

CHARACTERIZATION OF LYME DISEASE SPIROCHETES ISOLATED FROM TICKS AND VERTEBRATES IN NORTH CAROLINA

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ABSTRACT: *Borrelia burgdorferi* isolates obtained from numerous locations and from different hosts in North Carolina, were compared to previously characterized strains of the Lyme disease spirochete and other *Borrelia* spp. The spirochete isolates were confirmed to be *B. burgdorferi sensu stricto* based on immunofluorescence (IFA) using a monoclonal antibody to outer surface protein A (Osp A [H5332]) and polymerase chain reaction (PCR) using a species-specific nested primer for a conserved region of the gene that encodes for flagellin. In addition, the isolates tested positive in Western blots with species-specific monoclonal antibodies for outer surface protein A and OspB (84c), and the genus-specific, monoclonal antibody to flagellin (H9724). Infectivity studies with several of these isolates were conducted using *Mus musculus* and *Oryzomys palustris* and the isolates exhibited markedly different levels of infectivity. This study demonstrates that *B. burgdorferi sensu stricto* is present and naturally transmitted on the Outer Banks and in the Coastal Plain and Piedmont regions of North Carolina.

Key words: *Borrelia burgdorferi*, isolate characterization, Lyme disease, ticks, vertebrates.

INTRODUCTION

Lyme disease (LD) is a tick-borne zoonosis accounting for more than 90% of all cases of vector-borne illnesses currently reported in the United States (Dennis, 1993). In 1996, the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) received 16,455 case reports from 44 states and North Carolina contributed 66 cases to the total number reported (Myra et al., 1997). The etiologic agent of Lyme disease is *Borrelia burgdorferi*, an anaerobic, helically shaped bacterium in the family Spirochetales (Johnson et al., 1984). Lyme disease may progress in three distinct stages that are characterized by the development of a pathogenic rash, and cardiac, neurologic and oligoarticular sequelae (Kalish, 1993).

In the northeastern United States, the risk of infection with *B. burgdorferi* is dependent on the prevalence of the spirochete in the vector, *Ixodes scapularis* (Steere and Malawista, 1979). *Ixodes scapularis* larvae acquire the agent from in-

fective white-footed mice (*Peromyscus leucopus*) (Levine et al., 1985), and other reservoir hosts (Anderson, 1989). After molting to the nymphal stage, *I. scapularis* subsequently transmit *B. burgdorferi* to noninfected reservoir hosts (Anderson, 1989). In the Northeast, seasonal nymphal activity and feeding precedes seasonal larval activity and feeding on the same host species. This feeding sequence helps sustain transmission. Although white-footed mice serve as the primary reservoir in the Northeast, *B. burgdorferi* has been isolated from other vertebrate hosts, including the marsh rabbit (*Sylvilagus floridanus*), the cotton rat (*Sigmodon hispidus*), and a variety of other vertebrates (Oliver et al., 1993).

North Carolina supports a diverse group of potential *B. burgdorferi* reservoirs. *Ixodes scapularis* is abundant and focally distributed in the Coastal plain and Piedmont regions of the state (Apperson et al., 1990). However, the geographic distribution of LD cases in North Carolina does

not correlate precisely with the state-wide distribution of *I. scapularis* (Levine et al., 1991).

Lyme disease was first diagnosed in North Carolina in 1982 (Pegram et al., 1983). Diagnoses have been based on the Centers for Disease Control and Prevention case definition standards, which have consistently included an erythema migrans-like rash, confirming serology, and the presence of secondary sequela (Dennis, 1993). Isolation of the etiologic agent is considered the "gold standard" in documenting *B. burgdorferi* infection in patients and has been achieved often in the northeastern United States. Although human cases and infected ticks have been documented to occur in North Carolina, *B. burgdorferi* has not been isolated from suspected case-patients in the state (Kirkland et al., 1997). These circumstances surrounding southeastern United States diagnoses have led some investigators to suspect that another pathogen is responsible for a LD-like disorder transmitted by the lone star tick, *Amblyomma americanum* (Barbour, 1996; Kirkland et al., 1997). However, numerous *B. burgdorferi* isolates have been obtained from *I. scapularis* (J. F. Levine and C. S. Apperson, unpubl. data) and from various vertebrates (Ouellette et al., 1997; Kirkland et al., 1997) collected in North Carolina.

We characterized, through conventional biochemical and biomolecular methods, the antigenic and genomic characteristics of a representative sample of these North Carolina *B. burgdorferi sensu lato* isolates.

MATERIALS AND METHODS

Isolates tested and antigen preparation

The North Carolina isolates selected for this study were derived from ticks collected from vegetation and hosts trapped during previously conducted field studies on the Outer Banks (J. F. Levine and C. S. Apperson, unpubl. data), Coastal Plain region (Ouellette et al., 1997), and Piedmont region (Kirkland et al., 1997) of North Carolina. The North Carolina isolates were low-passage (≤ 2 times), and all isolates were cryopreserved ($< -90^{\circ}\text{C}$) prior to use (Ta-

ble 1, Fig. 1). All isolates selected for this study were preliminarily identified as *B. burgdorferi sensu lato* by indirect fluorescence antibody microscopy with a species-specific monoclonal antibody (H5332) to outer surface protein (*Osp*) A. Three strains of *B. burgdorferi* previously isolated from *I. scapularis* ticks, were used as positive controls. These included, the ATCC type strain 35210 (or B31), a clone of JD1 (C2) and a Valhalla isolate (AN 1.2) (Valhalla, New York, USA) (Levine et al., 1995). The Valhalla isolate was reisolated after passage in a Carolina anole (*Anolis carolinensis*). *Borrelia anserina* (BA2) and an isolate (FCB) recovered from a febrile dog in Florida, USA, believed to be *B. turicatae* (T. G. Schwan, unpubl. data), were used as negative controls. All isolates were grown at 35 C in BSK-H (Sigma Chemical Co., St. Louis, Missouri, USA; lot #124H4645) supplemented by the addition of kanamycin (Sigma Chemical Co.) and Fungizone (Gibco BRL, Gaithersburg, Maryland, USA) to a density of 2×10^8 cells/ml. The cultures were pelleted at 10,000 g for 10 min and washed three times with phosphate buffered saline/5 mm MgCl_2 (PBS/M). The final pellet was resuspended in a minimal volume of PBS/M to give an optical density reading of 2.0 at 600 nm in a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, New York, USA). Whole-cell lysates were prepared by mixing equal volumes of the cellular suspension with 2X SDS-PAGE sample buffer and boiling the mixture for 5 min.

SDS-PAGE and western blot analysis

The whole-cell lysate from each isolate was applied to a 12.5% bis-acrylamide gel (Fisher Chemical/Fisher Scientific, Pittsburgh, Pennsylvania, USA) and subjected to electrophoresis (Model 200, Aquebogue Machine Shop, Aquebogue, New York, USA) at a constant current of 15 mAmps until the dye front was 1 cm from the end of the plate (Laemmli, 1970; Maupin et al., 1994). Protein bands from the gel were transferred to nitrocellulose (Towbin et al., 1979) and immunoblotted using species-specific monoclonal antibodies for outer surface proteins (*Osp*'s) A and B (H5332 and 84C) and a genus-specific, monoclonal antibody (H9724) to the flagellar protein as described previously (Bundoc and Barbour, 1989).

Total genomic DNA isolation

One ml of the cellular suspension was used to extract total genomic DNA by a protocol described previously (Ferdows and Barbour, 1989). DNA fractions from each strain were screened in a 0.4% agarose gel. Whole lambda bacteriophage DNA (47 kb) and the 23- and

TABLE 1. Strain designation, number of in vitro passages, isolate host source and location of host collection, of the characterized North Carolina isolates of Lyme disease isolates.

Strain designation	Passage number	Host	Location isolated
C0064	2	blacklegged tick ^a	Pine Island North Carolina, USA (36°8' N, 75°28'W)
C0154	2	blacklegged tick ^a	Buxton Woods North Carolina, USA (35°9'N, 75°19'W)
C0219	2	white-footed mouse ^b	Pine Island North Carolina, USA (36°8'N, 75°28'W)
C0235	1	raccoon ^c	Camp Lejeune North Carolina, USA (34°42'N, 77°21'W)
C0242	2	marsh rice rat ^d	Pine Island North Carolina, USA (36°8'N, 75°28'W)
C1297	2	marsh rabbit ^e	Pine Island, North Carolina, USA (36°8'N, 75°28'W)
P3	1	white-footed mouse ^b	Pittsboro North Carolina, USA (35°45'N, 79°10'W)
P10	1	white-footed mouse ^b	Pittsboro North Carolina (35°45'N, 79°10'W)
B31	>40	blacklegged tick ^a	Shelter Island New York, USA (41°3'N, 72°21'W)
JD1 C2 ^a	2	Syrian hamster ^f	Ft. Collins, Colorado, USA (40°35'N, 105°7'W)
AN 1.2 ^a	2	green anole ^g	Valhalla New York, USA (41°4'N, 73°46'W)
<i>B. anserina</i> 2 ^a	2	chicken ^h	Ft. Collins, Colorado, USA (40°35'N, 105°7'W)
Florida Canine <i>Borrelia</i> (FCB)	2	dog	Sumter County, Florida, USA (28°44'N, 82°3'W)

^a *Ixodes scapularis*.^b *Peromyscus leucopus*.^c *Procyon lotor*.^d *Oryzomys palustris*.^e *Sylvilagus floridanus*.^f Initially isolated from *Ixodes scapularis*, passage in *Mesocricetus auratus*.^g Initially isolated from *Ixodes scapularis*, passage in *Anolis carolinensis*.^h *Callus domesticus*.

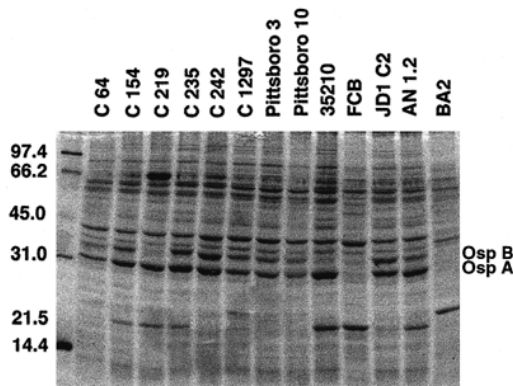


FIGURE 1. SDS-PAGE protein profiles for North Carolina isolates and control strains (35210, JD1, C2, AN 1.2) of *Borrelia burgdorferi* and other strains of *Borrelia*: FCB (Florida Canine *Borrelia*) and BA2 (*B. anserina*) (13 × 16 cm protein gel).

9.6-kb *Hind*III restriction fragments of lambda (Sigma Chemical Co.) were used as size markers. Gels were stained with ethidium bromide (Fisher Biotech/Fisher Scientific, Pittsburgh, Pennsylvania, USA) and examined with an ultraviolet transilluminator (TM-36, Ultra-violet products, San Gabriel, California, USA).

Polymerase chain reaction

The isolates were assayed by polymerase chain reaction (PCR), using a species-specific nested primer for the *B. burgdorferi* flagellin gene and standard annealing and amplifying cycles in a Perkin-Elmer-Cetus (Norwalk, Connecticut, USA) thermal cycler (Johnson et al., 1992). The template to be amplified was derived by taking 5 µl of a 1:10,000 dilution of the total genomic DNA preparations previously mentioned. The PCR products were analyzed in a 2% agarose (1% NuSieve GTG from FMC Bioproducts, Rockland, Maine, USA; 1% Ultra pure from Gibco BRL, Gaithersburg, Maryland, USA) gel. A *Hinf*I digest of pBR322 was used as marker DNA to identify the resultant 390 base pair product. Positive samples were digested with *Pvu*II (Promega Corporation, Madison, Wisconsin, USA) in accordance with manufacturer's instructions to yield the two corresponding fragments of 247 and 143 base pairs (Johnson et al., 1992).

Plasmid profiles

One ml of the cellular suspension was subjected to the QIAGEN (Chatsworth, California, USA) plasmid extraction kit (tip 20) protocol to obtain plasmid DNA. The eluted plasmid fractions were concentrated by ethanol precipita-

tion and applied to a 0.4% agarose gel. Plasmid DNA samples were subjected to a constant voltage of 12 volts for 16 hr in 40mM tris acetate-2mM EDTA (TAE) buffer. A 5-kb linear DNA ladder preparation (Bio-Rad Laboratories Inc., Hercules, California, USA) was used as marker DNA. The gel was stained with ethidium bromide and examined with a UV transilluminator.

Infectivity studies

Standard methods for inducing *B. burgdorferi* infection by needle inoculation (Piesman, 1993) and reisolation of spirochetes from urinary bladder (Levin et al., 1995) and ear tissue (Piesman, 1993) were used throughout this study. Intradermal inoculations (Gern et al., 1993) of culture derived spirochetes of several isolates (C0235, C0064, C0219, and JD1) were given to white mice (*Mus musculus*, outbred ICR strain) and marsh rice rats (*Oryzomys palustris*, pathogen-free outbred colony at the Naval Dental Research Laboratory, Great Lakes, Illinois, USA). Inoculum concentrations were adjusted by the method of Stoenner (1974) using a Petroff-Hausser cell chamber (Hausser Scientific Horsham, Pennsylvania, USA). Inoculations were performed with a wide range of cell densities (10^2 – 10^8 cells/ml) so that minimum infectious dose (MID) determinations could be estimated by the method of Reed and Muench (1938). The method facilitates estimation of the 50% end-point when this point lies between two dilutions. After a period of 28 days, the animals were euthanized by carbon dioxide intoxication and samples of urinary bladder and ear tissue were aseptically removed and placed in BSK-H media. Cultures were incubated at 35 C and checked every 48 hr by dark-field microscopy until spirochetes were obtained.

RESULTS

SDS-PAGE and western blot analyses

All North Carolina isolates in this study presented protein profiles that were highly comparable to the positive control strains, B31 (35210), JD1 C2 and AN 1.2 by SDS-PAGE (Fig. 1). Some variability was evident among the isolates in the relative migration and amount of *OspA* (31-kDa), and *OspB* (34-kDa), as well as some other lower molecular mass polypeptides between 18,000 and 23,000. However, the most obvious differences were in *OspB* as demonstrated further in immunoblotting.

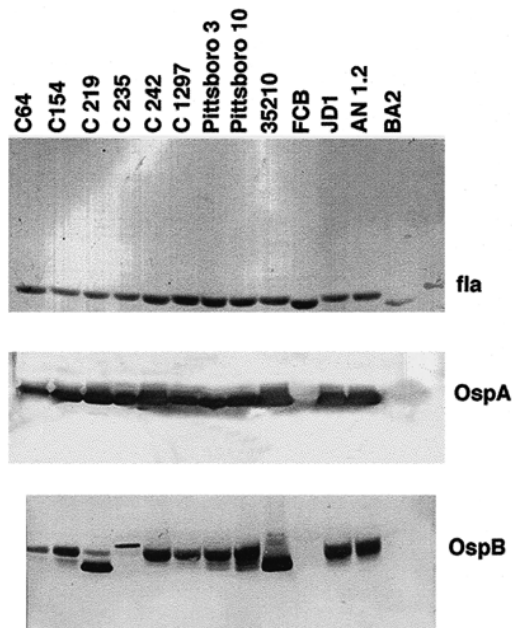


FIGURE 2. Western blots of North Carolina isolates of *B. burgdorferi* and positive control strains (35210, JD1, AN 1.2), and other *Borrelia* strains (FCB, BA2) using genus-specific H9724 (anti-flagellin), species-specific H5332 (anti-*OspA*), and 84C (anti-*OspB*) monoclonal antibodies (13 × 16 cm protein gel).

All 13 strains reacted positively when immunoblotted with the genus-specific, monoclonal antibody H9724 (Fig. 2). The flagellin protein in all isolates, except FCB and BA2, correspond to an apparent molecular weight of 41-kDa. The two negative controls flagellin protein migrated with an apparent molecular weight of approximately 40-kDa. All eight NC isolates reacted positively to immunoblots with species-specific monoclonal antibodies H5332 (anti-*OspA*) and 84C (anti-*OspB*). Some heterogeneity in *OspB* expression was demonstrated by patterns of reactivity with the monoclonal antibody 84C for all eight North Carolina isolates (Fig. 2).

Polymerase chain reaction

All NC isolates and the positive controls tested gave a positive signal after amplification with the nested primer to the flagellin gene (*fla A*). Negative controls (mi-

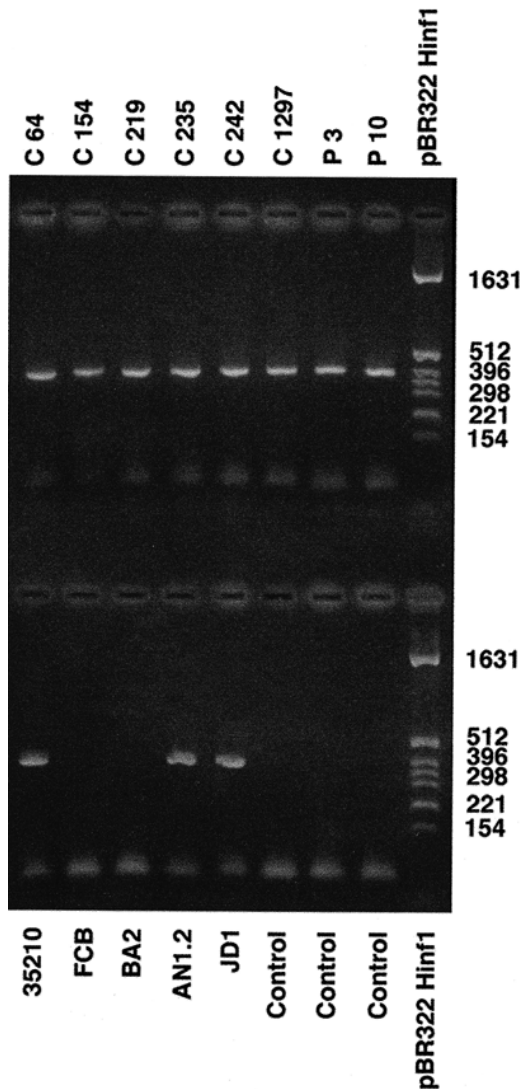


FIGURE 3. PCR analyses of North Carolina isolates and positive control strains (35210, JD1, AN 1.2) of *B. burgdorferi*, other *Borrelia* spp. strains (FCB, BA2), and minus template controls with a nested primer specific for a conserved region of the flagellin gene (11 × 14 cm agarose gel).

nus template) and the strains BA2 and FCB gave no positive signal. The resultant PCR product appeared to have a length of 390 base pairs when compared to the 400 base pair fragment of the pBR322 *HinfI* marker DNA (Fig. 3). The PCR products from all positive reactions were cut with *PvuII* into two fragments that corresponded to 247 and 143 base pairs (Fig. 4).

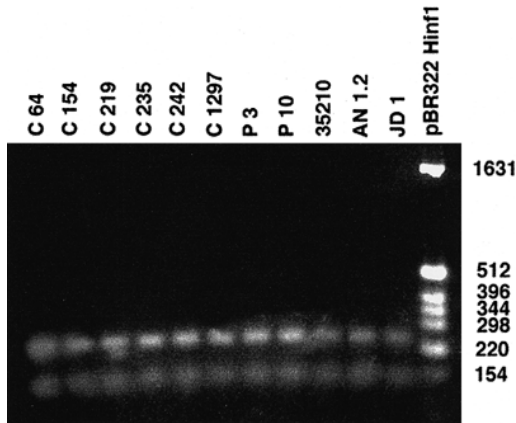


FIGURE 4. *Pvu*II restriction enzyme digests of positive PCR analyses products from all NC isolates and the positive control strains (3520, AN 1.2, JD1) (11 × 14 cm agarose gel).

Plasmid profiles

Plasmid DNA was recovered from QIA-GEN extractions. These samples, when applied to a 0.4% agarose gel, presented dramatically different plasmid profiles (Fig. 5). The number of plasmids detectable in each strain varied from 6 to 12. Plasmids isolated in these gels exhibit an apparent linear mobility of 4 to 50 kilobases. Four strains (C0064, C0154, P3 and P10) harbored an 8-kb supercoiled plasmid. However, there was no apparent correlation between plasmid composition and geographic location or host-isolate origin. These findings are consistent with the positive controls used in this study, and previously reported ranges and plasmid profile compositions reported for *B. burgdorferi* in other such studies (Schwan et al., 1993).

Infectivity studies

Two strains, C0064, and C0235 were inoculated into *M. musculus* and *O. palustris* (Table 2). Twenty-four animals of each species were inoculated with each strain. The tick isolate (C0064) required an intradermal dosage of 2×10^6 organisms to be able to isolate spirochetes from urinary bladder from 50% of the experimental animals 28 days post-inoculation. The MID

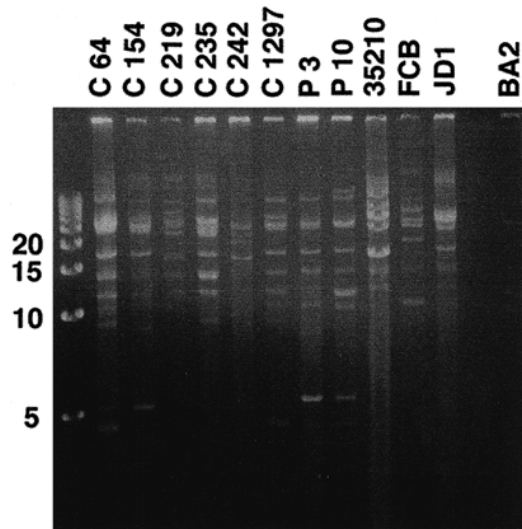


FIGURE 5. Plasmid profiles of the North Carolina isolates and positive control strains (35210, JD1) of *B. burgdorferi* and other *Borrelia* strains (FCB, BA2) (11 × 14 cm agarose gel).

estimated for the raccoon isolate (C0235) was 2×10^4 spirochetes. The same experiments repeated in marsh rice rats produced similar results. In marsh rats C0064 performed equally as it did in white mice, but C0235 was even more infectious; 100% of the animals were infected at 10^4 and 25% of the animals were infected with as little as 100 organisms (Table 2).

DISCUSSION

The combined results of the presented immunoblotting, PCR, and plasmid pro-

TABLE 2. Minimum infectious dose^a from intradermal inoculations of the JD1, C219, C64, and C235 strains of *Borrelia burgdorferi* in *Mus musculus* (outbred ICR strain) and *Oryzomys palustris* (inbred NDRL strain).

Strain designation	Minimum infectious dose in <i>Mus musculus</i>	Minimum infectious dose in <i>Oryzomys palustris</i>
JD1 C2	2×10^4	not determined
C219	2×10^8	not determined
C0064. ^b	2×10^6	2×10^6
C235 ^c	2×10^4	$2 \times 10^{2.4}$

^a Number of spirochetes required to infect 50% of the animals tested ($n = 24$ animals per strain, each species).

^b *Ixodes scapularis*.

^c *Procyon lotor*.

files indicate that all eight North Carolina isolates tested in this study were *Borrelia burgdorferi* sensu stricto. Lyme disease spirochetes from the southeastern United States, specifically Sapelo Island (SI, Georgia), were first characterized by Oliver et al. (1993, 1995). Although not molecularly characterized, *B. burgdorferi* has been identified by IFA from ticks collected in Alabama, USA (Luckhart et al., 1991). In the present study, the antigenic profiles of the NC isolates were more consistent with California (Schwan et al., 1993) and Illinois (Picken et al., 1995) *B. burgdorferi* isolates than the SI isolates (Oliver et al., 1993) or isolates obtained from mammals and ticks collected in the northeastern United States. The most predominant proteins in the antigenic patterns of *B. burgdorferi* isolates from California (Schwan et al., 1993) and Illinois (Picken et al., 1995) were *OspA* (about 31-kDa) and *OspB* (about 34-kDa). Only occasionally was *OspC* reported as a dominant protein in several of the California and Illinois isolates. Oliver et al. (1993) reported a major protein with an apparent molecular weight of 22.5-kDa (*pC*) as a principal feature of all SI isolates. This protein was most likely *OspC*. However, *OspC* had not been described and characterized until after the SI study (Padula et al., 1993). The antigenic trends we found were consistent with recent findings that *B. burgdorferi* isolates from the southeastern and western United States are also more genetically divergent than isolates from the northeast (Oliver, 1996).

Our study of North Carolina spirochete isolates, obtained from numerous locations and different hosts, document that *B. burgdorferi* sensu stricto is present and naturally transmitted in the Outer Banks, Coastal Plain, and Piedmont regions of North Carolina regions where Lyme disease is diagnosed. These findings further strengthen the argument that *B. burgdorferi* sensu stricto is found and naturally transmitted in the southeastern United States.

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