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 alternative 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 phylogenetic 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).
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 from New Jersey and Texas. 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).

**Table. Ticks collected in Choctaw County, Alabama**

| Ticks analyzed | *Amblyomma americanum* | *A. maculatum* |
|----------------|------------------------|----------------|
|                | Adult male | Adult female | Nymphs | Adult male | Adult female |
| Controlsa      | 11b        | 2            | 44      | 6          | 12           |
| Caseb          | 1          | 5b           | 139     | 7          | 1            |
| Total          | 12         | 7            | 183     | 13         | 13           |

aDenotes ticks collected near the homes of persons not diagnosed with southern tick-associated rash illness (STARI).

bPositive 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.

dDenotes 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. 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).

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-Stoener-Kelly (BSK-H) culture have been unsuccessful. Likewise, attempts to coculture *A. americanum* samples with an *I. scapularis* cell line (IDE2) failed to propagate *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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