ascertainment of sterilization to allow removal of infected amoebae from BSL-3, antibiotic efficacy in the presence and absence of amoebae, effect of the bacterial dye and lysis buffer on *F. tularensis, B. pseudomallei,* and MRSA, and the time course for encystment and excystment of each amoebal strain.

5.2.2.1 Sterilization Procedure

A vial of each microbe, containing approximately 10^8 cfu, was thawed and centrifuged at 10,000 rpm for 10 minutes in the microcentrifuge tube. Supernatant was removed, the pellet was re-suspended in 100 µL of 4% paraformaldehyde (PFA), and incubated at room temperature for 5 minutes. The sample was again centrifuged at 10,000 rpm for 10 minutes and supernatant removed. Following one wash with sterile PBS, bacteria were re-suspended in 200 µL of PBS and plated neat on the respective agar plates for each bacterial species. A positive control vial was treated identically to the protocol above but PBS was used instead of 4% PFA.

5.2.2.2 Antibiotic Efficacy in the Presence of Amoebae

Amoebae were seeded in a 24-well plate at $1x10^5$ amoebae/well and inoculated with 100 µL of *F. tularensis*, *B. pseudomallei,* or MRSA containing $1x10^6$ organisms. The interaction of amoebae and bacteria was allowed to proceed for 2 hours at 28ºC before the addition of 100 µg/mL gentamicin to the wells inoculated with *F. tularensis* or MRSA and 50 µg/mL ceftazidime to the wells inoculated with *B. pseudomallei*. Incubation in antibiotic-containing medium was continued for 2 hours at 28ºC and serial

dilutions of the supernatant were plated on MMH agar for *F. tularensis* or BHI agar for *B. pseudomallei* and MRSA.

5.2.2.3 Effect of Cell Membrane Stain on Bacteria

Verification that PKH26 was not harmful to the bacteria was confirmed by thawing two vials of each pathogen, performing the staining protocol on one vial using the dye and the other using PBS only, and diluting to a countable concentration before plating. Following a 24 to 48 hour incubation, colony counts from the two plates were compared.

5.2.2.4 Effect of Amoebal Lysis Buffers on Bacterial Viability

Saponin, an amphipathic glycoside, and Triton X-100, a non-ionic surfactant, are both used to permeabilize cell membranes, and have been shown to be useful compounds for lysing trophozoites; however, we wanted to ensure that they did not cause lysis of the bacterial cells. Three vials of each bacteria were thawed, diluted to a countable concentration, and one was exposed to 1% saponin (Sigma-Aldrich, St. Louis, Missouri, USA) in 1X PYG for 5 minutes, another was exposed to 0.2% Triton X-100 in 1X PYG for 5 minutes, and the last was exposed only to PBS (the control). The vials were plated and colony counts compared.

5.2.2.5 Kinetics of Encystment and Excystment for Each Amoebal Strain

Amoebae can be forced into a cyst state using encystment buffer and return to a trophozoite state via the addition of genera specific 1X media. In order to evaluate the time course of encystment and excystment, we placed 1 mL of amoebae culture from each of the 4 strains, containing $1x10^5$ amoebae, in a microcentrifuge tube, centrifuged at 1,000 x g for 5 minutes, removed the supernatant, and re-suspended in 1 mL of encystment buffer. The amoebae and encystment buffer were transferred to a 24-well plate and imaged every 24 hours out to 96 hours to determine the time course of encystment. The inverse of this experiment was performed to determine the time course of excystment by pipetting up and down

vigorously to remove any adherent cysts before transferring the cysts and encystment buffer to a microcentrifuge tube. Following centrifugation at 1,000 x g for 5 minutes, the supernatant was removed, the pellet was re-suspended in 1X media, and the cysts and media were transferred to a 24-well plate. Images were taken every 24 hours until 96 hours after the addition of 1X media.

5.2.3 Main Experiment

5.2.3.1 Bacterial Uptake, Rates of Encystment, and Pathogen Replication Within Amoebae

Amoebae, passaged twice, were seeded in a 24-well plate at a concentration of $1x10^5$ in starvation media (1/5 PYG or PYNFH for *Acanthamoebae* or *Hartmannella*, respectively) overnight at 28ºC to promote phagocytosis. The inoculum of PKH26-labeled *F. tularensis*, *B. pseudomallei*, or MRSA was prepared at MOI=10 in 100 µL of media, such that $1x10^6$ organisms was delivered to each infected well. Control wells were included for each strain of amoebae and were inoculated with 100 µL of sterile PBS. The infection was carried out at 28ºC for 4 and 24 hours to evaluate bacterial uptake and intra-amoebal replication, respectively. At the 4 hour time point, 100 ug/mL gentamicin was added to all wells (including those designated for the 24 hour time point) of *F. tularensis* and MRSA and 50 µg/mL ceftazidime was added to all *B. pseudomallei* wells and incubation continued at 28ºC for 2 hours. Amoebae from each well were collected by vigorous pipetting, then transferred to 2 micro-centrifuge tubes and centrifuged for 5 minutes at 1,000 x g. Supernatant was removed from both vials and the tube with the pellet destined for lysis was re-suspended in 1% saponin with 1X PYG for *F. tularensis* and 0.2% Triton-X100 for *B. pseudomallei* and MRSA diluted in 1X PYG. Dilutions of the lysate were made following a 5 minute incubation period at room temperature. Plates of the lysate were incubated at 37ºC with 5% CO_2 and counted after 24-48 hours. The second vial was used for confocal microscopy and the pellet was re-suspended in 100 µL of 4% PFA. Following a 15 minute incubation period at room temperature, the vial was centrifuged at 1,000 x g for 5 minutes, washed once in PBS, re-suspended in 100 µL of PBS and stored in the dark until evaluation under the microscope. The 24 hour wells were

maintained in the incubator overnight until the time point was ascertained and the exact protocol from above was repeated.

A Zeiss LSM 510 META laser scanning confocal microscope was used to assess bacterial uptake by amoebae as well as amoebal morphology (cyst vs. trophozoite). Zen software automatically identifies the microscope settings and the objectives used, control movements, and measurements carried out by the system occur with high precision. The images obtained from the microscope were used to determine the number of amoebae positive for bacterial uptake. Two hundred cells were counted per sample, except in rare instances where 200 cells were not found in the sample, in that case, all cells present were counted. A cell considered positive was found to have a spot or clump of bacteria observed within as a red color. Positive cells were further differentiated as trophozoites or cysts and if a cyst, was further classified to include whether the bacteria was dispersed throughout the cyst or between the double membranes.

5.3 Results:

5.3.1 Pilot Experiments

Ensuring proper sterilization is of the utmost importance when working with BSL-3 agents and making transfers to a lab of lower containment. Exposure of *F. tularensis*, *B. pseudomallei*, or MRSA to 4% PFA for 5 minutes resulted in complete decontamination **(Figure 5.1)**.

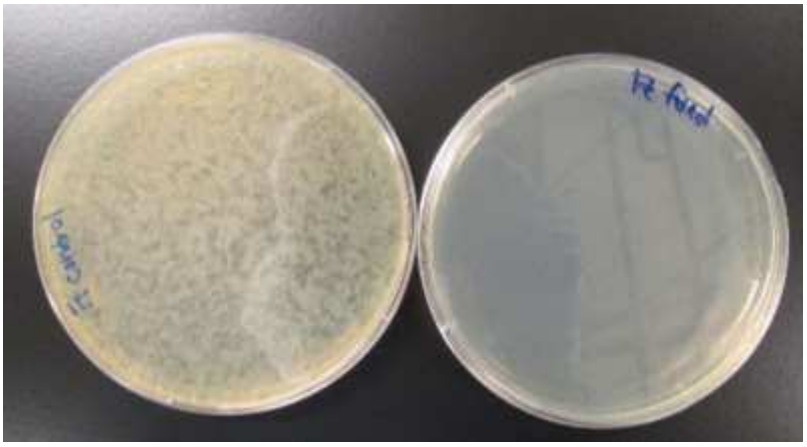


Figure 5.1: Complete sterilization of *F. tularensis* following a 5 minute exposure to 4% PFA can be observed on the right plate and a lawn from the untreated vial can be seen on the left plate.

Gentamicin at a concentration of 100 µg/mL was found to be highly efficacious in killing extracellular *F. tularensis* and MRSA in the presence of any of the 4 amoebae strains used in these experiments (data not shown). Furthermore, 50 µg/mL ceftazidime was sufficient to remove extracellular *B. pseudomallei* in the presence of all amoebal strains. The PKH26 stain did not reduce numbers of viable *F. tularensis*, *B. pseudomallei*, or MRSA. Triton X-100 severely reduced the numbers of viable *F. tularensis* whereas 1% saponin did not affect viability; thus, all experiments involving lysed trophozoites were conducted using 1% saponin in 1X PYG for *F. tularensis*. 0.2% Triton X-100 in 1X PYG did not effect *B. pseudomallei* or MRSA and thus, was used to lyse amoebae containing either of these pathogens. Following suspension in encystment buffer, all amoebae strains attained 90-100% encystment within 96 hours. Re-animation following exposure to 1X media resulted in excystment occurring within 48-72 hours **(Table 5.1)**. Large differences in these rates of encystment and excystment were not observed; however, it does appear that the *H. vermiformis* strain is prone to rapid encystment following a sub-optimal environment and is slightly reluctant to return to a trophozoite stage.

Table 5.1: Encystment and excystment kinetics for each strain of amoebae.

Amoebae strain	Encystment (hrs)	Excystment (hrs)
A. castellanii	48-72	48
A. lenticulata	96	48
A. polyphaga	48-72	48
H. vermiformis	24-48	48-72

5.3.2 Main Experiment

5.3.2.1 Bacterial Uptake, Rates of Encystment, and Pathogen Replication Within Amoebae

The average percentage of amoebae observed via confocal microscopy to contain intracellular bacteria at 4 and 24 hours is depicted in **Table 5.2**.

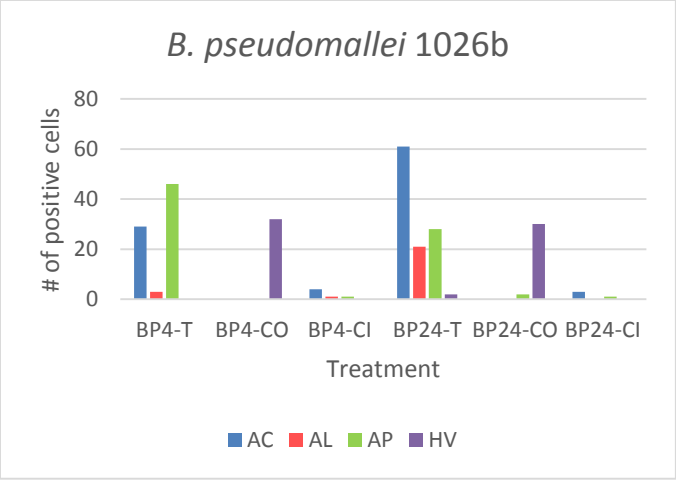
Table 5.2: Percent of amoebae with intracellular bacteria at each time point.

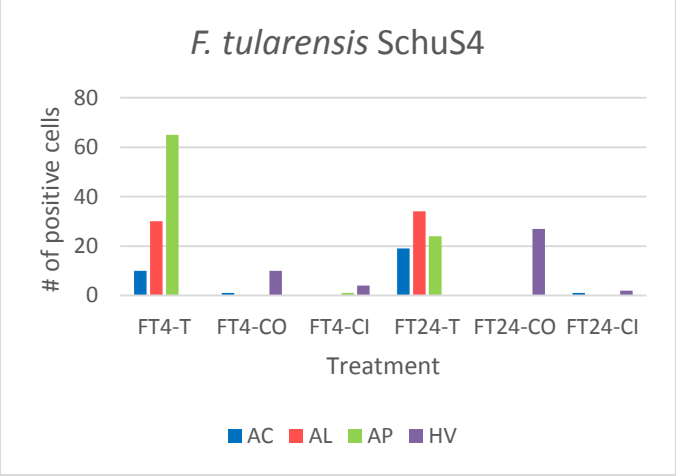
Bacterial strain	Time point	Range (%)	Average (%)	Standard deviation
B. pseudomallei 1026b	4 hours	4.5-23.5	16.8	7.7
	24 hours	15.5-32	20.6	6.7
F. tularensis SchuS4	4 hours	7-33	18.3	9.5
	24 hours	14-52	27	14.7
Methicillin resistant *S. aureus*	4 hours	5.5-26.5	16.5	7.6
	24 hours	11.5-31.5	18.5	8.1

A graphic representation of the data obtained via confocal microscopy is provided in **Figure 5.2.** *A. castellanii* and *A. polyphaga* were found to uptake *B. pseudomallei* with relative frequency at the 4 and 24 hour time points in trophozoites (BP4-T) as well as *A. lenticulata* at the 24 hour time point (BP24-T). **Figure 5.3** shows an *A. polyphaga* cyst with *B. pseudomallei* sequestered between the double membranes at 4 hours post-infection. *H. vermiformis* was found to have bacteria sequestered between the double membrane of the cyst at both the 4 and 24 hour time points (BP4-CO and BP24-CO). Very little *B. pseudomallei* was found to be dispersed within the cyst at either time point (BP4-CI and BP24-CI).

A polyphaga trophozoites, and to a much lesser extent, *A. castellanii* and *A. lenticulata,* were found to be highly efficient for uptake of *F. tularensis* at the 4 hour time point (FT4-T) as 33% of all cells were observed to be positive. *A. castellanii, A. lenticulata,* and *A. polyphaga* were found to be frequently infected as trophozoites at the 24 hour time point as well (FT24-T). **Figure 5.4** depicts an *A. castellanii* cyst infected with *F. tularensis* 24 hours after infection. *H. vermiformis* were found to have moderate to low levels of infection at the 4 hour time point both between the double membrane of the cyst and dispersed within (FT4-CO and FT4-CI) as well as at 24 hours between the double membranes (FT24-CO).

A.lenticulata and *A. polyphaga* trophozoites were found to uptake MRSA at moderate to high levels at both the 4 and 24 hour time points (MRSA4-T and MRSA24-T). As compared to amoebae infected with *B. pseudomallei* or *F. tularensis,* a much smaller number of positive amoebae cells were found to be encysted.





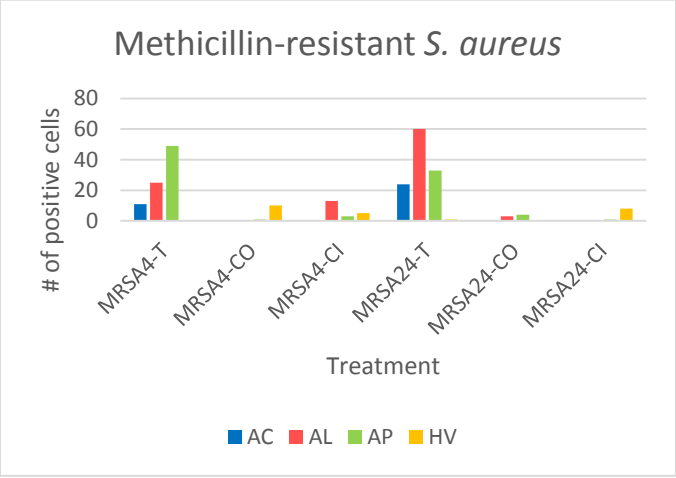


Figure 5.2: Number of amoebae from each species found to be positive for each bacterial species via confocal microscopy. The treatment labels are designated as the initials of the bacterium, followed by the time point (4 or 24), and the amoebal state/bacterial location if encysted: T= trophozoite; CO=encysted amoebae with bacteria between the double membranes; CI= encysted amoebae with bacteria dispersed within the cyst.

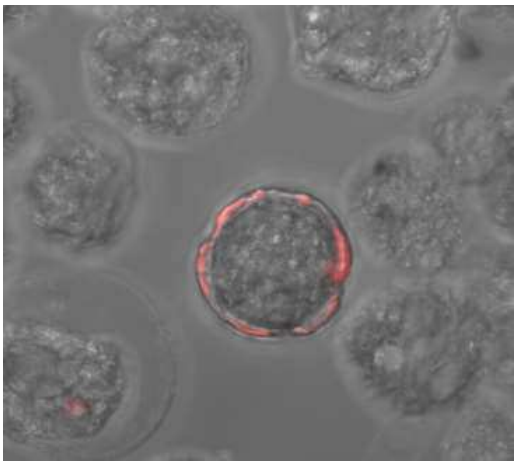


Figure 5.3: *A. polyphaga* cyst with *B. pseudomallei* sequestered between the outer double membranes at 4 hours post-infection (BP4-CO).

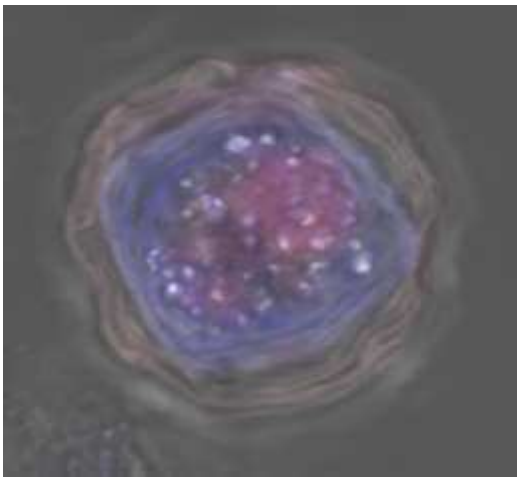


Figure 5.4: *A. castellanii* cyst with *F. tularensis* dispersed within at 24 hours post-infection (FT24-CI).

B. pseudomallei and *F. tularensis* were recovered via direct culture from the lysate of all strains of amoebae at both time points, whereas MRSA was not recoverable in all instances. The culture results from the lysis with all strains of amoebae and each pathogen can be found in **Tables 5.3, 5.4, and 5.5.** TNTC indicates plates that were too numerous to count.

Table 5.3: *B. pseudomallei* culture results for lysate.

Lysate		**4 HOURS** Dilution factor				**24 HOURS** Dilution factor			
		0	-1	-2	-3	0	-1	-2	-3
Amoebae strain	*A. castellanii*	TNTC	768	137	19	334	40	6	2
	A. lenticulata	TNTC	672	109	4	86	21	1	0
	A. polyphaga	TNTC	656	161	19	1260	72	11	1
	H. vermiformis	TNTC	TNTC	235	29	72	9	1	0

Table 5.4: *F. tularensis* culture results for lysate.

Lysate		**4 HOURS** Dilution factor		**24 HOURS** Dilution factor	
		0	-1	0	-1
Amoebae strain	*A. castellanii*	624	134	4	0
	A. lenticulata	612	108	2	0
	A. polyphaga	632	89	7	0
	H. vermiformis	TNTC	253	19	8

Table 5.5: MRSA culture results for lysate.

Lysate		**4 HOURS** Dilution factor		**24 HOURS** Dilution factor	
		0	-1	0	-1
Amoebae strain	*A. castellanii*	252	10	2	0
	A. lenticulata	67	37	0	0
	A. polyphaga	TNTC	174	154	29
	H. vermiformis	203	37	2	0

5.4 Discussion:

If amoebae can serve as a reservoir for natural infections and as a safe house for various pathogens in dynamic environments, this would change our thinking about several aspects of microbial ecology. For Tier 1 Select Agent pathogens, which includes both *F. tularensis* and *B. pseudomallei*, a

clear understanding of the interaction of amoebae with the pathogen is of supreme importance to manage environmental contamination and to determine the risk of transmission. Furthermore, this knowledge is important to further comprehend the selective pressures that the pathogens may be facing and could provide a new channel for microbial management through biocides targeted toward amoebae. MRSA, albeit not a concern for bioterrorism, is a bacterium with serious public health consequences and is often isolated in and around hospitals. Sewage lines and other waterways associated with hospitals provide an excellent platform for various MRSA isolates to interact and potentially be uptaken by an amoebae.

Results from this study mirror that which has been indicated by previous work relating to the capacity for *A. castellanii* (Abd *et al.,* 2003; El-Etr *et al.,* 2009) to become infected with *F. tularensis* and further suggests that at least 3 other species of amoebae are capable of uptaking this pathogen. *B. pseudomallei* was found to enter and replicate in all 4 strains of amoebae as well as MRSA which was cultured from all four strains of amoebae following the 4-hour infection and from several strains following the 24-hour infection.

The highly varied responses between each of the three pathogens in terms of bacterial uptake and replication within amoebae suggests that there are a wide variety of factors that influence the interaction of these two biological systems. Amoebal passage number will surely effect the results obtained as continued laboratory perpetuation has been shown to cause amoebae to lose genes necessary for encystment and presumably others essential for survival in nature but unnecessary in a laboratory capacity (W. Wheat, personal communication, March 13, 2016); in the same way many virulent bacterial pathogens attenuate following extensive laboratory propagation (Fux *et al.,* 2005). This work demands much further evaluation especially that related to persistence within trophozoite and cyst amoebal forms. The ultimate demonstration that amoebae are capable of serving as a reservoir species for any pathogen will involve the induction of disease in a mammal or bacterial uptake of a vector species following exposure to a subset of amoebae infected with the organism of interest.

CHAPTER 6: CONCLUDING REMARKS

F. tularensis has been studied extensively in laboratory animals to evaluate pathogenesis, routes of transmission, infectious and lethal doses, therapeutic options, and vaccine candidates, but our understanding of the dynamics and ecology of infection in hosts relevant to natural infections is impoverished. The overarching goal of this dissertation research was to assess the pathogenesis of *F. tularensis* infection in mammalian species and protozoa that have been implicated in environmental maintenance and transmission.

The present study is the first to report acute mortality in cottontail rabbits following a low-dose intradermal inoculation with type A strains of *F. tularensis,* along with associated high tissue burdens and distinct gross and histopathological lesions that reflect rapid bacterial replication and a highly active host inflammatory response. The protective effects of infection with type B strains on subsequent exposure to a highly virulent type A strain of *F. tularensis* were also evaluated, again using cottontail rabbits, which may encounter both types of this pathogen due to overlapping geographical regions for both cottontail rabbits and *F. tularensis* isolates. Host mortality was the most common response when inoculation with a type A strain is administered following a type B strain; however, the time to death was lengthened and in some cases mitigated altogether. Furthermore, bacteriuria was detected in 2 rabbits that received a type B inoculation followed by a type A inoculation which was noteworthy as this was the first instance in which this clinical sign was detected in cottontail rabbits with a tularemia infection. The discovery of contaminated excreta in cottontail rabbits may be supremely important in a natural setting, providing an opportunity for environmental contamination which could allow for transmission events or phagocytosis by amoebae. Taken together, these findings suggest that cottontail rabbits are a highly susceptible mammalian species that are likely to be involved in environmental maintenance and may play an important role as a sentinel species to alert public health agencies of the potential for exposures and thus help prevent human cases of disease. Furthermore, they may be more appropriately deemed a competent amplification host due to acute susceptibility and high organ burdens.

Oral inoculation of prairie voles failed to demonstrate infection at any level; however, intranasal inoculation resulted in acute mortality in 100% of voles infected with the OR96-0246 strain of *F. tularensis.* Interestingly, a high dose ($1x10^7$ organisms) delivered via the alimentary tract in an oral inoculation failed to result in any detectable level of infection; however, a much lower dose, 350-650 organisms delivered intranasally resulted in a lethal infection in all voles tested. Furthermore, only 1 of the 4 voles (25%) inoculated intranasally with the KY99-3387 strain succumbed to infection. This finding is inconsistent with what we found in cottontail rabbits as both type B strains were found to be very similarly virulent. Considerably more work is required in order to understand the role that voles may play in environmental maintenance and transmission. Based on our findings, in conjunction with findings by Bell and Stewart in 1975 and 1983, experimental infections that assess multiple strains of type B *F. tularensis* is essential to evaluate tolerance or susceptibility in several strains of voles and via multiple routes of infection.

Inoculation of four strains of amoebae with *F. tularensis* allowed detection of viable organism in all amoebae strains at both the 4 and 24 hour time points, via direct culture methods. Furthermore, confocal microscopy allowed for the visualization of pathogen uptake as well as amoebae morphology and bacterial distribution within the amoebae. The amoebae work presented here was preliminary in nature and by no means comprehensive. The ultimate goal of this work is to determine if amoebae are capable of harboring *F. tularensis* and transmitting the pathogen to a susceptible vector or mammalian host; this work provides first-step knowledge that is essential in order to evaluate the fundamental question in a laboratory setting.

The research presented in this dissertation contributes to our collective knowledge of *F. tularensis* pathogenesis and immunology in natural mammalian and protozoal hosts. The work provides a strong

framework from which further investigations of *F. tularensis* ecology may be conducted with the ultimate goal of preventing environmental amplification and transmission events.

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