EXPERIMENTAL COLONIZATION AND PERSISTENCE OF *FRANCISELLA TULARENSIS* IN *DERMACENTOR VARIABILIS* AND *AMBLYOMMA AMERICANUM*

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EXPERIMENTAL COLONIZATION AND PERSISTENCE OF *FRANCISELLA TULARENSIS* IN *DERMACENTOR VARIABILIS* AND *AMBLYOMMA AMERICANUM*

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CHAPTER I

INTRODUCTION

Francisella tularensis is a highly infectious, gram-negative, coccobacillus and causative agent of the disease tularemia in wild rodents and rabbits. The disease is occasionally transmitted to incidental hosts including humans. The genus *Francisella* has two species, *F. tularensis* and *F. philomiragia*. It has been recently proposed that *Francisella*-like endosymbionts of ticks and certain fish pathogens also be included in the genus *Francisella*. *Francisella philomiragia* is an opportunistic pathogen and is reported to cause pneumonia and systemic disease in immunocompromised individuals. *Francisella tularensis* has two main subspecies, type A (ssp. *tularensis*) and type B (ssp. *holarctica*). Type A is the more virulent form and is seen exclusively in North America, whereas the less virulent type B is found in North America and Eurasia. *Francisella tularensis* type A has a terrestrial life cycle; the bacterium is enzootic in wild rodents and rabbits. This transmission cycle is vectored by arthropods, mainly ticks and biting flies, and accounts for 90% of human tularemia cases in North America. *Francisella tularensis* type B has a waterborne life cycle in which the bacterium is enzootic in water-associated rodents and beavers and accounts for 10% of human tularemia cases in North America.

Francisella tularensis can be easily aerosolized and is highly infectious; therefore, the Centers for Disease Control and Prevention has classified *F. tularensis* as a category A Select Agent. Much of the research in recent years has focused on pathogenesis and vaccine development. Over the last 10 years, our understanding of the immune response to the bacterium as well as the genetic aspects of the pathogenesis of *F. tularensis* has improved considerably. However, our understanding of the ecology of this bacterium, its persistence in nature as well as the biology in the vector is still lacking.

The first two studies reported herein describe the systematic study of *F. tularensis* colonization in the tick vectors *Dermacentor variabilis* and *Amblyomma americanum* as well as describing the capillary feeding method of colonizing the different stages of these ticks with *F. tularensis*. The role of these ticks as a possible reservoir of *F. tularensis* is also discussed. The final chapter of this dissertation addresses the possible role of chitin as a nutrient source for *F. tularensis* in the tick life cycle.

CHAPTER II

LITERATURE REVIEW

Abstract

Francisella tularensis is a highly infectious pathogen that causes the disease tularemia. *Francisella tularensis* is susceptible to more than 300 different species including mammals, birds, amphibians and invertebrates. The animals which are important in maintaining the natural infection in enzootic foci are wild rodents and rabbits along with their arthropod vectors including ticks and flies. In this chapter the history and epidemiology of *F. tularensis,* ecology of vector borne transmission of *F. tularensis,* and artificial feeding methods for infecting ticks are reviewed.

Introduction

Francisella tularensis is non-motile, non-sporulating, facultative intracellular gram-negative bacteria that cause the disease tularemia in a wide variety of animals including wild and domestic animals and humans (Ellis *et al.,* 2002; Goodman *et al.,* 2005; Sjostedt, 2007). However, the environmental persistence of *F. tularensis* is mainly due to the tick transmitted enzootic cycle of tularemia in wildlife including rabbits and rodents (Eisen, 2007; Eisen *et al*., 2008; Jellison, 1974).

In the first half of the 20th century, human tularemia cases in the United States were relatively high, and the disease was transmitted mainly through contact with infected rabbits. In the latter half of the 20th century the number of human cases declined and the major mode of disease transmission was by tick bites (Eisen, 2007). In the last 50 years, the south-central United States comprised of Arkansas, Missouri, Oklahoma, and Kansas, has become the focal point of human tularemia outbreaks with 60% to 70% of these cases being attributed to infection from tick bites (Eisen, 2007).

The forest/shrub land ecology of the south-central tularemia endemic region is well suited for abundant tick populations (Eisen, 2007; Eisen *et al.*, 2008; Sonenshine, 1991). *Dermacentor variabilis* and *Amblyomma americanum* are the main vectors for *F. tularensis* in this region. Experimental studies have shown that ticks may serve as biological vectors of *F. tularensis,* and that it can be transstadially transmitted from one tick stage to the other (Bell, 1945; Francis, 1927; Hopla, 1953). Although experiments done in the 1930s and 1940s have shown transovarial transmission in *D. variabilis, D. andersoni,* and *Hemophysalis leporipalustris,* more recent studies have failed to confirm this result (Burgdorfer and Varma, 1967; Bell, 1945). *Francisella*-like endosymbionts (FLE) have been shown to colonize the reproductive tissues of *Dermacentor* species, and this association indicates an ancient link between *Francisella* and ticks (Baldridge *et al*., 2009; de Carvalho *et al*., 2011; Machado-Ferreira *et al*., 2009; Niebylski *et al*., 1997; Sun *et al*., 2000).

Host seeking activity of *D. variabilis* and *A. americanum* ticks peaks in early spring and summer in the south-central tularemia endemic region coinciding with the

annual seasonal tularemia outbreaks in humans. A tick-small mammalian hosts enzootic cycle is major reason for the persistence of tularemia in this region. Field studies have shown that *D. variabilis* adults and larvae can survive the winter and resume host seeking activity in the following spring, whereas the nymphs are not known to survive the winter months (Burg, 2001; Kollars *et al.*, 2000a; Sonenshine, 1972; Sonenshine, 1991). In contrast for *A. americanum*, it is the nymphs and adults that are known to overwinter (Brown *et al.*, 2011; Eisen, 2007; Kollars *et al.*, 2000a; Sonenshine, 1991). *Francisella tularensis* infected ticks that overwinter may carry over the infection from one enzootic cycle to the next and thus maintain the persistence of this bacterium in nature.

In recent decades, much progress has been made in our understanding of the pathogenesis and virulence of *F. tularensis* especially in regard to mouse infection models and vaccine development (Clinton *et al*., 2010; Lindgren *et al*., 2011; Meibom and Charbit, 2009; Santic *et al*., 2009); however, with regard to the biology of *F. tularensis* in the tick vectors and the molecular level interactions at the vector-pathogen interface very little is known (Eisen, 2007; Petersen *et al*., 2009). *Francisella tularensis* was shown to disseminate from the gut to the hemolymph in *D. andersoni*, but evidence of bacterium in the salivary gland or in the saliva has not been reported for any tick species (Francis, 1927; Petersen *et al.*, 2009). The glass capillary tube method of feeding ticks with pathogens has been shown to be an excellent method of infecting various tick species with pathogens (Broadwater *et al.*, 2002; Kocan *et al.*, 2005; Young *et al.*, 1996). Environmental persistence of *F. tularensis* has been reported especially for *F. tularensis* ssp. *holarctica* and *F. tularensis* ssp. *novicida* in aquatic environments, and in addition,

the latter was shown to form biofilms in the presence of chitin (Berrada and Telford III, 2011; Hazlett and Cirillo, 2009; Margolis *et al*., 2009).

History and Epidemiology of Tularemia

Tularemia was first described by G. W. McCoy in ground squirrels in Tulare County in California, and a year later the bacterium was isolated by McCoy and Chapin (McCoy and Chapin, 1912). Two years later the first human case of tularemia was described in Ohio (Wherry and Lamb, 1914). However, a disease of similar nature had been described in Utah in 1908, and even earlier, there were reports of similar disease description from Norway called lemming fever and also from Japan called Yato byo (wild hare's disease) (Morner, 1992; Ohara, 1954; Pearse, 1911). The bacterium was initially designated as *Bacterium tularense*, the term coined after Tulare County, California where McCoy first described the disease. In 1921, Edward Francis showed the experimental transmission of the bacterium in deerfly (*Chrysops discalis*) and later in several arthropod vectors including ticks (Francis, 1927; Francis and Mayne, 1921). The bacterium was briefly classified under the genus *Brucella* and *Pasteurella* because of the serological similarity (Topley and Wilson, 1937). In 1947, the name *Francisella* was given to the genus in recognition of the extensive work of Edward Francis on this

bacterium, and in 1961 Philip and Owen proposed the name *Francisella tularensis* as the type species for this genus (Olsufjev, 1970; Philip and Owen, 1961).

After the initial description of the disease in the 1920s, numerous tularemia cases were reported from North America, Europe, Russia and Japan. A distinct difference in the virulence of the isolates seen in North American outbreaks from those seen in Europe and Asia was reported. In 1961, Olsufiev and colleagues suggested *F. tularensis* biovar tularensis for the more virulent North American isolates and *F. tularensis* biovar palaearctica for the less virulent European and Asian isolates. Later the two biovars were designated as *F. tularensis* ssp. *tularensis* and *F. tularensis* ssp. *holarctica*, respectively (Olsufiev *et al.,* 1959; Olsufjev, 1970). In another classification based on the susceptibility of the *holarctica* subspecies for erythromycin, biovar I Ery^S and biovar I Ery^R was proposed (Olsufjev and Meshcheryakova, 1982). W. L. Jellison used the designation type A for ssp. *tularensis* and type B for ssp. *holarctica*. Owing to its simplicity, this nomenclature is still used today (Jellison, 1974; Olsufjev and Meshcheryakova, 1982). In 1950 a new bacterium similar to *Francisella* was isolated from water samples from Ogden Bay, Utah. This new bacterium was distinct from *F. tularensis* in that it could ferment sucrose, and the name *Pastuerella novicida* sp. nov. was designated and later categorized as a ssp. of *F. tularensis* (Larson *et al*., 1955; Olsufjev and Meshcheryakova, 1982). Another *Francisella* isolate was reported from Central Asia and was included as ssp. *mediasiatica.* Currently, there are four ssp. of *F. tularensis* namely *tularensis*, *holarctica*, *novicida* and *mediasiatica* (Olsufjev and Meshcheryakova, 1982).

Johansen and colleagues in 2004, reported two subdivisions within the virulent *tularensis* ssp. by analyzing 25 variable number of tandem repeat (VNTR) loci using the multi locus VNTR analysis (MLVA) method and designated them as A1 and A2 (Johansson *et al.*, 2004). Following this VNTR analysis, Staples and colleagues found similar genetic diversity among the type A isolates in the United States by analyzing pulsed field gel electrophoresis (PFGE) subtyping and the geographic location of the isolates (Staples *et al.*, 2006). They gave the designation type A East and type A West to the A1 and A2 subtypes and reported that the A1 isolates were more virulent and mainly found in the south-central States and along the Atlantic Coast, whereas the less virulent A2 subtype was seen in the arid regions of the Rocky Mountains west to the Sierra Nevada Mountains of California (Staples *et al.*, 2006). Kugeler and colleagues in 2009 identified four distinct genotypes of type A, namely A1a, A1b, A2a, A2b and also type B in the United States using PFGE, and they also identified the case fatality rates of these genotypes as 4%, 24%, 0%, 0%, and 7%, respectively (Kugeler *et al.*, 2009).

In the 1940s and 1950s, Russian researchers were able to develop several attenuated vaccine strains of *F. tularensis* using several methods including repeated culture in artificial media (Tigertt, 1962). In 1956, a mixture of these attenuated strains were transferred to the United States, and from this mixed culture, a type B strain was selected based on intradermal vaccine safety and efficacy (Eigelsbach and Downs, 1961; Tigertt, 1962). This strain was called the Live Vaccine Stain (LVS); although less virulent in humans, it still retained virulence in mice (Conlan *et al.*, 2002). The molecular mechanism by which the LVS is attenuated is not known, and the vaccine is not fully effective against higher inocula of virulent *F. tularensis.* Therefore, it has been approved

only for use in people who are at high risk of exposure, particularly research workers (Burke, 1977; Friend, 2006; Conlan and Oyston, 2007). Recent progress in identifying the possible genes responsible for the attenuation may lead to the development of a stable and effective vaccine (Salomonsson *et al.*, 2009).

Following the initial description of the disease in 1911, more than 35,000 human tularemia cases have been reported in the United States of which 90% are caused by the ssp. *tularensis* (Choi, 2002). *Francisella tularensis* have long been considered as a potential biological warfare agent because of the highly infectious nature of this bacterium and the ease of aerosolization (Dennis *et al.*, 2001). This pathogen was one of the biological agents used by the Japanese Imperial Army at Unit 731 during the Second World War and is also one of the biological agents developed by the United States and former Soviet Union as part of their biological weapons development program (Dennis *et al.*, 2001). The primary disease presentation for human tularemia include ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal, and septicemic forms (Dennis *et al.*, 2001). The different presentations of the disease depend mainly on the route of entry of the bacterium. The most common form, the ulceroglandular presentation, occurs due to the entry of *F. tularensis* through the bite of an arthropod vector or through cuts or abrasions of the skin while handling infected animals (Ellis *et al.*, 2002). The oculoglandular form is rare and can develop when bacteria comes in contact with the conjunctiva. The ingestion of contaminated food or water leads to oropharyngeal presentation. The virulent *F. tularensis* ssp. *tularensis* can often lead to typhoidal or septicemic forms of the disease because of the invasive nature of the bacteria, and this presentation can be fatal. The most acute form of the disease is

the pneumonic form, which can sometimes occur as a secondary complication of any of the above presentations.

The unusually high number of cases of tularemia in the first half of the 20th century in the United States was most likely due to the exposure to infected animals, particularly infected rabbits. Most of these cases were ulceroglandular in presentation (Jellison, 1974). The increased awareness of the disease and urbanization may have caused the number of tularemia cases to decline after 1950s, and this decline was more pronounced, especially in the number of winter cases of tularemia that accompanied the rabbit hunting season (Evans *et al.*, 1985). Tularemia in eastern and central States including Illinois, Kentucky, Ohio, Tennessee and Virginia continued to decline whereas the cases in the south-central States comprised of Arkansas, Missouri, Oklahoma and Kansas remained constant (Eisen, 2007; Jellison, 1974). Subtyping of the genotypes seen in the south-central endemic region has indicated that these infections are due to the virulent A1 subtype (Eisen, 2007; Kugeler *et al.*, 2009). Another focus of recent tularemia outbreaks is on Martha's Vineyard, an island off the coast of Massachusetts (Feldman *et al.*, 2001; Teutsch *et al.*, 1979). *Francisella tularensis* is believed to have been introduced to the island in the 1930s by the importation of infected rabbits for game hunting from the endemic areas of Arkansas and Missouri. Consistent with this conjecture, it is the A1 subtype that is found on the island (Kugeler *et al.*, 2009; Matyas *et al.*, 2007b). The first case of pneumonic tularemia on Martha's Vineyard was in 1978, and in the following years, cases have been continuously reported (Feldman *et al.*, 2001; Matyas *et al.*, 2007b). This unusual route of transmission is thought to occur due to

landscaping activity, especially due to the aerosolization of tularemia infected rabbit carcasses or rabbit nests by lawn mowing (Agger et al., 2005; Matyas et al., 2007b).

Ecology of Vector-Borne Transmission of *F. tularensis*

Tularemia is often described as a disease seen exclusively in the Northern Hemisphere (Sjostedt, 2003). Outbreaks of the disease in humans and other mammals have occurred in a variety of geographical locations. In the former Soviet Union, investigators had identified several endemic areas of tularemia which encompassed a large variety of natural foci including swamp-floodland, grassland- meadowland, woodland, steppe, cisalpine-stream and desert flood land (Olsufiev, 1966). In these various ecotones, *F. tularensis* was isolated from several ixodid ticks, rodents (*Arvicola terrestrius*, *Micotus* spp., *Mus musculus* and *Clethrionomys* spp.), Arctic hare (*Lepus timidus*), mosquitoes (*Aedes cinereus* and *Ochlerotatus excrucians*), and flies (*Tabanidae*) (Olsufiev, 1966). Olsufiev and colleagues have also demonstrated that *F. tularensis* can survive in tabanid fly gut for up to 56 hours (Olsufevn and Golovd, 1936). Although arthropods including tabanids, deer-flies and mosquitoes have been associated with *F. tularensis* transmission, they have not been shown to be biological vectors of *F. tularensis*, and this bacterium has never been isolated from their salivary glands (Keim et al., 2007; Sjostedt, 2007). Flies can act as mechanical vectors, and it is suggested that the

proximity of watershed areas in tularemia outbreaks may indicate water contaminated with *F. tularensis*, which may serve as a potential reservoir of infection for flies and mosquitoes, likely infecting them at the larval stage (Lundstrom, 2011; Olsufiev, 1966; Palo et al., 2005; Sjostedt, 2007; Svensson et al., 2009). These observations in Russia along with reports of tularemia in Scandinavia and other parts of Europe indicate that *F. tularensis* type B in Europe and Russia follows an aquatic life cycle, where *F. tularensis* infects semi-aquatic rodents, ticks, and flies, with human and animal transmission occurring via contact with infected rodents or by tick and fly bites (Keim *et al.*, 2007). The number of human tularemia cases in Russia has greatly decreased after the Second World War. This has been correlated to the decrease in rodent populations, particularly water rat populations (Efimov *et al.*, 2003).

In the United States there are distinct differences between *F. tularensis* type A and type B based on the geographic distribution, host association and vectors involved. In the United States *F. tularensis* type B has a similar aquatic life cycle as that seen in Europe and human tularemia cases caused by *F. tularensis* type B are concentrated along the upper Mississippi River and in areas of high rainfall including the Pacific Northwest. The major animal hosts involved in the enzootic cycle are the semi-aquatic rodents including muskrats (*Ondatra zibethicus)* beavers (*Castor canadensis),* and voles (*Microtus* sp.) (Jellison, 1974; Staples *et al.*, 2006).

Based on the genotyping data on the *F. tularensis* isolates, the geographical location of the isolates, and based on case histories, Staples and colleagues have speculated that the A2 subtype of *F. tularensis* has a terrestrial life cycle, which can

infect rabbits via ticks (*D. andersoni*) and flies functioning as arthropod vectors. Human cases involving the *F. tularensis* A2 subtype are seen in the arid regions to the west of Rocky Mountains to the Sierra Nevada Mountains (Staples *et al.*, 2006). Deer fly (*Chrysops discalis*) was one of the first vector linked to the transmission of *F. tularensis*, and the term "deer fly fever" has often been associated with tularemia (Jellison, 1974). These flies function as mechanical vectors of *F. tularensis*, and they transmit mainly the type A West (A2) subtype of *F. tularensis* which is seen to the west of Rocky Mountains primarily Idaho, Wyoming, Utah, and Nevada, with recent outbreaks in Utah (Petersen *et al*., 2009; Staples *et al*., 2006).

Francisella tularensis type A1 has a terrestrial transmission and is seen predominantly in the south-central States of Arkansas, Missouri, and Oklahoma and also along the Atlantic Coast. Rabbits and hares, including cottontail rabbits (S*ylavilagus* sp.) are the major hosts associated with *F. tularensis* type A1. However, these lagomorphs are not considered a stable reservoir host of *F. tularensis* because their populations are highly susceptible to the disease during epizootic outbreaks which result in high death rates (Jellison, 1974; Keim *et al.*, 2007; Shoemaker *et al.*, 1997). Ticks are the major vectors involved in the transmission of *F. tularensis* type A1 in humans and may also play a important role in maintaining the enzootic cycle in nature.

***Dermacentor* species.**

The importance of ticks as vectors for *F. tularensis* were perhaps know even during the initial discoveries of the bacterium in California ground squirrels. One of the infected squirrels collected during a study had an engorged tick and was a possible

contributor to the disease in that animal (Jellison, 1974). Several *Dermacentor* ticks are known to be vectors of *F. tularensis*. The two *Dermacentor* species that are important for tularemia infections in humans and animals in the United States are *D. variabilis* and *D. andersoni*. The first case of *D. andersoni* (Rocky Mountain Wood tick) transmitted glandular type of tick fever was later shown to be tularemia, and *F. tularensis* was subsequently isolated from *D. andersoni* ticks in nature (Jellison, 1974; Parker *et al.*, 1924). These ticks are known for their vector role in epizootics of tularemia in sheep in Idaho and Montana where during the first half of the 20th century, large number of *D. andersoni* infestations on sheep especially during the lambing season caused high mortality in sheep due to tularemia (Bell *et al*., 1978; Jellison, 1974).

Dermacentor variabilis is one of the principle vectors of *F. tularensis* in the United States and the bacterium was first discovered in this tick by R. G. Green (Eisen, 2007; Goodman *et al.*, 2005; Green, 1931). During an investigation of an ulceroglandular tularemia outbreak in the Crow Indian Reservation in south-central Montana, it was found that tick bites were one of the causes of infection, and 8 out of 15 pools of *D. variabilis* ticks collected from dogs in this area were positive for *F. tularensis* (Schmid *et al*., 1983). In another outbreak of glandular tularemia on the lower Brule and Crow Creek Indian Reservation in South Dakota, *D. variabilis* ticks were identified as the vector, and 8 out of 46 pools of *D. variabilis* ticks collected from dogs were positive for either type A or type B subspecies of *F. tularensis* (Markowitz *et al*., 1985).

Dermacentor variabilis also plays a key role in transmission of *F. tularensis* in the south-central United States and is cited as a primary reason for the tularemia persistence in this region. High incidence of tularemia in people involved in outdoor

activities is thought to be due to the high risk of tick exposure in this region (Assal *et al.*, 1968; Eisen, 2007; Scofield *et al.*, 1992; Taylor *et al.*, 1991). The seasonal distribution of human tularemia cases in this region is mostly during spring and summer months, which also coincides with the high *D. variabilis* tick activity (Eisen, 2007). *Dermacentor variabilis* ticks are identified as an amplifying host and vector of *F. tularensis* in Martha's Vineyard, and the genetic diversity seen among the *F. tularensis* isolates from these ticks indicate long standing enzootic cycle of tularemia on the island (Goethert *et al.*, 2004). In a PCR done on host seeking ticks on the island, less than 1% to 5% of the ticks were reported positive depending on site and year of tick collection (Matyas *et al.*, 2007a). In 2010, Goethert and Telford reported a high concentration of *F. tularensis* in infected ticks on the island with a median of 3.3×10^8 genome equivalents per tick (Goethert and Telford, 2010).

In the United States, *D. andersoni* is distributed throughout the Rocky Mountains at elevations above 1000m (Goodman *et al.*, 2005). Both larvae and nymphs of this tick species feed on a variety of small mammals including rodents and rabbits, and the adult ticks feed on larger mammals including humans, carnivores, game animals, cattle, sheep and horses (Burgdorfer, 1969; Jellison, 1974).

In the United States *D. variabilis* ticks are widely distributed in the Great Plains and Eastern States (Burgdorfer, 1969; Sonenshine, 1972; Sonenshine, 1991). The habitat range is restricted by a lack of suitable deciduous forest, brushy habitat and lack of adequate rainfall (Sonenshine, 1991). The prairie, sagebrush, and semidesert habitats to the west, the boreal forests to the north, and the narrow forested areas along the drainage basins of the Missouri, Arkansas, and Mississippi Rivers support abundant populations of

this tick species (Burgdorfer, 1969; Sonenshine, 1991). The immature stages of *D. variabilis* including larvae and nymph exclusively feed on smaller mammals including meadow mice (*Microtus* sp.) and white footed mice (*Peromyscus* sp.) and to a much lesser extent on rabbits, rats, squirrels and cats. These immature stages are usually seen questing at the ground level, while adult ticks quest higher in the vegetation and have a broader host range. The principle hosts include dogs and other larger mammals such as cattle, coyotes, wild cats, raccoons, foxes, skunks, deer and humans (Sonenshine, 1991). Kollar and colleagues, in a study to determine the host utilization of *D. variabilis* in Missouri, found that raccoons followed by opossums were the most preferred hosts for adult ticks, whereas voles and chipmunks were the hosts for nymphal ticks (Kollars *et al.*, 2000b). Marsh rice rats, white footed mice, and voles were the preferred hosts for the larval stages (Kollars *et al.*, 2000b).

Tick activity studies in Missouri showed that adult *D. variabilis* ticks continue their host seeking behavior from March through August, with peak activity from June to July (Kollars *et al.*, 2000b). The early year activity is from the spring cohort, which overwintered from the previous year, and the late year activity is usually from the summer cohort which molted from nymphs, that fed during the current year (Burg, 2001; Kollars *et al.*, 2000b; Sonenshine, 1972). In Missouri, *D. variabilis* nymphal activity is from April to October with the highest activity during May and June. Larval populations in Missouri have a bimodal activity, with one peak in late summer (September) and another during early spring (Kollars *et al.*, 2000b). Unfed larvae survive long periods of exposure in the environment and usually overwinter. Adults are also known to

overwinter; in contrast, overwintering of nymphs is rare (Burgdorfer, 1969; Sonenshine, 1972; Sonenshine, 1991).

Parker and Spencer showed in 1926 the transovarial transmission of *Bacterium tularense* in *D. andersoni* ticks. In this study, eight of the fifteen female ticks that engorged on infected hosts transmitted the infection to progeny. Two out of these eight positive ticks, had infection in eggs only, while in the other six infection was recovered form larvae or nymphs or both (Parker and Spencer, 1926). Parker concluded that transstadial transmission of *Bacterium tularense* does occur in *D. andersoni* and transovarial transmission can also occur, but not in all infected females. However, investigations in the 1950s and 60s failed to demonstrate the transovarial transmission in *D. andersoni* (Burgdorfer and Varma, 1967). Edward Francis reported detailed microscopic changes including bacterial multiplication in gut and dissemination into hemolymph in *D. andersoni* ticks infected with *Bacterium tularense* (Francis, 1927). He concluded that *D. andersoni* is a true biological host of tularemia, and the bacteria were seen in the feces, epithelial cells of the digestive tract, Malpighian tubes and the celomic fluid of these ticks. However, the bacterium was not observed in the salivary glands or the ovaries, and this observation led him to believe that the transmission was due to fecal contamination at the bite site (Francis, 1927).

Philip and Jellison demonstrated the experimental transmission of the bacterium to guinea pigs by infected *D. variabilis* (Philip and Jellison, 1934). The same authors confirmed the transovarial transmission in these ticks by demonstrating the infection of mice parasitized by larvae which hatched from eggs of an infected adult tick. However, larvae from the same lot failed to infect rabbits or guinea pigs. They also reported on the

evidence of transovarial transmission by injection of washed eggs of two infected female ticks intraperitoneally into guinea pigs; these animals later succumbed to tularemia. In the same article the authors observed that *Bacterium tularense* was not completely adapted for continuous colonization in *D. variabilis*, because of the mortality seen in heavily infected ticks, especially in ticks which fed on host during the peak of bacteremia (Philip and Jellison, 1934). In a detailed study in 1945, Bell reported the transmission of *Pasteurella tularensis* in *D. variabilis* ticks. He observed that certain ticks in the lot were more resistant to infection than the others. He also reported that vitality of the ticks were not affected by infection and reaffirmed the knowledge that bacteremia in the host (usually just occurring prior to the death of the animal) was required for ticks to become infected (Bell, 1945). During his study, Bell did not observe any mortality in ticks feeding at the time of death of the host, as observed by Philip and Jellison (Bell, 1945; Philip and Jellison, 1934). He did not observe any difference in fecundity between infected and uninfected ticks and also did not observe the transovarial transmission in these ticks (Bell, 1945). Higher (37°C) or lower (15°C) holding temperatures for up to 9 days of the infected nymphal ticks also did not affect their infectivity (Bell, 1945). Recently, Reese and coworkers evaluated the ability of the nymphal *D. variabilis* ticks to transmit A1b, A2 and type B stains of *F. tularensis* (Reese *et al*., 2010). Transstadial transmission from larva to nymph was observed with all three strains, but there were significant decrease in feeding success and significantly higher mortality in infected nymphs. Low transmission rates to mice were also seen in A2 and type B infected ticks, and infection of mice with the A1b infected ticks were not observed (Reese *et al.*, 2010).

A number of *Dermacentor* species are known to be principle tick vectors of *F. tularensis* in the former Soviet Union including *D. pictus* and *D. marginatus* (Hopla, 1974). In 1960, Petrov showed that *F. tularensis* infected *D. marginatus* larvae were capable of transmitting the bacterium and observed that feeding of ticks is accompanied by an increase in the number of bacteria, but the process of molting decreases bacterial numbers in ticks. In addition, Petrov observed that ticks had to be adequately infected for transmission from one stage to the other (Petrov, 1960). In 1966, Petrov showed that bacteria could penetrate the gut of the tick into hemolymph and the salivary gland of *D. marginatus* and also reported the transovarial transmission in this tick (Hopla, 1974). However, he could not reproduce the transovarial transmission in *D. marginatus* during later repetition of his experiments (Hopla, 1974).

In recent years, a *D. variabilis* associated endosymbiont has been discovered and phylogenetic analysis shows that it is closely related to pathogenic *Francisella* species (Goethert *et al.*, 2004; Matyas *et al.*, 2007a). The *Francisella*-like endosymbionts (FLE) and *Dermacentor* ticks show little sign of co-speciation owing to the less degree of phylogenetic divergence between FLEs of different *Dermacentor* species, indicating the relation between the endosymbiont and *Dermacentor* ticks is of recent origin (Niebylski *et al.*, 1997; Scoles, 2004). It is speculated that a broad host permissible tick transmitted a FLE ancestor similar to *F. tularensis*, which may have spread among other ticks by a common infection pathway and secondarily adapted to a symbiotic life style (Niebylski *et al.*, 1997; Scoles, 2004). Another closely related *Francisella*-like symbiont, different from FLE, was found in 50% of *D. variabilis* ticks tested on the Martha's Vineyard Island (Goethert and Telford, 2005). These *D. variabilis Francisella* (DVF) demonstrated

transovarial transmission, and co-infection of FLE with DVF did not interfere with the transmission of either bacteria in these ticks (Goethert and Telford, 2005). In1997, Niebylski and coworkers characterized a *D. andersoni* symbiont as belonging to the genus *Francisella* based on 16s rDNA sequence data (Niebylski *et al.*, 1997). The symbiont was found to inhabit the ovarial tissues, especially in the phagocytic vesicles and was vertically transmitting in 95.6% of the female progeny of *D. andersoni* ticks (Niebylski *et al.*, 1997).

***Amblyomma americanum*.**

Amblyomma americanum, the lone star tick, was first identified as a vector for *F. tularensis* in 1943 when Warring and Ruffin reported an outbreak of tularemia among soldiers in a maneuver area in Tennessee (Jellison, 1974; Warring and Ruffin, 1946). Of the 50 cases reported, 32 had history of tick bite prior to the onset of the disease. The area was heavily infested with ticks, the primary lesions indicated tick bite in 42 of these cases, and *A. americanum* was the only tick discovered in the vicinity (Warring and Ruffin, 1946). In a survey of ticks in Arkansas, Calhoun found that one pool of lone star tick taken from four dogs in Marion County was infected with *Pasteurella tularensis* (Calhoun, 1954). In a following survey of ticks in high tularemia incidence counties in Arkansas, five pools of *A. americanum* ticks were found positive for tularemia infection, while none of the dog ticks, rabbit ticks or the black legged ticks were found to be positive (Calhoun, 1954). One of the positive pool from a drag collection included adult female ticks and a nymph. The other positive pools had adults, nymphs and larvae collected from cows and dogs. Calhoun and Alford reported the finding of infected *A. americanum* larvae in nature, which indicates the possible transovarial transmission of *F.*

tularensis in these ticks (Calhoun and Alford, 1955). However, an investigation to study the possibility of the transovarial route in *A. americanum* has not been performed (Eisen, 2007).

In a review on the "Transmission of tularemia organisms by ticks in the Southern States", Hopla states that among the three tick species that are known to bite humans in this region, *A. americanum* and *D. variabilis* are the most important vectors of *F. tularensis* because of the peak host seeking activity of these ticks coinciding with tularemia outbreaks in these states (Hopla, 1960). The one other tick species found in this region, *Ixodes scapularis* may not be a good candidate as a *F. tularensis* vector, because the human biting stage of these ticks has a peak activity during the winter season, which does not correlate with tularemia outbreaks in this region. He also suggests that among the two possible *F. tularensis* vector species in this region, *A americanum* may be the most important because of its activity during the summer months, abundance, and broad host range among all three stages of the tick (Hopla, 1960).

Along with being a vector for *F. tularensis, A. americanum* is also a reported vector of other infectious agents of both humans and animals. Extensive studies have been done over the years to better understand the activity and habitat of this tick species (Bowman and Nuttall, 2008; Goodman *et al*., 2005; Jellison, 1974). *Amblyomma americanum* is distributed from central Texas, eastern Oklahoma, southeastern United States and along the Atlantic Sea Coast (Burgdorfer, 1969). This tick is predominantly found in forested habitats, and an example of an ideal habitat is the scrub-brush dominated hills of the Ozark region of eastern Oklahoma (Sonenshine, 1991). Larvae and

nymphs usually feed on rabbits, squirrels, foxes, raccoons, skunks, and on a variety of ground dwelling birds particularly quail, turkey, and poultry, whereas adult ticks feeds on larger and medium sized animals. All stages of *A. americanum* are known to attack humans, deer, cattle, horse, and dogs (Sonenshine, 1991).

H. G. Koch in a study about the survivability of lone star ticks in southeastern Oklahoma reported that these ticks are fairly tolerant to the extremes of high temperature and low humidity, and the best environment for survival was the bottomland oak-hickory habitat (Koch, 1984). The study also found that all three stages of the tick could survive the winter. Larvae that feed to engorgement as well as unfed larvae only occasionally overwinter, whereas nymphs and adults overwinter in large numbers. However, Burgdorfer reported that larvae that take a blood meal usually overwinter, but those larvae which fail to find a host die (Burgdorfer, 1969). In southeastern Missouri, adult lone star tick questing activity was highest in May with a gradual decline through September (Kollars *et al.*, 2000a). A dual spring and midsummer peak of adult ticks in eastern Oklahoma was reported by Patrick and Hair (Patrick and Hair, 1977). Kollar and colleagues found that in Missouri, nymphal ticks were mostly active from April through September with peak activity in June, while larval activity continued from July through October with peak activity in September (Kollars *et al.*, 2000a). The authors also studied the host preference for the different stages of the lone star tick and found that the white tailed deer was the most important host for adult *A. americanum* ticks, whereas in areas were white tailed deer abundance is rare, foxes and raccoons can be the primary host for adult ticks (Cohen *et al.*, 2010). White tailed deer, raccoons, rabbits, and bobwhite quail were all important host for nymphs. For larval ticks, rabbits were the most infested host

(Kollars *et al.*, 2000a). In order to determine the potential risk of human exposure to human biting ticks in Missouri, Brown and coworkers studied the habitat association of different ticks and found that *A. americanum* was the most abundant tick species in the area, and also reported that adults and nymphs preferred forested habitat to grassland (Brown *et al.*, 2011). The same authors also found that 20% and 30% of the State had elevated risk of human exposure to nymphal and adult *A. americanum* respectively and this data positively correlated with the reporting of tularemia cases in these areas.

Hopla had reported the experimental transmission of *Bacterium tularense* in lone star ticks, and the ability of infected larvae to retain infection though nymphal and adult stage (Hopla, 1953). This observation was previously reported by C. B. Philip (Philip and Parker, 1934). Hopla also reported that no difference in mortality of ticks was observed at any stage of infection of the ticks, when compared to control ticks, and reported large variations in the amount of bacteria per tick (Hopla, 1953). Infected adult ticks which were fasted for six months contained *Bacterium tularensis* although at reduced numbers, but were still able to infect guinea pigs. The same author also reported that feeding infected ticks on *Bacterium tularense*-immunized rabbits did not reduce the infection of ticks after subsequent molting to adult stage, and this result was also true for infected ticks on resistant hosts including dogs and *Bacterium tularense*-immunized mice (Hopla, 1953). Although on a different tick vector, this finding contradicts the conclusion of Bell, who reported *D. variabilis* ticks cleared the bacteria upon feeding on an immunized host (Bell, 1945). In repeat experiments, Hopla also demonstrated the stage to stage transmission of *Bacterium tularense* in *A. americanum* and showed that the bacterium persisted in the adult tick even after five months of fasting, although mean bacterial

counts per tick was reduced when compared to that of recently molted adult ticks. He also suggests that these results indicate the possibility of these ticks overwintering and carrying over the infection to the next year (Hopla, 1960). He reasoned that the decrease seen during long starvation period may be due to bacteria being evacuated from the gut of these ticks during defecation (Hopla, 1955).

Artificial feeding methods for infecting ticks

Ticks can be infected with pathogens by a variety of artificial *in vitro* methods. These include feeding chambers with natural or artificial membrane infusion, enema infusion, hemocelic injection, and glass capillary feeding (Broadwater *et al.*, 2002; Burgdorfer, 1957; Sonenshine, 1991). Membrane feeding chambers have been used to infect a number of ixodid ticks including *Ixodes, Amblyomma* and *Dermacentor* (Barre *et al*., 1998; Howarth and Hokama, 1983; Waladde *et al*., 1995; Young *et al*., 1996). These membranes are expensive to prepare and often undergo decomposition and contamination (Broadwater *et al.*, 2002). Enema infusion technique has been used to introduce pathogens in adult ticks, but this technique often lead to tick injury and mortality (Broadwater *et al.*, 2002; Turell *et al.*, 1997). Intra-hemocel injection of pathogens into ticks has been employed to infect ticks with *Borrelia burgdorferi* and Venezuelan equine encephalitis virus in *D. variabilis* and *Amblyomma cajennese*, respectively (Johns *et al.*,

2000; Turell *et al.*, 1997). This method is not the most suitable method of infecting ticks, as the natural route of entry of pathogens in most cases is through the mouth and into the gut lumen (Burgdorfer and Varma, 1967).

Capillary feeding of ticks was originally reported by Chabaud in 1950, and later in 1954, Burgdorfer described the method in detail for infecting *D. andersoni* and *Amblyomma maculatum* with *Leptospira* Pomona and *D. andersoni* with rabies virus (Burgdorfer, 1957). In this method the glass tubes were pushed over the hypostome and chelicera of the ticks under a microscope and plasticine blocks were used to hold the capillaries in position. Several other researchers have recently used this method of infecting various tick species with pathogens (Bouwknegt *et al*., 2010; Inokuma and Kemp, 1998; Rechav *et al*., 1999; Willadsen *et al*., 1984). Broadwater and coworkers successfully used this method for feeding *Ixodes scapularis* ticks with *Borrelia burgdorferi* (Broadwater *et al.*, 2002). In 2001, Maculoso and colleagues used glass micro-capillary tube feeding for colonizing *D. variabilis* ticks with *Rickettsia* species and showed the transovarial transmission of these *Rickettsial* organisms in ticks upon feeding to repletion on rabbits (Macaluso *et al.*, 2001). Kocan and colleagues were able to capillary feed *D. variabilis* ticks with *Anaplasma marginale* and suggested the potential of this feeding method for identifying aspects of pathogen-vector interactions that are not readily recognized in naturally feeding ticks (Kocan *et al.*, 2005). The advantages of this feeding method is to introduce uniform inocula to ticks and the elimination of expense and mortality in laboratory animals, which is the standard method for feeding ticks (Broadwater *et al.*, 2002; Burgdorfer, 1957).

Saliva induction in ticks

Previous literature cites three methods of inducing salivary secretions in ixodid ticks. They are application of pilocarpine, intra-hemocelomic injection of dopamine, and tactile stimulation of the mouth parts of the ticks. In 1960, J. D. Gregson demonstrated the collection of saliva in *D. andersoni* ticks by appropriate tactile stimulation to the chelicerae and by fitting a capillary tube of critical diameter over the mouth parts (Gregson, 1960).

In 1967, R. J. Tatchell, reported that hypodermic injections of pilocarpine in saline into the hemocel of *Boophilus microplus* ticks produced six times more saliva than from tactile stimulation alone (Tatchell, 1967). Ribeiro and coworkers showed that pilocarpine induced saliva of adult female *Ixodes dammini* ticks inhibits the function of peritoneal-derived rat neutrophils (Ribeiro *et al.*, 1990). Pilocarpine application was also used to detect pathogens in tick saliva (Ewing *et al*., 1994; Gage *et al*., 1992). Gage and colleagues detected rickettsial organisms in tick saliva, which was induced by topical application of 5% pilocarpine solution resulting in collection of as much as 1-5 µL of saliva from partially engorged *D. andersoni* ticks (Gage *et al.*, 1992). *Borrelia burgdorferi* was detected from saliva of partially engorged *Ixodes scapularis* ticks by application of 2µL of pilocarpine (50mg/mL in 95% alcohol) to the scutum of these ticks (Ewing *et al.*, 1994).

The neurotransmitter dopamine is released from the salivary nerve exerts of ticks, which results in activation of adenylate cyclase and formation of cyclic AMP (Sauer *et al.*, 1995). Certain drugs and neurotransmitters have been known to induce salivary

secretions in ticks (Needham and Sauer, 1979). Treatment of dopamine or cyclic AMP and theophylline significantly increases saliva production in ticks (Sauer *et al.*, 1995). Jaworski and colleagues used dopamine and theophylline to induce saliva in *Amblyomma* and *Dermacentor*; 1mM dopamine, 1mM theophylline and 3% dimethyl sulfoxide in saline injected intracelomically every 15 minutes could induce saliva in these engorged ticks (Jaworski *et al*., 1995). This method was also used on *A. americanum* to induce saliva for studying its characteristics (Madden *et al.*, 2004). Detection of pathogens in saliva is essential to confirm whether the pathogen is present in the saliva or is restricted to other tissues of the tick. (Gage *et al.*, 1992).

Utilization of chitin by bacteria

Chitin is one of the most abundant biopolymers in nature, second only to cellulose. In nature chitin is produced by fungi, arthropods, and nematodes. Chitin is composed of monomers of N-acetyl-D-glucosamine (NAG) linked together by β-(1-4)-glycosidic bonds (Merzendorfer and Zimoch, 2003). Chitinase enzymes hydrolyze chitin polymer to chitobiosan (dimer) and then to NAG. Many marine bacteria utilize this abundant source of chitin. Marine bacteria depolymerize chitin with cell surface hydrolases to NAG (Zobell and Rittenberg, 1938). Both gram-positive and negative bacteria have NAG as a main constituent of their peptidoglycan cell wall, and it has been shown to be an excellent

source of nitrogen and energy. NAG has also been shown to be preferentially taken up by marine α-proteobacteria (Cottrell and Kirchman, 2000). Among non-marine bacteria, the studies on NAG transport have been performed in *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Vibrio furnissii* (Bassler *et al*., 1991; Imada *et al*., 1977; Plumbridge, 1990). *B. subtilis* was found to utilize 90% of the NAG intake for producing cell wall precursors, and possesses a high affinity transport system for NAG (Freese *et al.*, 1970; Mobley *et al.*, 1982).

Chitin functions as a structural scaffold in arthropods, and supports the cuticle, trachea, and peritrophic membrane lining the gut epithelium (Merzendorfer and Zimoch, 2003). Insect growth and molting depends on chitin production and remodeling. In ticks chitin is one of the important components of the cuticle, which provides physical strength, muscle attachment sites, and protection from dehydration, and plays an essential role in molting (Sonenshine, 1991). Tilly and coworkers have shown that chitin mutants of *Borrelia burgdorferi* were unable to utilize chitobiose due to dysfunctional chitobiosan transporter genes (Tilly *et al.*, 2001; Tilly *et al.*, 2004). Piesman and colleagues, based on their observation of *Borrelia* growth in *Ixodes scapularis* ticks speculated that the unusual decline in the spirochete number in molting ticks may be due to the depletion of chitin by ticks, making it unavailable for bacterial growth within these ticks (Piesman *et al.*, 1990). Heavy infections of *B. burgdorferi* in tick eggs may affects normal growth of the developing eggs and interfere with chitin deposition on the eggs (Burgdorfer *et al*., 1988; Piesman *et al.*, 1990).

Francisella sp. have been shown to form biofilms (Hassett *et al.*, 2003; Verhoeven *et al.*, 2010). Biofilm formed by bacteria play an important role in environmental persistence and disease transmission. *Francisella tularensis* subspecies encode two conserved putative chitinase genes, *ChiA* and *ChiB* (http://www.patricbrc.org/) (Snyder *et al.*, 2007). Margolis and colleagues have shown that biofilm formation and chitin utilization in *F. tularensis* ssp. *novicida* is diminished in *ChiA* or *ChiB* mutants when chitin was the sole source of available carbon (Margolis *et al.*, 2009). A number of *F. tularensis* strains have been isolated from different tick species, and chitin utilization may be important during the tick infection stage of this bacterium.

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CHAPTER III

BIOLOGY OF *FRANCISELLA TULARENSIS* SUBSPECIES *HOLARCTICA* STRAIN LVS IN THE TICK VECTOR *DERMACENTOR VARIABILIS*

Abstract

The γ-proteobacterium *Francisella tularensis* causes seasonal tick-transmitted tularemia epizootics in rodents and rabbits and incidental infection in humans. To study the biology of *Francisella tularensis* in the tick vectors, colony-reared larva, nymph, and adult *Dermacentor variabilis* were artificially capillary fed (CF) a meal containing 10^7 CFU/mL *F. tularensis* subspecies *holarctica* strain LVS (live vaccine strain) via fine bore capillaries tubes fitted over the tick mouthparts. After the feeding period, the level of colonization and distribution in tick tissue were determined. CF larva and nymph were initially colonized with $8.8\pm0.8x10^1$and $1.1\pm0.03x10^3$ CFU/tick, respectively, followed by a decline in colonization to less than 10^2 CFU per tick at molting. After molting, the colonization increased to $5.2 \pm 0.01 x10^4$ and $1.02\pm0.39x10^4$ CFU per molted nymph and adult tick, respectively, and persisted in 60% of molted adult ticks at three months post-CF (PCF). In the CF adult ticks, *F. tularensis* initially colonized the gut and disseminated to hemolymph and salivary glands by three weeks PCF and was found to persist up to 6 months PCF. When *F. tularensis* was introduced via intra-hemocelic (i.h.) injection in adult ticks, a minimum of one CFU per tick was required to establish colonization, and *F. tularensis* was detected in saliva four days post i.h. injection. *Francisella tularensis* in tick salivary glands was infectious to mice with an infectious dose 50% (ID_{50}) of 2 CFU via i.p. injection. In gravid female ticks infected with *F. tularensis* via the i.h. route, the bacteria disseminated to the ovaries and subsequently to the ova, but the pathogen was not recovered from hatched larvae. *Dermacentor variabilis* is a potential vector for *F.*

tularensis in the south-central tularemia endemic region of the United States, and the reason for the persistence of this disease is due to the maintenance of *F. tularensis* in nature by the annual enzootic cycle of tularemia in ticks and small mammalian species. This study demonstrated that *D. variabilis* is an experimental vector for *F. tularensis*. The long-term persistence of *F. tularensis* in *D. variabilis* adults suggests bacteria can potentially overwinter in the tick and may carryover the infection from one year to the next maintaining the persistence of tularemia in the region.

Introduction

Francisella tularensis is a highly infectious, gram-negative, coccobacillus that causes tularemia epizootics in small mammals and incidental infections in humans (Goodman *et al*., 2005; Jellison, 1974; Sjostedt, 2007). Although *F. tularensis* can infect a wide range of animal hosts, including reptiles and birds, maintenance of the agent in a particular endemic region involves small mammalian hosts, which maintain a significant tick or biting insect parasitic cycle acting as transmission vectors for *F. tularensis* (Goodman *et al*., 2005; Jellison, 1974). Ticks have been implicated as the primary vector for *F. tularensis* in many endemic regions, but biting flies and mosquitoes also can serve as primary vectors. Flies and mosquitoes are mechanical vectors of *F. tularensis*, but *F. tularensis* colonizes and persists in ticks, and can therefore serve as biological vectors (Jellison, 1950; Staples *et al*., 2006). *Francisella tularensis* have co-evolved with ticks as

demonstrated by the presence of *Francisella*-like endosymbionts in many tick species. These *Francisella*-like endosymbionts have a 100% penetrance in symbiotic tick populations, reproductive tissue predilection and vertical transmission (Baldridge *et al*., 2009; Niebylski *et al*., 1997; Scoles, 2004; Sun *et al*., 2000). Although ticks and their hosts are reported to maintain tularemia enzootic cycle in nature, little is known about the biology of *F. tularensis* in ticks.

Rodent and lagomorphs serve as primary hosts and their associated tick species act as vectors in the endemic foci of tularemia in the United States comprised of Arkansas, Missouri, and Oklahoma (Calhoun, 1954; Hopla, 1960). In Oklahoma, human tularemia occurs in a summer seasonal pattern likely mirroring the seasonality of tularemia in small mammals. The peaks of this seasonality are concurrent with tick questing activity of adult *Dermacentor variabilis* and nymphal and adult *Amblyomma americanum*. These tick species are thought to be the primary tick vectors for human *F. tularensis* in this region (Assal *et al*., 1968; Eisen, 2007; Scofield *et al*., 1992). The cross timbers and prairie-forest ecosystems in this hyper-endemic region are especially suitable habitats for these ticks and 60-70% of human tularemia cases have been attributed to tick bites (Eisen, 2007; Eisen *et al*., 2008). Both *Dermacentor* and *Amblyomma* species have been shown to be experimental vectors for *F. tularensis* with transstadial transmission from larva to nymph and nymph to adult (Bell, 1945; Hopla, 1953). Although *Francisella*-like endosymbionts are transmitted transovarially in ticks, studies examining transovarial transmission of *F. tularensis* in ticks have reported conflicting results (Eisen, 2007; Hopla, 1953, 1974; Philip and Jellison, 1934). Detailed studies regarding vector competency, tissue localization and *F. tularensis* multiplication inside the tick has not

been reported. Ticks can be experimentally infected with infectious agents by allowing them to feed on an appropriate host animal or by use of a variety of artificial feeding methods (Burgdorfer, 1957; Johns *et al*., 2000; Turell et al., 1997; Young *et al*., 1996). In the current study capillary tube feeding (CF) was used to colonize *D. variabilis* with *F. tularensis* ssp. *holarctica* strain LVS. This method provides a uniform dose for ticks and reduces the variability encountered when ticks are fed on infected animals (Broadwater *et al*., 2002; Kocan *et al*., 2005). In addition, it is often difficult to synchronize tick feeding with the bacteremia when using animals to establish colonization in ticks and thus insure the optimum acquisition of the pathogen (Bell, 1945; Eisen *et al*., 2009). In the current study, the artificial method of chemically-induced salivation was also used to detect the presence of *F. tularensis* in tick saliva. Although this is the first report of the use of tick oral secretions for detection of *F. tularensis,* this technique has been reported previously for detection of other pathogens (Ewing *et al*., 1994; Gage *et al*., 1992).

The present study was designed to determine the colonizing efficiency of *F. tularensis* for *D. variabilis* ticks infected by the CF method and to determine the tissue localization and transstadial transmission of *F. tularensis* in this tick vector. The results presented in this study indicate that *D. variabilis* is an experimental vector for *F. tularensis* and may play a role as an inter-epizootic reservoir for tularemia in nature.

Materials and Methods

Ticks, bacterial strain and growth conditions

Dermacentor variabilis larvae, nymphs, and adults were obtained from the Tick Rearing Facility, Department of Entomology and Plant Pathology, Oklahoma State University (Stillwater, OK). Larvae were collected following feeding to repletion on rabbits. Nymphs used were partially fed on sheep to approximately 4.5 mg/nymph. Adult ticks used were unfed (flat adults), and those used for saliva induction experiments were allowed to feed on sheep for five to six days (partially fed adults). Females used for the transovarial transmission studies were fed to repletion. Flat adult and partially fed nymphs were weighed before and after CF to assess the CF success, and only those with 0.4 mg weight gain were used.

Francisella tularensis ssp. *holarctica* strain LVS (ATCC 29684) was supplied by the Oklahoma State Department of Health. Green fluorescent protein (GFP) expressing pFNLTP6 *gro-gfp* plasmid (Maier et al., 2004) was a gift of Thomas C. Zhart (Medical College Wisconsin, Milwaukee, Wisconsin), and electroporated into *F. tularensis*. For making electrocompetent *F. tularensis*, Mueller-Hinton broth (Becton Dickinson, Cockeysville, MD, USA) cultures supplemented with 2% IsoVitaleX (Becton Dickinson) were grown to early-log phase (optical density at 550 nm, 0.5 to 0.6 or $1x10^{10}$ CFU/mL), washed two times with 0.5 M sucrose, and suspended in 1 ml of 0.5 M sucrose. For electroporation, 1 µl of plasmid DNA (100 µg/ml) was mixed with 200 µL of electrocompetent cells, incubated at room temperature for 10 min and electroporated

using a MicroPulser electroporation apparatus (BioRad, Richmond, California) at 2.9 kV, 25 µF capacitance and 600 Ω resistance for 5.3 msec. After electroporation the cells were suspended in 1mL of Mueller-Hinton broth supplemented with 2% IsoVitaleX and incubated at 37°C for 4 h. Transformed colonies were selected by plating on brain-heart infusion agar (Difco, BD Diagnostic Systems, Sparks, MD) supplemented with 1% hemoglobin and ampicillin (60 µg/mL) and kanamycin (25 µg/mL) for selection. The transformation efficiency was $1.3x10^3$ transformants/µg of plasmid DNA. The transformed *F. tularensis* was used to infect ticks and to visualize *F. tularensis* in tick hemolymph. *Francisella tularensis* was grown on chocolate agar plates (Hardy diagnostics, Santa Monica, CA.) at 37°C in 5% CO_2 for 72 h. The BBL Prompt Inoculation System (BD Diagnostics, Franklin Lakes, NJ) was used to prepare *F. tularensis* suspensions in the tick inoculum. All chemicals used in the study were purchased from Sigma (St. Louise, MO) unless indicated otherwise.

Capillary feeding of larvae, nymphs, and adult ticks

The ticks were surface disinfected by washing in 30% hydrogen peroxide, distilled water, and 70% isopropyl alcohol for 5 seconds each. After washing, the adult ticks were immobilized dorsal sides up on double-sided tape in a 100 mm x 15 mm Petri dish base. The ticks were then further immobilized by applying single sided tape over $1/4^{th}$ of their caudal portion. The larvae and nymphs were immobilized with their dorsal side down on double sided tape on a Petri dish base. For CF, 10 µL (internal diameter of 0.0219 inch), 9 µL (internal diameter of 0.0189 inch) and 35 µL (internal diameter of 0.0314 inch) glass capillary tubes (Drummond Scientific Company, Broomall, PA) were

used for larvae, nymphs and the adult ticks, respectively. The ends of the tubes were positioned over the hypostome of the tick while the other end rested on the edge of the Petri dish attached with a double sided tape (Broadwater *et al*., 2002). The feeding media for larvae, nymphs, and adult ticks was Minimum Essential Media (MEM) (GIBCO Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT.). The feeding medium was spiked with *F. tularensis* ssp. *holarctica* strain LVS at approximately 10^7 CFU/ ml. The tick meal was then introduced into the capillary tubes, and the ticks were allowed to feed for 12 to 24 hours at 30°C and 90% relative humidity. After feeding the ticks were either surface disinfected by washing as above and minced for determinations of CFUs or were maintained in microcentrifuge tubes capped with moistened cotton plugs for varying periods of time in a humidity chamber (relative humidity of > 90%) at room temperature (23°C) (unless specified otherwise) with automated artificial lighting to simulate a 12 h day night cycle. To determine the bacterial number in tick gut, salivary glands, and ovaries, these tissues were dissected under sterile conditions under a dissecting microscope. Hemolymph was collected from the cut ends of tick leg using sterile glass capillary tubes. The minced whole tick or the tick tissues were incubated in PBS containing 64 μg/mL ampicillin for 2 h at room temperature on a rotor platform mixer (Boekel Scientific, Feasterville, PA.), serially diluted in PBS/ampicillin solution and plated on chocolate agar plates. CFUs were determined following incubation at 37°C in 5% CO_2 for 72 h.

Immunohistochemistry and real-time quantitative PCR

For immunohistochemical analysis, both infected and uninfected (ticks CF with meal without *F. tularensis*) ticks were cut longitudinally in half on a median plane and ovaries from gravid females were fixed in Carsons fixative, embedded with paraffin, and sectioned and affixed to glass slides. After deparaffinizing, the sections were incubated with phosphate buffered saline with 0.05% Tween 20 (PBST) at RT for 15 min. and then incubated at 37°C for 1 h with *F. tularensis* antiserum (Beckton Dickinson, Sparks, Maryland) at 1:60 dilution in PBST. Adsorbed antiserum was used as negative control. After washing the slides with PBST five times followed by a final washing with distilled water, the sections were incubated with FITC conjugated secondary antibody in PBST at 37°C for 30 min (KPL, Gaithersburg, Maryland) at 1:60 dilution. The sections were then washed in PBST twice, PBS once and finally washed with distilled water. The slides were dried and visualized using Nikon Eclipse 50i epi-fluorescence microscope and a Nikon digital sight DS-5M-L1 digital camera. For visualizing *F. tularensis* in tick hemolymph, ticks were CF with GFP-expressing *F. tularensis*, hemolymph was collected and placed directly on glass slide with coverslip and visualized using the epi-fluorescent microscope at 1, 2, 3, and 4 weeks PCF. For RT-qPCR reactions a 97 bp product of *F. tularensis* insertion sequence-2 was amplified with the primers ISFtu2F and ISFtu2R (Versage *et al*., 2003). Each sample was analyzed using Fast SYBR green master mix on an AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA.). During each analysis a negative control (no template) was processed and the amplification product was confirmed by analyzing the dissociation curve. RT-PCR reaction (20 µL) had 10µL Fast SYBR green master mix, 6µl DNase RNase free water,

1µL forward primer (ISFtu2F), 1µL reverse primer (ISFtu2R) and 2µL template. Cycling conditions were 95°C for 20 seconds, followed by 34 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Genome equivalents (GE) were calculated based on standard curves obtained by plotting threshold cycle value and different concentrations of *F. tularensis* DNA. The final value for each sample is calculated by multiplying with the dilution factor. The samples used for PCR were total DNA from ticks (tick minceate in 100ul PBS) extracted using DNeasy Tissue Kit (Qiagen, Valencia, CA.), with a final elution volume of 50µL.

Intra-hemocelic injection and saliva induction in ticks

To determine the lowest infectious dose for ticks and to colonize gravid females and partially fed adult ticks, 1µL of the inoculum containing approximately 10^7 CFU/mL of *F. tularensis* ssp. *holarctica* strain LVS in PBS was injected i.h. in the ventral region of the tick, medial to the caudal most coxa using a 10µL custom made Hamilton syringe with a 0.5 inch, 33 gauge needle (Hamilton Company, Reno, NV.). Injection of gravid females was done in the left or right spiracles. For the detection of *F. tularensis* in tick saliva, partially fed adult ticks were injected i.h. with *F. tularensis*, and the ticks were held in a humidity chamber at 27°C. For collecting the saliva, partially fed *F. tularensis* infected adult ticks were immobilized dorsal side up on double sided tape. Ticks were then injected with approximately 4 µL of 1 mM dopamine, 1 mM theophilline and 3% dimethyl sulfoxid in PBS (pH 7.3) (Jaworski *et al.*, 1995) every 15 min. for 1 h (at RT=23°C). Saliva was collected in 10 µL glass capillary tubes (Drummond Scientific

Company, Broomall, PA) placed over the hypostome of the tick. The capillary tube for collecting the saliva was held in place using modeling clay (Fig. 1).

Infective dose 50 in BALB/c mice

To determine the infectivity of *F. tularensis* recovered from ticks, salivary glands from four partially fed adult ticks (infected with *F. tularensis* four days previously via i.h. route) were excised by dissection under sterile conditions and minced in 200 µL PBS containing 64µg/mL ampicillin at four days post injection (PI). The salivary glands were diluted in PBS-ampicillin to make the appropriate inoculum size. Five experimental groups of BALB/c mice (six mice in each group) were injected i.p. with 0.05 CFU, 0.5 CFU, 5 CFU, 71.3 CFU, and 493 CFU respectively. Control group of five mice was injected with uninfected tick salivary gland in PBS-ampicillin.To determine the infective dose 50 of laboratory cultured *F. tularensis*, four experimental groups of BALB/c mice (six mice in each group) were injected i.p. with 1.3 CFU, 12.9 CFU, 64 CFU, and 129 CFU, respectively. Control group of four mice was injected with PBS alone. PI, mice were observed twice daily, and mice showing clinical symptoms (ruffled haircoat, huddling, lethargy, and decreased mobility) were euthanized. The liver and spleen were aseptically removed from the mice, weighed and homogenized. Blood was collected from the heart immediately after ethanization and serial 10-fold dilutions were made and plated on chocolate agar plates. CFUs were counted after 72 h of incubation at 37°C and 5% CO_2. The data from the experiment was used to calculate ID_{50} using the Reed-Muench method (Lennette and Schmidt, 1964).

Statistical analysis

Francisella tularensis colonization in different groups of *D. variabilis* ticks during adult colonization, transstadial transmission from larva to nymphs, and nymph to adult were compared by using 1-way analysis of variance on log-transformed data followed by pairwise multiple comparison of mean CFU values using Holm-Sidak tests. Overall significance level for Holm-Sidak tests was $P = 0.05$. The same method was also used to compare *F. tularensis* tissue colonization of two months PCF adult ticks. Mann-Whitney Rank Sum test was performed to determine the statistical difference in the mean CFU/infected tick between molted adult male and female *D. variabilis*. All statistical analyses were performed with SigmaPlot v11.0 software package (Systat Software Inc., Chicago, IL).

Results

Francisella tularensis colonization of *D. variabilis* larvae and transstadial transmission to nymphs.

Significant difference in the colonization of *F. tularensis* at different days PCF was observed (ANOVA: $F = 7.96$, degrees of freedom (df) = 6, 69, $P < 0.001$). The degree to which larvae took in the capillary fed meal was not ascertainable by weight change in larvae, so only the ticks which were positive for *F. tularensis* was used for the experimental analysis. At one-day PCF, 15.8% of larvae exhibited colonization with a

mean colonization level of 8.8±0.8x10^1 CFU/larva. This % colonization by capillary feeding was the lowest among the various stages of ticks used in this study. This low percent colonization may reflect a technical difficulty for larvae to acquire colonization by *F. tularensis* rather than a biological factor. At our experimental holding conditions (RH=95% and mean room temperature of 20˚C), the larvae molted to adults in 18 days PCF. Post molting, *F. tularensis* CFU/tick increased, reaching 5.2 ± 0.01 x10^4 CFU/tick after five weeks, demonstrating the transstadial transmission of *F. tularensis* (Fig. 2). Significant difference in the mean colonization levels between two weeks and five weeks PCF (unadjusted $P < 0.001$) was observed indicating bacterial multiplication within the ticks after molting. When the ticks were held at 23˚C, mortality of larvae was approximately 50% irrespective of *F. tularensis* colonization, and the number of *F. tularensis* colonized (molted) nymphs was extremely small. However, when the holding temperature was reduced to 20˚C, the percentage mortality was decreased and the number of *F. tularensis* colonized (molted) nymphs was higher (data not shown).

Francisella tularensis **colonization of** ***D. variabilis*** **nymphs and transstadial transmission to adults.**

No significant difference in the colonization of *F. tularensis* at different days PCF was observed (ANOVA: F =1.2, df = 12, 194, P = 0.29). The degree to which nymphs fed was ascertained by comparison of pre-feeding and post-feeding weights. Approximately 30% (>3mg/nymph weight gain post-feeding) of CF ticks were colonized with a mean level of 1.1 ± 0.03 x10^3 CFU/nymph at one day PCF. Considering only those nymphs that ingested the initial inoculum, 100% remained colonized for 14 days, but the

infections declined towards molting to adults at 28 days, at which time 7/15 were negative for *F. tularensis* by culture indicating either the colonization was too low to be detected or these ticks may have cleared the infection. Interestingly, 7/9 nymphs molting at 28 days had cleared *F. tularensis* colonization and only 2/8 colonized nymphs molted by 28 days, suggesting that colonization may prolong the time to molting. A progressive decline in the level of colonization between day 1 PCF and molting at day 28 was seen. Following molting to adults, the percent of molted colonized adults remained relatively constant at 60% for PCF between days 42 to 84, and the mean colonization level was 34 ± 45 at PCF day 28 and changed to $1.02 \pm 0.39 \times 10^4$ CFU/molted adult at day 49 PCF. The level of colonization varied considerably in molted adults and ranged from 10^1 to 10^9 CFU/tick (Fig. 3 A & B). No correlation between weight gain in nymphs after capillary feeding and *F. tularensis* colonization level in nymphs up to 7 days PCF was observed. The majority of the nymphs were colonized with an average value of 10^4 CFU/nymph irrespective of the weight gain (Fig. 4). Correlation between weight gain in nymphs and *F. tularensis* colonization level in molted adult ticks after 2 months PCF was also not observed (Fig. 5). Among the molted adults, the number of female ticks colonized (60%) was more than males (48%), however, the average CFU/tick in both males and females were similar ($P = 0.94$) (Table 1).

Francisella tularensis colonization of _D. variabilis_ adults and lack of transovarial transmission to hatched larvae.

For unfed adults, CF resulted in 57% becoming colonized with *F. tularensis* at 10^2 to 10^4 CFU/tick for 28 days PCF (Fig. 6). Those ticks not colonized appeared to have

not ingested the inoculum, because their weights did not increase after feeding. No significant differences were observed in the overall mean colonization levels for adult male versus female ticks at time up to 28 days PCF and difference in colonization levels in ticks at different days PCF (ANOVA: $F = 2.7$, df = 4, 74, $P = 0.07$) was also not observed. Tick mortality of approximately 10% was observed in both colonized and non-colonized adult ticks (data not shown). In order to examine persistence of colonization, one batch of 30 unfed adults were CF *F. tularensis* and held in a humidity chamber for 182 days PCF. Of these 12/30 survived to 182 days PCF of which only 3 were colonized with 10^3 to 10^6 CFU/tick. The survival rate of adult ticks CF *F. tularensis* was similar to that for ticks CF meal lacking *F. tularensis*.

To test whether transovarial transmission of *F. tularensis* to larvae occurs, adult female *D. variabilis* ticks were allowed to feed to repletion on sheep and subsequently inoculated i.h. with approximately 10^5 CFU/tick. The ticks were held in a humidity chamber until oviposition was completed, and the eggs hatched into larvae. After dissection of the infected gravid female ticks at 14 days PI, *F. tularensis* was detected in hemolymph, gut, Malpighian tubules, and ovaries (Fig. 7); but only 1/11 egg masses were positive for *F. tularensis* by culture or RT-qPCR. However, in a second experiment in which the ambient holding temperature of the colonized female ticks was changed from 23°C to 27°C, 7/8 of the egg masses were colonized at $3.20 \pm 0.02 \times 10^3$ CFU/egg mass and the female ticks post-ovipositing were colonized at 3.98×10^{10} CFU/tick. *Francisella tularensis* was not detected in ova deposited during the initial five days of oviposition, but became positive for *F. tularensis* from around 7 days after the start of egg laying (Table 2). Eggs hatched to larvae between 20 and 30 days post-ovipositing, but

transovarial transmission of *F. tularensis* to larvae was not detected by either culture or RT-qPCR in these larvae. At high levels of colonization, *F. tularensis* infection may interfere with hatching as demonstrated by one egg mass colonized with 10^7 CFU/100 eggs from which no larvae hatched (Table 3). To better understand how ova were colonized, but hatched larvae were not, ova were examined microscopically using immunohistochemical technique for detection of *F. tularensis*. The bacteria were demonstrated in the outer tunica propria and shell of ova, but not in the ova cytoplasm (Fig. 8 A-D). The fecundity of ticks was not affected by *F. tularensis* infection when ticks were held at 23˚C as compared to uninfected ticks. However, the mean egg mass weight of *F. tularensis* from infected ticks was lower (164mg) when compared to uninfected ticks (320mg) when ticks were held at 27˚C (Table 3).

Determination of inoculum dose of *F. tularensis* necessary to establish colonization in adult *D. variabilis* ticks.

As shown in Table 4, an inoculum dose of only 1.5 CFU/tick (n = 5) was sufficient to establish colonization in ticks by day 14 PI compared to higher inoculation doses which established colonization in 100% of the ticks at day one PI. The level of colonization in ticks inoculated with 1.5CFU/ tick was 4.36 ± 13 CFU/tick. Mortality was similar to that of the controls up to 14 days post i.h. for tick inoculated with 10^6 CFU *F. tularensis*/tick (data not shown).

Tissue localization of *F. tularensis* in adult *D. variabilis* ticks.

To determine the tissue dissemination in capillary fed adult *D. variabilis* ticks, ticks were dissected at various times PCF and the level of *F. tularensis* present in gut,

hemolymph, and salivary glands determined. As shown in Fig. 9, the primary site of *F. tularensis* colonization was the tick gut through day 14 PCF. By day 21 PCF, *F. tularensis* colonization extended to the hemolymph and salivary glands. Microscopic examination of these tissues using immunohistochemical staining showed hemocytes in the hemolymph were heavily colonized by *F. tularensis* (Fig. 10 A-D). The bacteria were observed free in hemolymph and also seen colonizing the granulocytes and plasmatocytes. Adult ticks colonized as nymphs exhibited colonization primarily of gut tissue ($8.9x10^5$CFU/tissue/tick) and salivary glands ($1.6x10^3$CFU/tissue/tick) with only low levels of colonization of hemolymph ($1.9x10^1$CFU/tick) at 2 to 3 months PCF (ANOVA: $F = 11.12$, df = 2, 29, $P = < 0.001$) (Fig. 11).

Infectivity of mice from *F. tularensis* colonized in tick salivary glands.

As shown in Fig. 12, dissemination of *F. tularensis* from the hemolymph into gut and the salivary gland occurred within two days PI, and *F. tularensis* was secreted into the saliva of 2/5 ticks after four days PI with a mean level of $1.0 \pm 0.04x10^4$ CFU/µL saliva/tick and 3/5 ticks with a mean level of $1.12 \pm 0.1x10^3$ CFU/µL saliva/tick after 6 days PI respectively. The ID_{50} for *F. tularensis* in tick salivary gland in BALB/c mice by IP injection was 2 CFU as compared to 43 CFU for laboratory cultured *F. tularensis* (Table 4 & 5). Liver, spleen and blood of the euthanized mice at their clinical end point was harvested and mean bacterial counts determined which were $1.8x10^7$CFU/g, $1.6x10^7$CFU/g, and $2.8x10^5$CFU/ml, respectively (Fig. 13 A). Immunostained sections of diseased mice liver and spleen showed that liver hepatocytes and splenic cells were heavily colonized by *F. tularensis* (Fig. 13 B & C).

Discussion

Colonization of both *D. variabilis* larvae and nymphs with *F. tularensis* by CF was demonstrated. The efficiency of CF for partially fed nymphs was 30%, whereas the feeding efficiency of engorged *D. variabilis* larvae and nymphs was less than 10%. The higher percentage of feeding and subsequent colonization of partially fed nymphs as compared to engorged larvae and nymphs could be due to the fact that the partially fed nymphs were not fed to repletion on sheep making them more responsive to CF, whereas the engorged larvae and nymphs were fed to repletion on rabbit and sheep, respectively, making them less accepting of CF. We did not observe any increase in mortality for infected nymphs or larvae. We also observed *F. tularensis* transstadial transmission from larva to nymph as well as from nymph to adult. Although the transstadial route of transmission was reported previously in *D. variabilis* ticks by feeding on *F. tularensis* infected lab animals (Bell, 1945; Philip and Jellison, 1934), this is the first report of this mode of transmission after infecting the tick by CF.

Long term maintenance of *F. tularensis* in *D. variabilis* ticks was also explored to determine whether ticks could maintain infection throughout the overwintering period. The overwintering periods in ticks will vary with latitude, but in eastern and central United States this period may last up to 5-6 months (Burg, 2001; Kollars *et al*., 2000;

Sonenshine and Mather, 1994). The life stages of *D. variabilis* ticks which overwinter in the natural environment are the larvae and the adults (Burg, 2001; Sonenshine, 1972). Molted adults which were infected with *F. tularensis* as partially fed nymphs were shown to maintain *F. tularensis* for 3 months (time at which study was discontinued), while unfed adults infected with *F. tularensis* maintained the colonization for 5.5 months. The high level of *F. tularensis* colonization per molted adults was also reported in naturally infected *D. variabilis* ticks collected from Martha's Vineyard, Massachusetts (Goethert and Telford, 2010). These results suggest that adult *D. variabilis* ticks could maintain the bacteria through the winter period, and therefore may be an important inter-epizootic reservoir for tularemia in its enzootic area. Although we did not hold the ticks under extreme cold conditions simulating natural overwintering period, ticks survivability and *F. tularensis* maintenance was higher at colder temperatures (data not shown). This higher level of infection based on the holding temperature has also been reported by Bell and colleagues (Bell, 1945).

Transovarial transmission of *F. tularensis* in *D. variabilis* ticks was first reported in 1936, but could not be replicated in later experiments (Bell, 1945; Francis, 1927). We have demonstrated temperature dependent transmission of *F. tularensis* to the tick egg shell and the tunica propria, but not to the cytoplasm of the ova which may explain the lack of infection in any of the hatched larvae. Negative results on RT-qPCR also confirmed that the hatched larvae were not infected with *F. tularensis*. The failure of hatching of one of the heavily infected egg masses also suggests that the high number of *F. tularensis* may harm egg development, a phenomenon also seen in *Borrelia* infected ixodid ticks (Burgdorfer *et al*., 1988). Further studies on transovarial transmission in ticks

using recently characterized subpopulations among *F. tularensis* (Petersen and Molins, 2010), by holding the infected gravid females at different temperatures, and by infesting the hatched larvae on ecologically relevant host may shed more light in the matter.

The infective dose of *F. tularensis* for *D. variabilis* by CF method was not determined because it was not practical to determine the exact amount of tick meal taken in by each tick. Even though i.h. is not a natural route of infection, the low infective dose for this route would indicate that these ticks could be susceptible to infection by feeding on infective blood which has been reported to reach high levels of bacteremia (Eisen et al., 2009).

Quantitative determination of *F. tularensis* dissemination in tick tissues suggested that after the initial multiplication of *F. tularensis* in the gut of tick, bacteria disseminated into the hemocel, infecting hemocytes and other tissues including Malpighian tubules, ovary and salivary glands. However, the long term association of *F. tularensis* was primarily in the gut tissues. The current study is the first report of the systematic quantification of *F. tularensis* infection in tick tissues. Edward Francis in 1927 described the qualitative microscopic changes in *D. andersoni* ticks after *F. tularensis* infection and found that *F. tularensis* was not disseminated to the ovary and salivary glands (Francis, 1927), which may be due to differences in tick species or subspecies or subpopulation of *F. tularensis* used in that experiment. Although *F. tularensis* is known to be transmitted via tick bites, this is the first report of *F. tularensis* in the saliva of a tick vector. The chemically-induced saliva secretion technique used herein to demonstrate *F. tularensis* in saliva of *D. variabilis* ticks has been used previously to study physiological components

of tick saliva and also for detection of other tick borne pathogens (Ewing *et al*., 1994; Gage *et al*., 1992; Jaworski *et al*., 1995; Madden *et al*., 2004). Finally, the virulence of *F. tularensis* recovered from the tick salivary gland was also determined by calculating the ID_{50} which was found to be one log less than for laboratory cultured *F. tularensis*. Components of tick saliva have been shown to increase the virulence of various tick pathogens including *F. tularensis* by suppressing host immune response (Bowman *et al.*, 1997; Horka *et al*., 2009; Krocova *et al*., 2003).

The CF method of infecting the ticks is an efficient method of colonizing ticks with their pathogens and is useful in understanding the basic biology and molecular interactions involved in the tick-pathogen interaction. This method was especially useful with *F. tularensis* which is highly pathogenic to laboratory animals used as host for feeding the ticks. The data herein suggests that *D. variabilis* is a biological vector for *F. tularensis*. Bacterial multiplication was clearly demonstrated within these ticks, and the bacterium was transmitted to a susceptible host via bite/saliva of the tick. For ticks to be considered a natural reservoir of a causative agent, the agent has to be maintained in the tick population permanently, and in ticks this means it has to transmit the agent transovarially. Transovarial transmission was not seen in our experiments; however, these studies demonstrated that the adult stage of this tick species was able to carry *F. tularensis* for long periods, thus suggesting that this tick may be an inter-epizootic reservoir in which infected adult ticks survive the winter and initiate another enzootic cycle in the spring. *D. variabilis* adults often feed on large mammals, including dogs and humans (Eisen, 2007; Markowitz *et al*., 1985; Schmid *et al*., 1983; Sonenshine, 1991). Adult infected ticks with high number of bacteria/tick could likely function as a bridging

vector for *F. tularensis*, transmitting *F. tularensis* from the enzootic cycle to incidental hosts. Further research is needed to define the biology of virulent subpopulations of type A and type B *F. tularensis* in *D. variabilis* ticks as well as in other relevant tick vectors.

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Table 1. *Francisella tularensis* colonization in molted adult *D. variabilis*.

D. variabilis ticks	Number of ticks	Number of infected ticks	Percentage of infected ticks	Mean Log* CFU±SD/tick
Male ticks	66	32	48.5%	4.9±2.3
Female ticks	62	37	59.7%	4.9±2.1

*Mean Log CFU ± standard deviation/tick in infected *D. variabilis* ticks.

Table 2. Transmission of *F. tularensis* to *D. variabilis* eggs.

Tick	Inoculum CFUs/gravid tick	CFUs/100 eggs*	CFUs/100 eggs†
1	$3x10^5$	0	$1x10^1$
2	$6x10^5$	0	$1x10^4$
3	$6x10^5$	2	$1x10^3$
4	$3x10^5$	0	$3x10^2$
5	$3x10^5$	0	$6x10^3$
6	$3x10^5$	0	$6x10^4$

*Egg masses oviposited between day 1 to day 7 of oviposition.
†Egg masses oviposited between day 8 to day 15 of oviposition.

Table 3. Comparison of *F. tularensis* infected and uninfected *D. variabilis* ticks held at 23°C and 27°C.

Tick (holding temperature)	Inoculum/gravid adult tick	Weight of tick before ovipositing (mg)	Weight of tick after ovipositing (mg)	Weight of total egg mass (mg)	CFU/mg adult ticks after ovipositing	CFU/100 eggs	CFU/100 larvae
1 (23°C)	$1x10^5$	585	270	265	$5x10^8$	Nil	Nil
2 (23°C)	$1x10^5$	650	374	233	$1x10^8$	Nil	Nil
3 (23°C)	$1x10^5$	449	227	188	$8x10^8$	Nil	Nil
4 (23°C)	Control (PBS)	465	186	238	Nil	Nil	Nil
5 (27°C)	$2x10^4$	766	423	228	$2.4x10^8$	$2.3x10^4$	Nil
6 (27°C)	$2x10^4$	796	505	166	$1.2x10^8$	10^7	Eggs did not hatch
7 (27°C)	$2x10^4$	846	586	100	$3.4x10^5$	0.2	Nil
8 (27°C)	Control (PBS)	No data	No data	266	Nil	Nil	Nil
9 (27°C)	Control (PBS)	No data	No data	375	Nil	Nil	Nil

Table 4. Determination of infectious dose of *F. tularensis* necessary to establish colonization of adult *D. variabilis* ticks.

Days PI	% Colonized per inoculum dose		
	1.5 CFU/tick	12.5 CFU/tick	140 CFU/tick
Day 1	0% (n=5)	40% (n=10)	100% (n=10)
Day 7	0% (n=5)	80% (n=10)	100% (n=10)
Day 14	40% (n=5)	ND*	ND*

* Data not collected

Table 5. ID_{50} of laboratory cultured *F. tularensis* via i.p. inoculation in BALB/c mice.

Group	Inoculum (CFUs of *F. tularensis*	Fraction of dead or sick mice	Percentage of dead/sick mice
A	1.3	0/6	0%
B	12.9	0/6	0%
C	64.3	5/6	83.3%
D	129.0	6/6	100%
Control	Nil*	0/4	0%

* Control group was inoculated with sterile phosphate buffered saline.

Table 6. ID_{50} of *F. tularensis* from *D. variabilis* salivary glands via i.p. inoculation in BALB/c mice.

Group	Inoculum (CFUs of *F. tularensis*	Fraction of dead or sick mice	Percentage of dead/sick mice
A	0.05	0/6	0%
B	0.5	2/6	33.3%
C	5	5/6	83.3%
D	71.3	6/6	100%
E	493	6/6	100%
Control	Nil*	0/5	0%

* Control group was injected with uninfected *D. variabilis* salivary glands.

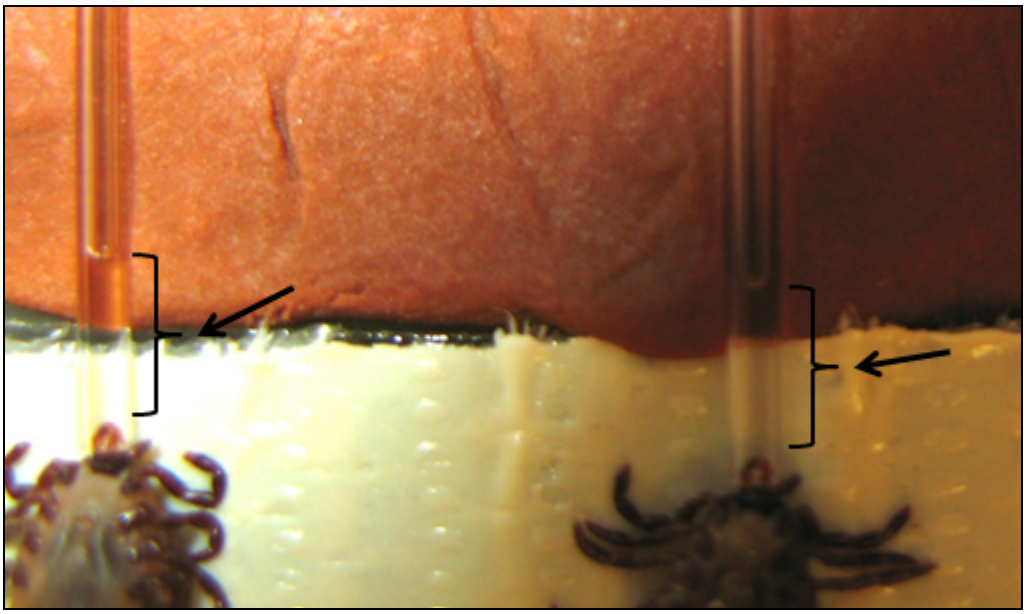


Figure 1. Saliva induction in *D. variabilis* ticks. The black arrows with brackets indicates the saliva collected in the capillary tubes.

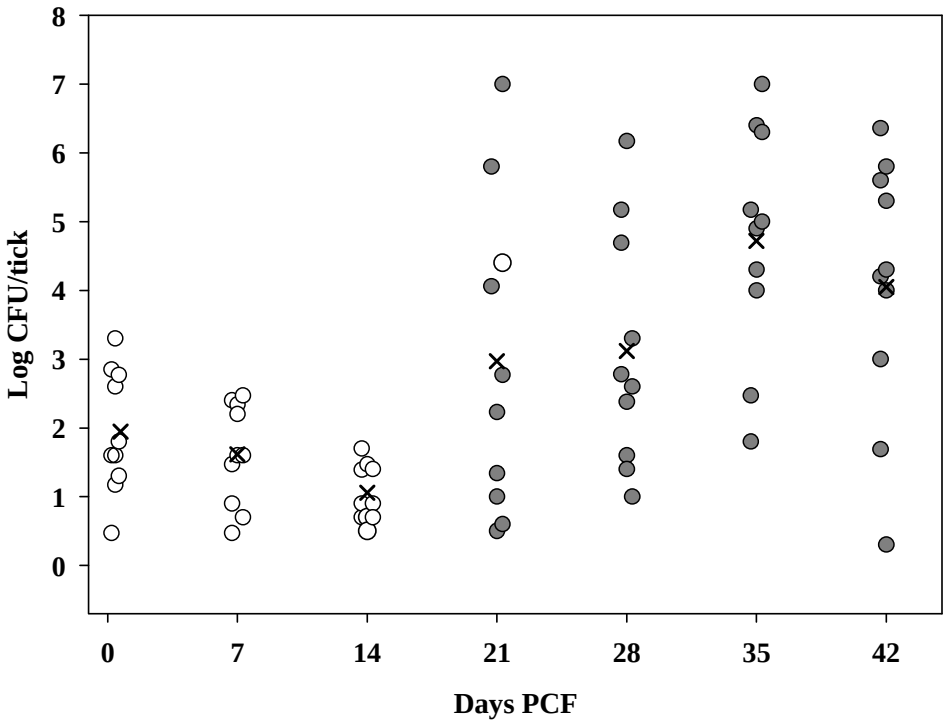


Figure 2. *Francisella tularensis* is transmitted transstadialy from larvae to nymphs. Open circles are infected larvae and filled circles are the molted nymphs. x = mean CFU/tick for each time point. For each time point the n was 10.

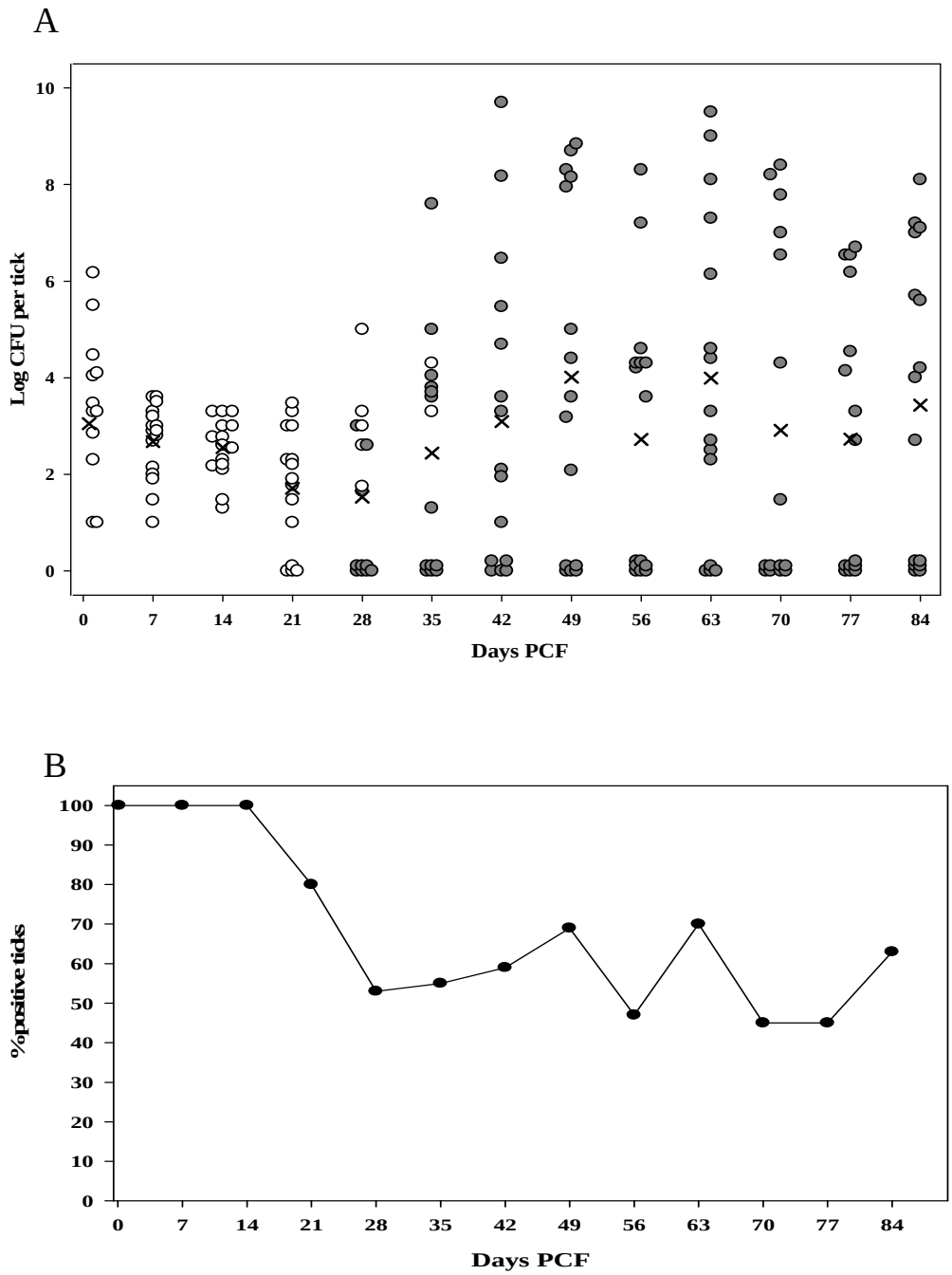


Figure 3. (A) *Francisella tularensis* is transmitted transstadially from nymphs to adults. Open circles are capillary fed nymphs and filled circles are molted adults For each time point the n was 15. x = mean CFU/tick for each time point.

(B) Percentage of infected ticks in the same experiment.

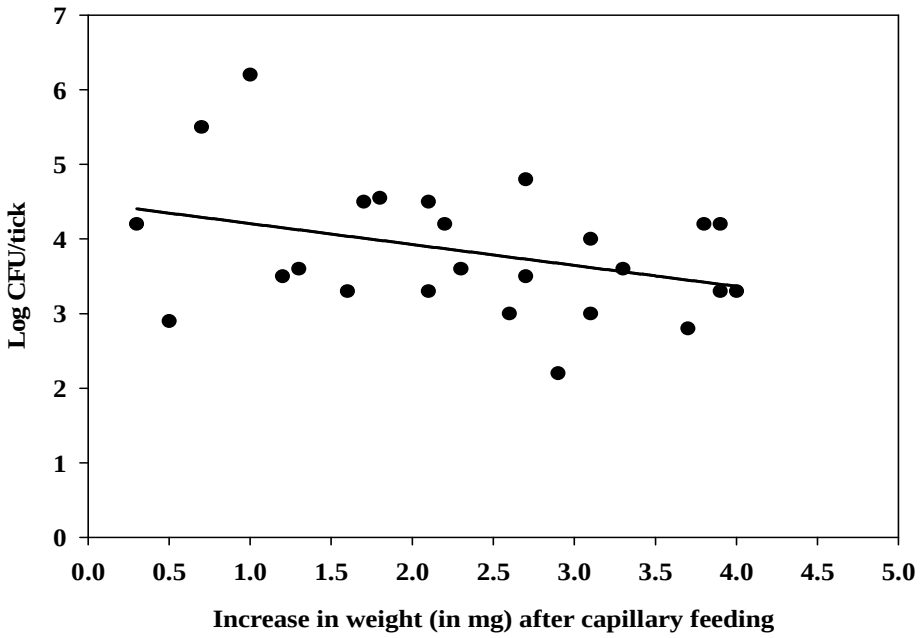


Figure. 4. Correlation between weight gain after capillary feeding of *D. variabilis* nymphs and *F. tularensis* colonization in nymphs up to 7 days PCF. Filled circles represent individual CF nymphs ($R^2 = 0.13$) (n=25).

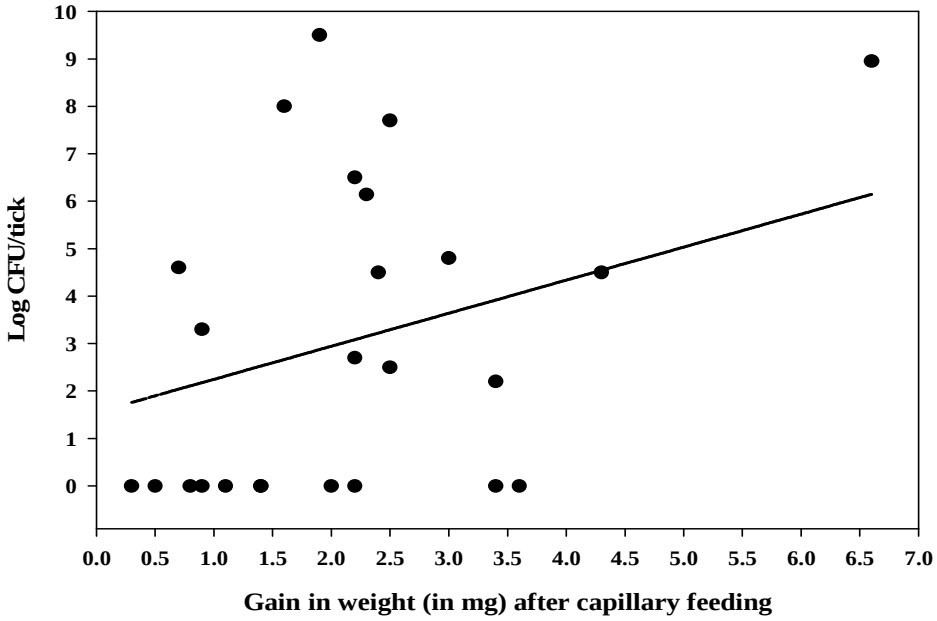


Figure 5. Correlation between weight gain after capillary feeding of *D. variabilis* nymphs and *F. tularensis* colonization in molted adult ticks after 2 months PCF. Filled circles represent individual CF adults. (R^2 = 0.16) (n=25).

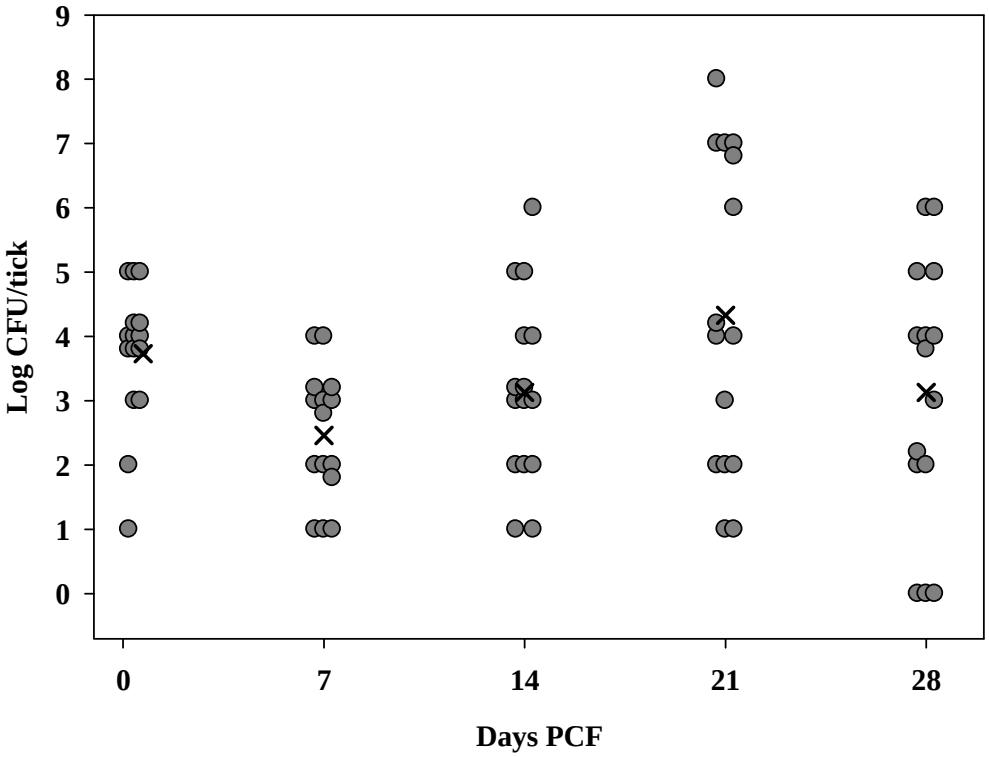


Figure 6. *Francisella tularensis* colonization of adult *D. variabilis* ticks.

The filled circles are capillary fed adults. For each time point the n was 15.

x = mean CFU/tick for each time point.

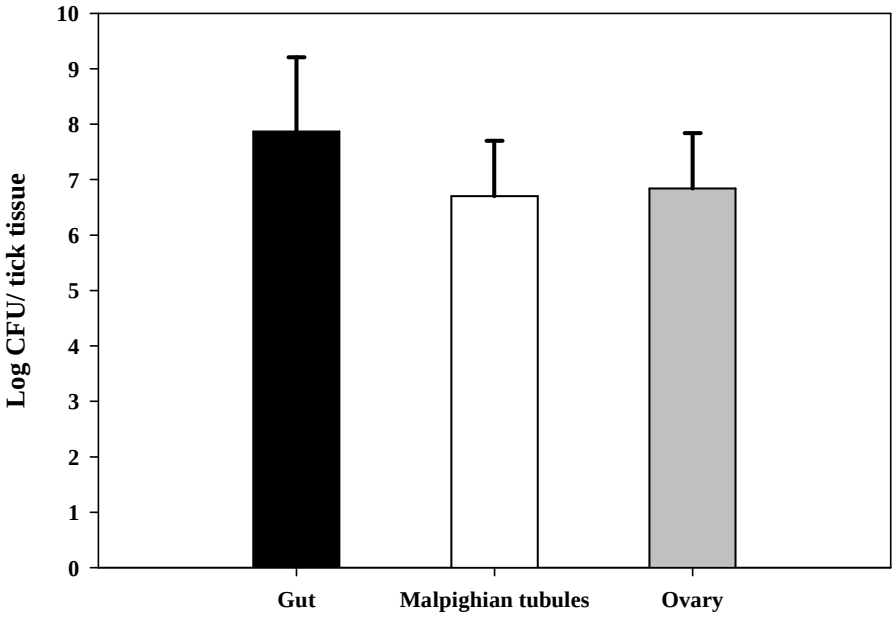


Figure 7. *Francisella tularensis* colonization of gravid female *D. variabilis* ticks PI. For each time point the n was 5. Error bars indicate standard deviation.

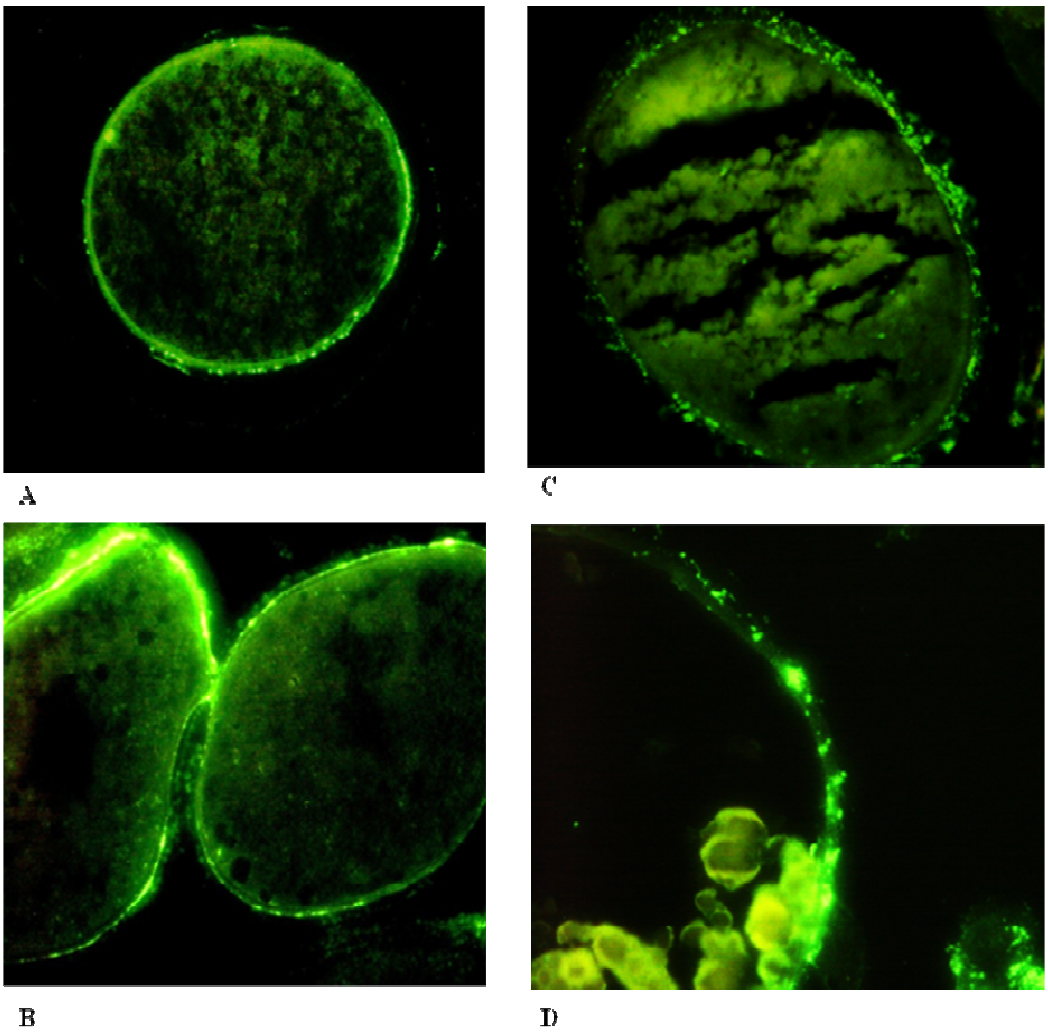


Figure 8. Immunostained sections of infected tick ova. *F. tularensis* colonizing the tunic propria and shell of *D. variabilis* ova. (A & B) 400x magnification. (C&D) 500x magnification.

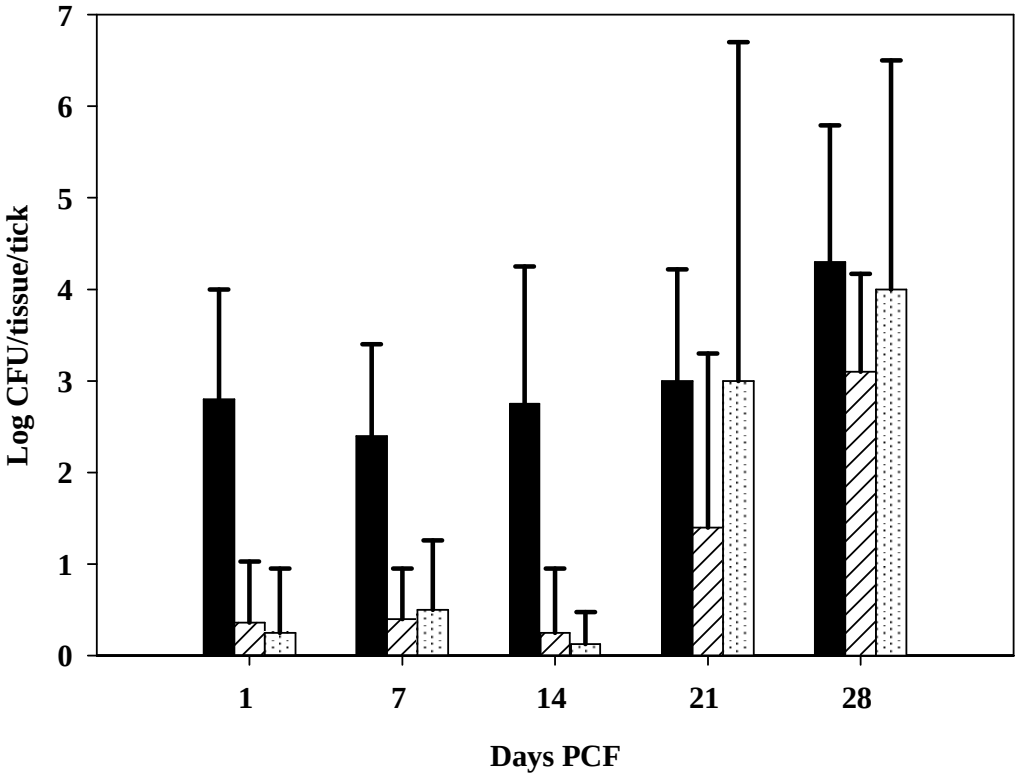


Figure 9. Tissue dissemination of *F. tularensis* in adult *D. variabilis* ticks PCF. Solid black bar- gut, white bar with diagonal lines- salivary gland, white bar with dots- hemolymph. For each time point the n was 5. Error bars indicate standard deviation.

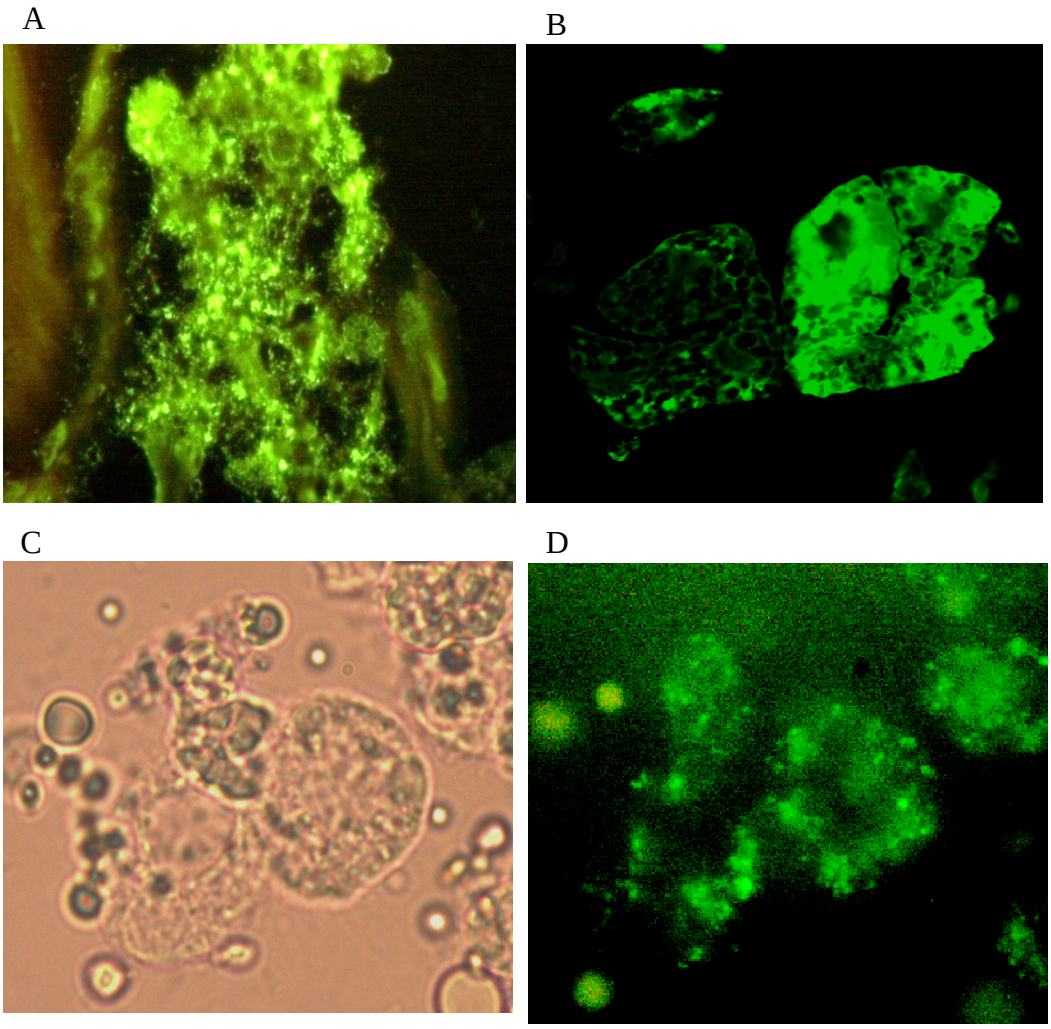


Figure 10. (A) Immunostained sections of infected adult tick gut infected with *F. tularensis*. (B) Immunostained sections of infected adult tick salivary gland acini. (C) Overlapped image of tick hemocytes infected with GFP expressing *F. tularensis*. (D) Fluorescent image of the same tick hemocytes. (400x magnification)

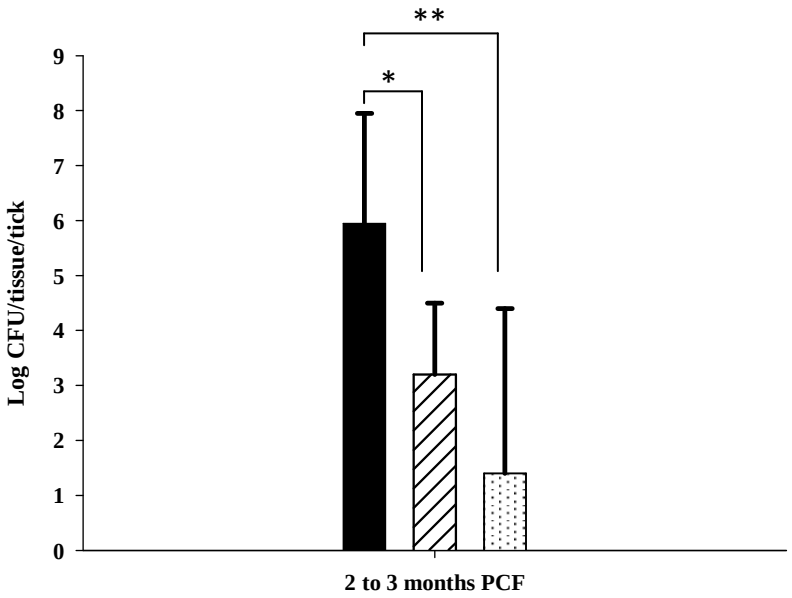


Figure 11. Tissue dissemination of *F. tularensis* in adult *D. variabilis* 2-3 months PCF**.** Solid black bar - gut, white bar with diagonal lines - salivary glands, white bar with dots - hemolymph. For each time point the n was 10. Error bars indicate standard deviation. * Unadjusted $P = 0.008$, ** unadjusted $P < 0.001$.

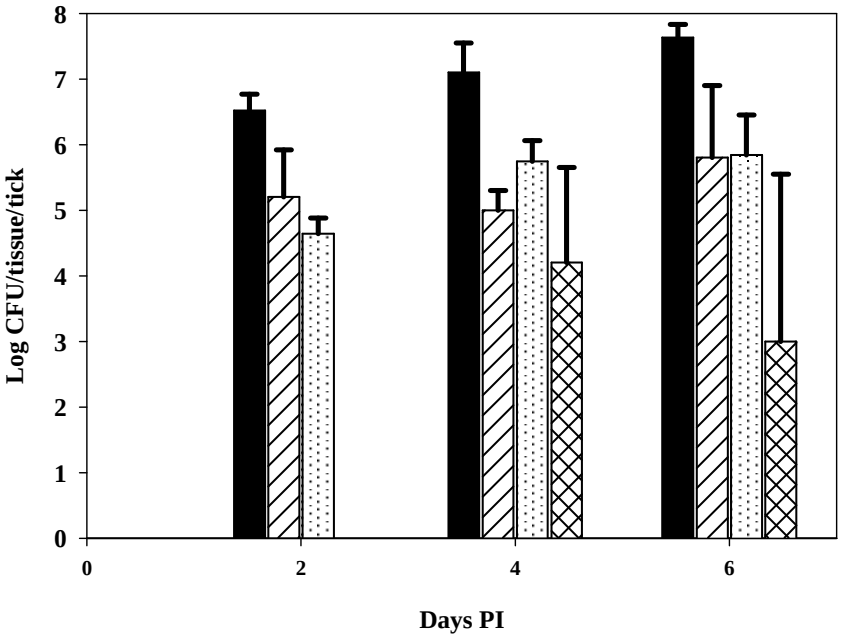
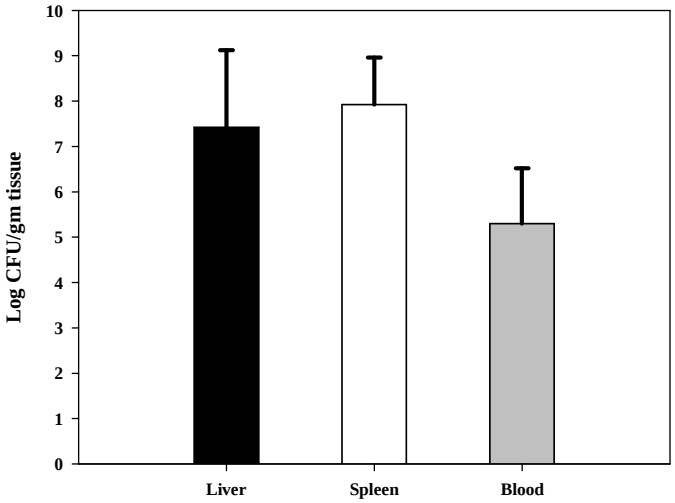


Figure 12. Tissue dissemination of *F. tularensis* in adult *D. variabilis* ticks PI**.** Solid black bar - gut, white bar with diagonal lines - salivary glands, white bar with dots – hemolymph and white bars with cross marks – saliva. For each time point the n was 5. Error bars indicate standard deviation.

A



B C

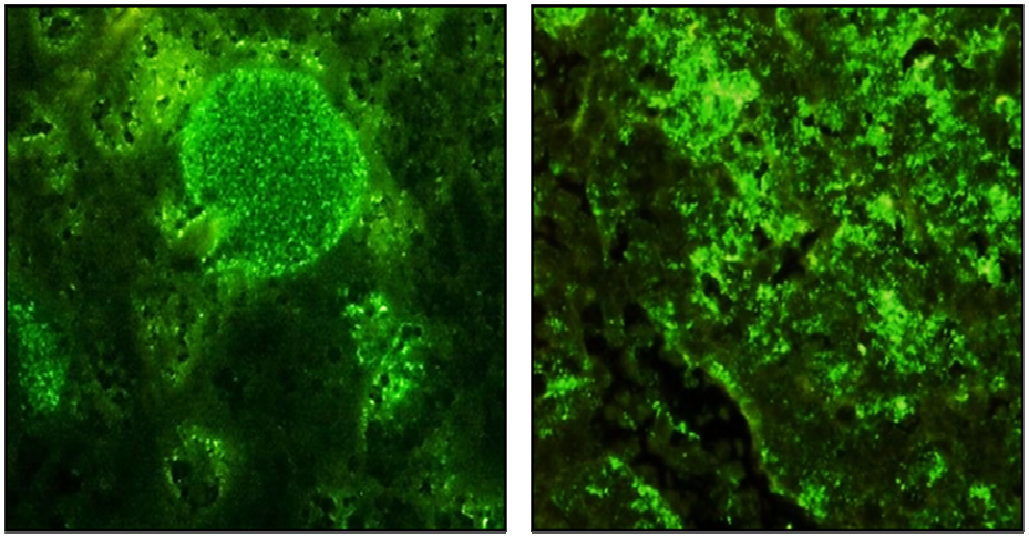


Figure 13. (A) *Francisella tularensis* in infected mice tissues. For each tissue n was 5. Error bars indicate standard deviation. (B) Liver hepatocyte filled with *F. tularensis*. (C) Spleen section filled with *F. tularensis* (immunostained liver and spleen section, 400x magnification).

CHAPTER IV

BIOLOGY OF *FRANCISELLA TULARENSIS* SUBSPECIES *HOLARCTICA* STRAIN LVS IN THE TICK VECTOR *AMBLYOMMA AMERICANUM*

Abstract

Francisella tularensis is the causative agent of tularemia, a tick transmitted disease of rodents and rabbits in its natural habitat and also causes occasional outbreaks of the disease in higher mammals including humans. The current the focus of human tularemia outbreaks in the United States is mainly seen in certain endemic areas of which the major one is located in the south-central United States comprised of Arkansas, Missouri, eastern Oklahoma and southeastern Kansas. All of the factors responsible for the persistence of *F. tularensis* in this highly endemic area are not known. *Amblyomma americanum* is the most abundant tick species in this tularemia endemic region, and it is known to be a vector for *F. tularensis*. The objective of this research is to investigate the persistence, dissemination and stage to stage transmission of *F. tularensis* in the tick vector *A. americanum*. For this study, colony-reared larva, nymph, and adult *A. americanum* ticks were artificially capillary fed (CF) a tick meal containing approximately 10^7 CFU/mL *F. tularensis* via fine bore capillaries tubes fitted over the tick mouthparts. After the feeding period the level of colonization and tick tissue distribution were determined. CF larva and nymph were initially colonized with $1.5x10^4$ and $1.3\pm0.01x10^4$ CFU/tick, respectively. For larval ticks colonization of *F. tularensis* declined to less than 10^2 CFU per tick at molting. After molting *F. tularensis* colonization per tick continued at approximately 10^2 CFU/nymph and then at two months post-CF (PCF) increased to $1x10^5$ CFU/ nymph. *Francisella tularensis* persisted in 50% of molted nymphs after 168 days PCF with $1.0\pm1.9x10^3$ CFU/nymph. For nymphal ticks PCF, *F.*

tularensis colonization continued to decline after molting, and *F. tularensis* was recovered from only one adult tick out of 25 live ticks at 84 days PCF. In the CF adult ticks, *F. tularensis* initially colonized the gut and disseminated to hemolymph and salivary glands by 24 h PCF. When *F. tularensis* was introduced via intra-hemocelic injection (i.h.) in adult ticks, a minimum of one CFU per tick was required to establish colonization, and *F. tularensis* was detected in saliva after 48 h post i.h. injection. Injection of *F. tularensis* via i.h. route into gravid females resulted in recovery of bacteria from the ovaries. However, transmission of *F. tularensis* to eggs was infrequent and the level of colonization was low. Transovarial transmission to hatched larvae was not observed. This study demonstrated that *A. americanum* is an efficient experimental vector for *F. tularensis*, and the long term persistence of *F. tularensis* in *A. americanum* nymphs suggests that infected nymphs could potentially overwinter and carryover the infection to the following spring in the endemic area.

Introduction

The epidemiology of human tularemia in the United Sates has changed in terms of both the transmission route as well as the geographical distribution in the last 100 years (Eisen, 2007; Hopla, 1974; Jellison, 1974). In the first half of the twentieth century tularemia was mainly seen as a disease of rabbit hunters or in people who came in contact

with infected rabbits by other means. The prevalence of tularemia was high in eastern and central States including Virginia, Tennessee, Georgia, Kentucky, Ohio, Mississippi and Louisiana (Jellison, 1974). The current focus of human tularemia is mainly in the south-central United States comprised of Arkansas, Missouri, eastern Oklahoma, and the southeastern Kansas. The primary mode of transmission of human *F. tularensis* in this region is by tick vectors rather than through contact with infected rabbits (Eisen, 2007).

A number of ixodid and argasid ticks have been shown to transmit *F. tularensis*, and stage to stage transmission has also been reported. However, demonstration of experimental transovarial transmission of *F. tularensis* in these ticks has been inconclusive (Bell, 1945; Francis, 1927; Parker and Spencer, 1926; Petrov, 1960; Philip and Jellison, 1934). Among the common tick species seen in this south-central tularemia endemic region, *A. americanum* is by far the most abundant (Brown *et al*., 2011; Eisen, 2007; Hopla, 1960). *Amblyomma americanum* is a three-host tick with the larval stage feeding on small rodents and rabbits, whereas nymphs and adults feed on larger mammals mostly deer, raccoon, and fox (Koch, 1984; Kollars *et al*., 2000; Patrick and Hair, 1977). *Amblyomma americanum* ticks are not strictly host specific at any stage of its life cycle. All three stages of the tick can feed on larger mammals including humans (Hopla, 1960). *Amblyomma americanum* ticks have been found naturally infected with *F. tularensis* in the south-central tularemia endemic region (Calhoun, 1954; Hopla and Downs, 1953). The reports of infected larvae in nature also raise the possibility of transovarial transmission in these ticks (Calhoun and Alford, 1955). C. E. Hopla studied experimental transmission of *F. tularensis* in *A. americanum* and reported that *F. tularensis* can be transstadially transmitted in *A. americanum* from larvae to nymph and from nymph to

adult. In addition *F. tularensis* were shown to persist in the ticks after six months of starvation (Hopla, 1953; Hopla, 1955).

Francisella tularensis has four subspecies of which type A (ssp. *tularensis*) and type B (ssp. *holarctica*) are most frequently associated with tularemia outbreaks in the United States. *Francisella tularensis* ssp. *holarctica* is moderately virulent and is often associated with water-borne tularemia outbreaks, while *F. tularensis* ssp. *tularensis* is highly virulent and follows a terrestrial transmission. The major *F. tularensis* spp. seen in the south-central tularemia endemic region is ssp. *tularensis*. With the recent findings of several subpopulations within *F. tularensis* subspecies and their possible different relationship with various hosts and vectors along with recent improvements in molecular level studies of *F. tularensis*, provide a favorable research environment to study tick vector-*F. tularensis* interactions at a molecular level. (Staples *et al.*, 2006; Bina *et al*., 2010; Rodriguez *et al*., 2009). In view of this a model for colonizing *F. tularensis* ssp. *holarctica* stain LVS (live vaccine strain) in *A. americanum* ticks was developed to characterize the biology of the bacteria in the tick vector.

The aim of this study was to systematically characterize the biology of CF *A. americanum* ticks and to determine the vector capacity of this tick to transmit *F. tularensis*. We conclude that *A. americanum* nymphs are able to maintain *F. tularensis* for longer time and this stage in the life cycle of the tick may play a role in the transferring the infection from one year to the next and help in maintaining the enzootic cycle in the nature. The capillary feeding model of colonizing ticks with *F. tularensis*

appear to be an excellent platform to study the molecular level interactions of *F. tularensis* and the tick vectors.

Materials and Methods

Ticks, bacterial strain and growth conditions

Amblyomma americanum larvae, nymphs, and adults were obtained from the Tick Rearing Facility, Department of Entomology and Plant Pathology, Oklahoma State University. Larvae were collected following feeding to repletion on rabbits. Nymphs used were partially fed on sheep to approximately 4.5 mg/nymph. Adult ticks used were unfed (flat adults) and those used for saliva induction experiments were allowed to feed on sheep for five to six days (partially fed adults). Females ticks used for the transovarial transmission studies were fed to repletion. Flat adult and partially fed nymphs were weighed before and after CF to assess the CF success and only those with 0.4 mg weight gain were used for the experiments.

Francisella tularensis ssp. *holarctica* strain LVS (ATCC 29684) was supplied by the Oklahoma State Department of Health. Green fluorescent protein (GFP) expressing pFNLTP6 *gro-gfp* plasmid (Maier *et al.*, 2004) was a gift by Thomas C. Zhart (Medical College Wisconsin, Milwaukee, Wisconsin), and electroporated into *F. tularensis*. For making electrocompetent *F. tularensis*, Mueller-Hinton broth (Becton Dickinson,

Cockeysville, MD, USA) cultures supplemented with 2% IsoVitaleX (Becton Dickinson) were grown to early-log phase (optical density at 550 nm, 0.5 to 0.6 or $1x10^{10}$ CFU/mL), washed two times with 0.5 M sucrose, and suspended in 1 ml of 0.5 M sucrose. For electroporation, 1 µl of plasmid DNA (100 µg/ml) was mixed with 200 µL of electrocompetent cells, incubated at room temperature for 10 min and electroporated using a MicroPulser electroporation apparatus (BioRad, Richmond, California) at 2.9 kV, 25 µF capacitance and 600 Ω resistance for 5.3 msec. After electroporation, the cells were suspended in 1mL of Mueller-Hinton broth supplemented with 2% IsoVitaleX and incubated at 37°C for 4 h. Transformed colonies were selected by plating on brain-heart infusion agar (Difco, BD Diagnostic Systems, Sparks, MD) supplemented with 1% hemoglobin and ampicillin (60 µg/mL) and kanamycin (25 µg/mL) for selection. The transformation efficiency was $1.3x10^3$ transformants/µg of plasmid DNA. The transformed *F. tularensis* was used to infect ticks and to visualize *F. tularensis* in tick hemolymph. *Francisella tularensis* was grown on chocolate agar plates (Hardy Diagnostics, Santa Monica, CA.) at 37°C in 5% CO_2 for 72 h. The BBL Prompt Inoculation System (BD Diagnostics, Franklin Lakes, NJ) was used to prepare *F. tularensis* inocula. All chemicals used in the study were purchased from Sigma (St. Louise, MO) unless indicated otherwise.

Capillary feeding of larvae, nymphs and adult ticks

The ticks were surface disinfected by washing in 30% hydrogen peroxide, distilled water and 70% isopropyl alcohol for 5 seconds each. After washing, the adult ticks were immobilized dorsal side up on double sided tape in a 100 mm x 15 mm Petri

dish base. The ticks were then further immobilized by applying single sided tape over $1/4^{th}$ of their caudal portion (Fig. 14 A). The larvae were immobilized with their dorsal side down on a double-sided tape on a dental pad, and the nymphs were similarly placed on pipette tip box cover cut at one end (Fig. 14 B & C). For CF, 10 µL (internal diameter of 0.0219 inch), 9 µL (internal diameter of 0.0189 inch) and 35 µL (internal diameter of 0.0314 inch) glass capillary tubes (Drummond Scientific Company, Broomall, PA) were used for larvae, nymphs and the adult ticks, respectively. The ends of the tubes were positioned over the hypostome of the tick while the other end rested on the edge of the Petri dish attached with a double sided tape (Broadwater *et al*., 2002). The feeding media for larvae, nymphs, and adult ticks was Minimum Essential Media (MEM) (GIBCO Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT.). The feeding medium was spiked with *F. tularensis* at approximately 10^7 CFU/ ml. The tick meal was then introduced into the capillary tubes, and the ticks were allowed to feed for 12 to 24 hours at 30°C and 90% relative humidity. After feeding, the ticks were either surface disinfected by washing as above and minced for determinations of CFUs or were maintained in microcentrifuge tubes capped with moistened cotton plugs for varying periods of time in a humidity chamber (relative humidity of > 90%) at 23°C (unless specified otherwise) with automated artificial lighting to simulate a 12 h day night cycle. To determine the bacterial number in tick gut, salivary glands, and ovaries, these tissues were dissected under sterile conditions using a dissecting microscope. Hemolymph was collected from the cut ends of a tick leg using sterile glass capillary tubes. The minced whole tick or the tick tissues were incubated in PBS containing 64 µg/mL ampicillin for 2 h at room temperature on a rotor platform mixer (Boekel Scientific, Feasterville, PA.),

serially diluted in PBS/ampicillin solution and plated on chocolate agar plates. CFUs were determined following incubation at 37°C in 5% CO_2 for 72 h.

Immunohistochemistry and real-time quantitative PCR

For immunohistochemical analysis, both infected and uninfected ticks were cut longitudinally in half and ovaries from gravid females were fixed in Carsons fixative, embedded with paraffin, and sectioned and affixed to glass slides. After deparaffinizing, the sections were incubated with phosphate buffered saline with 0.05% Tween 20 (PBST) at RT for 15 min. and then incubated at 37 °C for 1 h with *F. tularensis* antiserum (Beckton Dickinson, Sparks, Maryland) at 1:60 dilution in PBST. Adsorbed antiserum was used as negative control. After washing the slides with PBST five times followed by a final washing with distilled water, the sections were incubated with FITC conjugated secondary antibody in PBST at 37 °C for 30 min (KPL, Gaithersburg, Maryland) at 1:60 dilution. The sections were then washed in PBST twice, PBS once and finally washed with distilled water. The slides were dried and visualized using a Nikon Eclipse 50i epi-fluorescence microscope and Nikon digital sight DS-5M-L1 digital camera. For visualizing the bacteria in tick hemolymph, ticks were CF with GFP expressing *F. tularensis*, hemolymph was collected and placed directly on glass slide with coverslip, and visualized using the epi-fluorescent microscope 1 and 4 weeks PCF. For RT-qPCR reactions a 97 bp product of *F. tularensis* insertion sequence-2 was amplified with the primers ISFtu2F and ISFtu2R (Versage *et al.*, 2003). Each sample was analyzed using Fast SYBR green master mix on an AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA.). During each analysis a negative control (no template) was

processed and the amplification product was confirmed by analyzing the dissociation curve. RT-PCR reaction (20 µL) - 10µL Fast SYBR green master mix, 6µl DNase RNase free water, 1µL forward primer (ISFtu2F), 1µL reverse primer(ISFtu2R) and 2µL template. Cycling conditions were 95 °C for 20 seconds, followed by 34 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Genome equivalents (GE) were calculated based on standard curves obtained by plotting threshold cycle value and different concentrations of *F. tularensis* DNA. The final value for each sample is calculated by multiplying with the dilution factor. The sample used for PCR was total DNA from tick (tick minceate in 100ul PBS) extracted using DNeasy Tissue Kit (Qiagen, Valencia, CA.), with a final elution volume of 50µL.

Intra-hemocelic injection and saliva induction in ticks

To determine the lowest infectious dose for ticks and to colonize gravid females and partially fed adult ticks, 1µL of the inoculum containing 10^7 CFU/mL of *F. tularensis* in PBS or appropriate dilutions were injected i.h. in the ventral region of the tick, medial to the caudal most coxa using a 10µL custom made Hamilton syringe with a 0.5 inch, 33 gauge needle (Hamilton Company, Reno, NV.). Injection of gravid females was done in the left or right spiracles. For the detection of *F. tularensis* in tick saliva, partially fed adult ticks were injected i.h. with *F. tularensis*, and the ticks were held in a humidity chamber at 25°C. For collecting the saliva, partially fed *F. tularensis* infected adult ticks were immobilized dorsal side up on double sided tape. Ticks were then injected with approximately 4 µL of 1 mM dopamine, 1 mM theophilline and 3% dimethyl sulfoxide in PBS (pH 7.3) (22) every 15 min. for 1 h (at RT= 23°C). Saliva was

collected in 10 µL glass capillary tubes (Drummond Scientific Company, Broomall, PA) placed over the hypostome of the tick. The capillary tube for collecting the saliva was held in place using modeling clay.

Statistical analysis

Francisella tularensis colonization in different groups of *A. americanum* ticks during adult colonization, transstadial transmission from larva to nymphs, and nymph to adult were compared by using 1-way analysis of variance on log-transformed data followed by pairwise multiple comparison of mean CFU value using Holm-Sidak tests. Overall significance level for Holm-Sidak tests was $P = 0.05$. Student's t-test was performed to determine the statistical difference in the mean CFU/infected tick between molted adult male and female *A. americanum*. All statistical analyses were performed with SigmaPlot v11.0 software package (Systat Software Inc., Chicago, IL).

Results

Francisella tularensis colonization of *A. americanum* larvae and transstadial transmission to nymphs.

Significant difference in the colonization of *F. tularensis* at different days PCF (ANOVA: $F = 4.1$, degrees of freedom (df) = 14, 224, $P < 0.001$) was observed. The degree to which larvae took in the capillary fed meal was not ascertainable by weight change in larvae, but at one-day PCF, 100% of larvae exhibited colonization with a mean

colonization level of $1.5x10^4$ CFU/larva (Fig. 15 A & B). This high efficiency of capillary feeding was better than the nymphal or the adult tick feeding. At our experimental holding conditions (RH=95% and mean room temperature of 20˚C), the larvae molted to nymphs in 21 days PCF. Around the time of molting the bacterial count declined to < 50 CFU ($2.5\pm1.8x10^1$)/tick (Fig. 15 A). *Francisella tularensis* colonization of molted nymphs were at approximately 10^2 to 10^3 CFU/nymph for up to 70 days PCF and then increased in level to reach $1x10^5$ CFU/nymph at 84 days PCF, and colonization was maintained for 168 days PCF, which was the longest time period in the study (Fig. 15 A). Significant difference in the mean colonization levels between three weeks and 12 weeks PCF (unadjusted $P < 0.001$) was observed. The percentage of colonization declined from 100% at one day PCF to reach 50% colonized nymphs by 50 days PCF. The number of *F. tularensis* colonized nymphs increased to 80-90% by 77 and 84 days PCF (Fig. 15 B), which indicate that some of the nymphs that seemed to clear the infection during days 21 to 70 may still be colonized with extremely low number of bacteria which may be below the sensitivity of the colony plating assay.

Francisella tularensis **colonization of *A. americanum* nymphs and transstadial transmission to adults.**

Significant difference in the colonization of *F. tularensis* at different days PCF (ANOVA: $F = 7.07$, df = 12, 194, $P < 0.001$) was observed. The degree to which nymphs fed was ascertained by comparison of pre-feeding and post-feeding weights in fed nymph which increased in approximately 65% (>3mg/nymph weight gain post-feeding) of ticks. Nymphs were colonized with a mean level of $1.3\pm0.01x10^4$ CFU/nymph (Fig. 16).

Considering only those nymphs that ingested the initial inoculum, 100% remained colonized for 14 days, but the infections continued to decline toward molting to adults at 35 days, at which time 7/15 were negative for *F. tularensis* by culture. However the remaining 8 molted ticks were still infected demonstrating the transstadial transmission of *F. tularensis* from nymphs to adults in *A. americanum* ticks (Fig. 16). The progressive decline in the level of colonization continued and by 84 days PCF only 1/15 tick was still infected with *F. tularensis* (Fig. 16). No correlation between weight gain in nymphs after capillary feeding and *F. tularensis* colonization in nymphs up to 7 days PCF was observed (Fig. 17). Correlation between weight gain in nymphs after capillary feeding and *F. tularensis* colonization in molted adult ticks after two months PCF was also not observed (Fig. 18). Difference in the number of *F. tularensis* colonized male and female ticks was not observed; however, the average level of coonization in female ticks (6.3±2.4 Log CFU/tick) was more than that for males (3.6±1.6 Log CFU/tick) (P = 0.007) (Table 7).

Francisella tularensis colonization of _A. americanum_ adults and lack of transovarial transmission to hatched larvae.

For unfed adults, CF resulted in 60% becoming colonized with *F. tularensis* at 10^2 to 10^4 CFU/tick for 28 days PCF (Fig. 19). Significant difference in the colonization of *F. tularensis* at different days PCF (ANOVA: $F = 5.7$, df = 4, 54, $P < 0.001$) and between one day PCF and three weeks PCF (unadjusted $P < 0.001$) was observed. Those ticks not colonized appeared to have not ingested the inoculum, because their weights did not increase after CF. Significant differences were not observed in either mean

colonization levels for adult male and female ticks at time up to 28 days PCF and tick mortality of approximately10% was observed for both colonized and non-colonized adult ticks (data not shown).

After dissection of some of the infected gravid ticks at 14 days PI, *F. tularensis* was detected in hemolymph, gut, Malpighian tubules, and ovaries (data not shown); but only 3/11 egg masses were positive for *F. tularensis* by culture or PCR. Of the 3 positive egg masses the number of *F. tularensis* counted were 45, 8.3, and 0.4 CFU (values normalized to CFUs/100eggs). In a second experiment in which the ambient holding temperature of the colonized female ticks was changed from 23˚C to 27˚C, 2/7 of the egg masses were positive for *F. tularensis* by culture or PCR. Of the 2 positive egg masses the number of *F. tularensis* detected was 12.5 and 0.5 CFU (values normalized to CFUs/100eggs). Eggs hatched to larvae between 30 and 40 days post-ovipositing, but transovarial transmission of *F. tularensis* to larvae was not detected by either microbial culture or PCR in these larvae. The fecundity of ticks was not affected by *F. tularensis* infection when ticks were held at 23˚C as compared to uninfected ticks (Table 8). However the fecundity of *F. tularensis* infected ticks was decreased ($P = 0.04$, n=3) when held at 27˚C (mean egg mass weight = 193mg) as compared to at 23˚C (mean egg mass weight = 345mg).This decrease in fecundity may have resulted from higher holding temperature rather than due to *F. tularensis* infection as one of the uninfected ticks also had low egg mass weight when held at 27˚C (Table 8).

Determination of infectious dose of *F. tularensis* necessary to establish colonization in adult *A. americanum* ticks.

As shown in Table 9, an inoculum dose of only 1.5 CFU/tick was sufficient to establish colonization in some ticks by day 14 PI compared to higher inoculation doses which established colonization in 100% of the ticks at day one PI. The level of colonization in ticks inoculated with 1.5 CFU/ tick was 10 ± 26 CFU/tick. Mortality of inoculated ticks was similar to that of the controls up to 14 days PI for ticks injected with 10^6 CFU *F. tularensis*/tick (data not shown).

Tissue localization of *F. tularensis* in adult *A. americanum* ticks and presence of *F. tularensis* in *A. americanum* saliva

As shown in Fig. 20, *F. tularensis* penetrated the gut and disseminated to the hemolymph and salivary glands within 24 hour of CF. Microscopic examination of these tissues using immunohistochemical staining showed hemocytes in the hemolymph were heavily colonized by *F. tularensis*, and the bacteria were also observed in hemolymph, granulocytes and plasmatocytes. For the detection of *F. tularensis* in tick saliva partially fed *A. americanum* females were injected with *F. tularensis* via the i.h. route. Two days PI, *F. tularensis* could be detected in the saliva of 4/5 ticks with a mean value of $1.0\pm0.1x10^3$CFU/µl of saliva (Fig. 21).

Discussion

The persistence of tick borne type A *F. tularensis* in the south-central United States is perpetuated by a tick-small mammalian host enzootic cycle of *F. tularensis* in the region (Hopla, 1960; Petersen *et al*., 2009). The most abundant tick species found in this region is *A. americanum* and consistently accounted for more than 90 % of the total ticks in a number of tick surveys done in this region (Brown *et al.*, 2011; Calhoun, 1954; Calhoun and Alford, 1955; Hopla, 1960). Adult *A. americanum* start host-seeking activity in April which peaks during May and July. Nymphal and larval questing activity starts slightly earlier in the spring and can continue through September and October (Kollars *et al*., 2000). Although all three stages have been shown to overwinter in the south-central region, adults and nymphs have been known to overwinter in large numbers (Hopla, 1960; Kollars *et al*., 2000). All the three stages are to known to parasitize humans, and as reported from history of tularemia outbreaks, the incidence of human tularemia in this endemic region coincides with peak tick questing activity in May, June, and July with 60 to 70% of the cases reported having a history of tick bites (Assal *et al*., 1968; Eisen, 2007).

The results reported in the current study indicates that *F. tularensis* can maintain colonization in all three stages of *A. americanum*. The adult ticks cleared *F. tularensis* in three months PCF. This finding differs from that of an earlier report, in which *F. tularensis* maintained colonization in adult *A. americanum* ticks for up to six months post infection (Hopla, 1953). The reason for this difference is not clear, but one difference

between the two studies is that we colonized the *A. americanum* ticks with LVS strain (type B) of *F. tularensis*, whereas the earlier study reports of using the virulent *F. tularensis* (type A). Another factor which also may have influenced the result is the holding temperature of the ticks. Tick immune system can clear infections by bacteria that it encounters, and previous reports also indicate that at higher temperatures the tick immune system and the phagocytic activity of hemocyte are more active (Bell, 1945; Goodman *et al*., 2005; Johns *et al*., 2000; Sonenshine, 1991).

We were able to colonize *A. americanum* larvae and nymphs by CF with an efficiency of feeding of 100% and 70%, respectively. The infected larvae which molted to nymphs were able to maintain the *F. tularensis* colonization for 168 day PCF, which was the longest time point in the study. In the natural habitat the nymphs are known to overwinter in large numbers, and this stage is also known to parasitize on larger mammals including humans (Eisen, 2007; Hopla, 1960; Kollars *et al*., 2000). Based on this information it can be inferred that the nymphal stage of *A. americanum* can be an inter-epizootic reservoir of *F. tularensis* carrying over the infection from one year to the next and a potential bridging vector transmitting the bacterium to incidental hosts

We also demonstrated the vector competency of *A. americanum* for *F. tularensis*. The bacteria penetrated the gut and reached the salivary glands within 24 hours post CF. The extrinsic incubation period, demonstrated by the presence of *F. tularensis* in the saliva of the infected *A. americanum* adult ticks was found to be as low as 48 hours post-injection via the i.h. route. This is the first report of presence of *F. tularensis* in saliva of *A. americanum* ticks. The chemically-induced salivation method, although used for the

first time to detect *F. tularensis* in tick saliva, has been used previously for detection of other pathogens (Ewing *et al.,* 1994; Gage *et al.,* 1992). We have also shown that an inoculum dose of approximately 1 CFU *F. tularensis*/tick is required for colonizing *A. americanum* adults via the i.h. route.

All three stages of *A. americanum* including adults, nymphs, and larvae have been found naturally infected with *F. tularensis* in nature (Calhoun, 1954; Calhoun and Alford, 1955). The presence of *F. tularensis* in unfed larvae raised the possibility of transovarial route of transmission in this tick species. We examined the possible transovarial transmission of *F. tularensis* in *A. americanum* and found that occasional transmission to eggs could occur although with much less numbers of *F. tularensis* per egg masses. However, the transmission of *F. tularensis* from eggs to the freshly hatched larvae could not be detected. The lack of finding of large number of infected larvae in nature also leads to the conclusion reached by previous researchers that transovarial transmission of *F. tularensis* in ticks is the exception rather than the rule in nature (Bell, 1945; Hopla, 1974).

We have demonstrated a highly effective capillary feeding method for infecting all three stages of *A. americanum* with *F. tularensis*. This method enables one to infect ticks with a uniform dose of inoculum and avoids the use of lab animals as host for feeding ticks with *F. tularensis*. Based on the observation of high abundance of nymphal and adult ticks in the south-central tularemia endemic region and associated tularemia risk, and based on the findings reported here it can be concluded that these tick stages are

efficient vectors of *F. tularensis* in this region and the nymphal stage can potentially serve as inter-epizootic reservoir of *F. tularensis*.

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Table 7. *Francisella tularensis* colonization in molted adult *A. americanum*.

A. americanum ticks	Number of ticks	Number of infected ticks	Percentage of infected ticks	Avg Log* CFU±SD/tick
Male ticks	60	8	13.3%	3.6±1.6
Female ticks	108	15	13.9%	6.3±2.4

*Mean Log CFU ± standard deviation/tick in infected *A. americanum* ticks.

Table 8. Comparison of *F. tularensis* infected *A. americanum* ticks held at 23°C and 27°C.

Tick (holding temperature)	Inoculum/gravid adult tick	Weight of tick before ovipositing (mg)	Weight of tick after ovipositing (mg)	Weight of total egg mass (mg)	CFU/mg adult ticks after ovipositing	CFU/100 eggs	CFU/100 larvae
1 (23°C)	$4.5x10^5$	743	290	400	$1.2x10^7$	0.083	Nil
2 (23°C)	$6x10^4$	900	552	364	$7.7x10^6$	Nil	Nil
3 (23°C)	$6x10^4$	820	550	272	$1x10^7$	Nil	Nil
4 (23°C)	Control (PBS)	697	310	367	Nil	Nil	Nil
5 (27°C)	10^4	No data	No data	180	No data	Nil	Nil
6 (27°C)	$4x10^5$	No data	No data	250	$2x10^9$ /tick	Nil	Nil
7 (27°C)	$4x10^5$	No data	No data	150	$1x10^8$ /tick	12.5	Nil
8 (27°C)	Control (PBS)	No data	No data	190	Nil	Nil	Nil

Table 9. Determination of infectious dose of *F. tularensis* necessary to establish colonization of adult *A. americanum* ticks.

Days PI	% Colonized per inoculum dose		
	1.5CFU/tick	12.5CFU/tick	140 CFU/tick
Day 1	0% (n=5)	40% (n=10)	100% (n=10)
Day 7	0% (n=5)	80% (n=10)	100% (n=10)
Day 14	60% (n=5)	ND*	ND*

* Data not collected

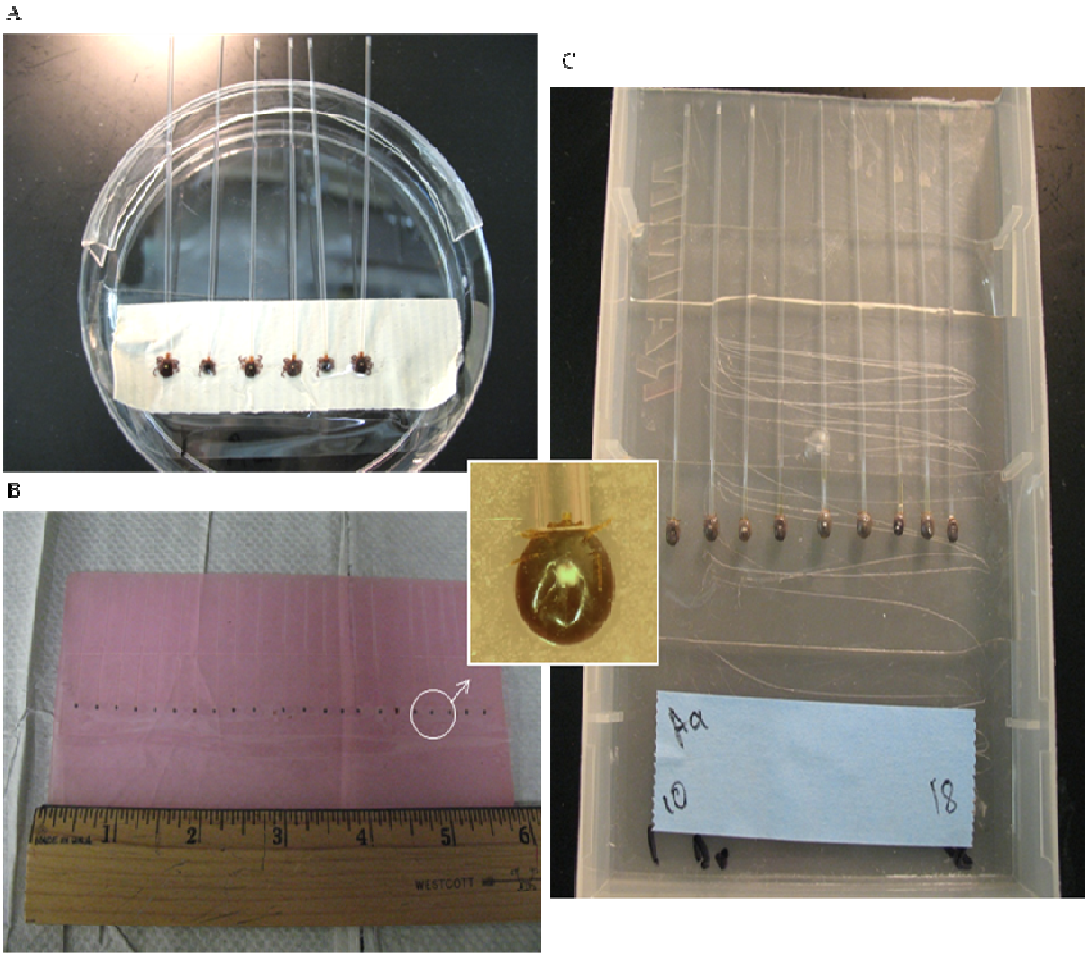

Figure 14. **(**A) Capillary feeding of adult *A. americanum* ticks. (B) Capillary feeding of *A. americanum* larvae. (C**)** Capillary feeding of *A. americanum* nymphs.

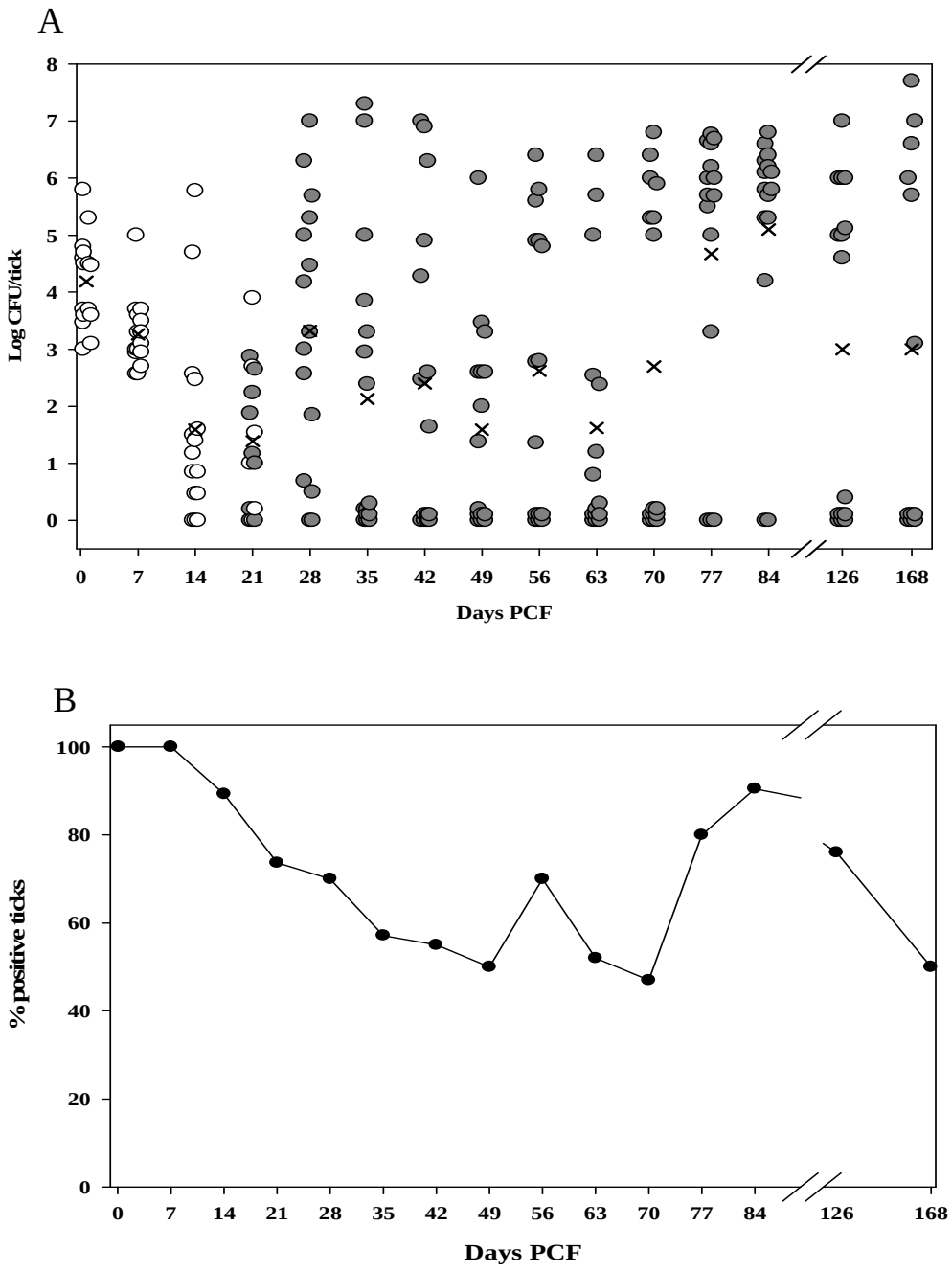

Figure 15. (A) *Francisella tularensis* is transmitted transstadially from larvae to nymphs. Open circles are capillary fed larvae and filled circles are molted nymphs. For each time point the n was 15. x = mean CFU/tick for each time point.

(B) Percentage of infected ticks in the same experiment.

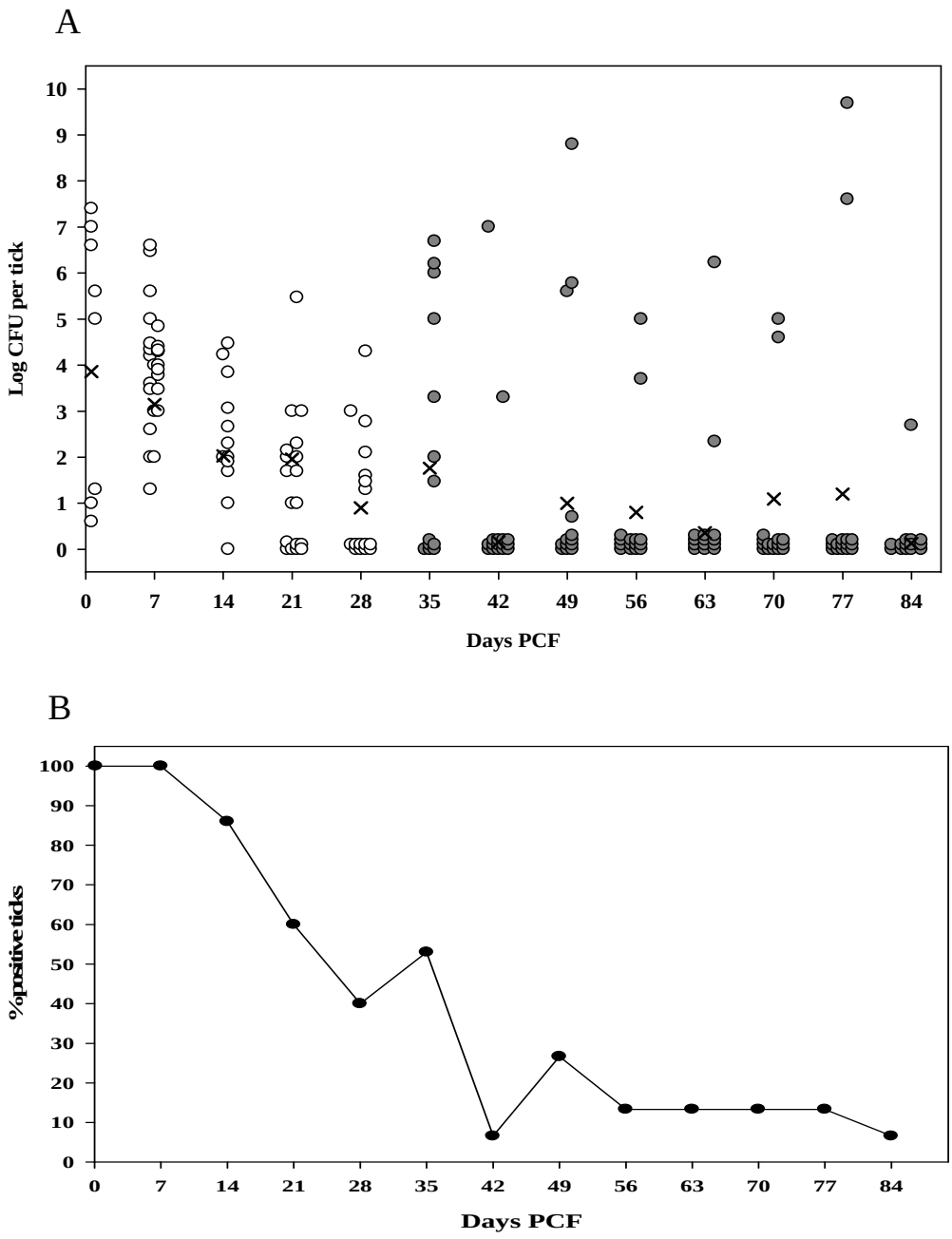

Figure 16. (A) *Francisella tularensis* is transmitted transstadially from nymphs to adults. Open circles are capillary fed nymph and filled circles are molted adults. For each time point the n was 15. x = mean CFU/tick for each time point.

(B) Percentage of infected ticks in the same experiment.

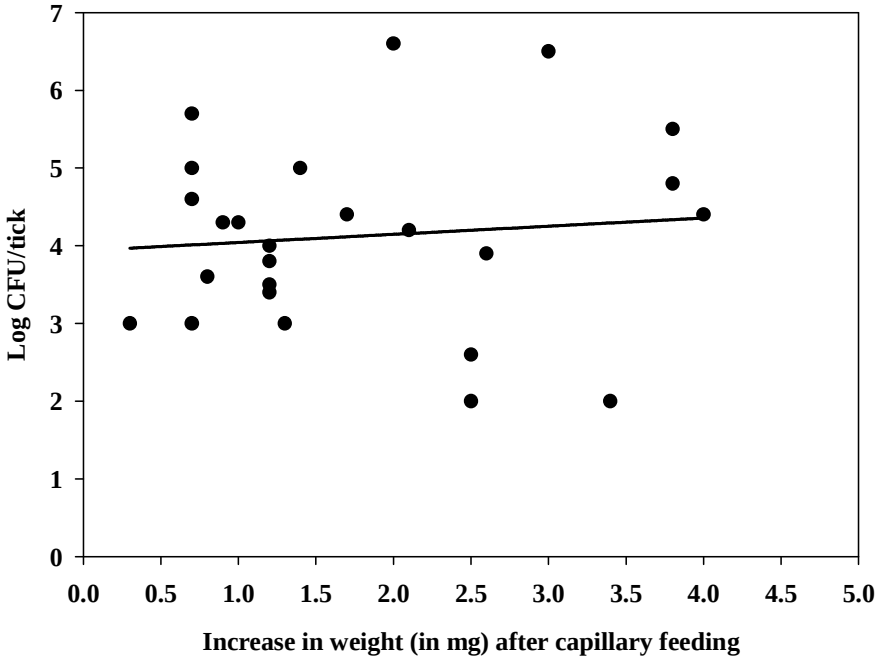

Figure 17**.** Correlation between weight gain after capillary feeding of *A. americanum* nymphs and *F. tularensis* colonization in nymphs up to 7 days PCF. Filled circles represent individual infected nymphs (R^2 =0.01) (n=25).

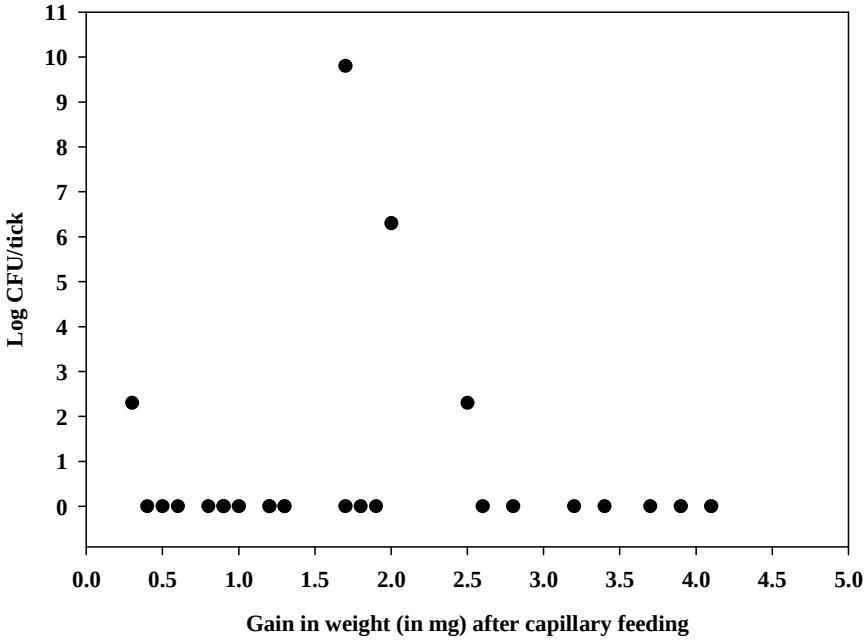

Figure 18. Correlation between weight gain after capillary feeding of *A. americanum* nymphs and *F. tularensis* colonization in molted adult ticks after two months PCF. Filled circles represent individual infected adults (R^2 = 0.001) (n=25).

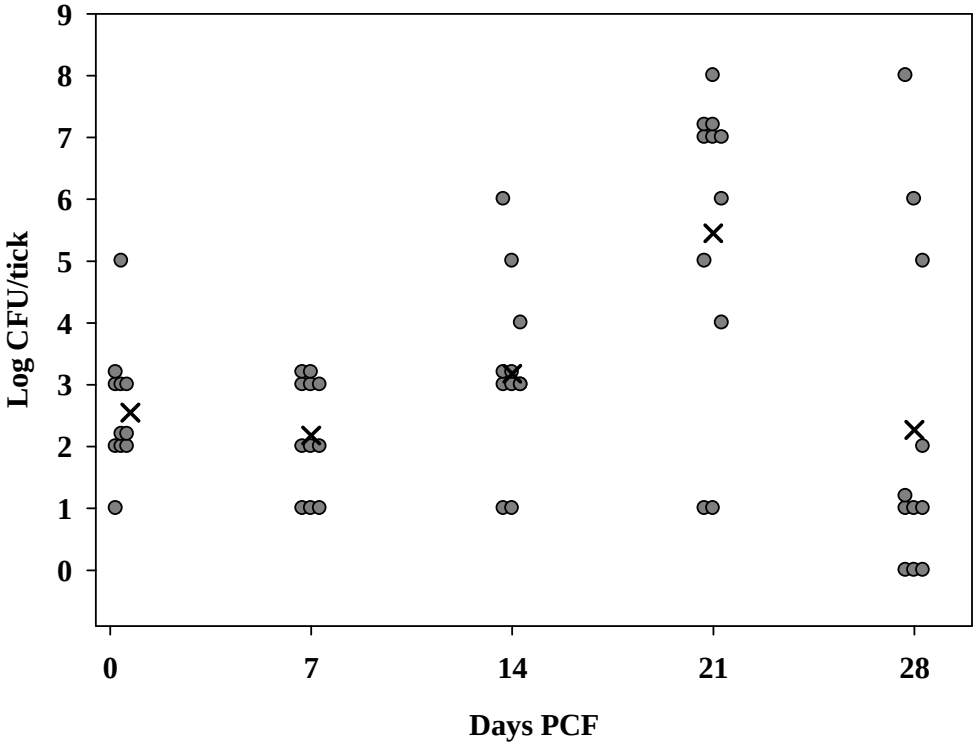

Figure 19. *Francisella tularensis* colonization of adult *A. americanum* ticks.

Filled circles are capillary fed adults. For each time point the n was 11.

x = mean CFU/tick for each time point.

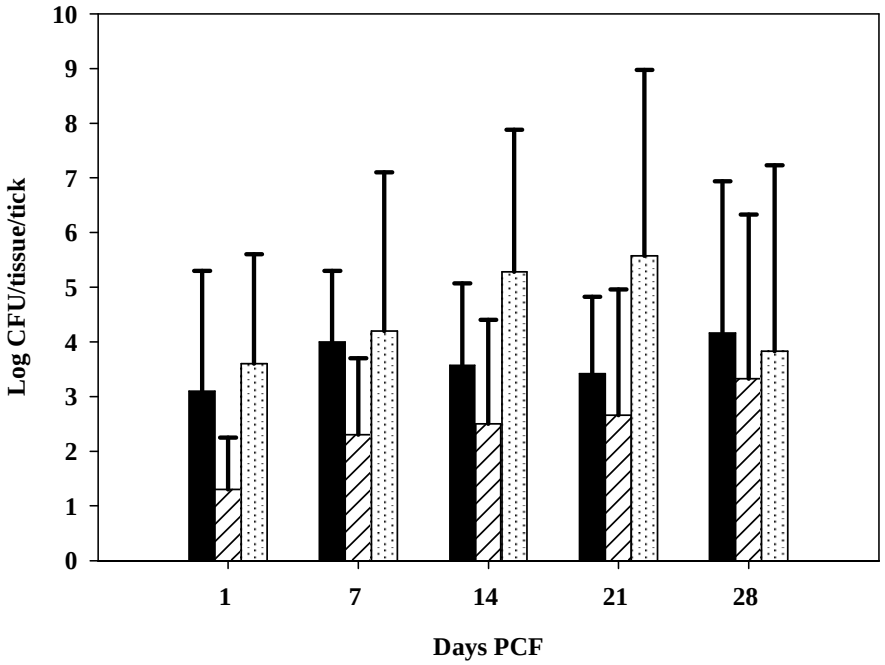

Figure 20. Tissue dissemination of *F. tularensis* in adult *A. americanum* ticks PCF. Solid black bar - gut, white bar with diagonal lines - salivary gland, white bar with dots - hemolymph. For each time point the n was 5. Error bars indicate standard deviation.

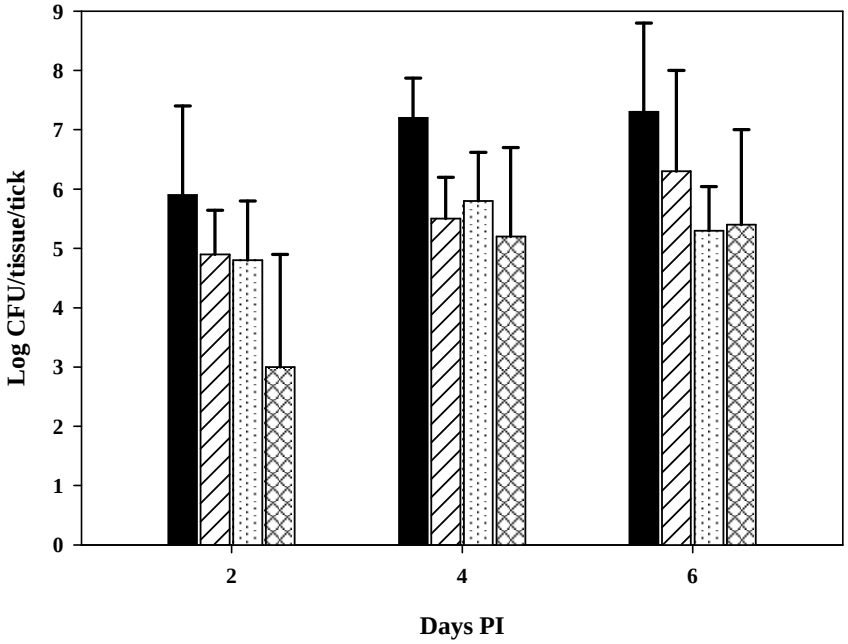

Figure 21. Tissue dissemination of *F. tularensis* in adult *A. americanum* ticks PI. Solid black bar - gut, white bar with diagonal lines - salivary gland, white bar with dots - hemolymph and white bar with cross marks - saliva (CFU/µl of saliva). For each time point the n was 5. Error bars indicate standard deviation

CHAPTER V

POSSIBLE ROLE OF CHITIN CATABOLISM IN *FRANCISELLA TULARENSIS* COLONIZATION OF TICK VECTORS

Abstract

Francisella tularensis is a facultative intracellular, non-spore forming, non-motile gram-negative bacterium that causes the disease tularemia in a number of vertebrates and invertebrates. *Francisella tularensis* ssp. *holarctica* is speculated to survive and persist in aquatic environments possibility in association with aquatic protists. In the United States, *F. tularensis* ssp. *tularensis* follows a tick-rabbit enzootic cycle and the previous study have demonstrated that *F. tularensis* can survive in *Dermacentor variabilis* and *Amblyomma americanum* ticks up to six months. This study explores whether *F. tularensis* can utilize chitin, a polymer of N-acetyl-D-glucosamine (NAG), abundant in the exoskeleton of ticks and aquatic protists as a nutrient. To accomplish this, we compared *in vitro* growth of *F. tularensis* ssp. *holarctica* strain LVS (live vaccine strain) in nutrient media and chemically-defined media supplemented with NAG, chitobiosan or chitin. We found that *F. tularensis* could utilize NAG, but not chitobiosan or chitin for enhanced growth. *Francisella tularensis* may utilize NAG at some phases of tick development as a nutrient.

Introduction

A number of bacteria have been shown to utilize chitin as a nutrient for extracellular as well as intracellular growth (Bassler *et al.*, 1991; Svitil *et al*., 1997). Some of these bacterial species including *Bacillus subtilis* have specialized chitin uptake mechanisms as well as chitinase enzymes (Imada *et al*., 1977; Plumbridge, 1990). Chitin is one of the most abundant biopolymers on earth, and utilization of this abundant source of nitrogen as well as energy gives a selective advantage to chitin-utilizing bacteria (Merzendorfer and Zimoch, 2003).

The genus *Francisella* is classified as a gamma-proteobacterium consisting of two species, *F. tularensis* and *F. philomiragia*. In addition, *Francisella*-like pathogens of fish and endosymbionts of ticks are now considered to be in this genus (Salomonsson et al., 2009; Scoles, 2004; Sjostedt, 2007). *Francisella tularensis* has four subspecies namely, *tularensis, holarctica, mediasiatica* and *novicida* (Sjostedt, 2007). The various species and subspecies of *Francisella* have adapted to unique ecological niches (Hazlett and Cirillo, 2009). *Francisella tularensis* ssp. *holarctica* can persist in fresh water and is thought to survive in water-associated unicellular organisms (Abd *et al*., 2003; Hazlett and Cirillo, 2009). Availability of chitin may be a common factor in *F. tularensis* persistence in fresh water as well as during its colonization in tick vectors.

Planktonic bacteria can initiate biofilm in response to environmental stress. Biofilms help bacteria to survive and persist in harsh environmental conditions (Davey and O'Toole G, 2000). *Francisella tularensis* subspecies encodes two conserved putative

chitinase genes. Subspecies *novicida* form biofilms on chitin surfaces, and it has been shown that chitinase genes in *F. tularensis* ssp. *novicida* are needed for the chitin association and subsequent biofilm formation (Margolis *et al.*, 2009). Subspecies. *holarctica* can also form biofilms in a static environment (Hassett *et al.*, 2003). *Francisella tularensis* may form biofilms during the prolonged nutrient starved conditions which it encounters during its colonization in tick vectors.

Francisella-like endosymbionts have been isolated from various tick species, and this symbiotic association between bacteria and ticks supports an evolutionary adaptation of *F. tularensis* to a tick associated life-style (Noda *et al.*, 1997; Sun *et al*., 2000). The tick cuticle, tracheal lining, and peritrophic membrane lining the gut are composed of chitin (Sonenshine, 1991).One of the most studied tick-pathogen interaction is the association between the Lyme disease agent *Borrelia burgdorferi* and its tick vector *Ixodes scapularis*. In *in vitro* experiments, *Borrelia* spirochete was shown to utilizes NAG and chitobiosan (Tilly *et al.*, 2001; Tilly *et al.*, 2004). Researchers have speculated that the spirochetes ability to utilize chitin in the *Ixodes scapularis* ticks would have positive impact on the spirochete in that it can utilize chitin as a nutrient source, but at the same time it could negatively influence the tick development if bacteria multiply inside the tick uncontrollably (Burgdorfer *et al*., 1988; Piesman *et al.*, 1990).

We have previously demonstrated the ability of *F. tularensis* to survive in ticks through the extended periods of starvation of ticks. In the current study we report the utilization of chitin precursor NAG by *F. tularensis* in nutrient and chemically-defined media when supplemented with NAG. Future research on chitin catabolism of *F.*

tularensis may better inform the role of chitin in the colonization of tick vectors by *F. tularensis* during its extended association with ticks.

Materials and Methods

Bacterial strain and growth conditions

Francisella tularensis ssp. *holarctica* strain LVS (ATCC 29684) was supplied by the Oklahoma State Department of Health. *Francisella tularensis* was grown on chocolate agar plates (Hardy diagnostics, Santa Monica, CA.) at 37°C in 5% CO_2 for 72 h. The BBL Prompt Inoculation System (BD Diagnostic Systems, Sparks, MD) was used for preparing inocula of *F. tularensis*. Initial *F. tularensis* inocula of $4x10^5$ CFU/mL was used to infect growth media. The nutrient media used were Mueller Hinton Broth (Becton Dickinson, Cockeysville, MD, USA) with or without IsoVitaleX (Becton Dickinson, Cockeysville, MD, USA) and Nutrient Broth (Difco, BD Diagnostic Systems, Sparks, MD), supplemented with Casitone (Becton Dickinson, Cockeysville, MD, USA). The ingredients for the chemically-defined media were purchased from Sigma-Aldrich (St. Louise, MO), except the MEM Essential Amino Acids Solution and MEM Non-Essential Amino Acids Solution which were purchased from Gibco (Gibco-Invitrogen, Rockville, Maryland) (Table 10). For the *in vitro* growth culture assay, *F. tularensis* was grown at 23, 27, 30, or 32°C at 180 rpm in an incubator shaker. Innova 4000 shaker incubator (New Brunswick Scientific Co., New Brunswick, NJ) was used for the cultures at 23°C, whereas Orbital Shaker incubator (Thermo Forma, Marietta, OH, USA) was used for the

growth of *F. tularensis* at 27 and 30°C. N-acetyl-D-glucosamine, chitobiosan and shrimp chitin were purchased from Sigma. The increase in growth of *F. tularensis* in culture was detected by OD_{550} reading on an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences) or by CFU counting on chocolate agar plates.

Growth of *F. tularensis* in media supplemented with NAG, Chitin or Chitobiosan

For growth of *F. tularensis* in Mueller Hinton broth (MH), 2% IsoVitaleX was added. For the Nutrient Broth (NB) culture studies *F. tularensis* was grown on either 0.72% (w/v) NB alone or with the addition of 1.76% (w/v) Casitone (pancreatic digest of casein) (NBC). The ingredients for the chemically-defined medium (CDM) were added to the buffer (0.25M NaCl, 0.8mM $MgSO_4$, 0.01mM $FeSO_4$, 10mM KH_2PO_4 and 8mMK_2HPO_4, pH 6.5) shown in Table 10. After adjusting the pH of the medium, all media were filtered using 0.2-µm pore size 75-mm Nalgene Fast PES filters (Nalgene, Thermo Fisher Scientific, Waltham, MA). NAG was added to the media at 10mM or 0.4mM concentration and chitobiosan was added at 0.2mM. Chitin was added to the media at a concentration of 0.4% (w/v).

Statistical analysis

Student's t-test was performed to determine the statistical difference between *F. tularensis* growth in NB and on addition of NAG to NB. The same test was also done to determine the difference in growth at 27°C versus 32°C. Statistical analyses were performed with SigmaPlot v11.0 software package (Systat Software Inc., Chicago, IL).

Results

Growth of *F. tularensis* in Mueller Hinton Broth and Nutrient Broth on supplementation of NAG, chitobiosan and chitin.

Growth advantage for *F. tularensis* when NAG, chitobiosan or chitin was supplemented to MH was not observed (Fig. 22 & 23). When *F. tularensis* was grown in NB alone or on addition of 10mM NAG there was no growth as detected by comparison of OD readings or CFU determinations. However, when casein was added to Nutrient Broth *F. tularensis* was able to use this media for growth (Fig. 24). Further addition of NAG to the Nutrient Broth resulted in significant increase in growth of *F. tularensis* by three days of culture at 27°C ($P = 0.003$) (Fig. 25). When either chitobiosan or chitin was added to NBC, no growth advantage was observed (Fig. 26). The increase in growth of *F. tularensis* on addition of NAG was seen when the culture conditions were at 27°C as well as 32°C and the growth advantage for *F. tularensis* on addition of NAG was more at 32°C when compared to 27°C with a $P < 0.001$ at two days of culture (Fig. 27).

Growth of *F. tularensis* in chemically-defined media on supplementation of NAG and chitobiosan.

Increase in growth of *F. tularensis* was not observed in the CDM in the absence of calcium pantothenate or spermine, but when these two nutrients were added and the pH of the medium was decreased from 7.3 to 6.5, *F. tularensis* was able to use the media for growth. Higher growth of *F. tularensis* was observed in the CDM on addition of 10mM NAG, but addition of chitobiosan had no growth advantage for *F. tularensis* (Fig. 28).

Discussion

In this study we report the significantly higher growth of *F. tularensis* in Nutrient Broth and chemically-defined media supplemented with NAG. Margolis and colleagues have reported the colonization of *F. tularensis* ssp. *novicida* on chitin surfaces and subsequent biofilm formation in carbohydrate starved environment (Margolis *et al*., 2009). The lack of increased growth of *F. tularensis* in Mueller Hinton broth supplemented with IsoVitaleX may be due to the high amount of starch in the broth and additional glucose in the IsoVitaleX enrichment, which may create a carbohydrate enriched environment making NAG supplementation NAG inconsequential. We also observed the higher growth of *F. tularensis* when the pH was of the chemically-defined media was decreased and on supplementation with calcium pantothenate as have been reported by Chamberlain (Chamberlain, 1965).

We have shown in previous studies that *F. tularensis* can persist in tick vectors for up to six months during starvation of these ticks. During this long term association, *F. tularensis* was primarily localized in the gut tissue. The ticks would be devoid of any blood-derived carbohydrate source during this period of starvation. This would be especially true with bacteria that survive the nutrient-depleted environment in ticks that overwinter in nature, which usually involve 5 to 6 months of tick diapause. *Francisella tularensis* can possibly utilize tick chitin as a nutrient source under these conditions. In addition, it was shown that *F. tularensis* colonization levels decline during molting. Remodeling of chitin during tick molting may make chitin less available for *F. tularensis*. Also, *F. tularensis* colonization of the tick ova suggests that *F. tularensis* may use chitin

for colonization of the ova surface. Whether the ability of *F. tularensis* to utilize NAG is beneficial during the tick life cycle and whether it could form biofilm in the tick gut could be answered in future studies.

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Table 10.

Ingredients for making 100mL of chemically-defined media

Buffer	70mL
MEM EssentialAmino Acids	10mL
MEM Non-Essential Amino Acids	20mL
Proline	160mg
Threnine	160mg
Cysteine	30mg
Thiamine	0.4mg
Ca pantothenate	0.2mg
Spermine	4mg

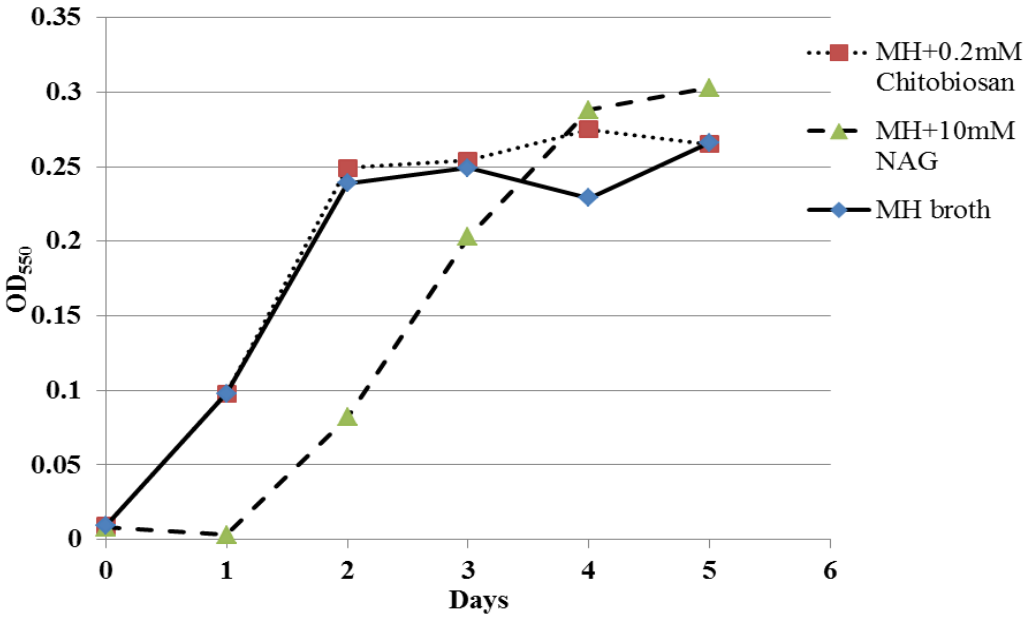

Figure 22. *Francisella tularensis* growth at 27°C in Mueller Hinton broth.

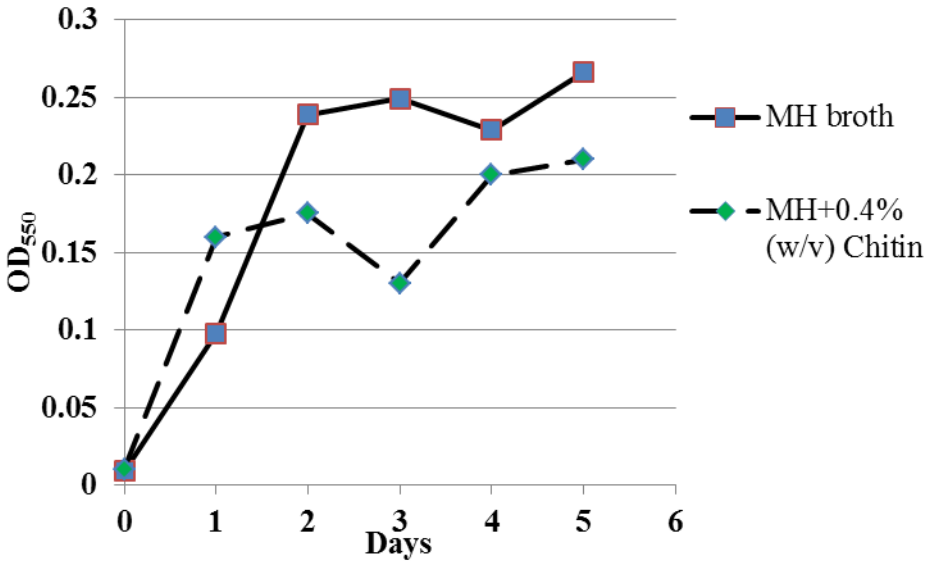

Figure 23**.** *Francisella tularensis* growth at 27°C in Mueller Hinton broth.

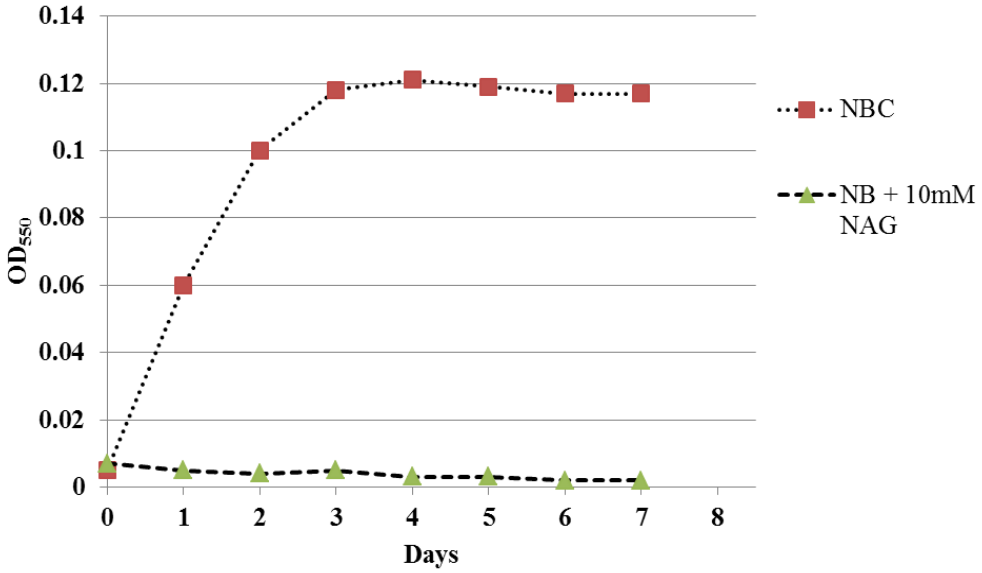

Figure 24. *Francisella tularensis* growth at 30°C in Nutrient Broth with addition of Casitone to the media.

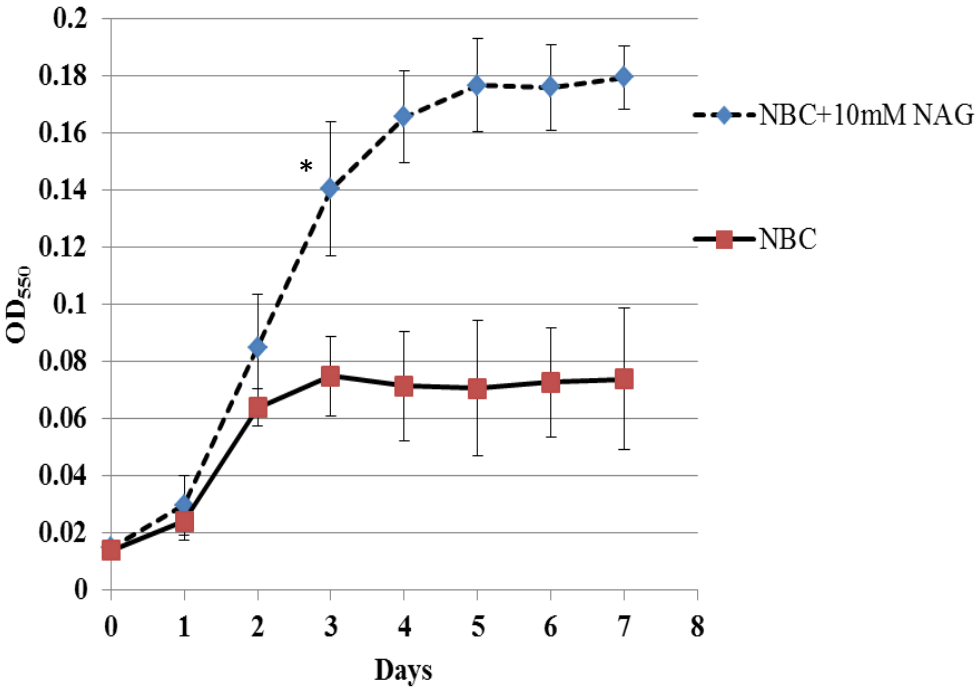

Figure 25. Significant increase in growth of *F. tularensis* growth at 27°C in Nutrient Broth plus Casitone. Error bars indicate standard deviation.

* $P = 0.007$.

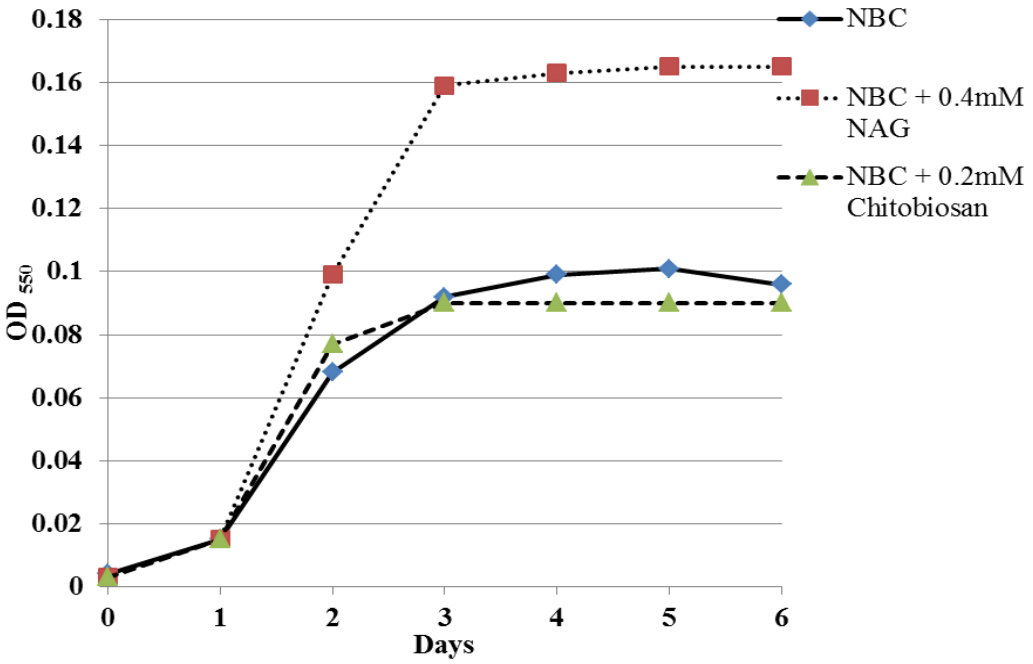

Figure 26. *F. tularensis* growth at 32°C in Nutrient Broth plus Casitone.

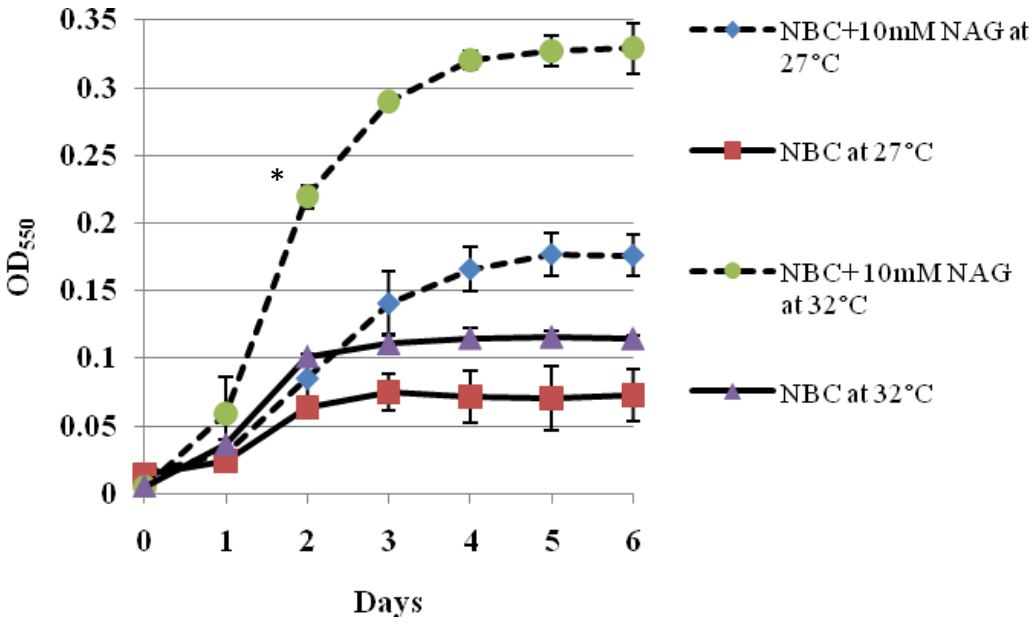

Figure 27. *Francisella tularensis* growth at 27°C and 32°C in Nutrient Broth plus Casitone. Error bars indicate standard deviation. $* P < 0.001$.

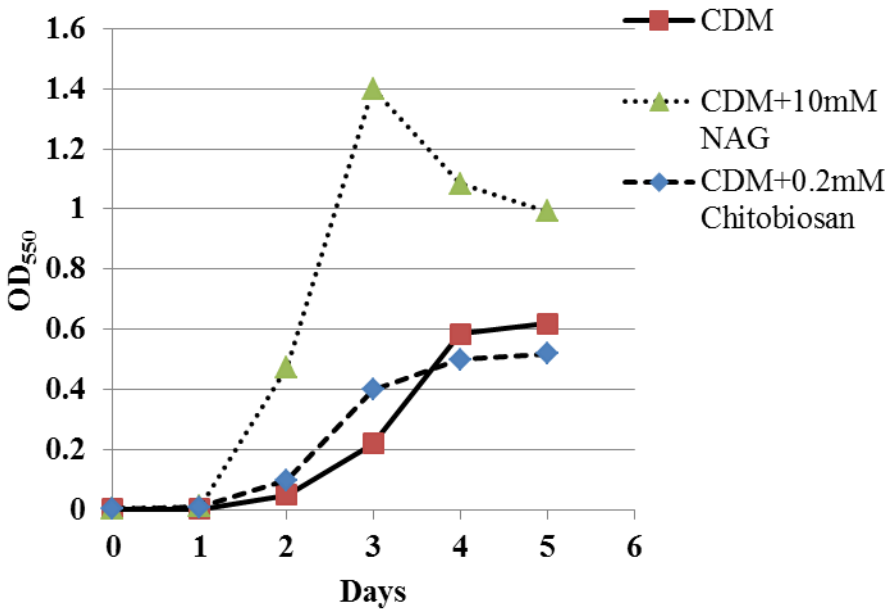

Figure 28**.** *Francisella tularensis* growth at 32°C in CDM.

CHAPTER VI

CONCLUSION

Environmental persistence of *F. tularensis* in tick vectors

Francisella tularensis is one of the most infectious bacteria known and exhibits a broad host range. *Francisella tularensis* has been recovered from more than 250 different species, including vertebrates and invertebrates (Keim *et al*., 2007). More than 400 different of *F. tularensis* have been identified as important for this high infectivity in mammalian species (Ellis *et al*., 2002; Friend, 2006). These genes include the ones that regulate intracellular growth, immune evasion, and dissemination (Meibom and Charbit, 2009). Among invertebrates, *F. tularensis* can infect a variety of tick, lice, bedbugs, fleas, mosquitoes, and flies (Steinhaus, 1946). In ticks, it can infect *Dermacentor* species including *D. occidentalis, D. variabilis, D. albipictus, D. marginatus, D. andersoni,* and other tick species including *Hemophysalis leporispalustris, H. cinnabarina, Ixodes pacificus*, *Rhipicephalus sanguineus* and *Amblyomma americanum* among ixodid ticks and *Ornithodoros parkeri,* and *O. lahorenis* among argasid ticks (Steinhaus, 1946). However, not much is known about infectivity and adaptation of this bacterium in arthropods hosts. The results presented here shows that all three stages, including larva, nymph and adult of both *D. variabilis* and *A. americanum,* can be colonized by *F. tularensis*. Based on the knowledge of longevity and host seeking behavior of these two tick species (Kollars *et al*., 2000a; Kollars *et al*., 2000b; Sonenshine, 1991), and also the results that we have presented here, it can be concluded that *A. americanum* nymphs and *D. variabilis* adults are likely the most suitable stages of these tick species that can maintain *F. tularensis* for extended periods of time.

Quantum of infection in capillary fed ticks

Ticks are obligate hematophagus arthropods, in which a bloodmeal is required for development and molting to the subsequent stage. *Amblyomma americanum* and *D. variabilis* are three host ticks with the larva, nymph and the adult taking blood meals on different host animals at each stage to complete its life cycle. The time period between feeding on one host, molting and initiation of feeding on the next host can vary from one month to more than one year in these tick species (Bowman and Nuttall, 2008; Sonenshine, 1991). Therefore, for *F. tularensis* to use ticks as a biological vector, it must be able to survive for extended periods of time in the tick for it to successfully transmit to the next host. The ability of *F. tularensis* to survive in *A. americanum* nymphs and *D. variabilis* adults may make them excellent vectors. During the transstadial transmission of *F. tularensis* in *D. variabilis* and *A. americanum* ticks, it was observed that soon after molting the level of *F. tularensis*/tick increases. This peak in *F. tularensis* colonization in molted nymph of *A. americanum* and molted nymphs and adults of *D. variabilis* may correlate with the host-seeking activity of these tick stages facilitating transmission of *F. tularensis* to new hosts. However, the advantage of the high *F. tularensis* numbers per tick is not clear since only low amount of bacteria is needed for infection in susceptible hosts. High number of bacteria per tick, especially in the gut and hemolymph, is likely to be required for *F. tularensis* to disseminate into the salivary glands and subsequent transmission through the saliva of the tick.

Effect of temperature on *F. tularensis* colonization in ticks

Ticks are poikilotherms in which ambient temperature may have a significant impact on the growth and transmission of tick-borne pathogens. Results from this study indicate that temperature can have an effect on *F. tularensis* growth inside the tick. In these experiments, infected ticks that were held at higher temperatures had decreased colonization of *F. tularensis*. This decreased survival of *F. tularensis* inside the tick at higher temperatures may be due to increased antimicrobial activity of ticks at higher temperatures or to *F. tularensis* general ability to better survive at low temperatures (Friend, 2006). *Francisella tularensis* can survive in cold conditions, and it is possible that colder temperatures may aid bacterial survival for longer periods of time in ticks. Therefore, cold winter temperatures may help *F. tularensis* to persist in the ticks during the long five to six months of winter rather than diminish the survival.

A significant decrease in colonization of *F. tularensis* was observed during the molting stage in both tick species. This decrease was pronounced at the molting of infected *D. variabilis* nymphs to adults and also during the molting of infected *A. americanum* larvae to nymphs. It is not known whether a decline in the availability of nutrients for bacterial growth or the unfavorable environment within the ticks during the ecdysial process may be the cause for this reduction. In nature, at lower temperatures the molting stage of the ticks are prolonged (Bowman and Nuttall, 2008), and this was also seen in our experimental holding conditions of the ticks. The increased molting period in ticks may result in reduced *F. tularensis* within ticks and may eventually lead to permanent clearing of the bacterium from these ticks. This may be one of the reasons why most of the *A. americanum* adults cleared the infection after molting. The molting

period of *A. americanum* nymph was more than 30 days which was the longest period seen in any stage of molting in both tick species. The most favorable environment for *F. tularensis* within ticks would be for the ticks to molt reasonably fast and subsequently to encounter a lower environmental temperature. These favorable conditions may occur in nature for the ticks colonized by *F. tularensis* in September and October. Molting of these ticks would then undergo a behavioral diapause as a resulting from a decreased temperature in November, enabling *F. tularensis* colonized ticks to overwinter and carryover the infection to the next spring. Future experiments, in which the infected ticks could be kept at controlled temperature conditions thus altering their duration of molting and the effect of this variation of molting period on *F. tularensis*, may further our understanding of this reduced colonization at molting.

Francisella tularensis dissemination in tick tissues

Another interesting difference between the two tick species was the longer time required for tissue dissemination in *D. variabilis* adults. More than two weeks PCF were required for dissemination of *F. tularensis* from gut to hemolymph and salivary glands in *D. variabilis* adults. In contrast, bacterial dissemination was seen within 24 hours PCF in *A. americanum* adults. When analyzing vector competency, this rapid dissemination of *F. tularensis* in *A. americanum* ticks may not be an advantage, because once the ticks become colonized by taking blood meal from an infected animal, the tick must detach, molt, and then feed on another susceptible animal to transmit the pathogen, a process that can take up to a month to complete. The rapid dissemination of *F. tularensis* in *A. americanum* ticks may have an advantage in male ticks which are often known to feed intermittently on hosts and thus can transmit the bacterium from one host to another

(Kocan *et al*., 2010). Ticks which become infected at the near death of the host animal (which has been shown to be the case with *F. tularensis* infection), feeding to engorgement may not be complete, and the rapid dissemination of *F. tularensis* from gut to saliva can be advantageous in bacterial transmission when these ticks feed on another host. *Francisella tularensis* was also observed to localize mostly in the gut of the ticks during long term persistence. Therefore, for the ticks which become colonized by *F. tularensis* in summer and fall and undergo overwintering, the bacterium might be most likely in the gut tissue. In these ticks which again start host seeking in the following spring it may take longer for *F. tularensis* to migrate from the gut to salivary glands. It would be interesting to study the dissemination of *F. tularensis* from the gut of these ticks to the saliva, including the time taken and the factors influencing the dissemination.

Host species and tick-borne tularemia

Availability of competent hosts is important for maintenance of the disease agent in tick vectors. This is especially true in *F. tularensis* transmission in ticks, because the transovarial transmission may not play a major role and the disease has to be maintained through repeated transstadial transmission. In such a scenario, the dilution effect of feeding on a non-competent host might reduce the *F. tularensis* infection rates in the tick population (Sonenshine and Mather, 1994). In the south-central tularemia endemic region of the United States comprised of Arkansas, Missouri, eastern Oklahoma and southeast Kansas, there are abundant deer populations which in turn support large populations of ticks. *Francisella tularensis* has not been recovered from deer in this region (Castellaw *et al.*) and deer may probably be resistant to tularemia infection and could be a non-competent host of *F. tularensis*. But this region also supports a variety of small

mammalian species including cotton tail rabbits and other small rodents which can function as competent hosts for tularemia (Brown *et al*., 2011; Kollars *et al*., 2000a; Kollars *et al*., 2000b). This wide range of host species, with deer population as tick amplifiers and small mammals as tularemia infected host, might be the reason for the persistence of tularemia in this region.

Effect of *F. tularensis* colonization on tick life cycle

In these experiments an increase in tick mortality during the infected adult stages of both species of ticks was not observed when compared to control ticks which were fed with tick-meal without *F. tularensis*. In addition, the high level of bacteria at 10^7 to 10^9 per tick was remarkable. In the face of this high colonization level, the ticks seemed to suffer no ill effects. *Dermacentor variabilis* ticks naturally infected with high number of *F. tularensis*/tick have been reported (Goethert and Telford, 2010). It would be interesting to see if these apparently healthy, heavily infected ticks would be affected in their feeding behavior. In contrast, increase in mortality in immature stages of *F. tularensis* infected ticks was observed, and this increase in mortality was reduced, when the holding temperature of the ticks was lowered. The wide variation in the level of *F. tularensis* colonization during different stages of development of the tick, including low numbers seen during molting and high numbers soon after molting can be either tick mediated or may be controlled by the bacterium itself. The recent developments in sequencing of tick genomes, availability of new techniques including gene silencing in ticks (Kocan *et al*., 2011; Nene, 2009), and with the recently reported improvements in genetic manipulations of *F. tularensis* (Barker and Klose, 2007; Bina *et al*., 2010;

Rodriguez *et al*., 2009), it may be possible to study the tick vector-*F. tularensis* interactions in greater detail in the future.

Francisella tularensis strains and tick-borne tularemia

The type A1 genotype of *F. tularensis* is the major genotype associated with human tularemia cases in south-central region of the United States. In this study *F. tularensis* type B LVS strain was used, and it is possible that these two strains can have different developmental patterns in tick vectors. Petrosino and colleagues compared the two strains and have found remarkable similarity gene content between their genomes (Petrosino *et al*., 2006). However, certain genes in type B were observed to be pseudogenes due to insertion and deletion events when compared to type A. Salomonsson and colleges have demonstrated that a type IV pilin gene and an outer membrane protein gene, which was active in type A and not in type B, was important for the infectivity of type A stains (Salomonsson *et al*., 2009). Most of the research involving tick vectors and *F. tularensis* were conducted almost 50 years ago, and it is not known which subspecies or strains of *F. tularensis* these researchers might have used. The capillary feeding model presents an excellent platform to study the interaction between different tick vectors and *F. tularensis* strains and would also be an ideal system to study the molecular epidemiology of these strains in the tick vectors.

Finally, these studies also demonstrated that *F. tularensis* can retain infectivity after it grow in inside the tick vector. The ID_{50} for *F. tularensis* recovered from salivary glands of *D. variabilis* ticks was one log lower than for culture grown *F. tularensis*, when bacteria was injected intraperitoneally in BALB/c mice. Results from our lab

(unpublished data) and others have shown that LD_{50} for LVS strain via intradermal (i.d.) route, which simulates the natural route of entry of bacteria by tick bite, is very high; however, i.d. LD_{50} for virulent type B and type A strains are less than 10 CFU in mice and humans (Saslaw *et al*., 1961a; Saslaw *et al*., 1961b; Conlan *et al*., 2005). Korocova and colleagues have shown that when LVS is mixed with salivary extracts of *Ixodes scapularis*, and introduced via i.d. route in mice, the proliferation and dissemination of LVS was increased (Krocova *et al*., 2003). They have also shown that the increased infectivity seen in this saliva-assisted transmission (SAT) was due to the immunosuppression of the host by the tick saliva. SAT studies with *D. variabilis* or *A. americanum* has not been done. Based on these results, it is reasonable to assume that the infective dose for virulent strains of *F. tularensis* in susceptible hosts via tick bite could be extremely low.

Ecology of tick-borne tularemia

Based on the low infective dose required for colonizing the tick, and the high *F. tularensis* colonization per tick, one would hope to find large number of infected ticks in the enzootic area; contrarily, the percentage of infected ticks in the enzootic area is extremely low, often less than 1% (Hopla, 1974; Matyas *et al*., 2007). Assuming that infective dose in susceptible vertebrate hosts is also quite low, epizootic outbreaks in the small mammalian population should also be expected throughout the tick activity season from spring to early fall. Such constant outbreaks in small mammals are also not seen. Small animal hosts may gradually acquire resistance to the infection, and the epizootics of tularemia seen in the fall, is probably due to infection of immunologically naïve juvenile cohorts of these animals. A thorough knowledge of the vertebrate hosts and

vector population dynamics is required to understand the persistence of the disease in enzootic regions (Sonenshine and Mather, 1994). *Francisella tularensis* is infective to multiple tick vectors and small mammalian hosts, so the disease dynamics in nature would probably involve multiple tick vectors and small mammalian species. However, with respect to transmission of *F. tularensis* to humans in the south-central tularemia endemic region, only three tick species maintain sufficiently high populations to be potential vectors for *F. tularensis*, namely *D. variabilis* and *Ixodes scapularis* adults, and nymphal and adult stages of *A. americanum.* Since the peak adult tick activity of *I. scapularis* is seen in the fall and winter months, which does not correlate with the human tularemia outbreaks, the *D. variabilis* and *A. americanum* ticks are the most likely vectors of human *F. tularensis* in the south-central United States. *Dermacentor variabilis* adults and *A. americanum* nymphs and adults were shown to be the vectors of *F. tularensis.* Although the percentage of infected *A. americanum* adults decreased over time, the infection of adult *D. variabilis* and nymphal *A. americanum* ticks persisted for longer duration.

Better understanding of the ecology, population dynamics of the tick vectors, susceptible vertebrate hosts, and resistant or dilution hosts in the endemic area, along with controlled experimental transmission studies to find the influence of abiotic and genetic factors influencing the transmission and persistence, will help to enhance predictive modeling of tularemia and also will enable development of control measures to reduce the risk of disease transmission.

Utilization of chitin and growth of *F. tularensis* in carbohydrate deficient media

Francisella tularensis subspecies have been shown to be associated with chitin-containing crustaceans (Anda *et al*., 2001; Diaz de Tuesta *et al*., 2001). The abundant source of chitin within ticks could be a source of nutrient for intra-tick *F. tularensis* growth during the prolonged association with the tick. Additional evidence for the possible chitin utilization of *F. tularensis* is the putative chitinase genes found in these bacteria (Margolis *et al*., 2009). The unusually lower *B. burgdorferi* colonization during the molting stages of infected *I. scapularis* ticks, and the morphological abnormality in seen in heavily infected tick eggs are thought to be the result of chitin catabolism by *B. burgdorferi* (Burgdorfer *et al*., 1988; Piesman *et al*., 1990). In our experiments with *F. tularensis* and tick vectors, it was observed that a similar decline in bacterial colonization occurred during tick molting. *Francisella tularensis* colonization on the chitinous outer shell of *D. variabilis* tick eggs was also observed. Based on these data it was hypothesized that *F. tularensis* could utilize chitin as a nutrient source for its growth. In the *in vitro* culture experiments, we have shown that *F. tularensis* can utilize NAG, the monomer of chitin, for its growth in Nutrient Broth as well as in Chamberlain's chemically-defined broth. However, the growth advantage was not seen when a carbohydrate saturated broth was used, indicating *F. tularensis* has an advantage in utilizing NAG only in a carbohydrate deficient environment. Growth advantage of *F. tularensis* in carbohydrate deficient broth was not observed when either chitin or chitobiosan was supplemented. Bacteria usually form biofilm in stagnant environments.

Francisella tularensis may attach to chitin and form a biofilm in a sessile state and subsequently utilize the chitin as opposed to in a freely moving culture condition.

While visualizing immunostained *D. variabilis* gut tissues, we could observe certain highly concentrated bacterial colonies in the gut epithelium. *Francisella tularensis* could possibility form biofilms during the extended association in the tick vectors. Several environmental and marine bacteria can form biofilms (Svitil *et al*., 1997; Verhoeven *et al*., 2010), and *F. tularensis* ssp. *novicida* was shown to be deficient in biofilm formations when the chitinase genes were knocked out (Margolis *et al*., 2009). *Francisella tularensis* ssp. *holarctica* has also been shown to form biofilms in stagnant conditions (Hassett *et al*., 2003). The biofilms enable these bacteria to survive harsh environmental conditions and can also aid in disease transmission in some pathogenic bacteria (Hinnebusch and Erickson, 2008). During the long starvation periods of tick vector, *F. tularensis* could possibly utilize chitin and form biofilms inside the tick. With the recent advances in genetic manipulations in *F. tularensis*, it would be interesting to study the growth of chitinase mutant strains of *F. tularensis* in the tick vectors. Another interesting study would be to investigate the formation of *F. tularensis* biofilms in tick vectors.

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VITA

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Francisella tularensis causes tick-transmitted tularemia epizootics in rodent and rabbit hosts and incidental infections in humans. The objective of this study was to develop a *F. tularensis* tick colonization model for elucidating the salient features of its biology in tick vectors. The first two studies reported herein describe the systematic study of *F. tularensis* ssp. *holarctica* strain LVS colonization in the tick vectors *Dermacentor variabilis* and *Amblyomma americanum* as well as describing the capillary feeding (CF) method of colonizing the different stages of these ticks. Post capillary feeding (PCF), level of colonization was determined by CFU determinations of tick minceate. Transmission of *F. tularensis* from larvae to nymph was seen in both tick species, but only *A. americanum* nymphs maintained *F. tularensis* for longer periods of time (168 days PCF). Transstadial transmission from nymph to adult was also demonstrated in both the tick species, but only *D. variabilis* ticks maintained *F. tularensis* colonization after molting from nymphs to adults. For CF adults, *F. tularensis* initial colonization of the gut disseminated to hemolymph and salivary glands in three weeks and 24 h PCF for *D.variabilis* and *A. americanum* respectively. Colonization of adult *D. variabilis* ticks persisted up to 6 months PCF (longest time point in study). Transovarial transmission was not observed in either tick species. However, colonized *D. variabilis* adult females transferred *F. tularensis* to surface layer of eggs but not to hatched larvae. The extrinsic incubation period (time to secretion in saliva), determined by intra-hemocel injection of *F. tularensis* in *D. variabilis* and *A. americanum* was 4 and 2 days, respectively. The ID_{50} for mice for intraperitoneal injection of *F. tularensis* from adult *D. variabilis* salivary glands versus laboratory culture *F. tularensis* were 2 and 43 CFU, respectively. Both tick species appear competent as experimental vectors for *F. tularensis* with *D. variabilis* adults and *A. americanum* nymphs better adapted for long term persistence of *F. tularensis*. The role of these ticks as a possible inter-epizootic reservoir of *F. tularensis* is also discussed. The final chapter of this dissertation addresses the possible role of chitin as a nutrient source for *F. tularensis* in the tick life cycle.

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