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Phylogenetic Analysis of *Borrelia burgdorferi* Species Detected in Small Mammals and Ticks in Florida

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Abstract

The infection prevalence of *Borrelia burgdorferi*, the etiological agent of Lyme disease, was estimated among small mammals and ticks at two sites in northern Florida using molecular methods. Analysis of the *B. burgdorferi* flagellin gene in samples extracted from the ticks and small mammals was used to construct a phylogenetic tree. The phylogenetic tree was used to compare partial flagellin gene sequences of the *Borrelia* strains from Florida with reference *Borrelia* strains. The infection prevalences of small mammals at the University of North Florida and Guana River State Park were 90% and 47%, respectively, with 6 of 7 small mammal species testing positive. Forty-two adult *Ixodes scapularis* ticks were also tested at each site: 20/42 tested positive at UNF (48%) and 5/42 tested positive at Guana River (12%). The neighbor-joining phylogenetic tree showed that the Florida *Borrelia* strains were 98-99% genetically similar to reference strains of *B. burgdorferi sensu stricto* (confirmed Lyme disease agent) and *B. bissettii* (pathogenicity undetermined). The findings confirm the presence of specific *Borrelia* species in northern Florida; however, their relationship to human Lyme disease in Florida is the subject of ongoing studies.

Introduction

With more than 15,000 cases reported each year, Lyme disease is the most

prevalent vector-borne disease in the United States [3]. It is most prevalent in the northeastern and north central USA, and northern California coastal areas, although cases have been reported all over the country [3]. From 1985 to October 2002, 555 cases were reported in Florida [13]. About half of these cases can be attributed to infection outside the state [12].

Lyme disease was named after Lyme, Connecticut, where arthritis was first noticed in a cluster of children in 1977 [22]. The most common clinical symptom is a circular ("bull's eye) rash known as erythema migrans [23]. The illness is caused by a spirochete bacterium, *Borrelia burgdorferi sensu stricto* (in the strict sense) [1,8]. The main vector for transmission to animals and humans is the blacklegged tick, *Ixodes scapularis* [2,17]. *B. burgdorferi sensu stricto* is part of a complex group of *Borrelia* known as *B. burgdorferi sensu lato* (in the broad sense) [1]. Along with *B. burgdorferi sensu stricto*, only two other subspecies are found in the U.S., *B. bissetti* and *B. andersonii*. *B. burgdorferi sensu stricto* is the only one of the 3 USA subspecies that is known to be pathogenic to humans. In the U.S., *B. bissetti* and *B. andersonii* have been isolated only from ticks and small mammals [16].

The other strains found to be pathogenic to humans are *B. afzelii* and *B. garinii*, which are both found in Europe [1]. Each pathogenic species is associated with certain clinical symptoms. *B. burgdorferi sensu stricto* is associated with arthritis, *B. afzelii* is associated with late dermatological problems, and *B. garinii* is associated with late neurological problems [24]. *B. burgdorferi sensu stricto* in the U.S. was found to be more genetically homogeneous compared to the European strains [1]. However, recent analysis of *Borrelia* found in the U.S., especially those found in the South, indicate that significant genetic heterogeneity exists among different strains [10, 11]. The medical importance of this finding is not yet clear.

Although Lyme disease is not highly prevalent in the South, hundreds of cases are reported from southern states each year [13]. Some patients have reported a mild illness associated with an erythema migrans rash that developed from the bite of the lone star tick, *Amblyomma americanum*, and not *Ixodes scapularis* [7,14]. *A. americanum* commonly bites humans in the South, but has been shown to be an incompetent vector for *B. burgdorferi* [15,19]. *A. americanum* is a vector for the relapsing fever group species, *B. lonestari*. *B. lonestari* is known to cause a Lyme-like illness called Master's disease or STARI, southern tick associated rash illness [7,14]. The clinical specimen cultures of patients bitten by lone star ticks have tested negative for *B. burgdorferi* [7]. These observations have caused much controversy over whether the Lyme-like illnesses in the southeastern USA are due to infection with *B. burgdorferi*, *B. lonestari*, or perhaps another previously undescribed *Borrelia* species.

The purpose of this research was to investigate *B. burgdorferi* infecting ticks and small mammals in northern Florida through PCR and DNA analysis. Specific objectives were to determine the presence and infection prevalence among small mammals and ticks in northeast Florida and to examine the genetic variability among the *Borrelia* strains. We amplified and studied an approximately 390-bp portion of the highly conserved flagellin gene of *B. burgdorferi*. The DNA sequence of this portion of the gene can distinguish different *B. burgdorferi* sensu lato species [5,9]. A phylogenetic tree was constructed to compare sequences derived from Florida vertebrates and ticks with reference strains from other areas of the USA.

Methods and Materials

Tick and Small Mammal Collection

Host-seeking ticks were collected along nature trails of the University of North

Florida, the south entrance of Guana River State Park, and several other public recreation sites in northern Florida. A 1-m² felt cloth was dragged along nature trails, firebreaks, and other woodland paths to collect host-seeking ticks. The drag was checked approximately every 15 paces, and examined. Ticks were stored in vials containing 70% ethanol. Dr. Kerry Clark previously collected the small mammals at UNF and Guana River State Park in 1999, using small Sherman® live-traps set in line transects in different habitat types. Captured animals were anesthetized with a ketamine hydrochloride/xylazine mixture injection. Animals were identified to species, weighed, measured, sexed, and examined for ticks and other ectoparasites. Several small ear punch biopsies were collected from each animal, and stored frozen. Upon recovery from anesthesia, all animals were then released unharmed at their capture site. All procedures involving vertebrate animals were conducted in accordance with protocols approved by the UNF Animal Care and Use Committee.

DNA Extraction and Amplification

DNA was extracted from vertebrate ear punches and individual adult ticks using a commercially available kit that utilizes a spin column format (Qiagen Dneasy™ Tissue Kit). Ticks and ear tissue samples were surface-sterilized with hydrogen peroxide and 70% ETOH. Samples were then placed in a microtube containing tissue lysis buffer, and chopped with a sterile scalpel. Proteinase K was added, and the samples were incubated overnight in a water bath at 55°C. After incubation, samples were mixed with ethanol, vortexed thoroughly, and pipeted onto the spin columns. Tubes were centrifuged, binding the DNA to the columns, and the flow-through and collection tube were both discarded. The spin columns were then washed twice with wash buffer. The DNA was finally eluted into clean tubes with 200

µl of TE buffer, and samples were stored frozen for use in PCR amplification.

Polymerase chain reaction (PCR) assays were performed in 50 µl volumes using a commercial PCR mastermix (Takara HS™) containing *Taq* DNA polymerase, 10X buffer, and dNTPs. Approximately 5 µl of each DNA extract was added as template, along with 0.5 µM of nucleotide primers designed to amplify a portion of the *B. burgdorferi* sensu lato flagellin gene. Each reaction included a sterile water negative control, and a *B. burgdorferi* sensu lato reference strain (MOK-1C or JD-1) as a positive control. Reactions were performed using an automated PCR thermocycler (Perkin Elmer). Positive flagellin PCR results were confirmed by amplification of a portion of the *B. burgdorferi* chromosomal p66 gene (21). The PCR products were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide, and photographed using a digital camera.

Purification of PCR products, DNA sequencing, and phylogenetic analysis

PCR products from representative *B. burgdorferi* flagellin-positive samples were purified using a Qiagen PCR purification kit. Samples were sequenced at the University of Florida Interdisciplinary Core for Biotechnology Research (ICBR). A BLAST

(basic local alignment search tool) search was performed using the National Center for Biotechnology Information (NCBI) website to obtain flagellin gene sequence data determined from *B. burgdorferi* s.l. strains in other studies to compare to our Florida strains. Sequences were aligned using the ClustalX program. Phylogenetic trees were constructed using neighbor-joining, maximum parsimony, and maximum likelihood with the MEGA (Molecular Evolutionary Genetic Analysis) and PAUP (Phylogenetic Analysis Using Parsimony and Other Methods) programs [6].

Results

PCR Analysis

Figure 1 shows typical results of nested PCR assays to detect *B. burgdorferi* flagellin gene DNA in Florida small mammal and tick DNA extracts. Some of the samples amplified the targeted gene fragment to detectable levels via agarose gel electrophoresis and ethidium bromide staining in the outer reaction (610 base pair product). However, all of the samples showed presence of the 390-bp inner/nested PCR flagellin gene product. The p66 PCR results (results not shown) verified those obtained with the flagellin gene assay.

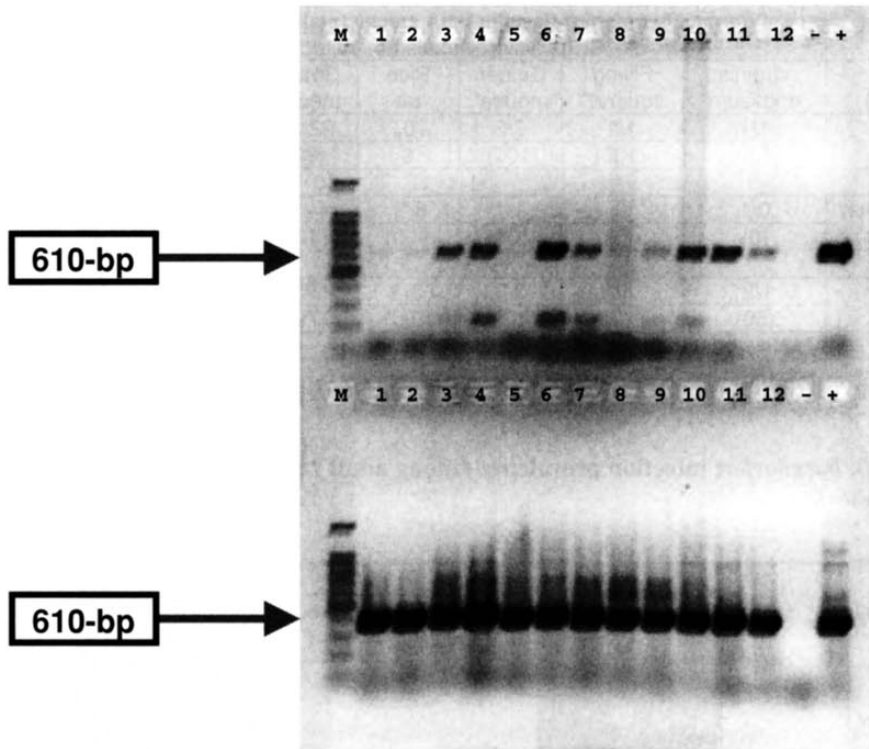


Figure 1. *B. burgdorferi* flagellin PCR
 The wells contain the following samples:
 M. 100-bp DNA marker,
 1. A.a4pool, 2. A.a15pool,
 3. FLAS1, 4. FLCL3,
 5. FLLS1, 6. FLTP1,
 7. RETKC18, 8. RETKC19,
 9. RETFL1, 10. RETFL9,
 11. RETFL27, 12. RETFL42,
 “-”. Negative control (water),
 “+”. Positive control (*Borrelia andersonii*)

Small Mammal Testing

Table 1 shows the prevalence of the flagellin DNA among small mammals collected in Florida in 1999. Six of the 7 small mammal species collected at UNF and Guana River tested positive for presence of the *B. burgdorferi* flagellin gene. The prevalence of infection among 31 samples tested at UNF was 90%. Fourteen of 30 samples (47%) from Guana River were positive.

Tick Testing

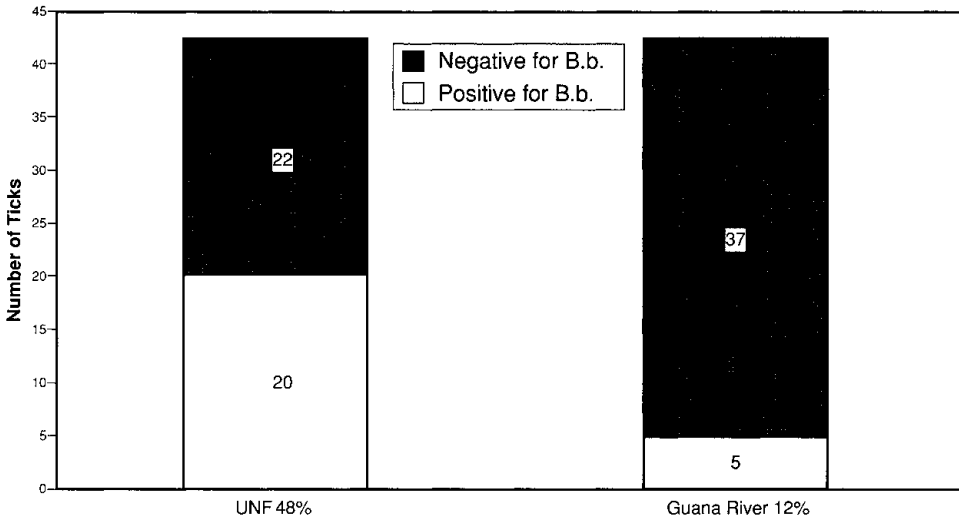
Three tick species tested positive for *B. burgdorferi*: *Ixodes affinis*, *Ixodes scapularis*, and *Amblyomma americanum*. Seven of 11 (64%) *I. affinis* ticks collected from 5 sites were infected. Overall prevalence among *I. scapularis* ticks collected from 3 coastal sites was 30% (38/126). Two *A. americanum* ticks of 138 (1.4%) collected from 5 sites also tested positive. The *B. burgdorferi* infection prevalence determined among *I. scapularis* ticks tested at UNF and Guana River is shown in Figure 2. At UNF, 20 of 42 ticks (48%) tested positive. At Guana River, 5 of 42 ticks (12%) tested positive.

Table 1: Prevalence of *Borrelia burgdorferi* flagellin DNA among small mammals collected in Florida, 1999.

Site (County)	No. of PCR positive animals/no. tested (%) of each species							Total
	Virginia opossum	Flying squirrel*	Golden mouse*	Rice rat	Cotton mouse	Cotton rat	Woodrat	
UNF (Duval)	1/1	1/1	2/2	0	22/25	2/2	0	28/31
	(100)	(100)	(100)	0	(82)	(100)	0	(90)
Guana River (St. Johns)	0/1	0	1/1	3/3	4/10	6/13	0/2	14/30
	(0)	(0)	(100)	(100)	(40)	(46)	(0)	(47)
Total	1/2	1/1	3/3	3/3	27/34	2/2	0/2	42/61
	(50)	(100)	(100)	(100)	(79)	(100)	(0)	(69)

* =New host record

Figure 2. *B. burgdorferi* infection prevalence among adult *Ixodes scapularis* from 2 sites in northeast Florida.



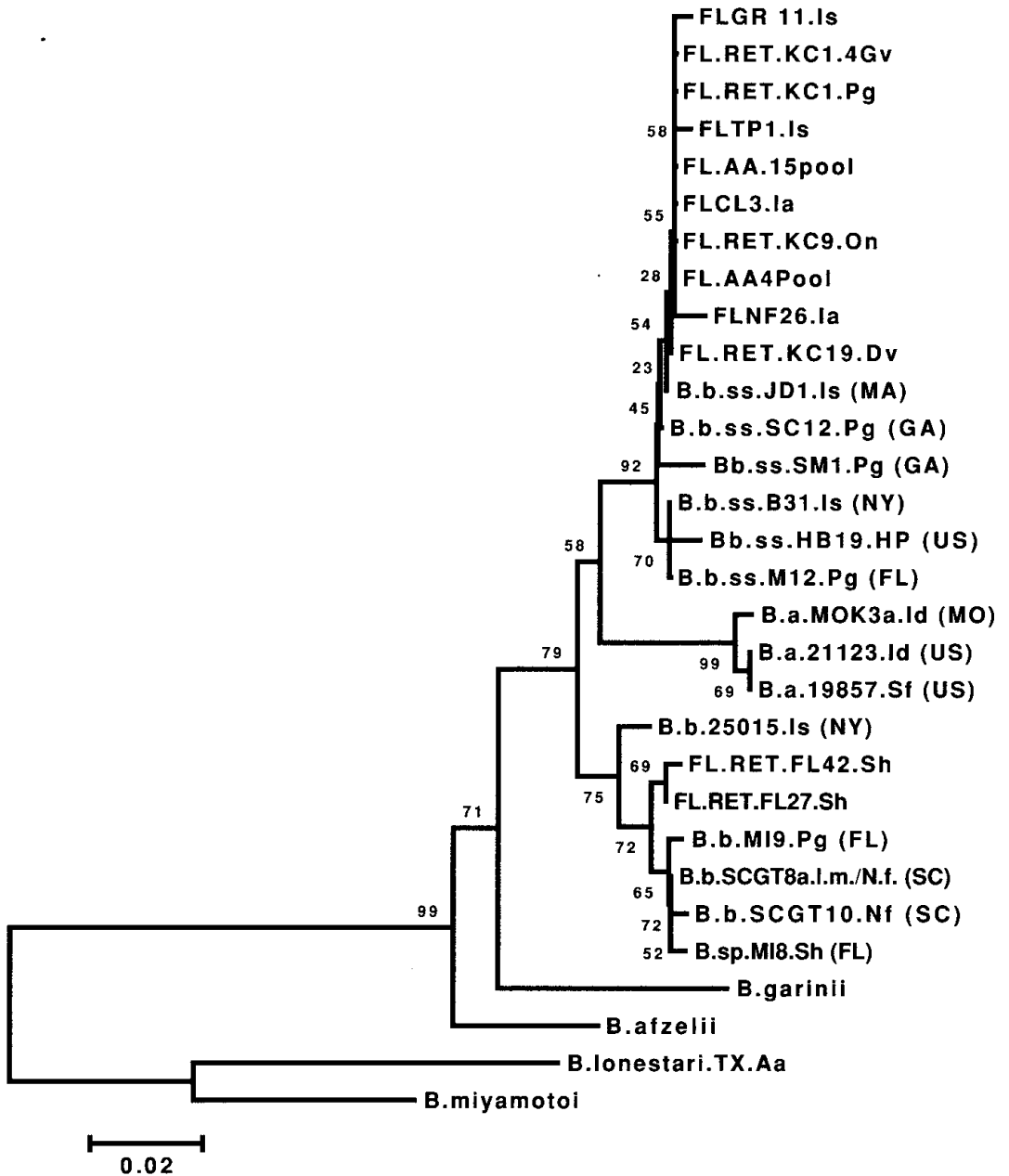


Figure 3. Phylogenetic tree of 12 Florida *Borrelia* strains and known reference strains of *B. burgdorferi* sensu lato on the basis of flagellin DNA sequences. The tree was constructed by neighbor-joining method. The tree was rooted with relapsing fever group *Borrelia* spp. The values in the tree represent bootstrap results.

Phylogenetic Tree

A neighbor-joining phylogenetic tree (Figure 3) was constructed to compare the flagellin gene sequences of Florida *Borrelia* strains to other *B. burgdorferi* sensu lato strains. The tree was based on 390-bp of the flagellin gene amplified from Florida small mammals and ticks. The tree was rooted with the relapsing fever group species, *B. lonestari* and *B. miyamotoi*. Bootstrap values represent percentages of 1,000 replications. The tree shows three main clades. The first main clade (represented by the top 16 strains) clustered the Florida *Borrelia* strains with known reference strains of *B. burgdorferi* sensu stricto. The second main clade (the next 3 sequences below the first clade) grouped known reference strains of *B. andersonii*; however, none of the Florida strains were clustered with this group. The third main clade (next 7 strains below the second clade) clustered two Florida strains with reference strains of *B. bissettii*, *B. afzelii* and *B. garinii*, two European strains pathogenic to humans, were separate from the 3 main clades from the U.S.

Discussion

Borrelia burgdorferi DNA was detected among small mammals and ticks at two sites in northern Florida. At two coastal sites, UNF and Guana River, 6 of 7 small mammal species tested positive with 69% prevalence overall (n=42/61). This includes the first documented detection of *B. burgdorferi* in the flying squirrel and golden mouse. The prevalence at these Florida sites was comparable to that reported by Clark et al. for rodents in the Coastal Zone of South Carolina [4], where prevalence of infection was between 50-89%. At UNF, prevalence of infection in small mammals was higher than at Guana River (90% vs. 47%). Possible reasons for this difference are: ecologic differences in habitats between the two sites that may favor greater density of

small mammals at UNF due to fewer predators; greater density of *I. scapularis* ticks at UNF; or fewer numbers of alternative hosts for *I. scapularis* ticks (i.e., lower species richness) at UNF compared to Guana River.

B. burgdorferi infection was detected in 47 of 275 ticks tested (17.1%). *Ixodes affinis* had 64% infection prevalence, which is much higher than the prevalence found in *I. scapularis* (30%). These results are also comparable to those reported in these two same tick species in South Carolina [4]. In South Carolina, these two species were the only ones found to be infected with *B. burgdorferi*. The prevalence of *I. affinis* (19/74, 25.7%) was also much higher than that of *I. scapularis* (12/864, 1.3%) in that state. The infection prevalence of adult *I. scapularis* ticks was higher at UNF (20/42, 48%) than at Guana River (5/42, 12%). This result is consistent with the higher infection prevalence of small mammals at UNF than at Guana River.

B. burgdorferi DNA was also found in 2 out of 138 *A. americanum* ticks (1.4%). Few states (Virginia, Alabama, Michigan, New Jersey, Georgia, Florida, and Texas) have reported finding *Borrelia* in this species [16]. However, *A. americanum* ticks have been found to be incompetent vectors for *B. burgdorferi* [15,19]. It is still unclear whether the *Borrelia* species found in this tick is a different species that may cause illness similar to Lyme disease.

The phylogenetic tree derived from flagellin gene sequences showed that Florida *Borrelia* strains were 98-99% genetically similar to other USA strains of either *B. burgdorferi* sensu stricto or *B. bissettii*. The Florida strains that clustered with *B. burgdorferi* sensu stricto were more than 99% similar to reference strains of that species. The Florida strains that clustered with *B. bissetti* were only slightly diverse with 98-99% similarity. Our results provide additional evidence to show that the flagellin gene is useful in *Borrelia* taxonomy.

Although the results of our analysis show that the Florida *Borrelia* are 98-99% genetically similar to other known reference *Borrelia* strains, this does not mean that they are identical in pathogenicity. Additional research is required to further analyze genetic variability and to determine pathogenicity. Two proteins, outer surface protein A (OspA) and outer surface protein C (OspC), have been studied to possibly differentiate *B. burgdorferi* species according to pathogenicity [24]. Both proteins show a high degree of heterogeneity. OspA is one of the major proteins expressed in *B. burgdorferi* and human isolates, and has been used in diagnostic testing as well as vaccine development [24]. OspC is not a major protein expressed in *B. burgdorferi*, but it is an immunodominant protein expressed during the early stage of human infection [10], and is believed to be important in pathogenicity.

In 2002, Tao Lin et al. conducted a study exploring the OspC gene of *B. burgdorferi* strains [10]. They tested the OspC genes of several southern *B. burgdorferi* sensu lato strains and compared them to other known OspC *Borrelia* sequences. There were many differences in the results when comparing their phylogenetic tree with ours. Both studies analyzed 11 of the same reference strains: SC12, SM1, B31, and M12 are *B. burgdorferi* sensu stricto; MOK3a, and 21123 are *B. andersonii*; and 25015, MI9, SCGT8a, SCGT10, and MI8 are *B. bissettii*. In the analysis by Lin et al., the *B. burgdorferi* sensu stricto, *B. andersonii*, and *B. bissettii* strains were distributed throughout different clusters of the tree. Our tree, based on sequences from a portion of the flagellin gene, grouped different strains of the same species together. The huge diversity in the two trees demonstrates that the pathogenicity of the *Borrelia* strains may be highly variable even among strains of the same species.

The question surrounding the etiology of the Lyme disease-like illnesses in Florida and other southern states remains. Dozens of laboratory confirmed cases of Lyme disease are reported in Florida each year. Cases of a Lyme-like illness have also been recognized in association with bites from the lone star tick, *A. americanum*, instead of the known vector of Lyme disease, *I. scapularis*. We found *B. burgdorferi* DNA in many *I. scapularis* and a few *A. americanum* ticks. *B. lonestari* could be responsible for some of the Lyme disease-like illnesses reported in Florida. Alternatively, it is quite possible that *B. lonestari* and *B. burgdorferi* are involved. Furthermore, *A. americanum* or *I. scapularis*, or both may be responsible for transmitting the causative agents to humans in this region.

Acknowledgments

I would like to thank Dr. Kerry Clark, my professor and mentor, and the other students in the Health Science Research Lab for their help and support. This project was supported in part by a 2002 UNF Biology Student Summer Research Award to Karen Overly; and a UNF College of Health Research Professorship award, and an American Lyme Disease Foundation Research Grant, to Dr. Kerry L. Clark.

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