Borrelia lonestari DNA in Adult *Amblyomma americanum* Ticks, Alabama

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Polymerase chain reaction analysis of 204 *Amblyomma americanum* and 28 *A. maculatum* ticks collected in August 1999 near the homes of patients with southern tick-associated rash illness and in control areas in Choctaw County, Alabama, showed *Borrelia lonestari* flagellin gene sequence from two adult *A. americanum*. The presence of *B. lonestari* in *A. americanum* ticks from Alabama suggests that this suspected pathogen may be widespread in the southeastern United States.

Lyme disease is the most commonly reported vector-borne disease in the United States. In the northeastern, midwestern, and western coastal states, the pathogen *Borrelia burgdorferi* sensu lato is well established and is maintained by *Ixodes scapularis* and *I. pacificus* in a variety of rodent reservoirs.

In the southeastern states, where *I. scapularis* is widespread but is less commonly found infected with *B. burgdorferi* or attaching to humans (1,2), isolations from humans are uncommon (3). However, a clinical condition similar to Lyme disease, termed southern tick-associated rash illness (STARI), has been described in humans in the southeastern region of the United States associated with the bite of *Amblyomma americanum* ticks (1,4,5). Moreover, a new spirochete, *B. lonestari*, was described from *A. americanum* on the basis of polymerase chain reaction (PCR) amplification of the flagellin and 16s rRNA genes (6,7). Virtually identical sequences have been found in ticks from geographic regions as disparate as New Jersey and Texas (6), suggesting this organism is widely distributed. Likewise, *Borrelia* spirochetes have been detected in *A. americanum* and *I. scapularis* in Alabama (8,9).

As part of an epidemiologic investigation of a reported cluster of STARI cases in Choctaw County, Alabama, we collected both *A. americanum* and *A. maculatum* ticks adjacent to the houses of suspected patients and in control areas, and the *B. lonestari* flagellin gene sequence was amplified from DNA extracted from *A. americanum*.

The Study

Ticks were collected with drag cloths in areas around the homes of persons with suspected cases, as well as in control areas. Clinical cases were defined as illness characterized by acute onset of an annular, expanding erythema migrans-like rash at least 5 cm in diameter, when no alterative explanation for the rash can be found; and there is a history of tick bite at the rash site or potential exposure to ticks within 14 days before rash onset. Ticks were identified to species by using standard taxonomic keys.

DNA was isolated from *Amblyomma* sp. by using an extraction procedure reported previously (10). Briefly, individual ticks were frozen in liquid nitrogen, macerated between metal plates, and then homogenized by adding 1 mL of DNA STAT-60 (Tel-Test, Friendswood, TX). The tick homogenate was then incubated with chloroform for phase separation of DNA, which was precipitated with 100% isopropanol. To ensure PCR-quality DNA, all tick extracts were tested for the presence of tick mitochondrial DNA (11). *Amblyomma* DNA was then subjected to a nested PCR procedure for *B. lonestari* by using primers FlaLL/FlaRL, then FlaLS/FlaRS (6). Flagellin-positive samples were further analyzed by using OspA primers as a control for possible flagellin false-positive samples. Subsequent DNA sequencing of positive samples for *Borrelia* sequence identification was done with a Taq Dyedeoxy terminator cycle kit (Applied Biosystems, Foster City, CA) and run on an Applied Biosystems 377 automated sequencer. The derived sequences were aligned with MegAlign (DNASTAR, Inc., Madison, WI) by using the clustal algorithm. Aligned sequences were transferred to PAUP (Sinauer Associates Inc., Sutherland, MA) for phylogentic analysis. Accession numbers D88295 (*B. afzelii*), X75201 (*B. anserina*), D82857 (*B. bissettii*), Y15097 (*B. burgdorferi*), D63372 (*B. garinii*), AF228034 (*B. hermsii*), D43777 (*B. miyamotoi*), D82863 (*B. parkeri*), U26705 (*B. lonestari,* New Jersey isolate), and U26704 (*B. lonestari,* Texas isolate) were used in this comparative genetic analysis.

Two hundred four *A. americanum* (21 adults and 183 nymphs) were collected: 13 adults and 44 nymphs from the properties of controls, and 8 adults and 139 nymphs near homes of persons meeting the STARI case definition. Twenty-nine *A. maculatum* adults were collected from control and STARI case areas. All but five ticks yielded PCR-quality DNA, as determined by PCR amplification of tick mitochondrial DNA (11).

Two (11%) of 19 of adult *A. americanum* ticks analyzed were positive for *B. lonestari* flagellin gene DNA (Table).

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Table. Ticks collected in Choctaw County, Alabama

Ticks analyzed	*Amblyomma americanum* Adult male	Adult female	Nymphs	*A. maculatum* Adult male	Adult female
Controls[a]	11[b]	2	44	6	12
Cases[c]	1	5[b]	139	7	1
Total	12	7	183	13	13

[a]Denotes ticks collected near the homes of persons not diagnosed with southern tick-associated rash illness (STARI).
[b]Positive results as demonstrated by amplification of the flagellin gene of *Borrelia lonestari.* A single positive tick was found among 11 adult male *A. americanum* males collected near the homes of controls and 1 of 5 adult females collected near the homes of STARI controls was positive.
[c]Denotes ticks collected near the homes of persons diagnosed with STARI.

Positive results for *B. lonestari* were confirmed by amplification of the 16s rRNA gene as described by Barbour et al. (6). All 183 nymphs and all 26 adult *A. maculatum* were PCR negative for *B. lonestari* flagellin gene DNA. Moreover, all tick DNA samples were PCR negative when analyzed for the *B. burgdorferi* OspA gene. Sequence analyses for both positive samples showed >99% homology with the published *B. lonestari* sequences from New Jersey and Texas. Alabama isolates numbers 1 and 2 were 100% homologous to *B. lonestari* NJ and differed by 1 bp when compared with published sequences of the Texas isolate of *B. lonestari*. Phylogenetic analysis, using maximum likelihood and bootstrap analysis with 500 replications of derived sequences (Figure), illustrated that both isolates clustered with reported sequences of the *B. lonestari* NJ and TX strains and demonstrated considerable divergence from *B. burgdorferi* sensu stricto, the only genospecies shown to cause disease in the United States.

The nucleotide sequences of the *B. lonestari* flagellin gene have been submitted to GenBank and assigned accession numbers AF298653 (Alabama isolate 1) and AF298654 (Alabama isolate 2).

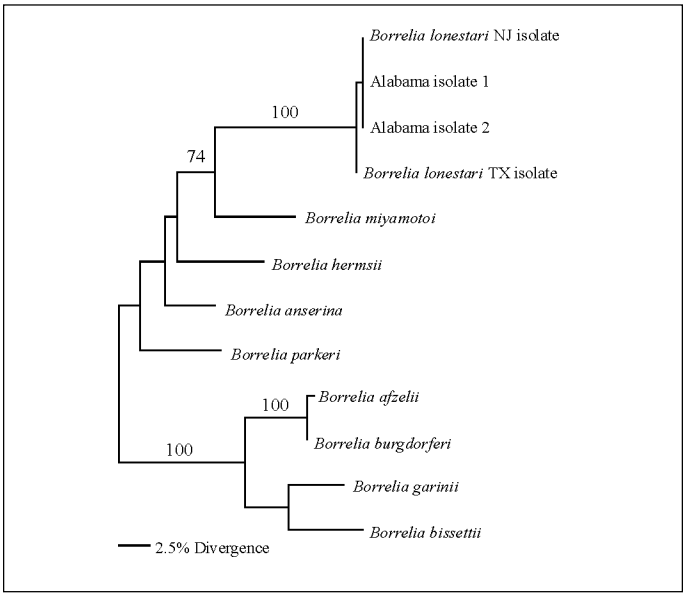

Figure. Maximum parsimony phylogenetic tree generated by using PAUP. Numbers indicate parsimony bootstrap scores for the branch. Only bootstrap scores >70 are included in the phylogenetic tree.

Conclusions

Spirochetes have been reported in *A. americanum* ticks from New York, New Jersey, Virginia, North Carolina, Alabama, Missouri, and Texas. In contrast to *B. burgdorferi* spirochetes, attempts to propagate these spirochetes from *A. americanum* in Barbour-Stoenner-Kelly (BSK-H) culture have been unsuccessful. Likewise, attempts to coculture *A. americanum* samples with an *I. scapularis* cell line (IDE2) failed to propogate *B. lonestari* (B. Johnson, pers. comm.). Thus, we used genetic analysis to determine the presence of this organism.

Erythema migrans associated with the bites of *A. americanum* has been reported from the southeastern United States, including Missouri (4) and North Carolina (5). Serum samples from these patients did not recognize *B. burgdorferi* antigens (4,5), and spirochetes from these cases have not been successfully cultured in BSK-H medium. Hence, these cases have been diagnosed as STARI. Recently, novel DNA sequences, amplified by PCR with primer sets recognizing the flagellin and 16s rRNA genes, have identified a new spirochete in *A. americanum* and *B. lonestari* (6). Nearly identical sequences have been amplified from *A. americanum* collected from Texas and New Jersey (2) and Maryland (1). *B. lonestari* is the suspected pathogen responsible for the STARI-related erythema migrans associated with bites by *A. americanum* (1). Moreover, *B. lonestari* was isolated from a patient in Westchester County, New York, who had traveled to Maryland and North Carolina and had an attached *A. americanum* at the site of an erythema migrans rash. This isolate differed only slightly from *B. lonestari* isolates reported in New Jersey and Texas (A. James et al., unpub. data).

Although the numbers of ticks we analyzed were small, our results suggest that adult *A. americanum* may transmit *B. lonestari* to persons in this area. Finding *B. lonestari* sequences in *A. americanum* from Alabama suggests that this spirochete is widely distributed in the United States. These first sequences from the southeastern United States are noteworthy because this region is a focus for numerous reports of erythema migrans associated with the bites of *A. americanum.* Further investigation is needed to formally isolate and propagate *B. lonestari*, as well as to determine its host reservoir.

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