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NORTH AMERICAN LYME BORRELIAE: NEW DISTRIBUTIONS AND AN INSIGHT INTO BORRELIA BISSETTII INFECTION, IMMUNE RESPONSE AND TRANSMISSION IN A MURINE MODEL

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

Veterinary Medical Sciences

by
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August 2014

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I dedicate this dissertation to my family. To my wife-to-be, Karine, who was there for me during some of the hardest moments of my work and who essentially takes care of me, as I seem to have the hardest time doing even the simplest tasks (laundry), je t'aime. To my parents, Mrs. Ann Beatty, Mr. David Beatty, Mrs. Cynthia Leydet, and Mr. Brian Leydet Sr., the support you have provided me has been essential to my success. I don't see myself here writing this without their unconditional love. To my brothers, Mr. David Leydet and Mr. Michael Leydet, even though I've lived miles away for the past seven years, I always know you are just a phone call or text away. To my grandparents, Mr. Robert Leydet, Mrs. Joan Leydet, Mr. James Coviello, and Mrs. Annmarie Coviello, thank you for the many years of smiles and encouragement during my long academic career. And finally to my future in-laws, the Posbic family, Jean, Chantal, Aline and Vincent, you guys have accepted me as one of your own and I am so grateful for that.

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ABSTRACT

Lyme borreliosis is an emergent threat to human health. It is estimated that 300,000 cases of Lyme disease are diagnosed annually in the United States. In recent years, non-traditional areas are starting to report more and more cases. While much research has gone into the tick and bacteria responsible for the disease, frequently new *Borrelia* species are being described and implicated in human illness. These species often go unstudied, and their threat to human health is not known. In the hopes of closing knowledge gaps on Lyme borreliosis in the US, we surveyed a non-endemic state, Louisiana, for Lyme *Borrelia* in ticks and mammals. We also assessed the potential for a second North American Lyme *Borrelia* species to cause mammalian disease by examining the infectivity and immune response of a non-*B. burgdorferi* sensu stricto North American isolate, *Borrelia bissettii*, in a murine model. Similarities between *B. burgdorferi* ss and *B. bissettii* prompted us to assess the ability for *Ixodes scapularis* to acquire and transmit *B. bissettii* to susceptible animals. Our research uncovered evidence for an enzootic cycle of *Borrelia burgdorferi* ss at a site in Louisiana between small mammals and *Ixodes scapularis* ticks. This, combined with broader evidence of multiple tick-borne pathogens in human biting ticks, highlights the need for tick-borne disease surveillance in areas not traditionally considered endemic. *Borrelia bissettii* may play a role in human disease in the US, especially in areas where *B. burgdorferi* ss is not common. We describe similarities between *B. bissettii* and *B. burgdorferi* ss murine infection; moreover, current Lyme diagnostic test could not differentiate infections. While *I. scapularis* could acquire *B. bissettii* spirochetes from infected mice, they existed at lower burdens compared to *B. burgdorferi* ss. *Ixodes scapularis* was also unable to transmit *B. bissettii* infection to susceptible animals, incriminating a different tick vector in the eastern

and southern US. These data support the need for continued work on the Lyme *Borrelia* species as a whole, especially in non-traditional areas and on understudied organisms.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. General Borreliology

In this section, information is presented that describes the characteristics of the bacteria within the *Borrelia* genus. More specifically information will be provided about the bacteria in the *Borrelia burgdorferi* sensu lato (sl) species complex (referred to as Lyme *Borrelia*) including basic biology and genetics. Following this overview, the current understanding of the Lyme *Borrelia* transmission cycle will be presented. While European and Asian Lyme *Borrelia* will be mentioned when relevant, focus will be on the Lyme *Borrelia* in North America. As such, the ecology and genetic diversity of North American Lyme Borreliosis will be described followed by a specific description and distribution of each of the Lyme *Borrelia* currently described in the United States. Next the mechanisms Lyme *Borrelia* utilize throughout infection in both the vector and vertebrate will be presented. Followed by the epidemiology of Lyme borreliosis in the United States and a review of the clinical disease caused by Lyme *Borrelia* infection including murine models of study and current methods in diagnostics.

The genus *Borrelia* are within the family Spirochaetacea under the order Spirochaetales. *Borrelia* are gram-negative spirochete bacteria [3]. Like other spirochetes they are uniquely defined by their high motility and flat-wave morphology due to several periplasmic flagella [3]. The *Borrelia* spirochetes are also unique in that they have a double membrane, which classifies them as gram-negative bacteria, yet lack the classical lipopolysaccharide and instead produce a slew of outer membrane associated lipoproteins [4, 5]. Spirochetes in the *Borrelia* genus share genetic similarities, which include a linear chromosomes ~900 kilobases in size which contains many housekeeping genes and is

conserved to gene composition and arrangement between *Borrelia* species [6]. Very unique to tis genus is the amount of plasmids these bacteria can harbor. To date, *B. burgdorferi* ss strain B31 has a recorded 21 plasmids [4]. Plasmids for these bacteria harbor many of the virulence and infection associated genes they also harbor many pseudogenes which are believed to be evidence of genetic decay. Surprisingly these plasmids can add an additional 400-650 kilobases to the spirochete's genome [7].

While the chromosome contains many housekeeping genes and is fairly conserved in regards to gene content and organization amongst the *Borrelia* spirochetes, the plasmids are highly variable and contain many psudo-genes and genes associated with virulence and the organism's specific enzootic cycle [6-8]. Although similar in many aspects, the *Borrelia* genus can be further divided into two major groups: the Relapsing fever *Borrelia* and the Lyme *Borrelia*.

1.1.1. Relapsing Fever *Borrelia*

The majority of relapsing fever borreliae are transmitted by ticks from the family Argasidae, commonly referred to as the soft ticks [6]. A single relapsing fever *Borrelia* is strictly associated with a louse vector. Recently a group of Ixodidae, or hard-tick, associated relapsing fever *Borrelia* have also been described [6, 9]. The relapsing fever *Borrelia* group also contain newly recognized reptilian associated species [10]. These *Borrelia* are distributed worldwide and infect a variety of animals [6]. Some of the spirochetes have been shown to be horizontally transmitted between vector and host, and vertically from female tick to pyrogeny [11, 12]. Clinically the relapsing fever *Borrelia* cause a relapsing fever induced by spirochetemia. This is roughly characterized by an initial three-day fever followed by a seven day relapse and then another three days of fever, this cycle repeats multiple times,

hence the group's name [13]. Louse borne relapsing fever was a disease of concern prior to the 17th century but is mainly eliminated today (except for areas of Africa) [14]. Tick borne relapsing fever is still a cause of human disease worldwide; however, case numbers pale in comparison to the Lyme *Borrelia*.

1.1.2. The Lyme *Borrelia* (*Borrelia burgdorferi* sensu lato)

Like relapsing fever *Borrelia*, Lyme *Borrelia* have been shown to infect many types of animals; however, they are only known to be transmitted by ticks from the Ixodidae family [15]. And while Lyme borreliae spirochetes most likely predate humans [16], Lyme borreliosis has only recently emerged as a significant cause of human morbidity [17]. *Borrelia burgdorferi* sensu stricto (ss) was first identified in 1982 [18]. Three decades later another 18 species of closely related spirochetes are now recognized to form the species complex, *Borrelia burgdorferi* sensu lato (sl) [19]. Members of this complex are distributed across the globe and have been identified on almost every continent (Figure 1.1). Twelve of

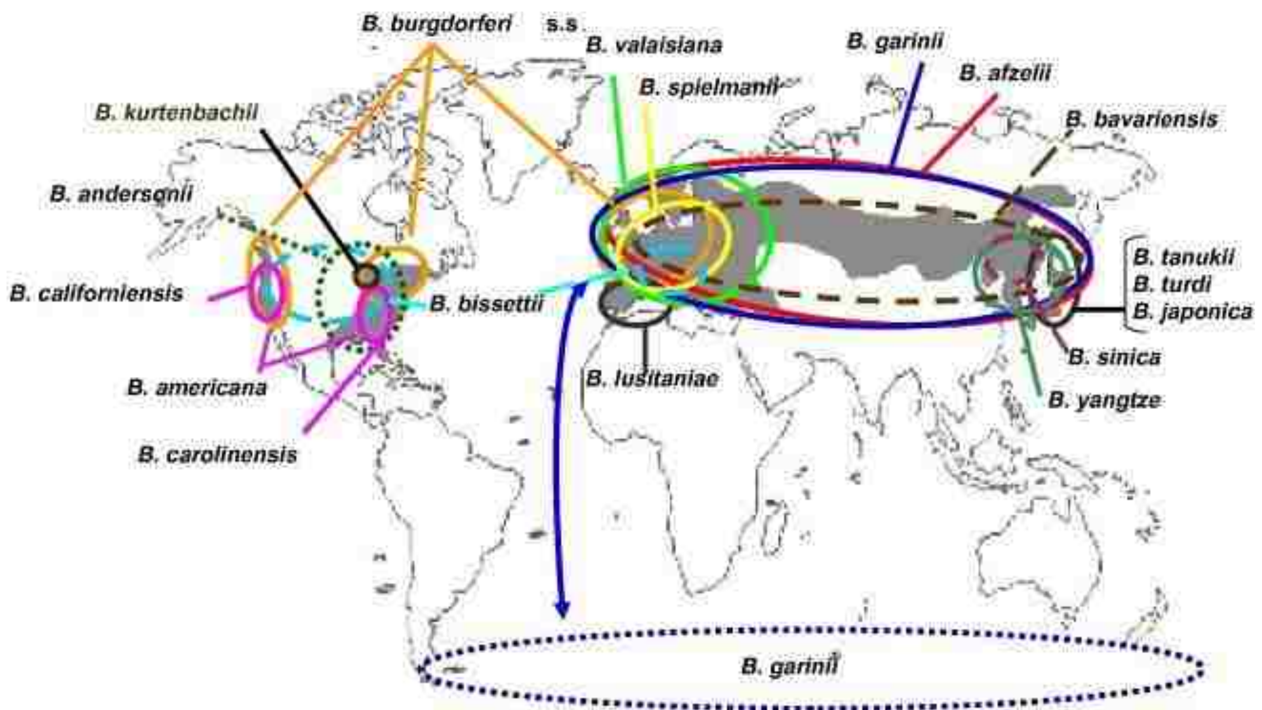

Figure 1.1. Worldwide geographic distribution of *Borrelia burgdorferi* sensu lato genotypes [1].

these spirochetes have been implicated in human illness, while pathogenicity of the six others is still unknown (Table 1.1). Currently seven species of *B. burgdorferi* sl have been described in the United States, yet only *B. burgdorferi* ss has widely been considered pathogenic to humans (Table 1.1). All the North American *B. burgdorferi* sl spirochetes are confirmed to be vectored by *Ixodes* genus ticks (Table 1.1). The vectoral capacity of other North American Ixodidae genera ticks has not yet been demonstrated.

1.2. Lyme *Borrelia* in the United States

Seven species of *B. burgdorferi* sl have been described in the United States. They are: *Borrelia americana*, *Borrelia andersonii*, *B. burgdorferi* ss, *Borrelia bissettii*, *Borrelia californiensis*, *Borrelia carolinensis* and *Borrelia kurtenbachii*. *Borrelia burgdorferi* ss is currently considered the only pathogenic Lyme *Borrelia* in the United States (Figure 1.2); however, evidence is growing that implicates more of these species in human illness (see probable pathogens [P] in Table 1.1). Here the ecology of Lyme *Borrelia* in the United States will be discussed followed by an overview of the current knowledge of each of the North American *B. burgdorferi* sl.

1.2.1. Ecology of Lyme *Borrelia*

Lyme *Borrelia* bacteria exist in enzootic cycles in nature. These cycles include vector ticks, reservoir animals and the bacteria itself. Many *Borrelia* have associations with certain vectors and specific enzootic cycles [15]. Because of these associations, the bacteria are often limited to hosts parasitized by the tick, which plays an important role in the bacterium's lifecycle. Defining the ecology of Lyme disease is a Sisyphean task.

Table 1.1. Currently recognized *Borrelia burgdorferi* sl species with their human pathogenic classification, distribution, known host species and tick vectors [1].

Species (type strain)	Human Pathogen	Distribution	Host	Main vector	References for description
B. afzelii (VS461)	C	Europe, Asia	*Apodemus* spp., *Myodes glareolus*, *Sorex* spp., *Sciurus* spp., *Erinaceus* spp., *Rattus* spp.	*Ixodes ricinus,I. persulcatus,I. hexagonus*	[21]
B. americana (SCW-41)	P	North America	*Thryothorus ludovicianus, Pipilo erythrophthalmus*	*I. pacificus,I. minor*	[22]
B. andersonii (21038)	P	North America	*Sylvilagus* spp., (Passeriformes)	*I. dentatus*	[23]
B. bavariensis (PBi)	P	Europe, Asia (?)	*Apodemus* spp., *Myodes* spp., Microtus spp.	*I. ricinus,I. persulcatus(?)*	[24]
B. bissettii (DN127-cl9-2)	P	North America, Europe	*Neotoma* spp., *Peromyscus* spp., *Sigmodon* spp., EU: unknown	*I. pacificus,I. scapularis,I. spinipalpis,I. affinis,I. minor,*EU*:* unknown	[25]
B. burgdorferi (B31)	C	North America, Europe	*Peromyscus* spp., *Tamias* spp., *Neatoma* spp., *Sorex* spp.,*Sciurus* spp., *Sigmodon* spp., *Erinaceus* spp., *Rattus* spp., *Procyon lotor, Turdus migratorius*	*I. ricinus,I. hexagonus, I. scapularis,I. pacificus,I. affinis,I. minor,I. spinipalpis,I. muris*	[26]
B. californiensis (CA446)	U	Western US	*Dipodomys californensis*	Unknown	[27]
B. carolinensis (SCW-22)	U	Southeast US	*P. gossypinus, N. floridana*	Unknown *(I. minor?)*	[28]
B. finlandensis (SV1)	U	Finland, Norway	*Lepus timidus*	*I. ricinus*	[29]
B. garinii (20047)	C	Europe, Asia, Artic-Antarctic circles	*Turdus merula*, *T. philomelos*, *Parus major*, seabirds (Puffin, Guillemot, Kittiwake, Razorbill)	*I. ricinus,I. persulcatus,I. uriae*	[30]
B. japonica (HO14)	P	Japan	*Sorex unguiculatus*, *Apodemus* spp., *Eothenomys smithi*	*I. ovatus*	[31, 32]

Table 1.1. cont.

Species (type strain)	Human Pathogen	Distribution	Host	Main vector	References for description
B. kurtenbachii (25015)	P	North America, (Europe?)	*Microtus pennsylvanicus*, *Zapus hudsonius Peromyscus* spp.?	Unknown *(I. scapularis?)*	[2]
B. lusitaniae (PoTiB2)	C	Mediterranean basin	Lacertidae	*I. ricinus*	[33]
B. sinica (CMN3)	U	China	*Niviventer confucianus*	*I. ovatus*	[34]
B. spielmanii (PC-Eq17N5)	C	Europe	*Glis glis, Eliomus quercinus*	*I. ricinus*	[35]
B. tanukii (Hk501)	U	Japan	*Apodemus* spp., *Clethrionomys rufocanus, Eothenomys smithii*	*I. tanuki*	[36-38]
B. turdi (Ya501)	U	Japan	*Turdus* spp.	*I. turdus*	[36-38]
B. valaisiana (VS116)	C	Europe, Japan	*Turdus merula, T. philomelos, Parus major*	*I. ricinus,I. columnae*	[39]
B. yangtze (nd)	U	China	*Niviventer fulvescens, Apodemus* spp.	*I. granulatus, I. nipponensis*	[40]

*C=confirmed; P=probable; U=unknown

Many factors are known to contribute to the disease, and large ecological studies admit there are confounding unknown factors [20]. A general description of Lyme *Borrelia* ecology is best illustrated with the well-studied Northeastern *B. burgdorferi* sensu stricto-*I. scapularis* enzootic cycle.

Ixodes scapularis, commonly known as the "black-legged tick", are forest dwelling arthropods. They make use of the microhabitat found in the dense underbrush and leaf litter as they are highly susceptible to desiccation [41]. Once upper level environments are right, the tick climbs (quests) twigs, grass or plants and utilizes sensory organs (haller's organs) to detect movement, heat, carbon dioxide with the ultimate goal of latching onto animals that pass by [42]. The tick requires three bloodmeals to complete its lifecycle, which is represented by four stages: the egg, larvae, nymph and adult. As one can imagine, availability of suitable hosts are essential for the lifecycle of the tick; this is also critical for the lifecycle

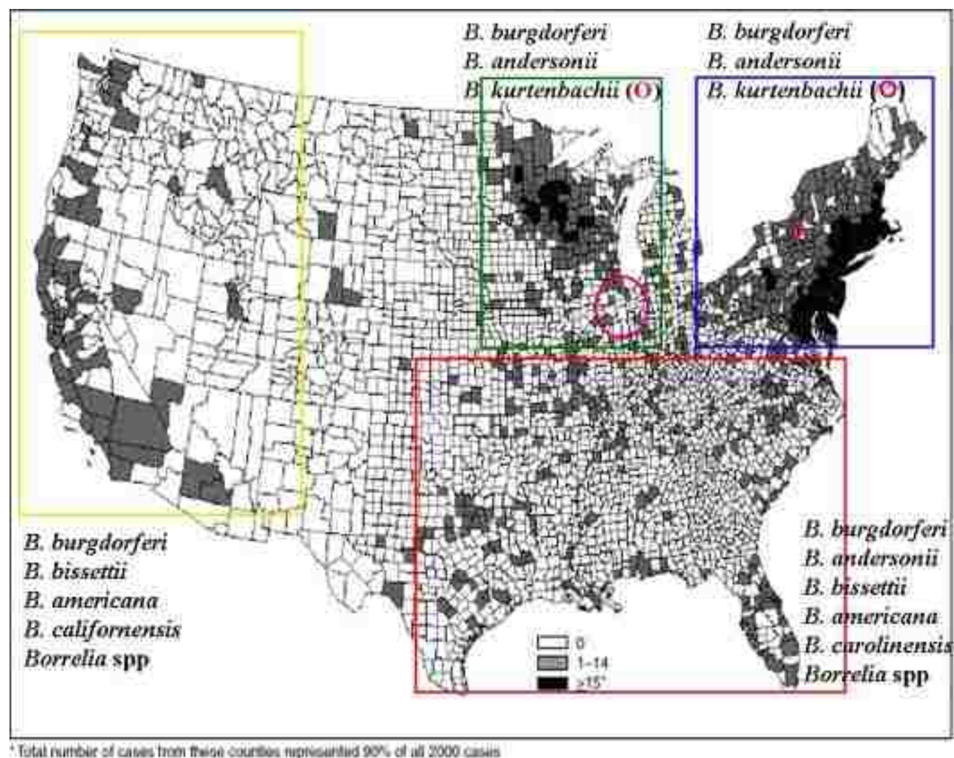

Figure 1.2. Distribution of North American Lyme *Borrelia* [2].

of the *Borrelia* spirochete. *Borrelia burgdorferi* sl spirochetes are not passed from mother to pyrogeny (transovarial); therefore, acquisition of the spirochete must come from an infected bloodmeal [12]. This requires susceptible and/or infected animals. *Ixodes scapularis* is a generalist parasite, meaning it will feed on a slew of different hosts, yet each life stage has a common host repertoire [43, 44]. For example, the larval and nymphal stages are more likely to be found feeding on small rodents (such as *Peromyscus* spp., mice) while the adults feed most often on deer [44]. This is important because it is believed that prior to major deforestation and subsequent devastation of natural deer populations, by hunting and habitat destruction by European settlers in the eastern US, both *I. scapularis* and *Borrelia* were widespread [45]. The emergence of Lyme is thought to coincide with the reforestation and deer population rebound in the 20th century and their close association with human populations [46]. These ideas are supported by experimental studies removing deer from Lyme endemic areas subsequently decreasing the abundance of infected ticks [47-49]. After deer populations rebounded, refugee population of *I. scapularis* and *Borrelia* "exploded" and today we have a Lyme epidemic. In areas like the Northeast US, small mammals (*Peromyscus* spp and *Sorex* spp) are the animal reservoirs for the Lyme bacteria [50]. Immature ticks (larva and nymph) feed on these animals and after molt are able to transmit the infection to other animals, perpetuating the cycle, or to humans, causing disease [50]. Humans are considered accidental and dead-end host for the bacteria. *Borrelia* does often exist in areas where major human vectors are not established or in low density. In these areas, the *Borrelia* spirochete is maintained in cryptic cycles, which involve reservoir animals and highly competent host-specific vector ticks (which rarely feed on humans) [51, 52]. These enzootic cryptic cycles perpetuate infectious *Borrelia* spirochetes; however, if a human biting

vector were to establish, the bacteria would eventually become epizootic and “spill out” into the human population causing illness. This is currently the case in many parts of North America [53, 54]. Active surveillance of vector tick populations and *Borrelia* prevalence in hosts and ticks is essential to understanding the evolving human risk in traditionally non-endemic areas of Lyme disease.

1.2.2. Genetic Diversity of Lyme *Borrelia*

Lyme *Borrelia* are genetically very fascinating. In 1997, the genome of *Borrelia burgdorferi* (B31 strain) became one of the first microbial genomes to be fully sequenced [5]. Since the advent of next generation sequencing, the total number of publically available genomes within the *B. burgdorferi* sl species complex at this time stands at eight complete (chromosomes & plasmids), three chromosomes (only), 14 plasmid profiles and another 66 scaffold/contigs (http://www.ncbi.nlm.nih.gov/genome/?term=borrelia). The genome of Lyme *Borrelia* spirochetes all consist of a linear chromosome around 900 kilobases in size. This chromosome is highly conserved in both gene content and order between the different *B. burgdorferi* sl species; this is due to the fact it contains many essential housekeeping genes [55]. In addition to the chromosome, Lyme *Borrelia* spirochetes contain a repertoire of linear and circular plasmids ranging from 9 kb to over 50 kbs which can add up to an additional 600 kbs of DNA [7]. Some strains contain up to 21 of these plasmids [7]. The plasmids themselves contain the majority of the genetically diverse genes, many of which are involved in the bacteria’s pathogenesis, tick-host lifecycle, and a host of pseudo-genes thought to be a sign of genetic decay [4, 7, 8]. As previously mentioned, the *B. burgdorferi* sl complex consists of 19 currently recognized species, seven of which have been identified in the United States. Multilocus sequence typing (MLST) and whole genome phylogenetics have

allowed for the delineation of the phylogenetic structure of the complex (Figure 1.3). This has immensely aided in the identification of shared and unique genes between and within species, which has ultimately facilitated in developing better diagnostic tests and identifying potential vaccine targets.

1.2.2.a.*Borrelia americana*

Spirochetes now recognized as *B. americana* were first isolated in 1993, by Schwan et al. from pools of adult *I. pacificus* [56]. The two isolates (CA-8-89, CA-29-91) were later determined by multilocus sequence analysis to represent an unknown species of *Borrelia* and temporarily named genomospecies 1 by Postic et al. [27]. In 1994, spirochetes (*B. americana*) were isolated from nymphal *Ixodes minor* ticks collected off Carolina wrens (*Thryothorus ludovicianus*) and Eastern towhees (*Pipilo erythrophthalmus*) in Charleston, South Carolina [57, 58]. It wasn't until 2009 that the *B. americana* species was delineated after multiple strains were subjected to multilocus sequence analysis [22]. Like many of the North American Lyme *Borrelia*, little is known about this group of spirochetes. The bacteria has been detected in California and South Carolina, in *I. minor* and *I. pacificus* ticks [56, 57, 59]. Its suggested reservoirs are birds and small mammals. Human pathogenicity is unknown; however, the organism has been detected in human clinical samples by molecular methods [60, 61]. To date no laboratory infection studies have been published. The type strain is SCW-41.

1.2.2.b.*Borrelia andersonii*

Borrelia andersonii was first isolated in 1989 by Anderson et al. from *Ixodes dentatus* ticks and a rabbit kidney in New York [62]. Marconi et al. (1995) proposed the delineation of these isolates from *B. burgdorferi* ss by sequence polymorphisms in the rRNA genes, and

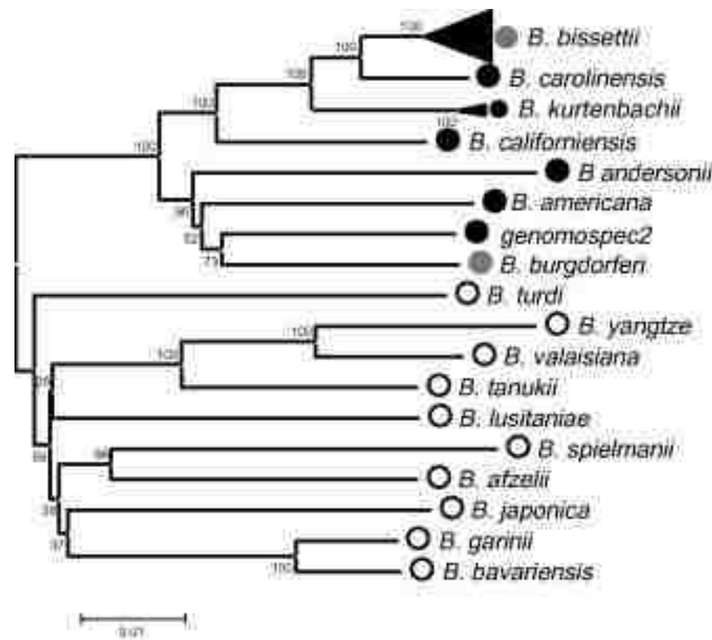

Figure 1.3. Neighbor-joining tree using concatenated MLSA genes showing the phylogenetic relationship of Lyme *Borrelia* group spirochetes. Black dots indicate species that occur in North America, circles are species that occur in Eurasia and grey don't are species that occur in both old and new world [1].

named the species after its discoverer Dr. John Anderson [23]. These conclusions were supported by Lin et al. (2001) when even more *B. andersonii* strains, which were isolated from ticks feeding on rabbits in Missouri, were added to the phylogenetic analyses [63]. *Borrelia andersonii* has been identified in multiple tick species, including: *Ixodes dentatus*, *Amblyomma americanum*, *I. scapularis* and *Haemaphysalis leporispalustris* [23, 64].

Field work suggest the reservoirs for *B. andersonii* are cotton tail rabbits, birds and possibly lizards [23, 63, 65]. *Borrelia andersonii* has been detected in multiple states in the US (FL, SC, GA, MO, MI, NY) [15]. Only one study has assessed the ability of *B. andersonii* (termed *B. burgdorferi* ss in the paper). Anderson et al. found 0 of 3 mice to be culture positive for *B. andersonii* (Strain 48080) spirochetes [66]. The pathogenic potential of *B. andersonii* to humans is unknown; however, it has been molecularly detected in human clinical samples [60, 61]. The type strain is 21038.

1.2.2.c.*Borrelia bissettii*

Spirochetes now known as *Borrelia bissettii* were first isolated in 1987 by Bissett and Hill from an *I. pacificus* tick [67]. In 1998 after the repeated isolation of more phenotypically similar spirochetes, Postic et al. described the group as a novel Lyme *Borrelia* spirochete, *B. bissettii* [25]. The range of this group of spirochetes is quite large, and like *B. burgdorferi* ss is a Lyme *Borrelia* spirochete extensively described both in the New and Old World. *Borrelia bissettii* spirochetes have been detected in many states across the US, including SC, GA, FL, NC, MI, LA, CO, CA [58, 59, 65, 68-70]. While *I. scapularis* is a suspected vector, both *I. pacificus* and *I. spinipalpus* have been experimentally demonstrated competent vectors [51, 71]. *Borrelia bissettii* has been described in hosts, including small rodents and lizards [51, 65, 72]. *Borrelia bissettii* has been implicated by numerous studies in human illness, and one study has investigated *B. bissettii* infection and pathology in murine models of borreliosis [73]. Mice develop pathology within the bladder, heart and in some cases femorotibial joint similar to *B. burgdorferi* ss. *Borrelia bissettii*-like spirochetes have been isolated from human patients in Europe, and detected by polymerase chain reaction in serum, blood, skin and cardiac tissue in both the US and eastern Europe [74-77]. The type strain is DN127.

1.2.2.d.*Borrelia burgdorferi* sensu stricto

Borrelia burgdorferi was the first Lyme *Borrelia* spirochete described. It was discovered by researchers in 1982 [18]. Initially isolated from an *I. scapularis* tick from Connecticut, the spirochete was formally described by Johnson et al. in 1984 and named after one of the researchers that discovered it, Dr. Willy Burgdorfer [26]. The spirochete is distributed worldwide and is the most commonly implicated *Borrelia* spirochete in human

Lyme borreliosis cases in the US [78]. It is a generalist spirochete and has been detected in a plethora of animal and tick species [15]. Its major vectors in the US are *I. scapularis* (Eastern US) and *I. pacificus* (Western US). *Borrelia burgdorferi* ss also exists in cryptic cycles. These cycles involve reservoir hosts and nidicoulus ticks that rarely encounter humans. These cycles can be extremely important as they facilitate the perpetuation of *B. burgdorferi* ss infection in wildlife [51, 79, 80]. The type strain of *B. burgdorferi* ss is B31.

1.2.2.e.*Borrelia carolinensis*

Between 1994 and 1997, peculiar *Borrelia* isolates were cultured from multiple small rodents (*Peromyscus gossypinus* and *Neotoma floridana*) and an *I. minor* tick in South Carolina. Sixteen of these isolates were initially classified as *B. bissettii* based on restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE) [57, 58, 63, 81]. In 2009 Rudenko et al., utilizing MLST, defined these isolates as a distinct *Borrelia* species [28, 82]. Because all isolates were isolated in South Carolina, the bacteria was formally named *Borrelia carolinensis*. *Borrelia carolinensis* has also been detected in France in *I. ricinus* and in southern California in a cryptic cycle involving the endangered Armargosa vole (*Microtus californicus scirpensis*) and *I. minor* ticks [83, 84]. To date no experimental infection work has been completed on spirochetes from this species. Reservoir and vector data is solely based on survey work. *Borrelia carolinensis* has never been described in human clinical samples. The type strain is SCW-22.

1.2.2.f.*Borrelia californiensis*

Borrelia californiensis spirochetes were first isolated by Postic et al. in 1998 from a *Dipodomys californicus* [25]. In 2007, MLSA was utilized to identify the spirochete as a novel species [27]. *Borrelia californiensis* has only been detected in California in kangaroo

rats, mule deer and in three species of ticks (*I. pacificus*, *Ixodes jellisonii*, and *I. spinipalpus*) [25, 27, 85]. Its pathogenicity to humans is unknown, the type strain is CA446.

1.2.2.g.*Borrelia kurtenbachii*

Borrelia kurtenbachii was first isolated in 1987, in upstate New York, from an engorged *I. scapularis* larvae collected off a *Peromyscus lucupus*. Anderson et al. described it as an infectious but non-pathogenic *Borrelia* spirochete after testing it in an experimental mouse model [86]. A few years later, Fikrig et al. noted mild pathology when utilizing the spirochete to test a recombinant *ospA* vaccine [87]. A decade after its isolation, strain 25015 was named and categorized as a *Borrelia bissettii* spirochete because of its similarity by RFLP of its 5s-23s intergenic spacer region and 16s rRNA sequences [88]. In 2010, Margos et al. utilized MLSA and classified strain 25015 and others like it as a new species, the species was named after the late Dr. Klaus Kurtenbach [2]. Studies in the mid 1990's by Strle et al. and Picken et al. described isolation of spirochetes similar to strain 25015 from patients in Slovenia [89, 90]. Because further analysis has yet to be done on these isolates we can only speculate that this spirochete is a probable pathogen. The type strain is 25015.

1.3. Mechanisms of Lyme *Borrelia* Infections in Vectors and Vertebrates

Lyme *Borrelia* require both ticks and hosts to perpetuate in nature. Hard ticks have four distinct lifestages: the egg, larva, nymph and adult. After egg hatching a bloodmeal is required between each life stage. There is no transovarial transmission from female to egg; therefore, larval ticks are never infected with Lyme *Borrelia* [12]. Infection must be acquired through feeding on an infected host and to a lesser extent through co-feeding with infected ticks [50, 91]. Once infected, the tick will carry the infection transstadially (through molt) allowing competent vectors to be infected for life [92].

1.3.1. Tick Acquisition of Lyme *Borrelia*

When an uninfected larva feeds on an infected host, acquisition of spirochetes happen rapidly (within 12-24 hours) and by 48 hours post tick attachment, large numbers of spirochetes can be visualized in the tick midgut [93]. Colonization of *Borrelia* in the larval tick is understudied; however, it is understood that certain spirochetal proteins contribute to the successful colonization of the larva [94]. Once in the tick's midgut, spirochetes persist through bloodmeal digestion. This poses a challenge for the bacteria as the tick forms a temporary peritrophic membrane around its ingested meal [42]. Digestion of bloodmeal components is mostly intracellular, but it has been shown that byproducts of digestion may have an effect on the extracellular bacteria [95, 96]. After feeding is complete and bloodmeal digestion has occurred, the spirochetes face another harsh environment, the molt. Spirochetes persist to the next developmental stage in competent vectors, and during molt, yet tick gut epithelia cells store many macromolecules in endosomes making access to nutrients difficult for the extracellular spirochetes [42]. This environment also exposes spirochetes to temperature fluxes as seasons change. Therefore, *Borrelia* genomes contain many plasmid associated genes have been identified that facilitate colonization and persistence in the vector. The most studied of these include *ospA*, *ospB* and *bptA* [97-99]. Both *ospA* and *ospB* are co-transcribed on a single promotor and recombinant forms of these protein have been shown to bind to tick midgut [99, 100]. It has been shown that disrupting these *osp* genes does not affect acquisition but instead survival during the tick's digestion of the bloodmeal [99, 100]. *OspA* has been shown to have a more contributory effect to this than *ospB*. Indeed a specific receptor, tick receptor for *ospA* (TROSPA), has been described in the *I. scapularis* midgut [101]. *Borrelial* persistence in ticks *A* (*BptA*) is another gene that encodes a surface

exposed lipoprotein which has been shown to be essential for spirochete survival through larval molt. Recently chemotactic genes have been identified in the *B. burgdorferi* ss genome and work is currently being conducted to determine the importance these have for tick acquisition [102].

1.3.2. Tick Transmission of Lyme *Borrelia*

Transmission from an infected tick to a host has been studied intensely, as this is a step where vaccine development has sought to exploit. With the development of *in vivo* imaging, kinetics of *Borrelia* dissemination in engorging ticks has been extensively teased apart. An infected tick has a population of spirochetes in its midgut, presumably situated in the ectoperiotropic space [103]. During the bloodmeal, spirochetes in an infected tick undergo rapid cellular division, which increases the spirochete population in the tick some 100-fold and in turn produces a phenotypically heterogenous population [103]. This diversity allows a percentage of the spirochetes to penetrate the midgut epithelial wall. How this migration is accomplished is currently under debate. It has be suggested that active penetration by motile spirochetes may facilitate this process [104, 105], yet a more recent study suggests nonmotile spirochetes accomplish penetration via aggregated adherence to tick gut epithelial cells [103]. Only late into feeding do spirochetes become motile and traverse the hemolymphatic system. An even smaller percentage penetrates the salivary glands where they are subsequently transmitted to the host. This results in spirochete population bottlenecking and only the most fit organisms are transmitted to carry on the infection cycle [103].

Interestingly it is common that non-cultivatable spirochetes are detected in infected tick feces during bloodmeal digestion [106], but despite this, many spirochetes remain in the lumen, survive through molt, and hypothetically have the ability to be transmitted by the adult stage.

1.3.3. *Borrelia* Dissemination in the Vertebrate

Borrelia infection occurs after the bite of an infected tick. Spirochetes enter the host's tissue during the blood meal and encounter components of the host's immune system. Initial attachment of Lyme *Borrelia* to host tissue is facilitated by integrins on the spirochetes surface which bind to human platelets and endothelial cells [107]. To increase its chances of transmission from one host to another, via an arthropod, the spirochete will persist in the host's blood and tissue near the site of inoculation as long as possible [108]. Spirochetes then begin to disseminate through local tissue matrices by a number of processes. Lyme *Borrelia* possess decorin-binding proteins that are hypothesized to promote spread through the collagen/decorin-rich dermal tissue [109]. Lyme spirochetes are also known to bind human plasminogen, which is subsequently cleaved into plasmin in the presence of urokinase-type plasminogen activator (uPA) [110]. It is hypothesized that this membrane-bound plasmin may degrade fibronectin and release/activate matrix metalloproteases (MMPs) to help the spirochete disseminate to tissues via a transient spirochaetaemia [111, 112]. Because *Borrelia* are a very slow replicating bacteria, immune evasion is essential for the dissemination and survival of the bacteria. *Borrelia* accomplish this feat utilizing a combination of tick salivary factors and a repertoire of their own immune evasion processes.

1.3.4. Tick Saliva and Host Immunomodulation

The bloodmeal of the hard tick can last several days [42]. Because of this, the tick releases components in its saliva which facilitate successful feeding [113, 114]. These

components include molecules that inhibit hemostasis, block pain, affect angiogenesis and wound healing. Most importantly for *Borrelia*, tick saliva contains potent immunomodulators of the immune system [113, 114]. *Borrelia* spirochetes exploit many of these components by utilizing tick salivary anti-complement proteins (Isac, Salp20 & TSLPI), antioxidents (Salp25D & ISL1373) and immune suppressing proteins (Salp15) to facilitate establishment in the host [6, 115-117]. These exploits are important, as immune competent hosts elicit a strong immune response to infection with *Borrelia* spirochetes [118]. Many tick-borne pathogens have enhanced transmission in the presence of salivary components [119]. *Borrelia* spirochetes are no exception [120].

1.3.5. Mechanisms of Complement Evasion

Complement, an innate immune system component, is one of the first hurdles *Borrelia* spirochetes encounter upon entering a host. Complement plays a critical role in innate defense against bacterial pathogens and utilizes over 30 serum and cellular proteins to promote bacterial phagocytosis and lysis via opsonization and porin formation [121]. It is an enzymatic process which is activated by three distinct pathways: alternative, classical, and lectin [121]. To combat the complement threat *Borrelia* spirochetes not only utilize tick salivary secreted anti-complement proteins, but also have a repertoire of lipoproteins (complement regulator acquiring surface proteins [CRASPS]) which bind components of the serum factor H family [122]. These proteins inhibit alternative pathways activation. *Borrelia* also recruit C4b-binding protein which inhibit the classical and lectin pathways of complement [123].

Utilizing the aforementioned strategies, *Borrelia* are very successful in evading being killed by complement; however, these mechanisms have been shown to be host/*Borrelia*-

species specific, as many *Borrelia* species differ in their ability to survive in the presence of different host serum [124-128].

1.3.6. Innate Immune Recognition of *Borrelia* Lipoproteins

Initial cellular recognition of bacterial pathogens is accomplished through pattern recognition receptors (PRRs) like toll-like receptors (TLRs), nod-like receptors (NLRs) and c-type lectin receptors (CLRs). PRRs directly recognize bacterial pathogen associated molecular pattern (PAMPs). These receptors play an indispensable role in aspects of initial response to insult (innate immunity) and eventually the bacterial specific adaptive response of the immune system. Recognition of *B. burgdorferi* ss lipoproteins through TLR2 [129], NOD2 [130] and TLR8 when internalized in phagolysosomes [131] all play a critical role in recognition spirochetes and promotion of downstream immune system modulators such as pro-inflammatory molecules, cytokines and chemokines.

1.3.7. Lyme *Borrelia* and Phagocytes

Phagocytes also play a role in host defense against *Borrelia* infection. Monocyctes, macrophages, neutrophils and glial cells have all been identified as potent phagocytes of *B. burgdorferi in vitro* [132, 133]. *Borrelia* spirochetes are very motile and are able to escape phagocytosis, yet when unable, phagosomal digestion of the bacteria is a potent signal for downstream inflammatory cytokines [131].

1.3.8. Lyme *Borrelia* Interactions with T-cells, B-cells and Antibodies

T-cell involvement in Lyme borreliosis is still a young area of study. Mouse studies have demonstrated that CD4+ T cells play a role in controlling spirochatemia, while CD8+ T cells seem to promote disease process. Development of a specific anti-*Borrelia* humoral response is critical to clearing the pathogen [134]. Early production of IgM by B-Cells helps

control initial spirochete burdens [135] while B-cell antibody class-switching and production of specific IgG is quickly initiated and can be detected two weeks post infection. To counter antibody mediated killing *Borrelia* spirochetes have been demonstrated to employ an antigenic variation system, which begins with the initial down regulation of the major surface lipoprotein *ospC* and then the activation of the *vlsE* locus, which uses a repertoire of transposable antigenic cassettes to change its surface lipoprotein coat aiding in evasion of the host immune response [136-138].

1.4. Lyme Disease (Borreliosis)

Lyme borreliosis most likely plagued humans before it was discovered in the late 20th century. Historical records describe diseases such as "Montauk knee" afflicting people in New York and tick associated skin rashes in Europe. *Borrelia burgdorferi* has also been identified in a 5,300 year old ice man discovered in the Eastern Alps [139-141]. Recently, *Borrelia* spirochetes predating humans have also been identified in a 15 million year old amber-fossilized *Amblyomma* spp. tick discovered in South America [16]. However, clinical Lyme borreliosis was first described in the United States in 1977 by Steere et al., only after a concerned citizen recognized the large number of children afflicted by what was supposed to be a rare disease, Juvenile Rheumatoid Arthritis [142]. Dr. Alan Steere was a medical doctor studying arthritis at Yale medical school when he was contacted to look into the peculiar occurrence. Ticks from the area were coincidently being sent to Rocky Mountain Labs where Dr. Willy Burgdorfer was examining them for the presence of *Rickettsia*. It was there that Dr. Burgdorfer noticed spirochete bacteria in the tick's midgut. Five years later, *B. burgdorferi*, the causative agent of Lyme borreliosis was formally described [18].

1.4.1. Epidemiology of Lyme Borreliosis in the United States

Since the discovery of Lyme, the incidence of human disease has steadily increased (Figure 1.4) [78, 143]. Currently the Centers for Disease Control (CDC) estimates almost 300,000 cases of Lyme are diagnosed every year [144, 145]. The majority of Lyme disease is reported from two regions of the US, mainly the Great Lake states and the Northeast (Figure 1.5). For example in 2012, 95% of the total human Lyme cases were reported from 13 states (Table 1.2). Lyme borreliosis has a bimodal age distribution peaking in children age 5-10 and adults 40-55 years old (Figure 1.6). Lyme affects both genders equally (53% in males) but

Table 1.2. Number of reported Lyme disease cases in 2012 reported to the CDC by State.

does overwhelmingly affect Caucasians (94% of all reported cases) [78]. Disease is more common when vector ticks are active: June-August (Figure 1.7). The risk of contracting the disease increases with the amount of time spent outdoors in or near wooded areas [146].

1.4.1.a.Southern Lyme Borreliosis

Occurrence of human Lyme borreliosis outside of the traditional hyperendemic zones (Northeast and Great Lakes) is a topic of great debate. This is especially true in areas where the vector tick occurs in abundance yet human cases of Lyme occur infrequently. Areas like the southern US have traditionally supported large populations of *I. scapularis* [147] yet, as seen in Table 1.2, report relatively few cases of human Lyme yearly. Hypotheses attempting to explain this phenomenon have existed for decades; however, the scientific community has yet to pinpoint the exact mechanism. One observation widely accepted is that the *I. scapularis* nymph is responsible for transmitting the majority of Lyme *Borrelia* to humans. This is supported in part by the seasonal overlap of disease occurrence and phenology of the nymphal ticks in hyperendemic regions [148].

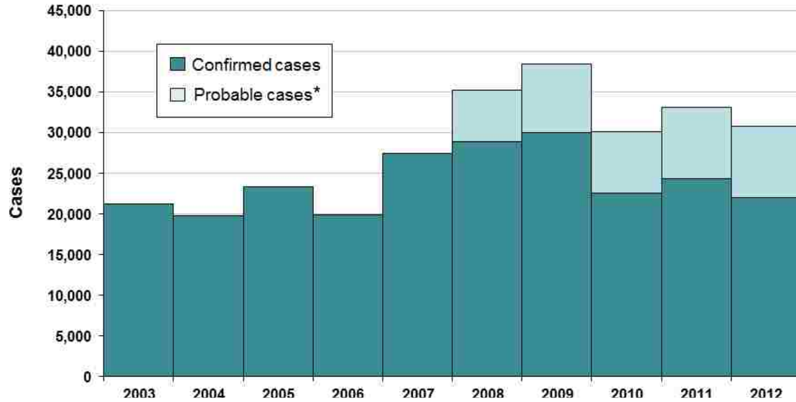

Figure 1.4. Number of human Lyme disease cases reported to the CDC yearly from 2003-2012. *Case definition was changed in 2008 to delineate probable cases from confirmed cases. http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html

Figure 1.5. Lyme disease cases reported in the US in 2012. One dot placed randomly within the county of residence for each confirmed case of human Lyme borreliosis. http://www.cdc.gov/lyme/stats/maps/map2012.html

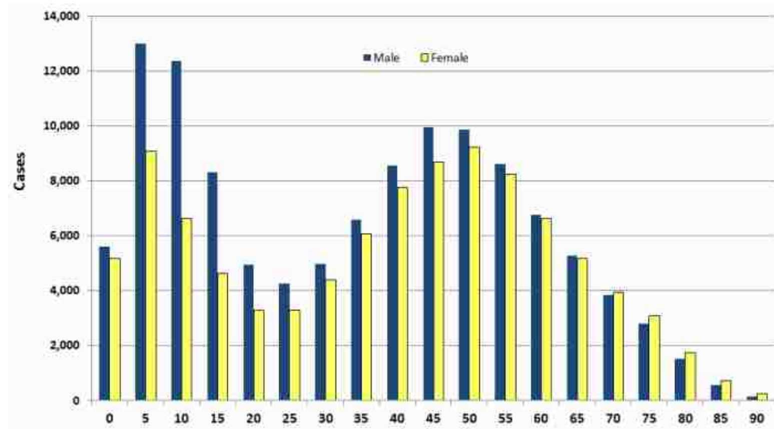

Figure 1.6. Cases of Lyme disease reported to the CDC from 2001-2010 defined by age and gender. http://www.cdc.gov/lyme/stats/chartstables/incidencebyagesex.html

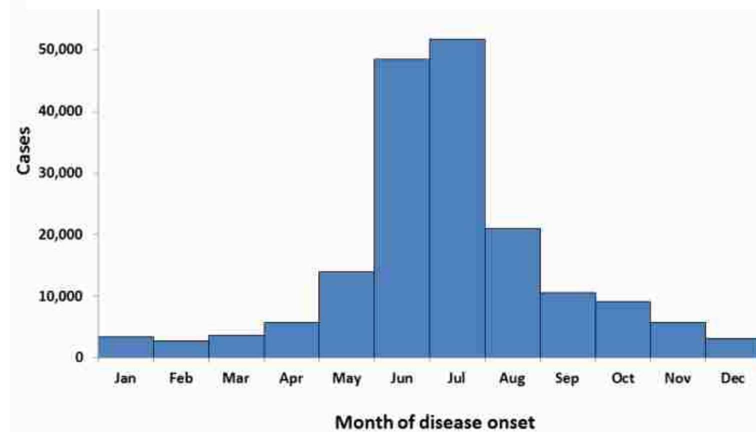

Figure 1.7. Month of disease onset as reported to the CDC in cases of Lyme disease from 2001-2010. http://www.cdc.gov/lyme/stats/chartstables/casesbymonth.html

Because of their small size, nymphs are also more likely to go undetected during attachment. In hyperendemic areas, nymphal ticks can be found in abundance, but this is not true in areas like the southern states (SC, GA, MS, LA, FL) where it is rare to encounter a nymphal *I. scapularis* [149]. This is peculiar because of the abundance of adult *I. scapularis* (Figure 1.8) [44].

Table 1.2. Number of reported Lyme disease cases in 2012 reported to the CDC by State.

State	**# of cases**
Top 13	
Connecticut	2657
Delaware	669
Maine	1111
Maryland	1651
Massachusetts	5138
Minnesota	1515
New Hampshire	1450
New Jersey	3616
New York	2998
Pennsylvania	5033
Vermont	522
Virginia	1110
Wisconsin	1766
Southern States	
Alabama	25
Arkansas	13
Florida	118
Georgia	31
Louisiana	7
Mississippi	1
North Carolina	122
South Carolina	44
Texas	25

*Data represents both confirmed and probable cases reported at http://www.cdc.gov/lyme/stats/chartstables/reportedcases_statelocality.html

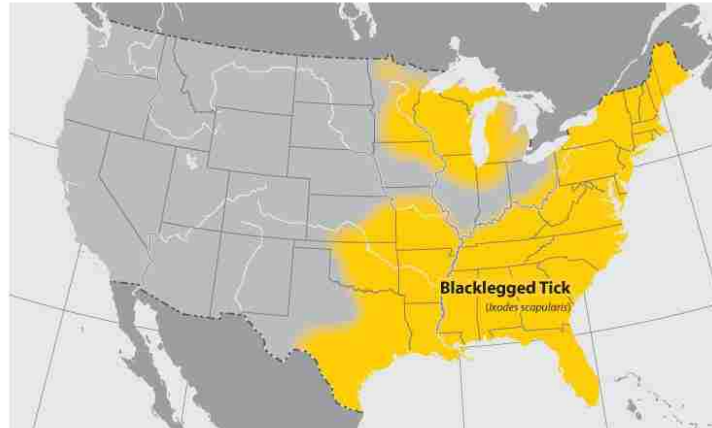

Figure 1.8. Known distribution of *Ixodes scapularis* in the United States. http://www.cdc.gov/ticks/geographic_distribution.html#blacklegged.

One of the first explanations of this phenomenon was that there existed two distinct species of ticks: one (*Ixodes dammini*) populated areas North of Maryland and another (*I. scapularis*) South of MD. *Ixodes dammini* was described as a novel species in 1979 based on morphological differences noted between southern and northern *I. scapularis* ticks [150]. The delineation of these two species would help explain the human borreliosis incidence differences by: 1) questioning vector competence of the southern *I. scapularis* [151] and 2) explaining observed nymphal human biting differences between the two species [152, 153]. Eventually; however, the conspecificity of the two species of ticks was proven by genetic, morphological and cross -breeding experiments [44, 154, 155]. While southern *I. scapularis* were confirmed competent vectors of *B. burgdorferi* ss [156-158], to date, the reduced human exposure to southern nymphal *I. scapularis* has yet to be elucidated. Large ecological studies in the southern US consistently show that populations of *I. scapularis* are infected with *B. burgdorferi* ss at low levels [159-163]. These findings have widely been attributed to the different feeding behavior exhibited by *I. scapularis* in southern states, where the

majority of data suggest that the immature life stages feed on *Borrelia*-refractory lizards [164, 165]. Reduction of the number of immature vectors on highly competent *Borrelia* reservoirs (*Peromyscus* spp.) directly decreases the prevalence of *Borrelia* in the ticks [166]. However, some evidence exists that at least some lizards are susceptible to *Borrelia* infection [167-169]. Experimental studies have not shown a distinct preference for feeding on lizards [170]; therefore, it is postulated that a general increase in host biodiversity has a negative correlation (dilution effect) on *Borrelia* infection in vector ticks [171]. Still, the dilution hypothesis has come under attack [166, 172]. Only will further research in these understudied areas really shine a light on what is actually occurring.

1.4.2. Human Lyme Borreliosis

Lyme Borreliosis is generally broken down into three stages: stage one being early infection, stage two termed early disseminated, and stage three referred to as late disease [173]. Stages one and two are considered the early phase of the disease and occur a few weeks to a couple months post infection and present with self-limiting signs and symptoms. Chronic or late disease occurs years after infection and can become progressive [173].

1.4.2.a.Early *Borrelia* Infection (Stage 1)

Initial infection (stage 1) causes a localized rash know as a bulls-eye rash or erythema migrans (EM) and limited systemic signs (fever, headache, malaise, and muscle aches) [174]. The EM rash (considered pathognomonic in endemic areas) grossly appears as a red, centrally-expanding rash with a diameter from 5 cm to sometimes > 20cm in diameter. This occurs one to four weeks after a tick bite (Figure 1.9 A&B) [174].

There exists no catch-all definition for the EM rash as they can present in many forms including ovid, circular, triangular, and generally lacking regular borders and may or may not show central clearing [175-177].

1.4.2.b.Early Disseminated *Borrelia* Infection (Stage 2)

Stage 2 or early disseminated disease is characterized by haemateogenous dissemination of the Lyme spirochete in an untreated host. This occurs within the first month post infection [173]. Clinical signs during this stage of infection include neurological manifestations (Lyme neurroborreliosis, LNB) and cardiac involvement (Lyme carditis), in 15% and 8% of individuals, respectively [178, 179]. The most common neurological sign in the United States is subacute meningitis, which can have associated transient facial paralysis (bell's palsy) (Figure 1.9 C) and peripheral neuropathy [180]. Signs and symptoms of these manifestations include fever, neck pain/tenderness, facial paralysis (unilateral or bilateral), and numbness or tingling in the extremities [180]. Neurological involvement is a result of haemateogenous dissemination of the spirochete into the leptomeninges, nerve roots, dorsal root ganglia, and cerebral spinal fluid (CSF) of the host [179, 180].

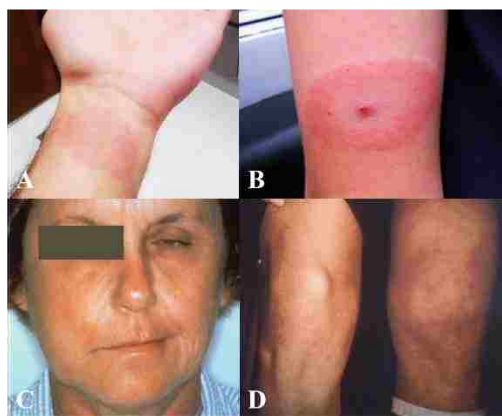

Figure 1.9. Clinical manifestations of Lyme borreliosis. A; Erythema migrans lacking central clearing, B; EM with central clearing and tick punctum, C; Bell's palsy, D; Lyme Arthritis.

Lyme carditis is another manifestation in the early disseminated phase of Lyme borreliosis. While neurological involvement is more common, cardiac signs and symptoms are implicated in patient morbidity and in some cases mortality [181, 182]. *Borrelia* spirochetes disseminate to the heart via blood vessels and can infect all layers of the myocardium [181, 183]. Lyme carditis can present as a magnitude of signs and symptoms, ranging from non-specific (light-headedness, syncope) to palpitations and even myocardial infarction [181]. However, the most common reported sign are cardiac conduction delays, most commonly atrioventricular blocks (2° Type 1 & 3°) [181]. Both cardiac and neurological manifestations are directly related to the presence of the bacteria, and upon bacterial clearance symptoms alleviate [179, 181].

1.4.2.c.Late *Borrelia* Infection (Stage 3)

The final stage of Lyme disease is termed late disease. This occurs months to years post infection in untreated individuals. In the US, Lyme arthritis is the most common manifestation at this point of the disease's pathogenesis, but can occur at any stage in the disease [173]. Unlike rapid arthritis seen in normal bacterial septicemia, the Lyme spirochete does not produce proteases, and degeneration of joints are due to monocyte/macrophage, immune complex and complement infiltration into the synovial fluid [184]. Lyme arthritis presents almost exactly like other chronic inflammatory arthritides with arthralgia intermittently affecting large weight bearing joints such as the knees (Figure 1.9 D) [173, 185].

1.4.2.d.Post Treatment Lyme Disease Syndrome (PTLDS) and "Chronic" Lyme Disease

Finally there exists two additional termed stages of Lyme disease, sometimes used interchangeably, that are not well understood and even controversial. The first is a condition

known as Post Treatment Lyme Disease Syndrome (PTLDS). This condition was first termed in the late 1990s but has recently gained more attention in the scientific community [186]. The syndrome itself occurs in around 10-20% of patients and it is marked by lingering symptoms, after the recommended antibiotic treatment regime, including but not limited to fatigue, pain, and joint or muscle aches [187-190]. The cause of PTLDS has not been determined and studies have posited many different explanations [191-194].

The second additional stage is loosely termed chronic Lyme disease. This stage, like PTLDS manifests months to years after antibiotic treatment. It is hypothesized that in chronic Lyme cases, antibiotic treatment is unable to "kill" all the spirochetes, possibly due to their dissemination to immune privileged sites such as the CNS and cartilaginous tissues [195]. While some animal studies support this notion [196-198] the debate continues.

1.4.3. Murine Model of Lyme Borreliosis

Like many diseases, the laboratory mouse model plays an important role in many aspects of Lyme *Borrelia* research. Mice (*Mus musculus*) have been shown to be susceptible to many species of *Borrelia* infection, yet different strains display a wide range of symptoms [6, 199, 200]. The murine model of Lyme borreliosis offers several advantages in the study of this disease process. 1) Murine models, being so common, are economical; they are also genetically and microbiologically defined. 2) Mice are susceptible to multiple species of *Borrelia* at all ages. 3) Many mice consistently develop both heart and joint disease during the course of infection. 4) Many of the disease manifestations seen in mice also parallel features of human infection. 5) Mice also develop a serological response, which can be helpful when developing and testing diagnostic tests. 6) Mice are also competent host for many of the tick species that serve as ecological vectors for Lyme *Borrelia,* which allow for

the study of the vector-host-pathogen interface, the triad of vector-borne disease research [201]. The availability of murine microbiological reagents, complete murine genome and availability of hundreds of transgenic and knockout mice allow for economical and expeditious experimentation. To date, mice have been shown to be susceptible to at least seven *B. burgdorferi* sl species of spirochetes [73, 86, 202-206]. And while some gross pathological signs, like arthritis, differ between strains of mice (C57BL/6 and BALB/c show less joint swelling then do C3H mice), disseminated infections are common in many models [201]. Severe combined immunodeficient (scid) mice are also commonly utilized in Lyme *Borrelia* research as they lack an adaptive immune response and are very susceptible to microbes, therefore, they are often utilized to test infectivity of even the weakest *Borrelia* species and mutants [201]. In comprehensive studies of *B. burgdorferi* ss infection done by Barthold et al. [201, 207, 208] dissemination of spirochetes was monitored as were clinical manifestations. Dissemination of spirochetes to blood and spleen is seen by day 3 post inoculation. By day 10, spirochetes colonize the ear tissues and invasion of joints occurs day 4 through 7 followed by heart colonization at days 7 through 10. Spirochete burdens peak at day 15 but persist for at least a year. Spirochetes showed a predilection for collagenous, heart, joint, arteries, nerves, muscles, skin and other tissues. Musculoskeletal pathology manifests as arthritis most often in the tibiotarsal joint. This is most likely due to edema secondary to infiltration of neutrophils and macrophages in response to large numbers of bacteria. Cardiovascular pathology occur at the aortic root, auricles and ventricular myocardium. With the exception of aortitis, due to neutrophilic infiltration, the major cause of pathology in the cardiac tissues is secondary to macrophage infiltration. Spirochetes can be visualized throughout the heart early, yet are mostly cleared by the immune system except

that some persist in the aortic wall at the base of the heart. The bladder can also exhibit lesions secondary to spirochete infection [201, 207, 208]. Mice develop a robust antibody response to many of the immunogenic proteins that the human immune system also recognizes [208]. This is of great use in testing and developing both serological diagnostic test and vaccine candidates. Lastly, mice serve as competent host for both immature stages of the human vectors for Lyme *Borrelia* and are susceptible to infection via tick bite [209, 210].

The murine model of Lyme borreliosis offers many benefits to researchers, however does come with limitations. Mice are not genetically similar to humans and to date, only non-human primates (NHP) have been shown to reliably mimic many of the human symptoms of Lyme infection (erythema migrans, arthritis, carditis and neurological manifestations) [211-215]. While the NHP models incur high costs, special facilities, and specific training they remain the closest model to human Lyme borreliosis described to date. Future work should utilized the invaluable NHP models to clarify human clinical sign and symptoms of the lesser studied Lyme *Borrelia*.

1.4.4. Diagnosis of Lyme Borreliosis

Considering Lyme disease is estimated to cause ~300,000 cases of human illness a year in the US, the research focused to Lyme Borreliosis diagnosis has been very substantial [145]. Diagnosis of Lyme disease in the United States is based on a combination of criteria that include the identification of clinical signs and symptoms, tick exposure history, and laboratory diagnostic tests [216]. However, many of the signs and symptoms of Lyme disease can vary between species and even strains of Lyme *Borrelia* infecting the individual [217, 218]. Therefore, both defining areas of endemic tick infection and laboratory diagnostic assays are a critical tool in the diagnosis of human Lyme borreliosis.

1.4.4.a.Two-Tier Serological Testing

Currently the Infectious Disease Society of America (IDSA) and the CDC recommend a two-tiered approach for clinical laboratory diagnosis. The first tier is a whole-cell IgM and IgG enzyme-linked immunosorbent assay (ELISA). If the ELISA is positive or equivocal it is followed by the second tier which is a western blot [219, 220]. Because the whole-cell ELISA has high sensitivity but lower specificity, a western blot is performed to confirm a positive or equivocal ELISA [216]. Criteria for a positive western blot are defined by the IDSA and CDC as

> "an IgM immunoblot be considered positive if two of the following three bands are present: 24 kDa (ospC), 39 kDa (bmpA), and 41 kDa (fla). An IgG immunoblot be considered positive if five of the following 10 bands are present: 18 kDa, 21 kDa (ospC), 28 kDa, 30 kDa, 39 kDa (bmpA), 41 kDa (fla), 45 kDa, 58 kDa (not groEL), 66 kDa, and 93 kDa. [219, 220]"

These recommended two-tiered diagnostic tests are based on proteins derived from the organism *B. burgdorferi* ss. While it has been demonstrated that this approach is adequate to diagnose infection with *B. burgdorferi* ss and a few strains of other Lyme *Borrelia* [216, 221, 222], problems arise when infection is caused by other know human pathogenic Lyme *Borrelia*. These problems can materialize as weak banding patterns or less than two (IgM) or five (IgG) bands present [221, 223-227].

Eurasia experienced problems with Lyme borreliosis diagnosis shortly after the initial description of the disease. The first serological tests were developed based on immunogenic proteins from North American *B. burgdorferi* ss organisms. Patients in Eurasia were presenting with different clinical manifestations than those in the US [228, 229]. Spirochetes isolated from patients and vector ticks were also phenotypically different from North American *Borrelia* species [229]. Eventually, multiple species of *Borrelia*, notably *Borrelia*

afzellii and *Borrelia garinii*, were confirmed as additional pathogenic spirochetes [19]. Serological test initially developed for *B. burgdorferi* ss were found to be less effective in the detection of these Eurasian pathogenic *Borrelia* [230, 231]. Subsequently, researchers and clinicians in these areas realized that to increase specificity and sensitivity of the diagnostic tests it would be to their advantage to use multiple species and local isolates of *B. burgdorferi* sl spirochetes [226, 232].

<u>1.4.4.b.C6 Peptide ELISA</u>

Another serological test that has been extensively compared with the standard two-tier testing methods is the C6 Peptide ELISA. The test is based on a 26-mer amino acid peptide developed against an invariant region (IR6) of the well-studied virulence associated outer surface vlsE protein [233]. This protein is largely responsible for antigenic variation during vertebrate infection [136]. This peptide is based on the *B. burgdorferi* ss vlsE protein sequence but has been shown to be conserved among the three major pathogenic *Borrelia* spp. (*B. burgdorferi* ss, *B. afzelii* and *B. garinii*) [234]. Multiple studies have concluded the C6 peptide ELISA is comparable if not more reliable than the current two-tier testing approach for Lyme borreliosis diagnosis [235-237].

Diagnosis of Lyme borreliosis is an evolving field. *Borrelia burgdorferi* sl infections can cause a wide variety of clinical signs [238, 239], overlap geographically [1, 240], and have differential reactivity to a variety of serological diagnostic tests [230, 231]; therefore, it is essential that we understand the infectivity and host response to all *Borrelia* species endemic to certain areas, as *Borrelia* infections could easily be overlooked.

1.5. Summary

Lyme borreliosis is a recently emergent vector-borne disease. Advancements in science have allowed researchers to elucidate minute details involving this organism and its disease. However, there remains much research to be done. An estimated 300,000 cases of Lyme disease are diagnosed each year in the United States and the zones of endemicity keep expanding. Much research has been focused on the *B. burgdorferi* ss species and its vector in areas of high endemicity. This most certainly was warranted; however, important aspects of the larger Lyme borreliosis picture were overlooked, as demonstrated with the clinical and diagnostic issues once faced in Eurasia. One can argue that we are seeing the same issue here in the US. It may have started with the *I. dammini*/*I. scapularis* debate but it continues with the "recent" spread of Lyme into non-traditional endemic areas (Virginia, Michigan and Canada). While descriptions of new Lyme *Borrelia* species seem to be published weekly, there continues to be a lacks of fundamental understandings into their infectivity, ecology and general biology. In this dissertation we take a broad approach to provide evidence that may explain, at least in part, some of these issues. We hypothesize that the Lyme borreliosis picture in the US is much more complex then we currently understand this is in part due to undescribed populations of Lyme *Borrelia* in states outside of hyperendemic areas and the presence of one or more additional pathogenic Lyme spirochetes in the US.

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CHAPTER 2. DETECTION OF HUMAN BACTERIAL PATHOGENS IN TICKS COLLECTED FROM LOUISIANA BLACK BEARS (*URSUS AMERICANUS LUTEOLUS*)

2.1. Abstract

There are four major human biting ticks in the noutheastern United States, which include: *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, and *Ixodes scapularis*. The black bear is a large mammal that has been shown to be parasitized by all the aforementioned ticks. We investigated the bacterial infections in ticks collected from Louisiana black bears (*Ursus americanus* subspecies *luteolus*). Eighty-six ticks were collected from 17 black bears in Louisiana from June 2010 to March 2011. All four common human biting ticks were represented. Each tick was subjected to polymerase chain reaction (PCR) targeting select bacterial pathogens and symbionts. Bacterial DNA was detected in 62% of ticks (n=53). *Rickettsia parkeri,* the causative agent of an emerging spotted fever group rickettsiosis, was identified in 66% of *A. maculatum*, 28% of *D. variabilis* and 11% of *I. scapularis*. The Lyme disease bacterium, *Borrelia burgdorferi*, was detected in two *I. scapularis*, while one *A. americanum* was positive for *Borrelia bissettii*, a putative human pathogen. The rickettsial endosymbionts *Candidatus* Rickettsia andeanae, rickettsial endosymbiont of *I. scapularis* and *Rickettsia amblyommii* were detected in their common tick hosts at 21%, 39% and 60%, respectively. All ticks were PCR-negative for *Anaplasma phagocytophilum*, *Ehrlichia* spp. and *Babesia microti*. This is the first reported detection of *R. parkeri* in vector ticks in Louisiana; we also report the novel association of *R. parkeri* with *I. scapularis.*

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Detection of both *R. parkeri* and *B. burgdorferi* in their respective vectors in Louisiana demands further investigation to determine potential for human exposure to these pathogens.

2.2. Introduction

Since Theobald Smith's seminal work describing ticks as the vectors for Texas cattle fever, the importance of arthropods as vectors for disease has surged to the forefront of both human and animal medicine [1]. By the turn of the 20th century many control measures were developed and deployed to combat the pests spreading these pathogens. Excellent reviews of these can be found in Hill et al. (2005) and Piesman & Eisen (2008). Even with advances in arthropod control and disease management the world has seen an emergence and re-emergence of zoonotic vector-borne diseases (ZVBD) within the past 30 years [2, 3].

Ticks are vectors for a plethora of zoonoses, which include the causative agents of anaplasmosis, babesiosis, erhlichiosis, Lyme and relapsing fever borreliosis, spotted fever group (SFG) rickettsiosis and a host of viral diseases [4, 5]. In the United States, ticks transmit more vector-borne diseases than any other arthropod. With over 80 known species of ticks in the US, opportunity exists for each one of the aforementioned diseases to affect humans and animals [6].

Not only is the US witnessing a steady increase in reported human cases of many tick-borne diseases, new tick borne pathogens are being discovered on a regular basis. For example in 1992, Lyme disease, Rocky mountain spotted fever (RMSF) and tularemia were the only nationally notifiable tick-borne diseases; approximately 20 years later the number has swelled to nine [7]. This increase amounts to three new tick-borne human diseases identified per decade. With the advent of better detection, diagnosis and surveillance, this number is likely to increase.

Despite the increase in research on tick and tick-borne diseases conducted elsewhere in the past several decades, comparatively less work has been done in Louisiana. With outdoor activity as the single most important risk factor for tick exposure [8], and Louisiana's recognition as "A Sportsman's Paradise," there is strong risk of tick exposure for both residents and visitors. In the southern US humans are most commonly parasitized by four ticks species: *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis* and *Ixodes scapularis* [9-11]. Each of these tick species have unique host preferences that, taken together, encompasses a range of host animals [12]. Black bears have been reported to harbor many of the same tick species as humans, and not surprisingly, pathogenic bacteria have been detected in ticks parasitizing bears [13-19].

Louisiana's state animal is the black bear, *Ursus americanus* subspecies *luteolus*, which has been listed as a threatened species by the United States Fish and Wildlife Service since 1992. The Louisiana department of Wildlife and Fisheries carefully monitors bear populations throughout the state and has collected ectoparasites during these monitoring efforts. The objective of our study was to investigate which ticks parasitize black bears in Louisiana, and to identify bacteria of interest that they harbor.

2.3. Materials and Methods

2.3.1. Bear Sampling and Tick Collection

From June 2010 to March 2011 ticks were collected from 17 black bears throughout Louisiana by personnel from Louisiana's Department of Wildlife and Fisheries (LDWF). Ticks collected off bears in this study were convenience samples, as the bears consisted of relocated and accidentally or illegally killed animals encountered by LDWF personnel. Bears were sampled from seven parishes: Concordia, Iberville, Livingston, Madison, Pointe

Coupee, St. Landry and St. Mary. Exhaustive searching of bears for ectoparasites was not conducted, leading to the collection of only easily visible adult ticks. Ticks were stored in 70% ethanol for transport, then identified and sexed using standard identification keys [20, 21].

2.3.2. Tick DNA Extraction

Ticks were surface sterilized by first soaking in a 10% bleach solution, followed by a phosphate buffered saline (PBS) wash, next soaked in a 70% ethanol solution, followed by a PBS wash and finally a soak in molecular biology grade water (MO BIO, Carlsbad, CA). Individual ticks were then bisected with a sterile scalpel and DNA was extracted from each tick using the GenElute DNA extraction Kit (Sigma, St. Louis, MO) following manufacturer's instructions with minor modifications. These modifications included: tick tissues were allowed to lyse overnight in a 55˚C water-bath and DNA was eluted in 100 µl of supplied elution buffer. DNA was stored at -20˚C until PCR was performed.

2.3.3. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on DNA extracts to detect the presence of selected bacteria. The PCR assays targeted the following genes: *msp2* for *Anaplasma phagocytophilum* [22], 18s *rRNA* for *Babesia microti [23]*, *flaB* for *Borrelia burgdorferi* sensu lato [24], 16s *rRNA* for *Ehrlichia* species [25, 26] and both 17-kDa antigen gene [27] and *ompA* [28] for SFG *Rickettsia*. Environmental controls were utilized for detection of DNA artifact contamination in all experiments this included: simultaneous extraction tubes with no tissue added for all DNA extractions and molecular biology grade water (MO BIO) used as a template in the downstream PCRs. Initial reactions contained 2.5 µL of DNA extract in a total reaction volume of 25 µL. TaKaRa Taq DNA polymerase

(TaKaRa-Bio Inc, Otsu, Japan) was utilized in all reactions. Each reaction had a final concentration of 1.25 U of Taq DNA polymerase, 45 mM KCL, 2.5 mM $MgCl_2$, 200 μM concentration of each deoxynucleoside, and 0.5 μM of the upstream and downstream primers. All reactions were carried out in a My Cycler thermal cycler (BioRad, Hercules, CA.) Nested reactions contained 1 μL of product from outer reaction under the same master mix concentrations. Thermocycling protocols were followed as published for each primer set.

2.3.4. DNA Purification/Sequencing/Analysis

PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Bands of the correct size were purified with a QIAquick PCR purification kit (Qiagen, St. Louis, MO) and bi-directionally sequenced on an automated ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA.) at LSU's Biommed Gene Lab. Sequences were aligned using ClustalX and compared with existing sequences in the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) [29, 30].

2.3.5. PCR Cloning

PCR-cloning was utilized to identify amplicon inserts in samples where multiple signals were detected in the sequencing chromatograph. Cloning reactions were performed according to manufacturer's instructions with an Invitrogen Topo TA cloning kit (Life Technologies, Grand Island, NY). Multiple colonies were picked and the universal primers M13F and M13R were used to sequence as previously described. The resulting sequences were aligned and analyzed as described above.

2.4. Results

2.4.1. Four Tick Species were Found Parasitizing Louisiana Black Bears

Eighty-six ticks were removed from a total of 17 bears. All ticks collected were adults; 51% (n=44) were females and 49% (n=42) were male. *A. maculatum* was the most commonly collected tick, comprising 44% of all ticks collected (n=38), *D. variabilis* represented 29% (n=18) of the total collected, *I. scapularis* was the next most abundant at 21% (n=15) and the least common was *A. americanum* at 6% (n=5) (Table 2.1).

Table 2.1. Number, species and gender of ticks (M=male, F=female) collected from each of the bears in this study

Bear	*A. americanum*	*A. maculatum*	*D. variabilis*	*I. scapularis*	Total
1	-	4 (M), 4 (F)	1 (M)	-	9
2	-	2 (M), 4 (F)	1 (F)	-	7
3	-	-	2 (M), 1 (F)	-	3
4	-	9 (M)	2 (M), 3 (F)	-	14
5	-	-	1 (M), 6 (F)	-	7
6	-	3 (M), 1 (F)	1 (M), 3 (F)	-	8
7	-	-	1 (F)	-	1
8	-	-	1 (F)	-	1
9	-	1 (M)	1 (M), 1 (F)	-	3
10	-	9 (M), 1 (F)	-	-	10
11	-	-	-	2 (M), 3 (F)	5
12	-	-	-	2 (M), 1 (F)	3
13	-	-	-	2 (F)	2
14	-	-	-	2 (F)	2
15	-	-	-	2 (F)	2
16	3 (M), 2 (F)	-	-	-	5
17	-	-	-	2 (M), 2 (F)	4
Total	5	38	25	18	86

2.4.2. Three Bacterial Pathogens were Detected in Ticks

DNA was extracted from all 86 ticks collected from Louisiana black bears. These extracts were tested for the presence of *A. phagocytophilum*, *B. burgdorferi* sensu lato, *B. microti, Ehrlichia* spp. and SFG *Rickettia* DNA by PCR. Pathogenic bacterial DNA was detected in 44% (n=38) of all ticks. *Rickettsia parkeri* was detected most frequently with 25 of 38 (65.7%) *A. maculatum*, 7 of 25 (28%) *D. variabilis* and 3 of 18 (11.1%) *I. scapularis* testing positive. *Rickettsia parkeri* sequences were 99% homologous with sequences in GenBank (Table 2.2). Evidence of *B. burgdorferi* sensu stricto was found in 2 of 18 (11%) *I. scapularis*, while one (20%) *A. americanum* had a sequence matching *Borrelia bissettii* (Table 2.2). No *A. phagocytophilum*, *B. microti* or *Ehrlichia* species DNA were detected in any of the ticks.

2.4.3. Three Rickettsial Endosymbionts were Detected in Ticks

Spotted fever group *Rickettsia* primers and subsequent sequencing identified rickettsial endosymbiont DNA in 20.9% (n=18) of their respective ticks. These endosymbionts included: *Candidatus* Rickettsia andeanae in 8 of 38 (21%) *A. maculatum*, rickettsial endosymbiont of *I. scapularis* in 7 of 18 (38.8%) *I. scapularis*, and *Rickettsia amblyommii* in 3 of 5 (60%) *A. americanum*. Endosymbionts were 99-100% similar to their respective reference sequences in Genbank (Table 2.2).

2.4.4. Multiple Bacteria Detected in Ticks

Ticks with evidence of mixed SFG *Rickettsia* DNA after sequencing were subjected to PCR cloning and sequencing of multiple clones to identify individual amplicons. Two ticks had the presence of multiple SFG *Rickettsia* DNA. This included one *A. maculatum* with both *Candidatus* R. andeanae and *R. parkeri*, and one *I. scapularis* with the rickettsial

endosymbiont of *I. scapularis* and *R. parkeri*. Two additional ticks had evidence of multiple bacteria by separate PCRs. In one *I. scapularis*, we detected both *B. burgdorferi* and the rickettsial endosymbiont of *I. scapularis* and one *A. americanum* had evidence of both *R. amblyommii* and *B. bissettii* DNA (Table 2.2).

2.5. Discussion

In this study we investigated which species of ticks were parasitizing Louisiana black bears and identified both pathogenic and symbiotic bacterial DNA in the arthropods. Human-biting ticks have been commonly recorded on bears in studies across the US dating back to the mid 70's [13, 14, 18, 19, 31]. A previous study in Idaho detected antibodies to multiple tick borne pathogens in black bears while a Wisconsin study isolated Lyme *Borrelia* spirochetes from these animals [15, 17]; more recently Yabsley et al. (2009) detected *Ehrlichia chaffeensis* and *R. parkeri* in ticks from black bears in South Georgia/ North Florida. Consistent with these previous reports, black bears in Louisiana harbor many common human-biting ticks; nearly half of these had PCR evidence of bacterial pathogens. Most importantly this is the first record of *R. parkeri* infection occurring in its natural vector in Louisiana, with almost 66% of the *A. maculatum* we tested having evidence of *R. parkeri*. This coincides with a recent publication detecting *R. parkeri* in the blood of dogs in Louisiana [32]. *Rickettsia parkeri* rickettsiosis is an emerging infectious disease. Detection of *R. parkeri* is becoming more common across the range of its primary vector, *A. maculatum*. Published infection rates of *R. parkeri* range from ~10-40% in populations of southeastern *A. maculatum* ticks [33-37]. Multiple ticks from multiple parishes in Louisiana had evidence of *R. parkeri* infection (Figure 2.1). *Rickettsia parkeri* was also detected in both *D. variabilis* and *I. scapularis.* This is the first record of detection of this SFG *Rickettsia* in *I.*

scapularis ticks. These ticks are not considered vectors for *R. parkeri* but are known to harbor other SFG *Rickettsia*. Interestingly, we detected *R. parkeri* in two male *I. scapularis,* considering male *Ixodes* ticks feed sparingly if at all, this finding raises the question of pathogenic SFG *Ricksttia* infection in *I. scapularis* [38]. Identifying such a large number of *R. parkeri* infected ticks associated with bears also proposes the question of the bear as a potential reservoir host. Lyme *Borrelia* spirochetes were detected in three ticks. *B. burgdorferi* sensu stricto was detected in two *I. scapularis* while *B. bissettii* was detected in a male *A. americanum*. *Borrelia burgdorferi* sensu stricto is the leading cause of the most common vector-borne disease in the US, Lyme borreliosis. *Borrelia bissettii* is a closely related Lyme spirochete that shares common vectors, hosts and geographic range, and more recently has been implicated in human disease [39-41]. Many species of ticks are associated with distinct endosymbiotic bacteria [46]. In this study we detected multiple symbiotic SFG *Rickettsia*. *Candidatus* R. andeanae is a recently isolated SFG *Rickettsia* and a putative endosymbiont of *A. maculatum* ticks [47]. It has been reported in multiple regions of the US where *A. maculatum* are found [34, 35, 48]. Our finding of *Candidatus* R. andeanae in 21% of *A. maculatum* is the first evidence of this bacterium in Louisiana and expands the known range of this recently described SFG *Rickettsia*. Detection of other SFG rickettsial endosymbionts, namely *R. amblyommii* in *A. americanum* and the rickettsial endosymbiont of *I. sapularis*, is not unexpected, and, these are the first published records of these SFG Rickettsiae in Louisiana ticks. Anecdotal reports have implicated *R. amblyommii* in human disease but the majority of these conclusions have yet to be substantiated in laboratory studies [49-53].

Table 2.2. Tick DNA samples positive by PCR in this study. Identical sequences are represented by identical accession numbers. DNA samples containing more than one bacterial species are denoted by asterisks.

Sample ID	Tick species	Accession number	Homology
S1AM1	*A. maculatum*	KC003477	99% *R. parkeri*
S1AM3, S1AM4 , S1AM5, S1AM6, S1AM8	*A. maculatum*	KC003475	100% *Candidatus* R. andeanae
S2AM3*	*A. maculatum*	KC003475	100% *Candidatus* R. andeanae
S2AM3*	*A. maculatum*	KC003476	99% *R. parkeri*
S2AM1, S2AM2, S2AM4 , S2AM5, S2AM6	*A. maculatum*	KC003477	99% *R. parkeri*
S3DV1, S3DV2	*D. variabilis*	KC003477	99% *R. parkeri*
S4AM1, S4AM2, S4AM3, S4AM, S4AM6, S4AM7, S4AM8, S4AM9	*A. maculatum*	KC003477	99% *R. parkeri*
S4DV1, S4DV2, S4DV3	*D. variabilis*	KC003477	99% *R. parkeri*
S5DV3	*D. variabilis*	KC003477	99% *R. parkeri*
S6AM1, S6AM3	*A. maculatum*	KC003477	99% *R. parkeri*
S6DV1	*D. variabilis*	KC003477	99% *R. parkeri*
S10AM1, S10AM2, S10AM3, S10AM4, S10AM5, S10AM6, S10AM7, S10AM8, S10AM9, S10AM10	*A. maculatum*	KC003477	99% *R. parkeri*
S10AM5, S10AM6	*A. maculatum*	KC003475	100% *Candidatus* R. andeanae
S11IS3*	*I. scapularis*	KC003478	99% *R. parkeri*
S11IS2, S11IS3*, S11IS4	*I. scapularis*	KC003474	100% Rickettsial endosymbiont of *I. scapularis*
S12IS1, S12IS2*	*I. scapularis*	KC003478	99% *R. parkeri*
S12IS2*	*I. scapularis*	KC003471	99% *B. burgdorferi*
S13IS1	*I. scapularis*	KC003470	99% *B. burgdorferi*
S14IS2	*I. scapularis*	KC003472	100% Rickettsial endosymbiont of *I. scapularis*
S16AA1, S16AA2*, S16AA4	*A. americanum*	KC003473	99% *R. amblyommii*
S16AA2*	*I. scapularis*	KC003469	99% *B. bissettii*
S17IS4	*I. scapularis*	KC003472	100% Rickettsial endosymbiont of *I. scapularis*
S17IS3	*I. scapularis*	KC003474	100% Rickettsial endosymbiont of *I. scapularis*

This study was conducted retrospectively on ticks collected from bears by Louisiana department of Wildlife and Fisheries personnel. Due to various limitations the collected ticks probably do not comprise the total tick population on each bear. It is expected that immature stages of ticks were overlooked during sampling, as ectoparasite collection was not the focus of the fish and wildlife personnel's work. In addition many of the female ticks collected and subjected to PCR were at different stages of engorgement, yet others had no obvious signs that a bloodmeal had begun. These limitations directly affect the interpretation of the data presented in that, while it is possible that detection of bacteria in these ticks may indicate infection, it is just as likely that these bacteria were imbibed during feeding on the host bear

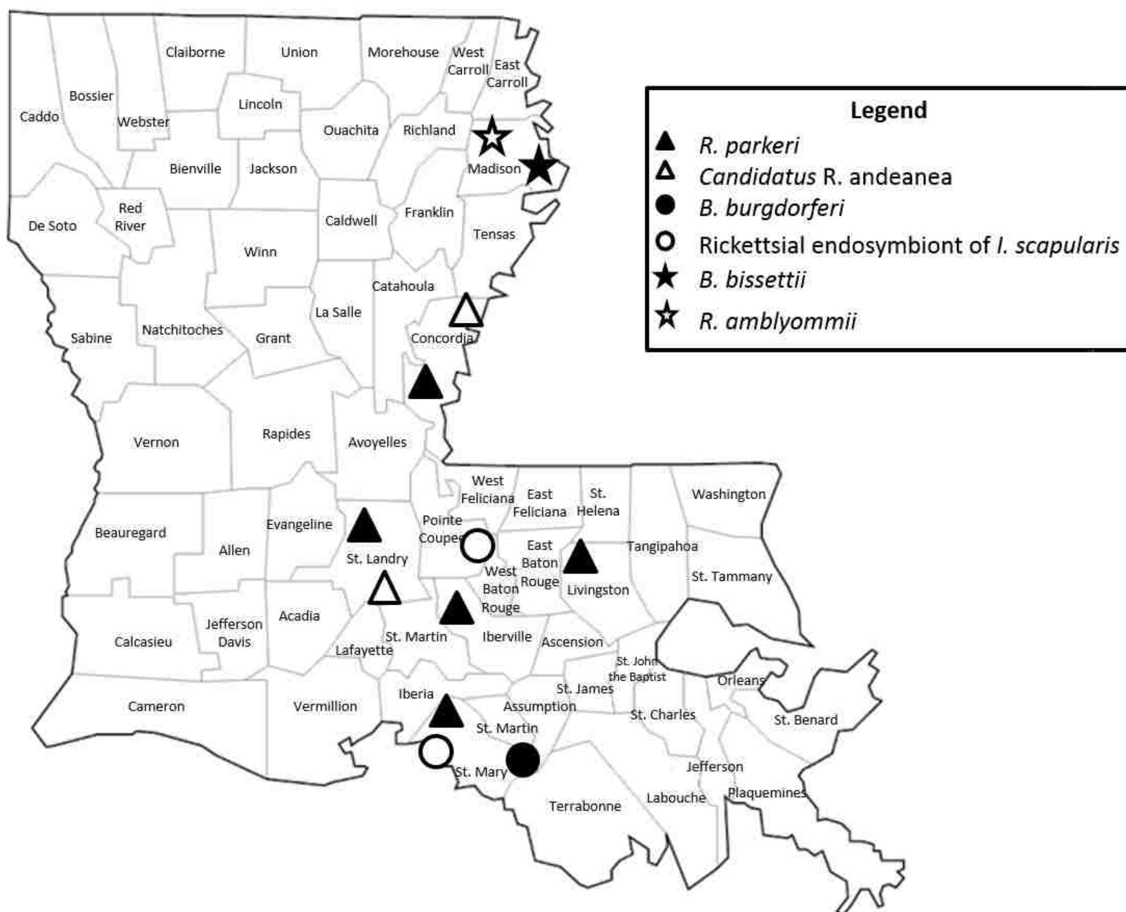

Figure 2.1. Location of ticks positive for bacterial DNA tested for in this study.

either by co-feeding transmission or by an infection in the bear. Future studies should ascertain infection status in questing; unfed, ticks in Louisiana.

With the resurgence of zoonotic vector-borne diseases in the past three decades, the importance of disease surveillance and control has become apparent. In the US, the recent discoveries of tick-borne zoonotic pathogens like *R. parkeri*, *Rickettsia* spp. 364D, *Ehrlichia muris*-like (EML), Panola mountain *Ehrlichia*, and Powassan virus combined with the increasing frequency of tick borne diseases like Lyme disease, SFG rickettsiosis, anaplasmosis, ehrlichiosis and babesiosis has more than proven a need to closely monitor tick-borne diseases [54-57]. Since little is known about the organisms circulating in Louisiana ticks, detection of bacteria in ticks that commonly parasitize humans improves our understanding of tick-borne disease risks to human and animals in the state. The detection of *R. parkeri* and *B. burgdorferi*, and the novel association of *R. parkeri* with *I. scapularis*, in ticks collected from black bears prompts more questions on the prevalence of tick-borne pathogens in questing and other host-associated ticks across Louisiana. Surveillance of pathogens in human-biting ticks in Louisiana is essential to educating health professionals, elucidating tick-borne disease risk and ultimately protecting the health of the public.

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CHAPTER 3. DETECTION OF LYME *BORRELIA* IN QUESTING *IXODES SCAPULARIS* (ACARI: IXODIDAE) AND SMALL MAMMALS IN LOUISIANA

3.1. Abstract

Lyme borreliosis is caused by spirochetes from the *Borrelia burgdorferi* sensu lato (s.l.) species complex. In the United States, *B. burgdorferi* sensu stricto (s.s.) (Johnson, Schmid, Hyde, Steigerwalt, and Brenner) is the most common cause of human Lyme borreliosis. With over 25,000 cases reported annually, it is the most common vector-borne disease in the US. While around 90% of cases are contained to the northeastern and Great Lake states, areas in Canada and some southern states are reporting rises in the number of human disease cases. Louisiana records a few cases of Lyme each year. While some are most certainly the result of travel to more endemic areas, there exists evidence of locally acquired cases. Louisiana has established populations of the vector tick, *Ixodes scapularis* (Say), and a wide variety of potential reservoir animals, yet Lyme *Borrelia* has never been described in the state. Using culture and polymerase chain reaction, we investigated the presence of Lyme *Borrelia* in both mammals and questing ticks at a study site in Louisiana. While culture was mostly unsuccessful, we did detect the presence of *B. burgdorferi* s.s. DNA in 6.3% (11/174) of ticks and 22.7% (5/22) of animal samples. To our knowledge this is among the first evidence documenting *B. burgdorferi* s.s. in Louisiana. Further investigations are required to determine the significance these findings have to human and animal health.

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3.2. Introduction

With over 25,000 human cases reported annualy, Lyme borreliosis is the most common vector borne diseases in the United States [1]. It is caused by spirochetes from the *Borrelia burgdorferi* sensu lato (s.l.) species complex (collectively referred to as Lyme *Borrelia*). In the US *B. burgdorferi* sensu stricto (s.s.) is the most common Lyme *Borrelia* spirochete implicated in human disease, its main vectors being *Ixodes scapularis* (Say) and *I. pacificus* (Cooley and Khols) [2]. The nymphal life stage of these ticks is overwhelmingly responsible for the majority of reported human cases [3]. Lyme borreliosis is a zoonotic disease and humans are considered dead end hosts, therefore reservoir hosts like mice, chipmunks and shrews are essential in perpetuating the spirochetes' enzootic transmission cycle [4].

Although the majority of Lyme borreliosis cases occur in northeastern and Great Lakes states [1], areas not historically considered Lyme endemic are starting to report more cases. For example, researchers in Canada have mapped the encroachment of *I. scapularis* and subsequently Lyme *Borrelia* spirochetes over the past two decades [5, 6]. On the southern side of this expansion, states like Virginia are becoming Lyme "hotspots" with a reported 1300% increase in cases from 1997-2007 [7, 8]. Louisiana reported 133 cases of Lyme borreliosis from 1998-2011, however for many of these cases it is impossible to determine if the patient had actually acquired the infection in Louisiana or from travel to more Lyme endemic areas [9]. In 2012, a young child was diagnosed with Lyme borreliosis by physicians associated with Tulane Medical Center. This case met the Centers for Disease Control's surveillance definition and had no history of travel outside the state (Black 2012; B.F.Leydet, personal communication). Like most other states in the southern US the lower

incidence of Lyme borreliosis in Louisiana is likely multifactorial. While Lyme *Borrelia* spirochetes are endemic to many states in the South, differences from the traditional northeastern Lyme *Borrelia* ecology probably play a significant factor in human exposure and disease diagnosis. For example, host diversity in the South is higher, leading to more refractory hosts and changes in the vector's ecology, multiple complex cryptic Lyme *Borrelia* cycles involving non-human biting ticks exist, and the population and overlap of *Amblyomma americanum* (L.) the vector of Southern-Tick Associated Rash Illness further complicates the situation [10-13]. These reasons amongst others likely play an integral role in the lower infection prevalence and significantly reduced human encounters with nymphal *I. scapularis* across the southern US [14, 15].

The spread of Lyme borreliosis highlights the importance of surveillance in areas once considered low risk. Surprisingly, even with established state-wide populations of *I. scapularis* [16], a plethora of potential reservoir hosts [17], and evidence that Louisiana populations of *I. scapularis* are competent vectors of *B. burgdorferi* [18], almost nothing exists in the literature regarding the presence of Lyme *Borrelia* in Louisiana. Therefore, this study investigated the presence of Lyme *Borrelia* in ticks and mammals at a site in Louisiana.

3.3. Materials and Methods

The study area chosen was Tunica Hills Wildlife Management Area in West Feliciana Parish, Louisiana. Forest cover was categorized as upland hardwood rolling bluff land which supports a large population of animals including species of small rodents, birds, wild turkey, and white-tail deer. Questing ticks were collected by flagging with 1-m^2 white felt cloth flags along defined hiking paths and animal trails. Flagging was conducted once or twice a week

for 2-3 hours throughout the year (2009-2010) but only *I. scapularis* were utilized in this study. Animal trapping was conducted when nymphal *I. scapularis* were expected to be feeding on hosts (from spring to early fall in 2010). Animals were trapped using Tomahawk live traps placed along animal tracks and trails. A total of thirty-three traps were set at night at least once a week during the aforementioned trapping period. This resulted in a total of 1056 trap nights. Captured animals were anesthetized with a combination of ketamine and dexmedetomidine, measured, weighed and sexed. Ectoparasites were removed and placed in tubes alive for later processing and identification using a standard key [19]. Two 2-mm ear punch biopsies (EPB) were taken, and blood was drawn for culture and PCR analysis. Animals were released at capture site following recovery from anesthesia. *Borrelia* culture isolation attempts were performed on blood, EPB, and questing ticks in BSK-H complete media (Sigma, St. Louis, MO) supplemented with rifampin 50 μg/ml, phosphomycin 50 μg/ml and amphotericin B 2.5 μg/ml as previously described [20, 21]. An indirect immunofluorescence assay (IFA) was performed on cultures with evidence of spirochete growth utilizing monoclonal antibodies specific for *B. burgdorferi* (H5332) as previously described [22]. DNA extractions were performed on 2-mm EPBs from small mammals and half of each adult black-legged tick using the GeneElute Genomic DNA Miniprep kit (Sigma). EPBs and tick halves were minced by single use sterile scalpel and DNA extracted as directed by the manufacturer. PCR and thermocycling were performed with a nested primer set targeting a 389-bp portion of the chromosomally located *flaB* gene as previously described [23]. *Borrelia flaB* PCR has been successful in the sensitive detection and delineation of *B. burgdorferi* s.l. species by sequence analysis of PCR products, from both field and culture samples [24-26]. Environmental controls utilized for detection of DNA

contamination in all experiments included simultaneous extraction tubes with no tissue added for all DNA extractions and molecular biology grade water used as a negative template in all downstream PCRs. PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following manufacturer's instructions. LSU's Biommed Gene Lab Bi-directionally sequenced the amplified PCR products. Sequences were aligned using CustalX and compared with existing sequences in the National Center for Biotechnology Information (NCBI) Genebank using the Basic Local Alignment Tool (BLAST) [27, 28]. In all aforementioned testing, appropriate positive controls included: a culture of clonal *B. burgdorferi* B31 (5A11) and extracted gDNA from the culture. Negative controls as previously described above repeatedly showed no evidence of contamination throughout this study.

3.4. Results

In total, 174 adult blacklegged ticks (*I. scapularis*), seven mice (*Peromyscus* spp.), six eastern wood rats (*Neotoma floridana* Ord), four raccoons (*Procyon lotor* L.), four eastern grey squirrels (*Sciurus carolinensis* (Gmelin) and one North American opossum (*Didelphis virginiana* Kerr) were screened by PCR and culture for evidence of Lyme *Borrelia*. PCR testing of ticks produced 11 (6.3%) borreliae sequences. Five (22.7%) of the animals had PCR positive EPBs; this included two mice (28.6%), a woodrat (16.7%), a raccoon (25%) and a squirrel (25%). No *I. scapularis* were collected from trapped animals. The 11 positive questing *I. scapularis* all had identical sequences (Table 3.1). Animal sequences shared some similarity with the ticks in one polymorphic site; however, other single nucleotide polymorphisms differed among samples. All sequences generated in this

study had nucleotide differences from *B. burgdorferi* strain B31, yet were 99% homologous when aligned (Table 3.1). One tick and one squirrel sample produced spirochetes in primary culture isolates; however, upon multiple passages the samples were lost to bacterial contamination. Aliquots of these cultures were subjected to IFA in which they reacted to *B. burgdorferi* specific OspA H5332 antibodies. These two cultures were also positive by PCR and had identical sequences to their respective tick half and EPB which were subjected only to PCR as previously described.

3.5. Discussion

Since 1998, Louisiana has recorded 133 cases of human Lyme borreliosis [9]. While a proportion of these are certainly acquired from travel to more Lyme endemic parts of the country, there is evidence for locally acquired cases. The enzootic cycle of *B. burgdorferi* has been well studied in areas of the Northeast, yet many states in the South continue to be understudied. Large scale US surveys have concluded that the prevalence of infected *I. scapularis* nymphs in the South is minuscule. However, these conclusions were based on very low numbers of ticks [12], and in Louisiana almost no work has been conducted investigating the presence of Lyme *Borrelia*. Conversely tick surveys across other southern states have identified *B. burgdorferi* infection in many populations of *I. scapularis* [29-37]. In addition to natural cycles of *B. burgdorferi* involving *I. scapularis,* cryptic cycles of Lyme *Borrelia* have been documented in southern states involving *Ixodes affinis* (Neumann)*, I. minor* (Neumann) and *I. dentatus* (Neumann) transmitting multiple *B. burgdorferi* s.l. species [11, 38]. Competent reservoir animals dispersed across the southern US have been shown to be infected with multiple species of Lyme *Borrelia* [11, 32]. Several ecological surveys conducted in southern states have led to the discovery of new Lyme *Borrelia* species,

vectors, and reservoirs, which have expanded our understanding of Lyme *Borrelia* ecology [29, 30, 39].

Along with a recent survey of ticks collected off black bears in Louisiana [40], these are the first records of *B. burgdorferi* in Louisiana ticks and wildlife. This discovery, while novel, was not unexpected. Like other southern states, the prevalence of *B. burgdorferi* in Louisiana ticks at our study site is lower (6.3%) than the more endemic areas in the Northeast where prevalence rates can be as high as 30-50% [12, 41, 42]. The lack of nymphal *I. scapularis* collected from animals and by flagging during this study is also consistent with reports from states across the southern US [43]. The decreased success in cultivating *Borrelia* from ticks and mammals in this study is a phenomenon seen in other studies outside areas of higher endemicity. Could this be due to a lower detection sensitivity of culture versus molecular methods [44]? Or are Lyme *Borrelia* spirochetes in the South less likely to grow in traditional media [29]? While this remains unknown further studies are warranted.

Because Louisiana consistently reports cases of human Lyme borreliosis each year, the detection of *B. burgdorferi* DNA in both vector ticks and competent reservoir hosts is significant. Nymphal *I. scapularis* are the main vector for *B. burgdorferi* in the eastern US, and while not detected in this study, can be found questing and attached to humans in Louisiana (B.L., unpublished data). It is also important to note that DNA evidence of bacteria does not confirm viability of the organism, and because cultures in this study were mostly unsuccessful, caution must be taken in the interpretation of these results. Still, molecular surveys demonstrating presence of a pathogen in known vectors and reservoirs are the critical first steps in our understanding of vector borne disease agents in different geographical areas.

Table 3.1. *B. burgdorferi flaB* sequences amplified from mammalian and tick gDNA samples in this study

Organism sampled	Common name	Homology to *B. burgdorferi* B31 (NC_001318.1)	Nucleotide differences from B31 (basepair site of polymorphism)
N. floridana	Wood rat	99%	T→C (738)
P. lotor	Raccoon	99%	T→C (738)
Peromyscus spp.	Field Mouse	99%	T→C (510); T→A (687) & T→C (738)
Peromyscus spp.	Field Mouse	99%	T→C (738)
S. carolinesis	Gray Squirrel	99%	T→C (510); T→A (687) & T→C (738)
S. hispidus	Cotton rat	99%	T→C (738)
I. scapularis (questing)	Black-Legged Tick	99%	T→C (738)

*Sequence results aligned to the full (1011 basepairs) *flaB* portion of the B31 chromosome (positions 147649–148659)

More importantly, studies like this ultimately help relay new information to researchers and healthcare providers who in turn help educate the public. Evidence from this study should prompt further state-wide surveys investigating the prevalence of Lyme *Borrelia* in Louisiana ticks and wildlife.

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CHAPTER 4. SIMILARITY OF *BORRELIA BISSETTII* AND *BORRELIA BURGDORFERI* SENSU STRICTO IN MURINE INFECTION AND IMMUNE RESPONSE

4.1. Abstract

Increasing numbers of *Borrelia burgdorferi* sensu lato (sl) species have been isolated in the United States since *Borrelia burgdorferi* sensu stricto (ss) was identified as the etiologic agent of Lyme disease. To date, many of these species remain understudied, even with forthcoming evidence associating them with human illness. Using a murine model, we investigated the infectivity, virulence, and humoral immune response to a North American isolate of *Borrelia bissettii* by culture, molecular and serological methods. Original *B. bissettii* cultures were unable to infect immunocompetent mice, but were confirmed infectious after adaptation in immunodeficient animals. The ID_{50} of *B. bissettii* was more than two logs higher than that of *B. burgdorferi* ss. Despite this, *B. bissettii* produced spirochete burdens similar to *B. burgdorferi* ss in skin, heart and bladder and only significantly lower in joint tissues. *Borrelia bissettii* induced an antibody response similar to *B. burgdorferi* ss as measured by immunoblotting and the C6 enzyme-linked immunosorbent assay. This study contributes to our understanding on the potentially pathogenic *B. bissettii* spirochete. Infection dynamics of *B. bissettii,* and especially its induced humoral response, are similar to *B. burgdorferi* ss, suggesting that this species may also cause human infection.

4.2. Introduction

In 1982, the causative agent of Lyme disease was isolated from an *Ixodes scapularis* tick [1]. The bacterium, a gram-negative spirochete, was named *Borrelia burgdorferi* after Dr. Willy Burgdorfer, one of the researchers who identified it. Three decades after this discovery, advancements in molecular biology have led to the delineation of the *B. burgdorferi* sensu lato (sl) species complex [2]. This complex is currently made up of 19

named species distributed around the globe [3, 4]. While genetically similar, many of these bacteria occur in specific tick-vertebrate enzootic cycles [5]. Some vector ticks commonly contact humans [6]; but, many of these *Borrelia* enzootic cycles involve ticks that rarely bite humans, which subsequently leaves the pathogenicity of many *B. burgdorferi* sl species in question [7, 8].

In 2011, 33,000 cases of human Lyme disease were reported to the Centers for Disease Control (CDC) [9]. However, the CDC has announced they estimate this statistic is well below the actual number of diagnosed cases (~300,000/year) [10]. Seven *B. burgdorferi* sl species have been culture isolated in North America [2]. Yet, only *B. burgdorferi* ss is widely considered responsible for cases of human Lyme disease in the US [2]. One of the species, *Borrelia bissettii*, is found overlapping the distribution of *B. burgdorferi* ss in the US and also in areas of Europe. *Borrelia bissettii* is phylogeneticly closely related to the human pathogenic *B. burgdorferi* ss species [11]. To date, three studies have demonstrated molecular detection of *B. bissettii* [12-14] in human clinical samples. *Borrelia bissettii* infection and pathology have been previously reported in laboratory animals [15, 16]; but, apart from these and general molecular surveys in ticks and wildlife, our understanding of *B. bissettii*, as well as the majority of non-*B. burgdorferi* ss in the US, is infantile at best.

Diagnosis of Lyme disease is an evolving field, often based on a combination of clinical signs (i.e., erythema migrans, facial palsy, and major joint arthralgia), exposure to vector ticks in endemic areas, and serological diagnostic tests [17]. It is well understood that *B. burgdorferi* sl spirochetes can cause a variety of clinical signs [18, 19], overlap geographically [4, 8], and have differential reactivity with serological diagnostic tests [20, 21]. Initial diagnostic tests (enzyme-linked immunosorbent assays, and IgM/IgG

immunoblots) were designed based on the *B. burgdorferi* ss organism; however, with our increased understanding of the genetic diversity of this species complex, many of these tests are proving to be less effective in their diagnostic abilities. This can be attributed to the highly diverse repertoire of *Borrelia* immunogenic proteins [22], ipso facto, studies have shown that utilization of multiple species in these serological tests greatly increases sensitivity [25-28]. If in North America multiple *B. burgdorferi* sl spirochetes have the potential to cause human illness, it is essential we have a complete understanding of their infection course and induced immune response in well characterized animal models.

In this study, we compared *B. bissettii* and *B. burgdorferi* ss infection in a murine model by examining the ID_{50} value, tissue bacterial load, and immune response. Although *B. bissettii* had a much higher ID_{50} value than *B. burgdorferi* ss, infection generated similar bacterial load between the two in most types of tissues examined. Moreover, *B. bissettii* induced a similar humoral response as *B. burgdorferi* ss.

4.3. Methods

4.3.1. *Borrelia* Species

Low passage (≤ 5) *B. burgdorferi* strain B31 (5A11) and *B. bissettii* strain CO275 were grown in Barbour-Stoenner-Kelly (BSK-H) complete (Sigma-Aldrich) media with supplemented antibiotics as previously described (*Borrelia* strain descriptions can be found in appendix) [23]. *Borrelia burgdorferi* B31 (5A11) is a clonal isolate from the original B. burgdorferi isolated from an *I. scapularis*, this clone has the full repertoire of 21 plasmids [24]. *Borrelia bissettii* CO275 is a bacterial isolate from a *Neotoma mexicana* in Colorado [25].

4.3.2. Murine Inoculation

Initially, six week old male C3H/HeJ (Jackson Labs) mice were randomly allocated into three groups of five mice. Intradermal inoculations were done in the caudoventral abdomen just right of midline with 10^6 mid-log phase *Borrelia*. Groups were inoculated with either *B. bissettii*, *B. burgdorferi* or BSK-H. Infection was allowed to develop for four weeks after which mice were humanely euthanized. Blood (intracardiac) and tissues (skin, heart, bladder and joint) were taken for subsequent serology, culture and qPCR. Tissues were cultured as previously described [26] and monitored weekly for eight weeks by dark-field microscopy for evidence of spirochete growth. Three BALB/c scid mice were inoculated with *B. bissettii* culture as described above. After three weeks, mice were sacrificed and tissues (ear punch, inoculation site, and heart) were cultured as previously described [26]. Cultures were grown up to mid-log phase and these host adapted spirochetes were utilized to repeat the experiment in immunocompetent C3H/HeJ mice increasing numbers of mice to 15 for *B. bissettii*. Animal procedures were approved by Louisiana State University's Institutional Animal Care and Use Committee.

4.3.3. ID_{50} Determination

Host adapted spirochetes were grown in culture as mentioned above. Cultures were 10-fold serially diluted in BSK-H media. Twenty mice were randomly allocated into four groups. Mice were inoculated with a single dose of spirochetes suspended in 100 µL of BSK-H. Doses ranged from 10^3 to 10^6 spirochetes. Mice were monitored for infection by culture of ear punch biopsies weekly for four weeks. Mice were then sacrificed and tissues were

subjected to culture. The ID_{50} values were calculated by the method of Reed and Muench [27].

4.3.4. DNA Extraction and qPCR

Mice tissue samples were subjected to gDNA extraction using GeneElute Genomic DNA Miniprep kit (Sigma) following manufacturer's supplied protocol. Extracts were stored at -20˚C until qPCR was performed. Primers and probes (Table 4.1) for *B. burgdorferi* and *B. bissetii* were developed based on known sequences of the decorin binding protein B (*dbpB*) gene located on lp54 (a single copy linear plasmid essential for the *borrelia* infectious cycle). Mouse primers and probes were adapted from Grasperge et al. (2012) [28]. qPCR reactions were set up in a 96-well plate; each reaction consisted of 17.5 µL of 2X Maxima Probe qPCR Master Mix (Thermo Scientific), 200 nM of each *Borrelia dbpB* primer and probe, 8 5nM of each mouse *cfd* primer, 200 nM of mouse *cfd* probe; molecular biology grade water, and 5 µL of template DNA.

Table 4.1. qPCR Primers and probes utilized in this study

Primer Name	Sequence	Reference
BbdbpB-F	5'-GGCTAGTCCACCACTTGTTACC-3'	This Study
BbdbpB-R	5'-GCAGCTCTTGAATCGTCCTC-3'	This Study
BbdbpB-Probe	5'-Cy5-CACCTTTTCCCGTGGCTTCTT-3'	This Study
BbissdbpB-F	5'-AAACGCACTCCCTTGTCAG-3'	This Study
BbissdbpB-R	5'-GGTTGCATGTAACGTTGGAC-3'	This Study
BbissdbpB-Probe	5'-HEX-TCCCTAGAAGATGATTCAAGCGC-3'	This Study
CFD1461	5'-CAGTTTCTTGCTGGCTATTGG-3′	26
CFD1570	5′-CCACGTAACCACACCTTCG-3′	26
CFD-Probe	5'-(6)FAM-TAGTGGCCCTGCCCTA-3'	26

Reactions were transferred in triplicate-10 µL aliquots to 384-well plates for subsequent qPCR. Standard curves for quantification were based on 10-fold serial dilutions of single copy gene plasmids. Plasmid constructs for standard curve serial dilutions were assembled

following methods previously described [29]. qPCR was performed with a Roche Lightcycler 480i. Thermocycling protocol included an initial denaturation step for 10 mins at 95˚C, followed by 40 cycles of a denaturation step for 15 sec at 95˚C and a 60 sec at 60˚C annealing step. Analysis of the reaction was conducted with the LightCycler 480i software.

4.3.5. C6 Peptide Enzyme-Linked Immunosorbent Assay (ELISA)

Individual infected mouse serum (diluted 1:200) was tested by C6 ELISA as previously described [30]. A TMB ELISA development kit (Kirkegaard & Perry laboratories) was utilized according to manufacturer's directions, and optical density (OD) values were measures at 450 nm on an ELISA plate spectrophotometer. Cutoff OD was defined as the mean OD ± 3 standard deviations of the negative control mice sera. All samples were assessed in duplicate and averaged ODs are reported.

4.3.6. Immunoblots

Immunoblots of whole cell *B. burgdorferi* B31 fractionated proteins were accomplished with the MarDx Marblot IgG kit (Trinity Biotech) following manufactures instructions with minor modifications. This included utilizing individual infected mouse sera as the primary antibody (1:200 dilution) followed by incubation with 1:1000 goat-anti mouse alkaline phosphatase labeled IgG (Kirkegaard and Perry Laboratories).

4.3.7. Characterization of *B. bissettii* (CO275) by Next Generation Sequencing

Borrelia bissettii spirochetes isolated from BALB/c scid mice were subjected to next generation sequencing on the Ion Torrent PGM platform utilizing a 316 chip, followed by denovo assembly with MIRA 4.0 [31]. MIRA processed 3,763,285 reads which produced 816 contigs with an average coverage of 150x. Contigs generated were then aligned to a reference genome (*B. bissettii* DN127; NC_015921) using Geneious version 7.1.2 this

resulted in 352 contigs mapped to the reference chromosome which provided almost complete coverage (99.6%) and a 98.5% homology. Protein coding regions (CDS) of the 352 chromosome contigs were identified and annotated by the NCBI Prokaryote Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and deposited in NCBI WGS server (Accession JNBW00000000). Non-chromosome contigs were aligned to *B. burgdorferi* B31 plasmids (NC_000948-000957, NC_001903-001904 and NC_001849-001857) and open reading frames (ORFs) were predicted with the program GLIMMER [32]. Geneious aligner was utilized to pairwise align and compare known *B. burgdorferi* B31 host associated virulence determinates with *B. bissettii* CO275 homologs.

4.3.8. Statistical Analysis

Data were analyzed by Kruskal-Wallis with Dunn's Multiple Comparison in GraphPad Prism version 5.00 for windows (GraphPad Software). P values ≤ 0.05 were considered significant.

4.4. Results

4.4.1. *Borrelia bissettii* is Able to Infect Immunocompetent Mice Only After Adaptation in Immunodeficient Mice

The initial infection study was performed by intradermal inoculation of C3H/HeJ mice with a single dose of 10^6 spirochetes of *B. bissettii* or *B. burgdorferi* ss grown from frozen stock cultures. As shown in Table 1, all the five mice that were inoculated with *B. burgdorferi* ss developed infection. In contrast, none of the mice that received *B. bissettii* produced a positive tissue cultures indicating that *B. bissettii,* in at least in this phase and form, are unable to infect immunocompetent mice (Table 4.2).

Borrelia bissettii was shown infectious in past studies [16, 33]. Lose of infectivity could result from long term storage [34]. To restore infectivity in immunocompetent mice,

cultured *B. bissettii* was first inoculated into BALB/c scid mice. As shown in Table 4.2, all tissues examined became positive four weeks post inoculation. Next, *B. bissettii* isolated from infected scid mice was utilized to infect immunocompetent mice. Like mice that were inoculated with *B. burgdorferi* ss, all mice inoculated with host adapted *B. bissettii* became infected (Table 4.2), indicating that *B. bissettii* infectivity was restored after adaptation in immunodeficient animals.

The original *B. bissettii* isolate used in this study was a low passage stock culture, which had never been subcloned. Because of the potential for a highly herterogeneous population of borreliae, passage through scid mice allowed us to select for a more homogenous population of infectious spirochetes, which we confirmed by next generation sequencing.

Table 4.2. *Borrelia bissettii* is able to establish infection in immunocompetent mice only after adaptation in immunodeficient mice.

Experiment	Inoculum (10^6 cells)	No. of positive specimens/no. of samples examined					All Tissues	No. of infected mice/no. of mice inoculated
		Ear	Skin	Heart	Bladder	Joint		
1	*B. bissettii*	0/5	0/5	0/5	0/5	0/5	0/20	0/5
	B. burgdorferi ss	5/5	5/5	4/5	5/5	5/5	24/25	10/10
2	*B. bissettii*	3/3	ND	3/3	ND	3/3	9/9	3/3
3	*B. bissettii*	15/15	15/15	11/15	15/15	13/15	69/75	15/15
	B. burgdorferi ss	5/5	5/5	5/5	5/5	5/5	25/25	5/5

***** Experiment 1: Ten C3H/HeJ mice were inoculated with 10^6 cells of original *B. bissettii* (n=5) or *B. burgdorferi* ss (n=5) cultures. Experiment 2: Three BALB/c scid mice were inoculated with 10^6 cells of *B. bissettii.* Experiment 3: Twenty C3H/HeJ mice were inoculated with 10^6 cells of either host-adapted *B. bissettii* cells isolated from scid mice (n=15) or infectious *B. burgdorferi* ss (n=5). Ear, skin (inoculation site), heart, bladder and joint specimens were collected four weeks post-inoculation, cultured for spirochetes and monitored for bacteria growth for eight weeks. ND, not determined.

4.4.2. *Borrelia bissettii* Infection Produces Similar Bacterial Loads in Skin, Heart and Bladder Tissues as *B. burgdorferi* ss but Lower Burdens in Joint Tissues

Tissue bacterial load was estimated by qPCR to compare virulence between *B. bissettii* and *B. burgdorferi* ss.

After four weeks, tissues (skin, heart, urinary bladder and joint) were harvested for DNA extraction from 15 C3H/HeJ mice infected with *B. bissettii* and 10 mice infected with *B. burgdorferi* ss. As shown in Figure 4.1, *B. bissettii* infection led to similar bacterial load as *B. burgdorferi* ss infection in skin, heart and bladder tissues. However, *B. bissettii* burdens were significantly lower in joint tissues (Figure 4.1).

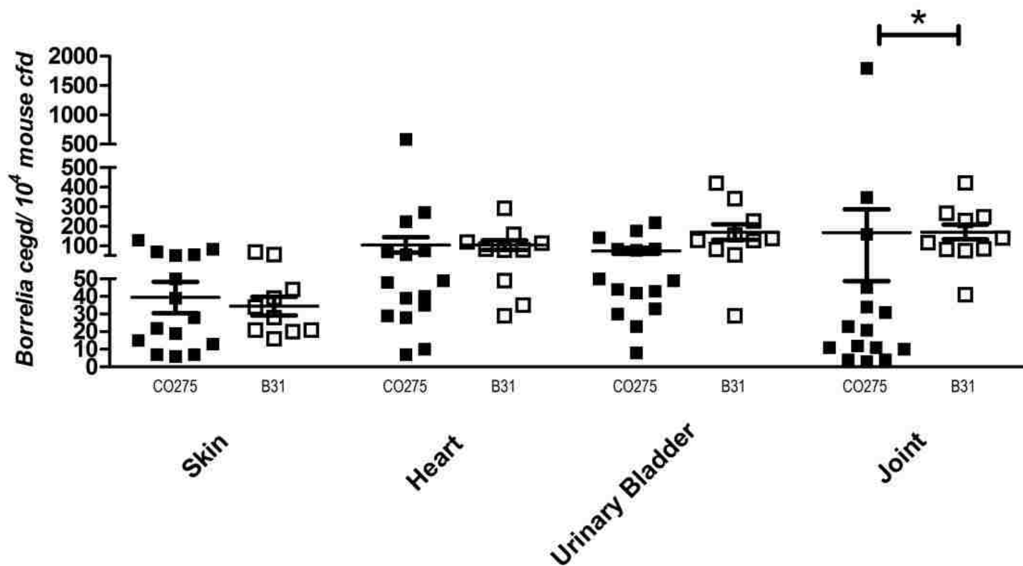

Figure 4.1. *Borrelia bissettii* infection generates similar bacterial loads in skin, heart and bladder tissues as *B. burgdorferi* ss but lower spirochete burdens in joint tissues. Twenty five C3H/HeJ mice were inoculated with 10^6 spirochetes of either host-adapted *B. bissettii* (n=15) or infectious *B. burgdorferi* ss (n=10) and sacrificed a month later. Skin (inoculation site), heart, bladder and joint specimens were harvested for DNA extraction and analyzed for tissue bacterial load by qPCR. The bacterial load is presented as *dbpB* gene copy number (copies of extracted gDNA, cegd) per 10^4 mouse cells. Data were analyzed by Kruskal-Wallis with Dunn's Multiple Comparison. Error bars define SEM and asterisks denote significance $p \leq 0.05$. (C0275= *B. bissettii*, B31= *B. burgdorferi*).

4.4.3. The ID_{50} Value of *B. bissettii* is Higher than *B. burgdorferi* ss

ID_{50} was determined as a measurement of *B. bissettii* virulence in the murine host. As shown in Table 4.3, the ID_{50} value was approximately $2.4x10^4$ organisms. This represents at least a 100-fold higher ID_{50} than *B. burgdorferi*, whose ID_{50} has been estimated at >200 organisms in numerous studies conducted by different groups including us [35-38].

4.4.4. *Borrelia bissettii* Elicits a Similar Murine Antibody Response as *B. burgdorferi* ss

Murine humoral immune response was analyzed and compared between *B. bissettii* and *B. burgdorferi* ss using a Lyme IgG immunoblot (Mardx Marblot). This commercial kit was chosen as it is commonly utilized in Lyme serology. As shown in Figure 4.2, similar banding patterns were detected in serum from mice regardless of *Borrelia* infection. Accordingly, when recommended diagnostic criteria were applied, as the manufacturer instructs, all infected mice were diagnosed as positive by Lyme serology.

Table 4.3. ID_{50} Determination of *B. bissettii*

Inoculum (No. of cells)	No. of culture positive samples/no. of specimens examined				**No. of infected mice/no. of mice inoculated**	**ID_{50} (no. of cells)**
	Ear	**Skin**	**Heart**	**Joint**		
10^3	0/5	0/5	0/5	0/5	0/5	$2.4X10^4$
10^4	1/5	1/5	0/5	1/5	1/5	
10^5	5/5	0/5	0/5	0/5	5/5	
10^6	5/5	4/5	2/5	2/5	5/5	

* Twenty C3H/HEj mice were inoculated with serial dilutions of host adapted *B. bissettii* cultures. Ear punches were cultured and monitored weekly for spirochete growth. All mice were sacrificed four weeks post inoculation and tissues were cultured and monitored by dark field microscopy for eight weeks for evidence of bacterial growth. The ID_{50} values were calculated by the method of Reed and Muench [25].

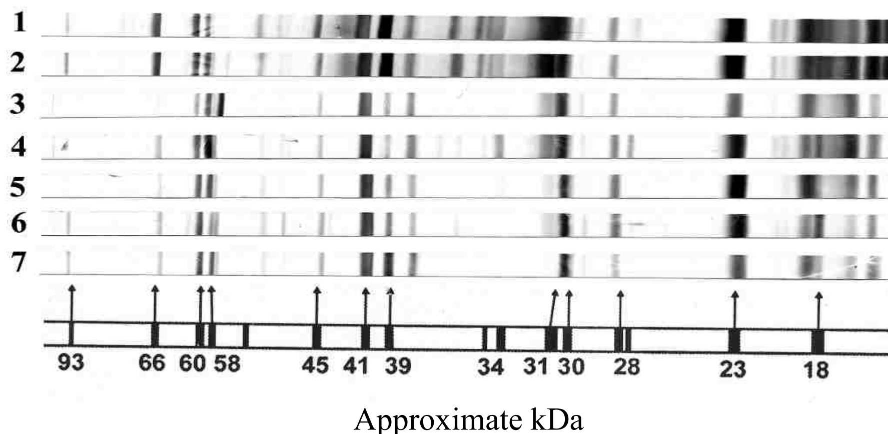

Figure 4.2. *Borrelia bissettii* humoral response is similar to *B. burgdorferi* ss during murine infection. Serum samples were collected from mice inoculated with 10^6 of either infectious *B. burgdorferi* ss (Lane 1 and 2) or host adapted *B. bissettii* (Lane 3-7) cultures four weeks post inoculation and analyzed for specific immune response using a commercial IgG immunoblot (MarDx Marblot)

4.4.5. *Borrelia bissettii* Elicits an Anti-C6 Antibody Response During Murine Infection

As all *B. burgdorferi* sl species identified, to date, as Lyme disease agents induce a strong C6 antibody response [39], the anti-C6 antibody response to *B. bissettii* infection was analyzed. As shown in Figure 4.3, the specific antibody response was strong in all mice infected with *B. bissettii*, indicating that the C6 ELISA also can detect infections with these particular species.

4.4.6. *Borrelia bissetti* Possess Homologous Genes of Many Major *B. burgdorferi* ss Virulence Determinates

The anti-C6 response demonstrated that *B. bissettii* must harbor a *vlsE* gene, but genomic data available for this species lack such a gene [40].To address this issue, DNA was prepared from our infectious isolate, and next generation sequencing was performed on the Ion Torrent PGM platform. In addition to a *vlsE* gene, many other critical virulence genes, such as *ospAB*, *dbpAB*, *bbk32, p66, ospC, crasp1, crasp2,* and multiple *erps* were found within the genome (Table 4.4).

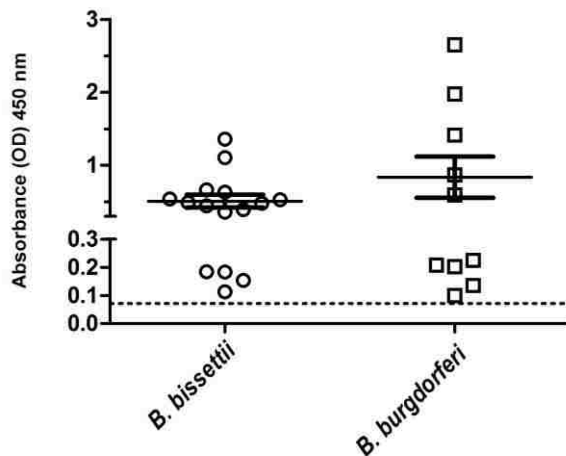

Figure 4.3. *Borrelia bissettii* induces an anti-C6 antibody response similar to *B. burgdorferi* ss during murine infection. Sera were collected from mice inoculated with 10^6 of either host adapted *B. bissettii* (n=15) or infectious *B. burgdorferi* ss (n=10) cultures four weeks post inoculation and analyzed for anti-C6 antibody by ELISA. Dotted line indicates a cutoff value as defined by the mean OD plus 3 standard deviations obtained from 10 sham inoculated mice.

4.5. Discussion

Areas in Eurasia experience first-hand the complexity that multiple pathogenic *Borrelia* can add to the epidemiology of human Lyme disease. With the forefront of molecular technologies, our initial understanding of this pathogen has drastically changed [2]. While these advances can work in our favor, in many ways they also complicate the situation. The idea of one spirochete species, *B. burgdorferi*, being the sole cause of human Lyme was challenged in Europe. European cases of human borreliosis have clinical manifestations that present differently when compared to Lyme cases in the US [41, 42]. In addition, spirochetes isolated from vector ticks and humans infections showed phenotypic differences when compared to *B. burgdorferi* [42]. Eventually multiple *Borrelia* species, most notably *B. afzellii* and *B. garinii*, were identified as additional spirochetes capable of causing human illness in Eurasia [2]. Subsequently, serological tests initially developed

Table 4.4. *Borrelia bissettii* CO275 contains many homologous genes to *B. burgdorferi* B31 host virulence determinates

***Borrelia burgdorferi* B31 Gene**	**CO275 Contig Location**	**Nucleotide Pairwise Homology (%)**
bbk32	c1220	94.6
crasp1	c303	68.5
crasp2	c1369	97.7
dbpA	c171	77.9
dpbB	c151	83
erpG	c265	78
erpK	c16	74.2
erpP	c155	77.3
ospA	c310/1165	89.3
ospB	c1166	84.8
ospC	c183	82.7
p66	c24	94
vlsE	c233	82

*****Contigs were mapped to B. burgdorferi B31 plasmids and ORFs were identified utilizing the program GLIMMER. Geneious aligner was utilized to compute pairwise alignment.

against North American *B. burgdorferi* ss were found to be less effective in detecting confirmed human borreliosis cases in these same areas [20, 21]. The United States, like Eurasia, has a diverse *Borrelia* fauna. While *B. burgdorferi* ss certainly accounts for the majority of human borreliosis cases [43], current serological methods have not been sufficiently evaluated with respect to their efficacy in detection of non-*B. burgdorferi* ss North American *Borrelia* infections It is also unknown what, if any, clinical presentations would be encountered in a non-*B. burgdorferi* ss North American borreliosis infection. What is known is that many areas, while not endemic for *B. burgdorferi* ss, do have an abundance of other *B. burgdorferi* sl species [4, 8]. In this study, we describe murine infection and antibody response to *B. bissettii*, a widespread yet understudied *Borrelia* species.

Interestingly, we were unable to infect immunocompetent mice with the *B. bissettii* culture grown directly from low passage frozen stocks. Because this isolate had been utilized in previous studies and was successful in murine infection [16, 33], we passed the cultures through immunodeficient scid mice to determine if over time these isolates had lost their infectious phenotype. The isolate caused disseminated infections in scid mice and re-isolated spirochetes were utilized successfully in repeat experiments with immunocompetent mice. This phenomena had never been experienced in our laboratory, but loss of infectivity after long term *Borrelia* storage has been described by at least one other research group [34]. This isolate had been in cold storage for over 15 years. Similarly, virulence associated plasmid loss has been described after long term storage of *Bacillus anthracis* [44]. In this study, by passing spirochetes through scid mice, we were able to restore the ability to infect immunocompetent animals. The objective of this study was not to investigate the reason behind this finding; however, the exact mechanism does warrant further study and this finding should be taken into consideration by researchers planning to utilize *Borrelia* isolates that has been stored for an extended period of time.

Borrelia bissettii like *B. burgdorferi* ss has been shown to cause similar pathology in murine hosts. This includes similar lymphocytic accumulations in the bladder interstitium, mononuclear cell infiltrates into great vessels and myocardium, resulting in atrial inflammation and myocardial necrosis and less commonly mild inflammation and lesions in the femorotibial joint [16]. Spirochete burdens reported in this study support the pathological findings reported by Schneider et al. *Borrelia bissettii* disseminates to tissues and has similar spirochete burdens compared to *B. burgdorferi* ss in the skin, heart and urinary bladder; yet, colonizes the joint tissues at lower numbers. Similar burdens seen in both the urinary bladder

and heart substantiate pathology seen in murine models of both *Borrelia* infections. Decreased joint burdens described in this study attest to the variable signs of joint pathology seen in a previous *B. bissettii* pathology study [16]. Spirochete burden data also supports evidence of human manifestations of *B. bissettii* infection which include reports of *B. bissettii* DNA detection in a cardiac tissue biopsy of a Lyme borreliosis case and in multiple instances, human serum [12-14]. Our findings, as well as growing literature reporting the pathogenic potential of *Borrelia bissettii,* demands further investigation.

Much research has been targeted at developing better diagnostic tests for human Lyme borreliosis infection. Current serological diagnostic methods recommended by the Infectious Disease Society of America consist of a two-tier approach [45]. This includes a Lyme whole cell ELISA followed by an IgM/IgG immunoblot. Another serological test, the C6 ELISA, has been shown to be comparable if not more reliable than the current two-tier testing approach [46-48]. Initial serological tests were directed towards detecting one species, *B. burgdorferi* ss, of the *B. burgdorferi* sl complex. However, the expanding role of other species in human illness has led to serological tests, especially in Eurasia, that utilize multiple *B. burgdorferi* sl species to better facilitate diagnosis [49, 50]. This has been shown to increase sensitivity and specificity of these tests in areas endemic for multiple human pathogenic borreliae [49, 50]. In this study, we investigated the ability of a Lyme IgG Immunoblot and the C6 ELISA to detect infection in our *B. bissettii* murine model. First, we assessed the ability of an FDA approved immunoblot test to detect *B. bissettii* infection in mice. Our results indicate that *B. bissettii* infected mice had similar immunoblot results compared to mice infected with *B. burgdorferi* ss. Therefore, murine infections with *B. bissettii* elicit an antibody response that is cross reactive with many *B. burgdorferi* ss

antigenic proteins. The second test we evaluated was the C6 peptide ELISA test. It is based on a 26-mer amino acid peptide developed against an invariant region (IR6) of the well-studied virulence associated outer surface vlsE protein [30]. This peptide was created against the *B. burgdorferi* ss vlsE protein sequence but has been shown to be conserved among the three major pathogenic *Borrelia* spp. (*B. burgdorferi* ss, *B. afzelii* and *B. garinii*); and, during infection with many of these species mice elicit a positive anti-C6 antibody response [39]. In our study *B. bissettii* infected mice elicited a similar anti-C6 antibody response as did mice exposed to *B. burgdorferi* ss. This is the first report of a North American *B. burgdorferi* sl species other than *B. burgdorferi* ss eliciting a mammalian anti-C6 antibody response. Considering the endemicity of *B. bissettii* in many states and a recent study [51] reporting canines in Colorado testing positive by a diagnostic C6 snap test; it is likely, contrary to the authors' conclusion, that these animals were exposed to and or infected by *B. bissettii*, further supporting the pathogenicity of this species of Lyme *Borrelia*.

With the continued development of next generation sequencing, the ability to identify similarity between bacterial species is becoming easier, faster and more cost effective. Because the only *B. bissettii* genome sequenced to date was lacking the *vlsE* gene and lacks experimental infection data, we subjected our isolate to next generation sequencing. We confirm the species identity of *B. bissettii* CO275 and identify homologs to many major *B. burgdorferi* B31 host associated virulence genes supporting the infectious phenotype seen in our laboratory studies.

Our results confirm that *B. bissettii* has the ability to cause disseminated infections in mice [15, 16, 33, 52]. More importantly, this is the first report of spirochete infection loads and anti-C6 antibody response during infection with *B. bissettii*. These data also describe the

ability of current serological tests to detect murine infection of *B. bissettii*. In mice, *B. bissettii* can cause a disseminated infection, and based on two serological diagnostic tests, has similar antibody profiles to *B. burgdorferi* ss infections. While the murine model is the first step at determining infectivity of *B. bissettii* to mammals, it by no means implies these organisms are infectious to humans; however, there is growing evidence that *B. bissettii* may cause human and possibly canine illness. Based on our work, current serological test are unable to distinguish between these two infections in mice. Future studies should assess specific antigenic profiles shared by *B. bissettii* and *B. burgdorferi* ss to facilitate detection of specific *Borrelia* infections. More human-like animal models, such as the non-human primate model, could be utilized to determine what, if any, clinical signs *B. bissettii* infections would manifest in humans. The spectrum on Lyme disease is changing; to combat this global complex of emerging and potentially emerging bacterial infections, we must fully understand the scope of the problem. Future work should be directed at assessing the ability for each *B. burgdorferi* sl species' potential to cause illness in humans.

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CHAPTER 5. *IXODES SCAPULARIS* IS ABLE TO ACQUIRE YET UNABLE TO TRANSMIT *BORRELIA BISSETTII* TO MICE

5.1. Abstract

It is estimated that 300,000 cases of human Lyme borreliosis occur annually in the United States. Although the US is home to a diverse fauna of Lyme *Borrelia* species, only one is considered responsible for human disease. However, evidence is surfacing implicating other species in human illness. And, while much research has focused on the *Borrelia burgdorferi* sensu stricto (ss)-tick interface, tick vectors for most of the other North American *Borrelia* species still remain unknown. In this study we assess the ability of *Ixodes scapularis* to acquire and transmit *Borrelia bissettii*, a potential human pathogen, in a murine model. *Borrelia*-free *I. scapularis* larvae were allowed to feed on mice with disseminated infections of *B. burgdorferi* ss or *B. bissettii*. Ticks that molted were then allowed to feed on naïve mice to assess for transmission. Additionally, ticks were collected at multiple time points throughout the experiment and spirochete infection and burdens were assessed by culture and qPCR. Although *I. scapularis* acquire *B. bissetii* spirochetes at similar proportions as *B.* burgdorferi ss, spirochete burdens were significantly lower in *B. bissettii* infected ticks. Furthermore, *I. scapularis* is unable to transmit infection to naïve animals. And although a single animal showed some evidence of spirochete exposure, serology supported the finding that infection did not develop in these animals. In the tick, *B. bissettii* spirochete levels modestly increase throughout the tick's developmental cycle until the adult stage where infection is not evident. *Borrelia burgdorferi* ss, unlike *B. bissettii*, underwent an exponential increase in spirochete numbers during the nymphal blood meal and retained similar infection levels as adults. In this model, *Ixodes scapularis* was unable to support *B. bissettii* throughout its life cycle, and while viable spirochetes can be detected in *B. bissettii*

infected ticks fed on naïve mice, infection is not transmitted. These data implicate another tick as the primary vector for *B. bissettii* in the eastern and southern US. However, this unique model may help assess details of the *Borrelia*-tick interface.

5.2. Introduction

Lyme borreliosis is a tick-borne zoonotic disease caused by spirochetes from the *Borelia burgdorferi* sensu lato (sl) genocomplex (collectively referred to as Lyme *Borrelia*). Spirochetes in this complex are responsible for an estimated 300,000 cases of human disease a year in the United States and at least another 85,000 cases a year in Europe [1, 2], making Lyme borreliosis the most common arthropod-borne disease in the Holarctic regions of the world [3, 4].

Nineteen species make up the *B. burgdorferi* sensu lato complex [5]. Lyme *Borrelia* spirochetes are transmitted by ticks from the *Ixodes ricinus* complex and are dependent on both ticks and reservoir hosts to perpetuate in natural enzootic cycles [6]. Not all *B. burgdorferi* sl species are associated with human disease; to date, 12 species are confirmed or probable human pathogens [5, 7]. Of the Lyme *Borrelia* associated with or known to cause human disease, generally, one of five species are most often implicated in human infection. Yet, *Borrelia afzellii*, *Borrelia garinii*, and *B. burgdorferi* sensu stricto (ss) are responsible for the majority of human Lyme borreliosis around the world [8]. *Borrelia burgdorferi* ss is distributed worldwide, while *B. afzellii* and *B. garinii* are solely old world pathogens [8]. Seven species of Lyme *Borrelia* are endemic to the United States [9]. However only, *Borrelia burgdorferi* ss, is considered responsible for human cases of Lyme borreliosis [10]. Three decades after the discovery of the Lyme *Borrelia* complex, our understanding of this group of bacteria is still limited. Research has been focused intensely on the three major

human pathogenic species, especially *B. burgdorferi* ss, while the remaining 16 species remain understudied. As the *Borrelia* species complex keeps expanding, little work is done to identify the pathogenic potential and vectors for newly discovered and described species. This leaves a critical gap in our grasp of the epidemiology of Lyme borreliosis. In the United States, 95% of human Lyme borreliosis cases occur in the Northeast and Great Lake states where the organism responsible is *B. burgdorferi* ss and the vector is *Ixodes scapularis [10]*. However, cases also occur in many other areas of the US [11], including states where *B. burgdorferi* ss has yet to be described.

One of the understudied North American Lyme *Borrelia* species is *Borrelia bissettii*. *Borrelia bissettii* was first isolated in 1987 by Bissett and Hill, and like many of the Lyme *Borrelia* spirochetes, was first described as an atypical *B. burgdorferi* ss strain [12]. It was not until 1998 that this atypical group of spirochetes received formal designation as a separate *Borrelia* species [13]. The range of this group of spirochetes is quite large, and like *B. burgdorferi* ss can be found both in the New and Old World [14]. *Borrelia bissettii* spirochetes have also been detected in many states across the US, including SC, GA, FL, MI, LA, CO, NC and CA [15-20]. Both *Ixodes pacificus* and *I. spinipalpus* have been experimentally demonstrated to be competent vectors to vertebrates; however, the ability of *I. scapularis* has only been suggested in the literature [21, 22]. *Borrelia bissettii* has been described in hosts including small rodents and lizards [13, 17, 22]. A few studies have investigated *B. bissettii* in experimental murine models [21, 23]. Mice develop pathology within the bladder, heart and occasionally in the femorotibial joint in similar ways as *B. burgdorferi* ss [23]. *Borrelia bissettii*-like spirochetes have been isolated from human

patients in Europe, and detected by polymerase chain reaction in serum, blood, skin and cardiac tissue in both the US and Eastern Europe [24-27].

Ixodes scapularis is arguably the most important human biting tick in the United States, serving as a vector for at least five tick-borne zoonosis. Even with recent evidence associating *B. bissettii* with human illness [24-28], detection of the organism in field collected ticks [16, 18-20, 22, 29-32], and the similarities between *B. burgdorferi* ss and *B. bissettii* infection in animal models, there exists no experimental evidence implicating *I. scapularis* as a vector for *B. bissettii*. This study tests the hypothesis that *I. scapularis* is able to acquire *B. bissettii* from, and subsequently transmit *B. bissettii* to a susceptible murine host.

5.3. Materials and Methods

5.3.1. Mice

C3H-HeJ mice, obtained from Jackson laboratory, were utilized in this experiment. All mice were 5-week-old males and were housed in microisolator cages with HEPA filtered air. Both food and water were provided ad libitum. All animal research was approved by the IACUC at Louisiana State University.

5.3.2. *Borrelia* Culture Preparation

Frozen stocks of low passage *B. burgdorferi* strain B31 and *B. bissettii* strain CO275 were cultivated in Barbour Stoenner Kelly (BSK-H) complete media (Sigma) supplemented with antibiotics and fungicide as previously described [33]. Cultures were grown to a concentration of 10^6 spirochetes per mL, centrifuged for 10 minutes at 10,000xG, resuspended in 100 µL of BSK-H and utilized for mouse inoculation. Original *B. bissettii*

cultures were inoculated into BALB/c scid mice and re-isolated for use in immunocompetent mouse infection experiments, as described in the previous chapter.

5.3.3. Mouse Infection

For initial infections, 15 mice were randomly assigned to three groups consisting of five mice. Intradermal inoculations were done in the caudoventral region of the abdomen just right of midline with a 27G syringe and 100μL of inoculum. Group 1 mice were inoculated with *B. burgdorferi* ss spirochetes. Group 2 mice were inoculated with *B. bissettii* spirochetes. Mice in group 3 served as a control and were inoculated with only BSK-H media. Infections were allowed to develop for four weeks. Infection status was monitored by taking ear punch biopsies (EPB) at seven day intervals. Ear punch biopsies were cultured in BSK-H media as previously described, and monitored weekly for eight weeks by darkfield microscopy for spirochete growth [34].

5.3.4. Tick Feeding

Borrelia-free larval *I. scapularis* were purchased from Oklahoma State University Tick Laboratory and maintained in an environmental chamber (Percival, Perry IA) with a 12:12 L:D schedule at ≥96% RH. Larval ticks were allowed to free feed until repletion on individual mice caged on a wire floor over water. Engorged larva from each mouse were allowed to molt, subjected to culture as described above, or stored in 100% ethanol at -20˚C until DNA extraction and qPCR could be performed. After molt, nymphal ticks were again subjected to culture, stored in 100% ethanol at -20˚C until DNA extraction and qPCR, or utilized for transmission experiments to naïve mice.

5.3.5 Transmission experiment

Nymphs derived from larva fed on infected mice were utilized to determine transmission rates to naïve mice. After molt, ticks were allowed to rest for a minimum of two weeks. Again 15 mice were randomly allocated into three groups of five mice. Each mouse was infested with 20 nymphal *I. scapularis* that had previously fed on an uninfected, *B. bissettii* infected or *B. burgdorferi* ss infected mouse. Engorged ticks were collected and subjected to culture, stored in 100% ethanol at -20˚C until DNA extraction and qPCR, or allowed to molt to adult stage. After molt the remaining adults were subjected to culture or DNA extraction and qPCR.

5.3.6. DNA Extraction and qPCR

Whole ticks were subjected to genomic DNA extraction using GeneElute Genomic DNA Miniprep kit (Sigma). Ticks were minced with a sterile scalpel in separate 1.5 mL sterile microcentrifuge tubes containing 180 µL of Lysis Solution T (Sigma) and 20 µL of a 20 mg/mL solution of proteinase K (Sigma). Tubes were vortexed thoroughly and placed in a water bath incubator at 55˚C overnight (~12 hours) until completely dissolved. Following incubation, gDNA extraction was carried out according to manufacturer's supplied protocol except extracts were eluted in 100 µL of supplied elution buffer. Extracts were stored at -20˚C until qPCR was performed. *Borrelia*-specific primers and probes for *B. burgdorferi* ss and *B. bissettii* were developed based on known sequences of the decorin binding protein B (*dbpB*) gene located on lp54 (a linear plasmid essential for infectivity) (Table 5.1). 17.5 µL of 2x Maxima Probe qPCR Master Mix (Thermo Scientific); 200nM of each *Borrelia dbpB* primer and probe combination, molecular biology grade water, and 5µL of template DNA.

Table 5.1. qPCR Primers and probes utilized in this study

Primer Name	Sequence	Reference
BbdbpB-F	5’-GGCTAGTCCACCACTTGTTACC-3’	This Study
BbdbpB-R	5’-GCAGCTCTTGAATCGTCCTC-3’	This Study
BbdbpB-Probe	5’-Cy5-CACCTTTTCCCGTGGCTTCTT-3’	This Study
BbissdbpB-F	5’-AAACGCACTCCCTTGTCAG-3’	This Study
BbissdbpB-R	5’-GGTTGCATGTAACGTTGGAC-3’	This Study
BbissdbpB-Probe	5’-HEX-TCCCTAGAAGATGATTCAAGCGC-3’	This Study

qPCR reactions were set up in a 96-well plate in 35 μL reactions; each reaction consisted of From 96-well plates reactions were transferred in triplicate-10 μL aliquots to 384-well plates for subsequent qPCR. Standard curves for quantification were based on 10-fold serial dilutions ($1x10^8$ copies to $1x10^0$ copies) of single copy gene plasmids of each *Borrelia dbpB* gene fragment. Plasmid constructs for standard curve serial dilutions were assembled following methods previously described [35]. Quantitative PCR was performed on a Roche Lightcycler 480i. Thermocycling protocol was as follows: an initial denaturation step for 10 mins at 95˚C, followed by 40 cycles of a denaturation step for 15 sec at 95˚C and a 60 sec at 60˚C annealing step. Analysis of reactions were conducted with the LightCycler 480i software.

5.3.7. Serological Tests

Both western blotting and an enzyme inked immunosorbent assay were utilized to detect exposure to *B. bissettii* spirochetes in tick exposed naïve mice. To prepare whole cell lysates for SDS-PAGE, 10 mL of late-log page *B. bissettii* culture was harvested by centrifugation. The cell pellet was washed three times with phosphate buffered saline (PBS) to remove remaining media after which we added 1 mL of Laemmli Sample Buffer (BIORAD) with DDT. Sample was loaded into a 12% Tric-HCl precaset Ready Gel (BIORAD) and separated by electrophoresis using a BIORAD MINI protean tetra system

(120v for 50 mins). Separated protein were then electrotransferred to a nitrocellulose membrane (Thermo Scientific). Membranes were blocked overnight in a 5% solution of dry milk (Carnation). The membrane was then transferred to a BIORAD MINI Protean 2 multiscreen. Primary antibody, murine serum (1:50), was added to each well and incubated for 1 hour at room temperature (RT). After incubation serum was removed and membrane was washed with a solution of PBS and 0.1% Tween 20 three times for five minutes each, after which the seconday antibody, Alexa fluor 680 donkey anti-mouse IgG (1:15000) (Invitrogen), was added to each well and incubated in the dark for an additional hour. Membranes were then removed from the slot blot and rinse as previously mentioned. Membrane were visualized on a LICOR Odyssey CLx imaging system and images were analyzed with Image Studio Version 3.1. C6 peptide ELISA was performed as previously described except with murine sera diluted at 1:50 [36].

5.3.8. *Borrelia* Isolation

Mouse tissue isolation was performed using sterilized instrument in a BSL-2 containment cabinet. Tissues were placed in BSK-H media supplemented with antibiotics and monitored for growth weekly by darkfield microscopy for eight weeks [34]. Tick isolation was performed with media described above. Ticks were surface sterilized by washing in 10% bleach, phosphate buffered saline (PBS), 70% ethanol, and finally rinsed in molecular grade water (MO-BIO). Ticks were minced up in BSK-H with a sterile scalpel and monitored for spirochetal growth.

5.3.9. Statistical Analyses

Mann-Whitney U tests were performed to compare spirochete burdens between tick infections, and a fisher's exact test was performed to compare infection proportions. Statistical tests and graphs were compiled with Graphpad Prism version 5.00. p values ≤ 0.05 were considered significantly different.

5.4. Results

5.4.1. *Borrelia bissettii* can be Acquired by *I. scapularis* as Successfully as *B. burgdorferi* ss but at Significantly Lower Burdens.

To examine whether *B. bissettii* can be acquired by *I. scapularis* ticks, groups of 5 C3H/HEj mice were inoculated with 10^6 cells of either *B. burgdorferi* ss or *B. bissettii*. Four weeks post-inoculation ear biopsies were taken and cultured to confirm infection. Approximately 200-300 *I. scapularis* larvae were allowed to free feed on each mouse. Upon detachment, ticks were surface sterilized and two tick pools (*B. bissettii*-infected and *B. burgdorferi* ss-infected) were generated for the entire study.

Twenty-five engorged larva randomly selected from each pool were cultured to determine acquisition rates. As shown in Table 5.2, 20 of the 25 ticks from the *B. burgdorferi* ss pool were positive for spirochetes, resulting in an 80% acquisition rate. In comparison, cultures were grown from 16 of the 25 ticks from the *B. bissettii* pool; an acquisition rate of 64% was obtained. These data allowed us to conclude that *I. scapularis* acquire *B. bissettii* as efficiently as *B. burgdorferi* ss ($p = 0.35$).

Next, tick spirochete loads were determined by qPCR. Fifty ticks were randomly selected from the *B. bissettii* infected pool and 40 ticks from the *B. burgdorferi* pool. *Borrelia bissettii* and *B. burgdorferi* were detected in 36 and 32 ticks, respectively. The resultant acquisition rates by qPCR were 72% for *B. bissettii* and 80% for *B. burgdorferi* (*P*

= 0.46), supporting acquisition rates as determined by culture. Ho wever, the *B. burgdorferi* spirochete load was eight times higher than that of *B. bissettii* ($P < 0.0001$) (Table 5.2).

Table 5.2. *Ixodes scapularis* is able to acquire *B. bissettii* but generates a significant lower load

Murine infection	No. of positive ticks/No. of ticks examined	*p*-value	Mean spirochete no./Tick	*p*-value
B. bissettii	16/25	0.35	2,305	< 0.0001
B. burgdorferi ss	20/25		18,467	

*Larvae were allowed to feed on mice infected with either *B. bissettii* or *B. burgdorferi* ss. Engorged ticks were examined for spirochete acquisition rates by culture and spirochete loads by qPCR. A Fisher's exact test and Mann-Whitney U test were performed to compare acquisition rates and spirochete burdens, respectively.

5.4.2. *Ixodes scapularis* are Unable to Transmit *B. bissettii* Infection to Mice.

Engorged larvae in the two tick pools were allowed to molt to nymphs. One hundred randomly selected nymphs from each pool were utilized to assess the ability to transmit infection. Groups of 5 naïve C3H/HEj mice were each infested with 20 nymphs. Based on the acquisition rate determined above, each mouse had at least 12 ticks that harbored spirochetes. Nymphs that had fed on naïve mice were collected and pooled by *Borrelia* species infection, with each sub-pool including 100 ticks. Infection was monitored by weekly ear biopsies, and at 4 weeks after tick initial detachment, mice were sacrificed and tissues were harvested and cultured for spirochetes. As shown in Table 5.3, all mice exposed to *B. burgdorferi* ss infected ticks were infected. In contrast, none of the mice exposed to the *B. bissettii* infected ticks had evidence of infection, indicating that *I. scapularis* is unable to transmit infection to mice. This negative finding promoted us to seek additional evidence of infection. As our previous study showed *B. bissettii* infection induced a strong anti-C6 response in mice, we first examined this specific antibody by the C6-peptide ELISA. None of

the 5 mice that were exposed to the *B. bissettii*-carrying ticks showed the specific response (data not shown), again indicating that no infection had been transmitted.

Table 5.3. *Ixodes scapularis* is unable to transmit *B. bissettii* infection to mice

Tick inoculation	No. of positive specimens/ no. of samples examined					
	Ear biopsy	Skin	Heart	Bladder	Joint	No. of mice infected/no. of mice inoculated
B. bissettii	0/5	0/5	0/5	0/5	0/5	0/5
B. burgdorferi ss	5/5	5/5	5/5	2/5	5/5	5/5

*Groups of 5 mice each were inoculated with 20 nymphs that were previously collected from mice infected with either *B. bissettii* or *B. burgdorferi* ss. Infection was monitored by weekly ear biopsy. Four weeks after tick repletion mice were sacrificed and tissues were cultured for spirochetes.

Serum samples from the 5 mice exposed to *B. bissettii* infected nymphs were also analyzed by immunoblotting using whole cell lysates. As shown in Figure 5.4, only one specimen had a single band detected at ~33 kDa, again supporting that mice had not developed an infection.

5.4.3. *Ixodes scapularis* is unable to support *B. bissettii* during its lifecycle

The dynamics of spirochete loads was investigated throughout the *I. scapularis* developmental cycle. As described in Table 5.2, the load of *B. bissettii* was 8 times lower than that of *B. burgdorferi* in engorged larvae. To investigate the next stage of the tick's life cycle, 50 nymphs that had molted from engorged larvae were randomly sampled and spirochete burden was quantified by qPCR. *Borrelia bissettii* burden in unfed nymphs increased by 2-fold from the larval stage ($P < 0.001$) while *B. burgdorferi* burden did not significantly change ($p > 0.05$), yet had a downward trend (Figure 5.2). Spirochete burdens

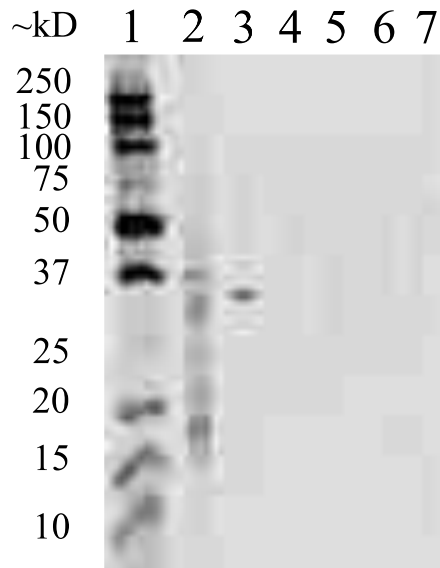

Figure 5.1. Immunoblot analysis of sera from mice exposed to *B. bissettii* infected nymphs. Four weeks after exposure to infected ticks mouse sera were diluted 1:50 and blotted against *B. bissettii* whole cell lysates for the detection of specific antibody responses. Lane 1; Proteins Standard Marker, Lane 2; serum from mouse infected via needle inoculation, Lanes 3-7 sera from mice exposed to *B. bissettii* infected nymphs.

between infection status significantly differed at this stage, as unfed *B. burgdorferi* infected nymphs had spirochete levels 2 times higher than *B. bissettii* nymphs ($P < 0.001$).

When 50 engorged nymphs randomly picked from each sub-pool were analyzed, the most dramatic change was observed. While the *B. bissettii* spirochete burden showed a significant, albeit modest, 13% increase from unfed to engorged nymph ($P < 0.0001$); the number of *B. burgdorferi* spirochetes increased 45-fold ($P < 0.001$) after the nymphal bloodmeal. This resulted in engorged nymphs harboring 100 times more *B. burgdorferi* spirochetes, on average, than *B. bissettii* ($p < 0.0001$) (Figure 5.2). After nymphs molted to adults, *B. bissetti* became undetectable by qPCR, a result that was further confirmed by culture (data not shown), indicating the all the spirochete of this species died out during this

tick developmental stage. In contrast, the *B. burgdorferi* load remained essentially unchanged during this specific developmental stage ($p > 0.05$) (Figure 5.2).

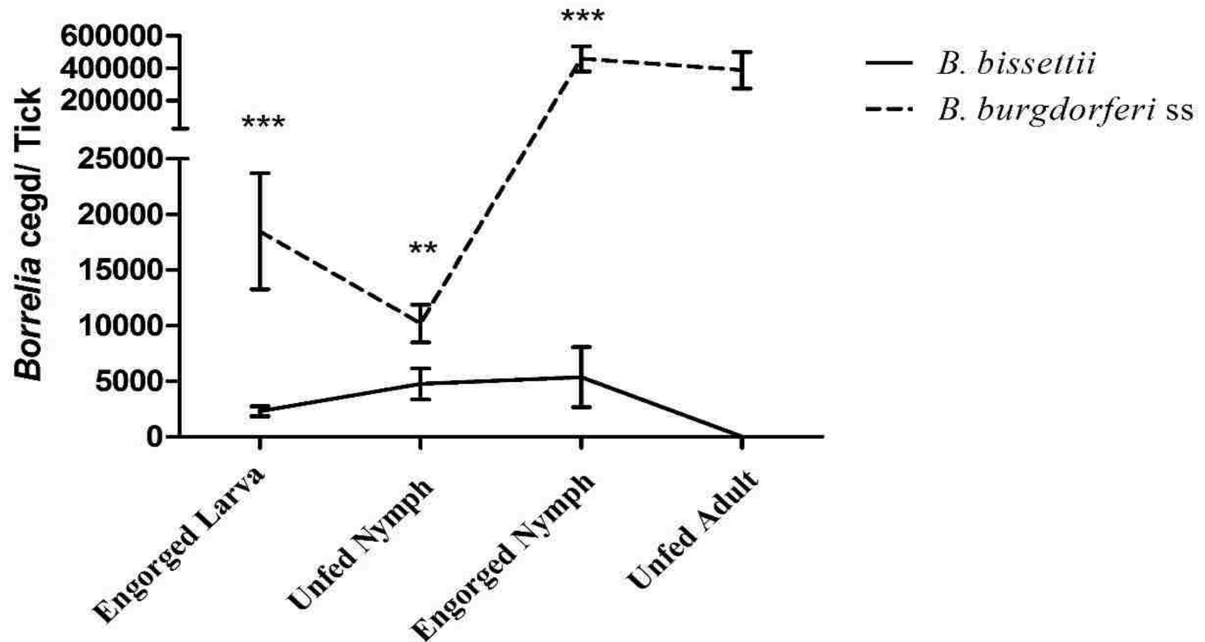

Figure 5.2. *Ixodes scapularis* harbors significantly less *B. bissettii* spirochetes than *B. burgdorferi* ss throughout the lifecycle. Whole ticks at each developmental stage were analyzed by qPCR to determine spirochete burdens. Data is presented as *Borrelia* copies of extracted gDNA (cegd) per tick. A Mann-Whitney U test was performed to compare between spirochete burdens at time points. *** $P < 0.0001$, ** $P < 0.001$. Error bars represent SEM.

5.5. Discussion

To truly understand Lyme disease is a Sisyphean task, one must understand the vector, the bacteria, the host, the environment and countless other unknown factors. As seen in the past 30 years, more and more research fuels additional questions. The United States harbors a diverse *Borrelia* fauna, and while only one species is widely believed to be responsible for the 300,000 human cases diagnosed annually, new evidence is challenging this belief. Three additional North American Lyme *Borrelia* spirochetes have been associated with human illness, some more recently than others [7, 27, 37].

Borrelia bissettii is a *B. burgdorferi* sl spirochete that has been implicated as a potential human disease causing agent in multiple studies [24-27]. In many respects, *B. bissettii* is very similar to the human disease causing *B. burgdorferi* ss spirochete. For example, in Europe and in many regions of the United States, both *B. bissettii* and *B. burgdorferi* ss geographically overlap [14, 38]. Both species have been recorded in many of the same hosts and ticks [13, 15, 17, 22, 39]. Infection with each species lead to similar infection dynamics and clinical manifestations in laboratory animal models [23]. *Borrelia bissettii* is also one of the closest related species to *B. burgdorferi* ss, phylogeneticaly [40]. Yet, unlike *B. burgdorferi* ss, only two tick species have been confirmed vectors for this group of spirochetes. In a single study, both *Ixodes pacificus* and *Ixodes spinipalpus* were able to acquire *B. bissettii* spirochetes from and transmit spirochetes to naïve dusky-footed woodrats and deer mice [21]. *Ixodes scapularis* is the common vector for many tick-borne pathogens including *B. burgdorferi* ss, throughout much of the US. Yet, its ability to experimentally acquire and transmit *B. bissettii* spirochetes remains unknown. Utilizing a well characterized tick-murine *Borrelia* infection model, we tested the hypothesis that *I. scapularis* can serve as a competent vector for *B. bissettii*. Unexpectedly, this was not the case.

The percentage of larva that acquired spirochetes during an infectious blood meal did not differ between *B. bissettii* and *B. burgdorferi* ss. However, the amount of spirochetes in the tick post blood meal was significantly lower in *B. bissettii* infected ticks. Most research involving the exact mechanisms behind tick acquisition and transmission of *Borrelia* spirochetes have focused on *B. burgdorferi* ss and *I. scapularis*. Many *B. burgdorferi* ss genes have been shown to be involved in this part of its lifecycle [41]. While genetic

manipulation of these genes may impede certain aspects of tick acquisition and/or subsequent transmission of *B. burgdorferi* ss spirochetes, tick/*Borrelia* species compatibility is extremely understudied. Despite this, *I. scapularis* has been shown to be capable of transmitting a variety of geographically and genetically diverse *Borrelia* species and strains [42-47].

After allowing *B. bissettii* infected *I. scapularis* to molt we assessed their ability to transmit *B. bissettii* infection to susceptible mice. While *B. burgdorferi* ss infected nymphs successfully infected all naïve mice via bloodmeal, feeding *B. bissettii* infected nymphs on naïve mice did not result in infection, as determined by culture of multiple tissues. We additionally utilized serology as a method of assessing infection in mice. Our previous work has shown *B. bissettii* infection in mice elicits a strong anti-C6 antibody response, yet mice infested with *B. bissettii* infected ticks showed no evidence of this specific response. Furthermore, immunoblots with sera form these same mice, even at low serum dilutions, supported the fact that *B. bissettii* infection was not transmitted to these mice. However, one mouse had evidence of a single band (~33kDa) by immunoblot indicating the potential of spirochete exposure. Nevertheless, cultures of engorged nymphs that had fed on naïve mice produced live spirochetes irrespective of *Borrelia* species infection. Non-competent vectors of *B. burgdorferi* ss have been demonstrated in multiple studies; however, unlike our results, spirochetes are often cleared shortly after larval acquisition and rarely cultivatable after molt [44, 45, 48, 49]. Indeed, true refractory ticks rarely have detectable bacteria even during infectious bloodmeals [50].

The detection of cultivatable spirochetes that were not transmitted in the *B. bissettii* infected *I. scapularis* was further explored by qPCR. We quantified the spirochete burdens in both infected cohorts of engorged nymphs and adults post molt. *Borrelia bissettii* infected

ticks did show modest, yet significant, increases in spirochete burdens through the ticks development cycle until the adult stage where evidence of spirochete infection was absent. In contrast, *B. burgdorferi* ss spirochete levels in infected ticks were not significantly different from acquisition to flat nymphs, however, did show a downward trend. The most striking difference was the increase in spirochete burdens in the fed *B. burgdorferi* ss infected nymphs. Spirochete burdens underwent exponential increases during nymphal blood meal in these ticks. This was not evident in *B. bissettii* infected nymphs. And unlike *B. bissettii* spirochetes, *B. burgdorferi* ss spirochetes levels were unchanged in adult *I. scapularis*. At each developmental stage of the tick *B. bissettii* spirochetes levels were significantly lower than *B. burgdorferi* ss levels in infected ticks. The most dramatic difference occuring at the replete nymphal stage (100-fold difference). Taken together these data indicate the inability for *I. scapularis* to support the *B. bissettii* spirochete throughout the tick's developmental cycle.

Mice exposed to *B. bissettii* infected ticks did not develop infection, yet in at least one case a mouse showed evidence of antibodies to *B. bissettii* lysate by western blot, which could indicate exposure to live spirochetes. Utilizing immundeficient mice in future experiments would confirm the exposure, as spirochetes reaching the immune compromised host would be much more likely to establish infection. Future work could also assess for bacterial presence at the infected tick bite site multiple time points throughout the feeding period. Despite limitations in the present study we believe our description of the lack of exponential increase in the spirochete population in *B. bissettii* infected nymphs during feeding is an important finding. Exponential increases of spirochetes in *B. burgdorferi* ss infected nymphal midguts during feeding has been reported by others, and is hypothesized to

assist in traversing the tick's midgut, a major barrier to *Borrelia* transmission [51, 52]. The lack of this exponential growth most likely played a factor in *I. scapularis'* inability to transmit *B. bissettii* to susceptible mice. However, the overall lower spirochete burdens in *B. bissettii* infected ticks at all stages indicates multiple factors may contribute to the tick's inability to transmit this spirochete. The lack of transmission in our study suggests *B. bissettii* enzootic cycles in the eastern and southern US may rely on a different tick vector. One possibility is the *Ixodes affinis* tick. These ticks have been described along the eastern and southern US and contribute to enzootic cycles of *B. burgdorferi* ss in these areas [22, 53-57]. Furthermore, at least one study has detected *B. bissettii* in these ticks however, this area remains understudied [58].

Assessing the vector competence of newly described *Borrelia* species should be an area of continued study, especially now that the Lyme *Borrelia* complex is expanding, and more and more species are being associated with human illness. We describe an interesting finding while assessing the ability of *I. scapularis* to transmit *B. bissettii*. While it appears that multiple factors may contribute to our observation that *B. bissettii* infected ticks are unable to transmit infection to susceptible hosts, the exact mechanisms behind this require further studies. We believe this "transmissionless" tick infection model may provide important information into the *Borrelia*-tick interface. With the advent of technologies like next generation sequencing and advanced microscopy techniques, this unique model may provide an important glimpse into questions of *Borrelia* vector competence.

5.6. References

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CHAPTER 6. DISCUSSION OF RESULTS AND FUTURE RESEARCH DIRECTION

6.1. Discussion

Lyme borreliosis is responsible for an estimated 300,000 cases of human illness annually in the United States [1]. Since the discovery of *Borrelia burgdorferi*, thirty years of research has led to an exceptional expansion of the Lyme *Borrelia* species complex. Each new discovery and description increases the complexity of this epidemic. Currently a total of 19 Lyme *Borrelia* species are recognized worldwide [2]. The United States harbors a diverse fauna of Lyme *Borrelia* spirochetes. Seven species have been described in the US; however, only one, *B. burgdorferi* ss, is widely considered responsible for cases of human Lyme borreliosis [3, 4]. Evidence is coming forward that implicates other species in human illness, yet the research into these organism remains infantile [5, 6]. Areas of the US reporting the majority of the human Lyme borrelisosis cases are hyperendmic for the *B. burgdorferi* ss spirochete and its vector tick, *Ixodes scapularis* [7]. Not consequently these are the causative agent and vector for the majority of human illness. However, areas not considered endemic for the spirochete can harbor large populations of the vector. Louisiana continually reports sporadic cases of human borreliosis yet investigation into the presence of Lyme *Borrelia* have not been conducted [8].

We conducted general molecular surveys investigating bacterial pathogens in known human biting ticks in Louisiana, an understudied state. Detection of bacterial pathogens in their respective vectors was not unexpected. We present the first report of two known tick-borne zoonoses in their vectors: *B. burgdorferi* ss in *Ixodes scapularis* and *Rickettsia parkeri* in *Amblyomma maculatum* [9]. We also report the first detection of *Borrelia bissettii* in Louisiana this was detected in an *Amblyomma americanum*, this tick is not a known vector

for Lyme *Borrelia* and given the fact it was collected off a host this may indicate *B. bissettii* infection in the bear. Detection of Lyme *Borrelia* in *I. scapularis* was further corroborated with field studies in Tunica Hills, Louisiana [10]. Tunica hills supports large populations of wildlife and multiple tick species. The successful detection of *B. burgdorferi* ss in *I. scapularis* adults and in known animal reservoirs at this site should be further investigated with larger studies across the state. While Louisiana is similar to most southern states in that only sporadic cases of human Lyme borreliosis are reported each year, we should not undermine the fact that locally acquired infections do occur [11]. Nymphal *I. scapularis* are the vector most commonly associated with human Lyme borreliosis cases in hyperendemic regions of the US [12]. In southern states, including Louisiana, it is rare to encounter this life stage of the tick. However, adult *I. scapularis* are competent Lyme *Borrelia* vectors and should not be overlooked as a human health threat. At Tunica Hills, *I. scapularis* are infected with *B. burgdorferi* ss at rates far lower than areas in the US were the majority of human Lyme cases are reported, but at rates consistent with other southern states. Obviously the risk of contracting Lyme disease in Louisiana is not as high as other areas in the US. Nevertheless the risk is still present and the diagnosis and subsequent treatment of infection requires knowledge of the presence of the pathogen in vectors. Lyme borreliosis is becoming more and more common in areas once considered devoid of the bacteria and tick [13, 14]. Reasons behind this “spread” are still a topic of debate; however monitoring the presence of vector borne diseases should be a top priority in areas where large populations of the vectors are present. Until a fool-proof method to protect against Lyme *Borrelia* infections is discovered, the approximately 300,000 cases of human Lyme disease diagnosed annually is steadily going to increase.

Of the seven North American Lyme *Borrelia* spirochetes, only *B. burgdorferi* ss is consistently studied. *Borrelia burgdorferi* ss is the purported spirochete behind all 300,000 annual cases of human Lyme borreliosis in the US. Yet, more and more people are getting infected in areas where the vector rarely encounters humans and where the pathogen is either not found or not yet detected [8]. Many of these regions do harbor many other Lyme *Borrelia* spirochetes whose human pathogenic status remains unknown. *Borrelia bissettii* is an understudied *Borrelia* spirochete that in many ways is very similar to *B. burgdorferi* ss. We analyzed the infection of this spirochete in a murine model. Our results corroborate and expand upon the few studies utilizing this spirochete [15, 16]. We report the similarities between infection with *B. bissettii* and *B. burgdorferi* ss in the mouse. Spirochete infection burdens support murine pathology reported by others and substantiate the few reports of this bacteria being detected in human clinical samples. Most importantly, we describe the murine humoral response to *B. bissettii* infection, reporting the cross reactivity of two common serological Lyme diagnostic assays. Over 3.4 million Lyme tests are performed each year in the US at an estimated cost of almost $500 million [17]. Other species like *B. bissettii* may be pathogenic to humans and present identical in Lyme diagnostic tests. Could the range of pathogenic Lyme *Borrelia* endemic areas be much larger than we realize? Further studies should focus on investigating non-*B. burgdorferi* ss *Borrelia* spirochetes and their potential to cause mammalian disease and cross reactivity with current Lyme diagnostic tests.

We report the first whole genome shotgun sequence of an infectious isolate of *B. bissettii*. The homology shared between *B. bissettii* and *B. burgdorferi* ss virulence genes support the infectious phenotype described in our murine model. The advent of next generation sequence technologies allows us to do comparative genomics between infectious

and non-infectious *Borrelia* species, yet many species still remain poorly characterized. Sequencing of representative *Borrelia* species would be of great benefit to the field.

The geographical overlap of *B. bissettii* and *B. burgdorferi* ss is very peculiar. Both are regularly detected in similar hosts and ticks, including the common *B. burgdorferi* vector (*I. scapularis*) [4, 18]. Yet vector competence of *I. scapularis* for the *B. bissettii* spirochete has not been demonstrated. We assessed the ability for *I. scapularis* to acquire and transmit the *B. bissettii* spirochete to susceptible mice. Our results indicate that *I. scapularis*, while susceptible to *B. bissettii* infection, is not capable of transmitting infection to naive mice. And while serological tests confirmed the inability to cause infection, one animal did had a single reactive band by western blot indicating exposure may have occurred, however, this band was only evident in highly concentrated sera in only one animal, which lends evidence to a potential non-specific cross reaction. Spirochete burdens throughout the tick's lifecycle are significantly lower in *B. bissettii* infections and absent in the adult. *Borrelia bissettii* fails to start replicating upon initiation of the nymphal blood meal, a trait important for successful transmission from *B. burgdorferi* ss infected ticks to susceptible animals [19]. These data indicate that another tick is most likely responsible for *B. bissettii* enzootic cycles in eastern and southern areas of the US. Although two species of ticks in the western US have been determined competent vectors for *B. bissettii,* future work should investigate the vector competence of other tick species in the eastern US [15]. The ability of *I. scapularis* to acquire *B. bissettii,* yet not transmit the organism, could facilitate more in depth studies on tick/*Borrelia* compatibility. Because of the genetic similarities between *B. burgdorferi* ss and *B. bissettii* deep sequencing technologies like RNA-Seq may successfully identify pan genomic differences between the two organisms throughout stages of tick infection.

The inability of *I. scapulairs* in this work to transmit *B. bissettii* infection to susceptible animals is confounding as the spirochete has been detected in both rodents and ticks in molecular surveys [20, 21]. One explanation for this is that there may be a cryptic vector of *B bissettii* in the eastern and southern US. Indeed, the nidicoulus *Ixodes affinis* has been found to be infected with *B. bissettii* [22]. Future work should assess the competency of ticks such as *I. affinis*. Another explanation to our finding is that our isolate was a Colorado isolate of *B. bissettii*, eastern and southern isolates of *B. bissettii* may differ. While this may be true, robust genetic analysis of *B. bissettii* in the Western and Eastern US have reliably concluded they are of the same species [23, 24]. And while one may argue that geographical isolation of the species may lead to strict vector association; this is not the case with other species of Lyme *Borrelia* and ticks [25, 26]. Future work should be done to tease out the unique mechanisms seen in this *I. scapularis*-*B. bissettii*-murine model.

In conclusion, this dissertation describes novel distributions of tick-borne bacterial pathogens in Louisiana, mainly *Borrelia burgdorferi* ss. It also assesses the pathogenic potential of the spirochete *Borrelia bissettii*, more specifically identifying similarities with *Borrelia burgdorferi* ss in a murine model. This is also the first evidence describing the cross reactivity of the C6-Peptide ELISA Lyme diagnostic test to a North American non-*B. burgdorferi* ss infection. We report the first whole genome shotgun sequence of an infectious North American non-*B. burgdorferi* ss spirochete. We lastly describe a peculiar tick/*Borrelia* interaction: the ability of *I. scapularis* to acquire but not transmit *B. bissettii* infection to susceptible mice.

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VITA

Brian Leydet Jr. was born in Virginia Beach, Virginia. He is the Son of Mr. Brian Leydet Sr. and Mrs. Ann Beatty, and has two younger brothers: Mr. Michael Leydet and Mr. David Leydet. Brian completed his training as an Emergency Medical Technician-Paramedic and passed national registry in 2004. While working as a paramedic, he continued his education at Old Dominion University in Norfolk, Virginia, and graduated in 2007 with a bachelor degree in health science (B.S.H.S). He then transplanted to Jacksonville, Florida to pursue graduate education. In 2009 he graduated from the University of North Florida with a master of public health (M.P.H). After completing epidemiological work on invasive *Haemophilus influenzae*, Brian left the sun and sand of Florida for the bayou and crawfish of Louisiana. He started his pursuit of a doctor of philosophy (PhD) degree in 2009 under the guidance of Dr. Fang-Ting Liang. Brian combined his love for the outdoors and scientific training, and completed both field studies and experimental infection work with Lyme *Borrelia* and its tick vector. Brian completed his dissertation and will graduate in August 2014. After graduation, Brian will continue research in the field of Lyme disease at the Trudeau Institute in Saranac Lake, New York.