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Isolation and transmission of the Lyme disease spirochete from the southeastern United States

(*Borrelia burgdorferi*/*Ixodes scapularis*/cotton mice)

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ABSTRACT **The isolation of the Lyme disease spirochete (*Borrelia burgdorferi*) from the southeastern United States is reported. Three isolates, two from cotton mice (*Peromyscus gossypinus*) and one from the black-legged tick (*Ixodes scapularis*), were recovered from Sapelo Island, Georgia, in July and September 1991. The spirochetes were characterized by indirect fluorescent antibody assay using a battery of five monoclonal antibodies, by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) of whole cell lysates, and by the polymerase chain reaction (PCR) assay using primers for three DNA target sequences found in *B. burgdorferi* reference strain B-31. Transmission experiments indicate that the three Georgia isolates can infect experimentally inoculated hamsters and mice. Tick transmission of one of the isolates has been attempted so far; *I. scapularis* transmitted isolate SI-1 from hamsters to mice, but the lone-star tick, *Amblyomma americanum*, did not.**

Lyme disease (LD) is a multisystemic illness caused by the spirochete *Borrelia burgdorferi* (1) and transmitted by ticks in the genus *Ixodes*, particularly those of the *Ixodes ricinus*/*Ixodes persulcatus* species complex (2, 3). Clinical manifestations include erythema migrans, arthritis, facial palsy, short-term memory loss, inattentiveness, loss of sensation, profound fatigue, cardiac arrhythmias, and other symptoms (4). LD is or has been known as Lyme borreliosis, Lyme arthritis, erythema chronicum migrans (ECM), erythema migrans, summer flu, and other names. It was first reported as ECM in 1909 in Sweden (5), but its debut in the United States was not reported until 1970 in Wisconsin (6). Seven years later during an outbreak of oligoarticular arthritis in Connecticut, researchers established the connection between ECM and Lyme arthritis (7, 8). Since then, the number of reported cases in the United States has increased dramatically. From 1986 to 1990, LD accounted for 81% of all reported cases of vector-borne diseases in the United States. A total of 9469 LD cases were reported by state health departments to the Centers for Disease Control and Prevention for 1991 (9). That number represented a 19% increase over 1990 and a 7% increase over the previous high year, 1989. More than 40,000 cases have been reported in the United States since 1982 (9). Most cases occurred in the northeastern United States, where much is known about LD; a considerable number of cases also originated in Ohio, Wisconsin, Minnesota, and California (9). A significant number of LD cases have been reported from Georgia and other southeastern states, but little is known about the disease in that region. Nevertheless, competent vector ticks are present in southeastern states (10–12), and serologic surveys of feral animals (13–16) suggest that the LD spirochete occurs in that region. However, skeptics still doubt whether *B. burgdorferi* and LD occur and are endemic there, since the presence of *B. burgdorferi* has not been confirmed through culture (17). We report here the isolation, characterization, and experimental transmission of *B. burgdorferi* from the southeastern United States.

MATERIALS AND METHODS

Cotton mice (*Peromyscus gossypinus*) were collected in May and September 1991, and black-legged ticks (*Ixodes scapularis*) in September 1991, from Sapelo Island, Georgia. Mouse tissues (urinary bladder, ear clips, and ear-punch biopsy specimens) and tick tissues (midgut and non-midgut samples) were cultured in Barbour–Stoenner–Kelly (BSK) II medium (18) containing 0.15% agarose (Seakem, FMC Bioproducts, Rockland, ME), antibiotics, and other compounds (19) by a modification of previously reported techniques (20). Before culturing, mouse ears were cleaned with 95% ethanol and ear clips were sliced into small pieces or several 2-mm punches were taken from each ear. Ear clips or punches were cleaned again in 95% ethanol followed by a rinse in a 1:1 mixture of 10% Clorox and 95% ethanol. Ear punches were then placed into 1.25 ml BSK II medium (18) and small ear slices were placed in 4.5 ml of medium. All cultures were incubated in a 5% CO_2 atmosphere at 33–34°C and examined for spirochetes by darkfield microscopy twice weekly for 2 weeks and, if spirochetes were not detected, weekly thereafter for 6 weeks. Spirochetal isolates were tested by indirect fluorescent antibody analysis (21, 22) using five monoclonal antibodies, including two *B. burgdorferi*-specific anti-outer surface protein A (OspA) monoclonals (H3TS and H5332), two *B. burgdorferi*-specific anti-outer surface protein B (OspB) monoclonals (H5TS and H6831), and a *Borrelia* (genus)-specific anti-flagellin monoclonal antibody (H9724).

Preparation of each spirochetal culture for characterization by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) involved whole spirochetal lysates (23, 24). Aliquots of 20 ml each were centrifuged at 10,000 × *g* for 30 min. Supernatants were removed and spirochetal pellets were suspended in 1 ml of phosphate-buffered saline (PBS: 120 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4/10 mM KH_2PO_4, pH 7.4) with 5 mM $MgCl_2$ by vortex mixing until evenly dispersed. Samples were centrifuged again at 10,000 × *g* for 15 min. After a second PBS/$MgCl_2$ wash, spirochetal pellets were resuspended in 0.1 ml of sterile distilled water and then frozen at −80°C for 30 min, thawed, and refrozen. After the final thaw, the suspended spirochetal pellets were vortex-mixed and an aliquot was then removed for total

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Abbreviation: LD, Lyme disease.

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protein determination by the Bradford method (25). Molecular characterization of each whole spirochetal lysate was partly determined by SDS/PAGE. Each sample was diluted to contain 5.0 μg of protein per 25 μl of distilled water to which an equal volume of SDS/PAGE dissolving buffer containing 0.05 M DL-dithiothreitol was added and heated at 100°C for 10 min. Samples were electrophoresed through a 12% separating gel with a 4% stacking gel, both of which were bonded to Gel Bond (FMC). Both prestained and nonstained low molecular weight protein standards (Bio-Rad) were simultaneously electrophoresed for extrapolation of the molecular weights. The SDS/polyacrylamide gel was stained with Coomassie brilliant blue R-250, destained, and stained again with $AgNO_3$ by using a Bio-Rad $AgNO_3$ stain kit according to the manufacturer's instructions.

The polymerase chain reaction (PCR) was used to detect several known DNA target sequences specifically found in *B. burgdorferi* reference strain B-31. Prior to PCR, the spirochetal lysates were centrifuged at 600 × *g* for 15 min to sediment any cellular debris, and protein assay was then performed using a small aliquot of the spirochetal protein supernatant (25). Before addition to the PCR mixture, 0.5 μg of spirochetal protein for each isolate in 10 μl of distilled water was heated at 100°C for 10 min to inhibit any proteolytic activity. The PCR mixture contained 2.5 units of *Taq* DNA polymerase, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, and 50 pmol of each appropriate oligonucleotide primer. Amplification of target sequences was performed in a Perkin–Elmer/Cetus thermal cycler with several denaturing, annealing, and extension times and temperatures (Table 1). One set of primers (supplied by Barbara Johnson, Centers for Disease Control and Prevention, Fort Collins, CO) was used to amplify a 600-bp target sequence found in the flagellin (*fla*) gene of *B. burgdorferi* B-31 strain (26). A second set of primers was used to amplify a 158-bp target sequence found in the B-31 strain *ospA* gene (23). PCR was also performed on whole spirochetal lysates by using a third set of primers (supplied by Patricia Rosa, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT) that amplify a 370-bp chromosomal target sequence present in 17 of 18 characterized strains of *B. burgdorferi* worldwide (27). For each PCR assay, pure genomic DNA from *B. burgdorferi* B-31 was used as a positive control and sterile distilled water as a negative control. The PCR-amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide. Polaroid photographs of the ultraviolet-transilluminated gels were taken for a permanent record.

Transmission experiments were conducted with three *B. burgdorferi* isolates (SI-1, SI-2, and SI-4) from Georgia; the northeastern isolate (SH2-82) served as a positive control. The needle-transmission experiment involved inoculation of SI-1 into six hamsters in duplicate doses of 1.0, 0.75, and 0.5 ml from a culture containing ≈9.2×10^7 spirochetes per ml. The control hamster received 0.75 ml of a culture of similar concentration containing the northeastern SH2-82 strain. SI-2 (0.5 ml) was injected into each of four cotton mice (*P. gossypinus*), and SI-4 (0.5 ml) was injected into four additional mice. Each dose of spirochetes was divided equally into a subcutaneous and intraperitoneal injection for each hamster and mouse. Infection of hamsters and mice was determined by xenodiagnosis and/or spirochetal isolation.

I. scapularis larvae (Georgia population) were fed on the two hamsters that received the 1.0-ml dose and, upon molting, the nymphs were fed on naive white mice. *Amblyomma americanum* larvae (Georgia population) were fed on the two hamsters that received the 0.75-ml dose and, upon molting, were fed on naive white mice. *I. scapularis* nymphs (Georgia population) were fed on the two hamsters that received the 0.5-ml dose and, upon completion of the nymphal feeding (≈35 days), one of these hamsters was subsequently fed on by *I. scapularis* larvae and nymphs (Massachusetts population formerly known as *Ixodes dammini*). After feeding, some of the larvae were preserved in formalin for subsequent analysis by PCR as were some of the resulting nymphs and adults. Some of the nymphs resulting from larvae fed on inoculated hamsters were fed on naive white mice. *I. scapularis* (Georgia population) nymphs and larvae were fed on the control hamster that was inoculated with the SH2-82 strain. Upon feeding and molting, some of the nymphs were fed on naive white mice; other nymphs and the adults were preserved in formalin for later PCR analysis.

RESULTS

Three spirochetal isolates from Sapelo Island, Georgia, were recovered from two cotton mice (*P. gossypinus*) and an unfed questing male *I. scapularis* tick. The first isolate (SI-1) was from the urinary bladder wall of a cotton mouse collected on Sapelo Island, Georgia, in May 1991. The second isolate (SI-2) was cultured from urinary bladder, ear clips, and ear-punch biopsy specimens of another cotton mouse from Sapelo Island, collected in September 1991. The fourth isolate (SI-4) was from the midgut and other tissues of a male *I. scapularis* collected during September 1991. The third isolate (SI-3) was from a cotton rat and will be reported later with other rat isolates.

The three spirochetal isolates reported here reacted positively with all five *B. burgdorferi* monoclonal antibodies tested. As noted in *Materials and Methods*, H6831, H3TS, H5332, and H5TS are species-specific whereas H9724 is genus-specific. The three isolates reacted negatively to *Borrelia hermsii* 9826 and *Borrelia coriaceae* F6F3, F6B3, and F6B11 antibodies. The major proteins identified by SDS/PAGE of the three Georgia isolates were identical in molecular weight to those of *B. burgdorferi* strains previously described (28). At least nine major proteins from whole spirochetal lysates of the Georgia isolates and of three New England isolates of *B. burgdorferi* (SH2-82, JD-1, and Guilford) were resolved with a $AgNO_3$-stained 12% polyacrylamide gel, including the 31-kDa OspA and the 41-kDa flagellin protein. The three Georgia isolates were identical in major molecular weight protein composition to the New England isolates with the exception of a prominent band estimated to be ≈22.5 kDa (Fig. 1). This 22.5-kDa low molecular weight protein was not visible by SDS/PAGE of

Table 1. Primers and thermal cycler settings for performing PCR on *B. burgdorferi* isolates

Gene	Primers		PCR conditions			No. of cycles
	Name	Sequence (5′ → 3′)	Denaturing	Annealing	Extension	
fla	S275	AAGTAGAAAAAGTCTTAGTAAGAATGAAGGA	94°C, 30 sec	55°C, 1 min	72°C, 2 min	35
	R855	AATTGCATACTCAGTACTATTCTTTATAGAT				
ospA	D608	GTTTTGTAATTTCAACTGCTGACC	95°C, 1 min	50°C, 45 sec	72°C, 2 min	45
	U609	CTGCAGCTTGGAATTCAGGCACTT				
Chromosomal	A	CGAAGATACTAAATCTGT	94°C, 1 min	37°C, 30 sec	60°C, 1 min	30
	C	GATCAAATATTTCAGCTT				

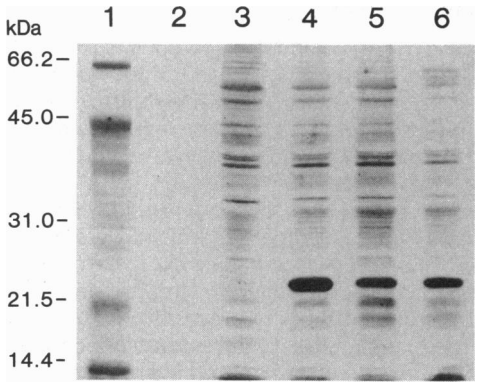

FIG. 1. SDS/PAGE of whole spirochetal lysates. Lane 1, low molecular weight standards (molecular masses in kilodaltons at left); lane 2, blank; lane 3, *B. burgdorferi* B-31; lane 4, SI-1 isolate; lane 5, SI-2 isolate; lane 6, SI-4 isolate.

whole spirochetal lysates of the three *B. burgdorferi* isolates from New England (data not shown).

The *B. burgdorferi* B-31 type strain from New York and the Sapelo Island isolates from Georgia consistently amplified *fla* and *ospA* gene sequences by PCR assay (Figs. 2 and 3). These amplified products were readily detected after 35 and 45 cycles, respectively. When PCR primers were used that recognize a conserved DNA sequence of 17 of 18 known *B. burgdorferi* strains worldwide, the specific 370-bp product also was amplified from each isolate (Fig. 4). Nonspecific PCR products were not detected in any of the PCR-amplified DNA target sequences.

The three Georgia spirochetal isolates reported here are readily transmitted by inoculation to hamsters and mice. Duplicate injections of three doses (1.0, 0.75, and 0.5 ml) of SI-1 resulted in all six hamsters becoming infected. SI-2 (0.5 ml per mouse) injected into four cotton mice and SI-4 (0.5 ml per mouse) inoculated into four additional mice resulted in all eight mice becoming infected. The northeastern strain SH2-82 (0.75 ml per mouse), used as a control, also caused infection in a hamster.

Tick transmission of SI-1 strain was successful with *I. scapularis* but unsuccessful with *A. americanum*. Feeding of *I. scapularis* larvae (Georgia population) on two hamsters inoculated with SI-1 produced 38 nymphs from one and 32

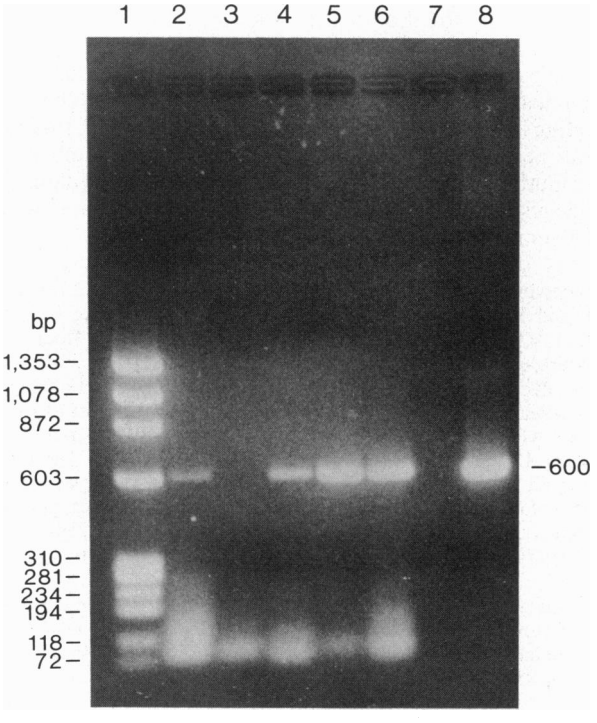

FIG. 2. Agarose gel electrophoresis of PCR products amplified with primers for the *fla* gene of *B. burgdorferi*. Lane 1, DNA size standards; lane 2, *B. burgdorferi* B-31; lane 3, sterile distilled water control; lane 4, SI-1 isolate; lane 5, SI-2 isolate; lane 6, SI-4 isolate; lane 7, blank; lane 8, *B. burgdorferi* genomic DNA (positive control).

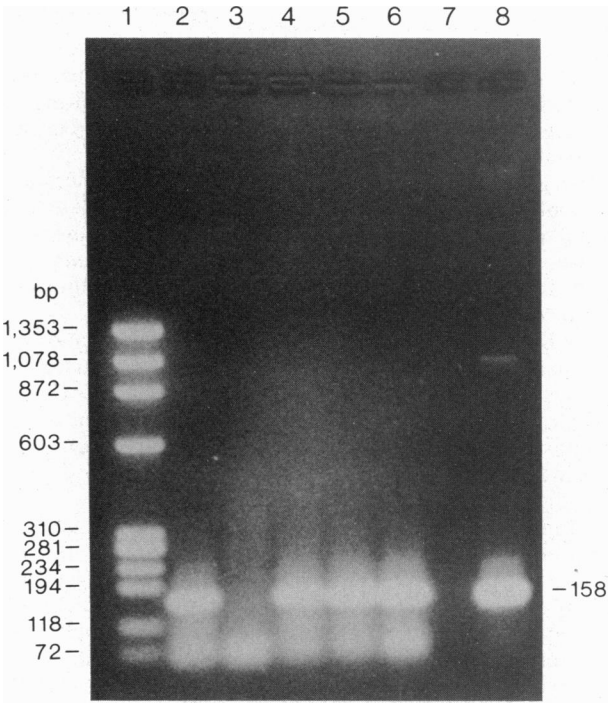

FIG. 3. PCR products amplified with primers for the *ospA* gene of *B. burgdorferi*. Samples and sample order are the same as in Fig. 2.

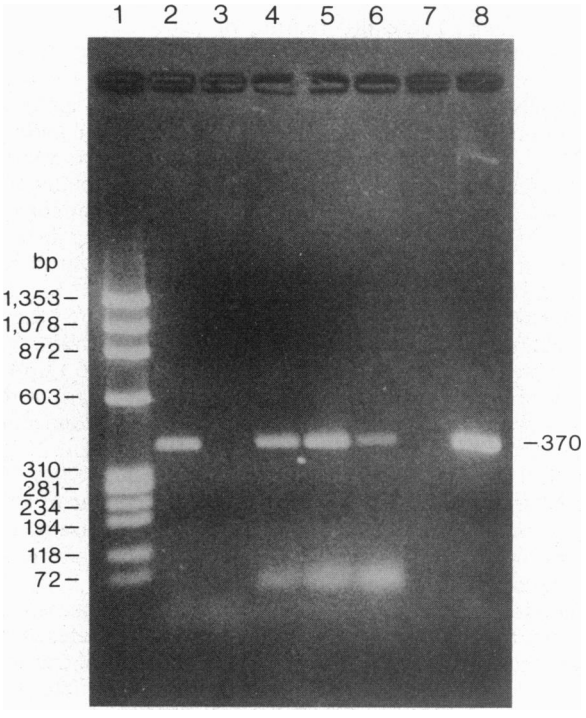

FIG. 4. PCR products amplified with primers for the chromosomal sequence conserved 17 of 18 *B. burgdorferi* species. Samples and sample order are the same as in Fig. 2.

nymphs from a second hamster. Each group of nymphs was then fed on one white mouse. The mice were necropsied 8 weeks after the nymphs had fed, and spirochetes were cultured from ear clips, heart, kidney, spleen, and urinary bladder of one mouse and from ear clips, spleen, and urinary bladder of the second mouse.

The 13 nymphal *I. scapularis* derived from larvae fed on the control hamster (inoculated with the SH2-82 New York strain) transmitted the spirochetes to a naive white laboratory mouse. Eight weeks after tick exposure, mouse ear clips, heart, spleen, kidney, and urinary bladder yielded cultures of *B. burgdorferi*.

Feeding *A. americanum* larvae (Georgia population) on two hamsters inoculated with SI-1 strain produced 153 nymphs from one hamster and 240 from the other. The 153 nymphs were fed on nine white mice (17 per mouse); the 240 nymphs were fed on six white mice (40 per mouse). All 15 mice were negative for spirochetes upon necropsy 8 weeks after the nymphs fed.

A third part of the tick transmission experiment involved injection of two hamsters with SI-1 isolate (0.5 ml) and subsequent feeding of *I. scapularis* nymphs (Georgia population) and *I. scapularis* larvae and nymphs (Massachusetts population) on them. The hamster with only nymphs on it died during tick feeding, and the partially fed nymphs were preserved in formalin for later PCR analysis. Spirochetes were reisolated from the dead hamster. The second hamster with nymphs and larvae attached to it survived, and 24 male and 34 female ticks molted from fed nymphs; 103 fed larvae molted to nymphs. The adults were preserved for later PCR analysis and the nymphs were fed on four white laboratory mice (40 per mouse). All four mice were positive for *B. burgdorferi* upon subsequent culture from ear clips, spleen, and urinary bladder wall.

DISCUSSION

The appearance of a 22.5-kDa low molecular weight protein in the spirochetal isolates from Sapelo Island, Georgia, and its absence from the New England isolates of *B. burgdorferi* is interesting. There are reports of major low molecular weight proteins in several tick-derived isolates of *B. burgdorferi* from California. Five of 11 such isolates had low molecular weight proteins in the 20- to 24-kDa molecular mass range, and the spirochetes bound monoclonal antibody 86 DN-1 in Western blot and IFA tests (29). There was also an unusual *B. burgdorferi* strain (DN127 cl 9-2), isolated from an *Ixodes pacificus* tick, that did not react with monoclonal antibodies to OspA and OspB, which are found in most strains from the United States. It had an abundant 25-kDa protein. Strains of *B. burgdorferi* with prominent low molecular weight proteins are uncommon in the United States, especially in the Northeast. Several European strains with low molecular weight proteins had no detectable OspA or OspB protein (30–32). One strain (2591) isolated from a white-footed mouse (*Peromyscus leucopus*) in Connecticut possessed OspA and OspB as well as a prominent 22-kDa protein (33). Our three isolates (SI-1, SI-2, and SI-4) from Sapelo Island, Georgia, appear to be more similar to strain 2591 from Connecticut than to some European strains with regard to surface proteins (OspA and OspB) and prominent low molecular weight proteins.

Data from monoclonal antibody screening, comparisons of proteins by SDS/PAGE of whole spirochetal lysates, DNA target sequence analyses by PCR, and spirochetal transmission by *I. scapularis* all indicate that the three isolates from Sapelo Island, Georgia, are *B. burgdorferi*. The high prevalence (64.5%) of antibodies (titers, 320 to >40,960) to *B. burgdorferi* among 31 *P. gossypinus* mice from the same island (15) as that of the spirochetal isolates described here provides further evidence for the presence and endemicity of *B. burgdorferi* on Sapelo Island. Unpublished data (J.H.O., F.W.C., A.M.J., and H.J.H.) on 13 additional spirochetal isolates that appear to be *B. burgdorferi* from Sapelo Island and four other geographic sites in Georgia and Florida, and the high prevalence of antibodies to *B. burgdorferi* in *P. gossypinus* on three nearby islands (21.4%, 57.7%, and 90.0%; ref. 15), indicate that *B. burgdorferi* is endemic in Georgia and Florida. Moreover, antibodies to *B. burgdorferi* in *P. gossypinus* from North and South Carolina, Alabama, and Mississippi suggest that *B. burgdorferi* might also be endemic in parts of those southeastern states as well (15). Indeed, indirect and direct fluorescent antibody assays of *I. scapularis* and *A. americanum* recovered from cotton mice and white-tailed deer, respectively, indicated the presence of *B. burgdorferi* in Alabama (34). Another report also indicated the presence of a *Borrelia* species in *I. scapularis* from deer in Alabama (35). Antibodies to *B. burgdorferi* have also been reported in several other mammalian species from other southeastern states (13, 14).

The occurrence of *B. burgdorferi* isolates from five geographic sites extending along a coastal region of over 200 miles in Georgia and Florida (unpublished data) and the serologic data from several southeastern states suggest that *B. burgdorferi* is widespread in the Southeast. Further evidence to support this contention is the isolation of *B. burgdorferi* from a second rodent species (unpublished data), the existence of naturally infected *I. scapularis*, and our demonstration that *I. scapularis* is a competent vector of a southern strain of *B. burgdorferi*. The recent report that the main vector of *B. burgdorferi* in the Northeast, *I. dammini*, is conspecific with *I. scapularis* (12) further emphasizes the need for additional research on LD in the South. Much research is needed to determine reservoir hosts to *B. burgdorferi*, prevalences of infected *I. scapularis* ticks, and vector competencies of other potential tick and insect species. The large number of lizards on which many immature *I. scapularis* feed in the Southeast, and the greater number of mammalian and avian species present as potential tick hosts and spirochete reservoirs, suggests the possibility of a more complicated zoonotic situation in the Southeast than that existing in the northeastern United States. Finally, there is a great need to obtain accurate data on the number and distribution of human cases throughout the United States, but especially in the South, to determine the ultimate human involvement in the LD equation.

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