SHORT COMMUNICATION

DNA Evidence of *Borrelia lonestari* in *Amblyomma americanum* (Acari: Ixodidae) in Southeast Missouri

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ABSTRACT *Amblyomma americanum* collected near Lake Wappapello, Missouri, tested positive for *Borrelia lonestari* using polymerase chain reaction and sequence analyses of *B. lonestari* 16S rRNA and flagellin (*fla*B) genes. Twelve pools containing a total of 214 nymph or adult ticks contained evidence of infection with *B. lonestari* (minimum prevalence 5.6%). These data suggest that persons in southeast Missouri are at risk for exposure to *B. lonestari* after *A. americanum* tick bite, a possible cause of erythema migrans-like rash illness in this region. Derivation of the complete coding sequence for *B. lonestari fla*B is also reported.

KEY WORDS *Borrelia*, flagellin, 16S rRNA, *Amblyomma*, Missouri

THOUSANDS OF HUMAN LYME disease cases have been reported from Missouri and other south central and southeastern states in the United States since Lyme disease became a nationally notifiable disease in 1991 (CDC 2001, CDC 2002, Wharton et al. 1990). Many cases were based on the presence of a physician-diagnosed Lyme disease-like rash (i.e., a 5 cm or greater erythema migrans [EM]) and exposure to the predominant human-biting tick in these regions, *Amblyomma americanum* or the Lone Star tick (Burkot et al. 2001, Campbell et al. 1995, Felz et al. 1999, Kirkland et al. 1997, Masters et al. 1998, Masters and Donnell 1996). But human infection with the Lyme disease spirochete, *Borrelia burgdorferi* sensu stricto, could not be confirmed by culture of EM specimens or by serologic testing in case control studies in Missouri and North Carolina (Campbell et al. 1995, Kirkland et al. 1997). While spirochetes have been observed in the midgut of field-collected Lone Star ticks (Feir et al. 1994, Luckhart et al. 1992, Rawlings and Teltow 1994, Schulze et al. 1984), *A. americanum* has been shown to be incompetent for transmission of *B. burgdorferi* to rodents in the laboratory (Piesman and Happ 1997, Ryder et al. 1992, Sanders and Oliver 1995). Ongoing research to determine whether these individuals actually contracted Lyme disease or suffered from a Lyme-like illness caused by infection with another microbe has suggested that *Borrelia lonestari* is the etiologic agent (Barbour 1996, Barbour et al. 1996, James et al. 2001).

Attempts to cultivate spirochetes from *A. americanum* in medium suitable for growing other *Borrelia* species (modified Barbour-Stoenner-Kelly [BSK] medium) have consistently failed. Therefore, researchers have relied on polymerase chain reaction (PCR) amplification and DNA sequence analysis of conserved borreliae genes to characterize these organisms. Using partial 16S rRNA and flagellin (*fla*B) gene sequence analyses first described by Barbour in 1996 (Barbour et al. 1996), *B. lonestari* DNA has been detected in *A. americanum* from several geographic regions, including Alabama, Texas, New Jersey (Barbour et al. 1996, Burkot et al. 2001), and Tennessee (Stegall-Faulk et al. 2003); in archived blood samples collected from white-tailed deer in Arkansas, Georgia, North Carolina, and South Carolina (Moore et al. 2003); and from the EM specimen and attached Lone Star tick from one human patient with possible exposure in Maryland or North Carolina (James et al. 2001). Even though spirochetes have been visualized in the midgut of *A. americanum* from Missouri by immunofluorescence with anti-*B. burgdorferi* polyclonal Ab, efforts to further characterize these borreliae have relied mostly on techniques that are biased toward *B. burgdorferi* sensu lato (i.e., culture in BSK, and PCR or DNA sequence analysis of *B. burgdorferi*-specific targets [Barbour et al. 1996, Feir et al. 1994, Oliver et al. 1998]), thereby hampering the ability to identify other borreliae, including *B. lonestari.* This report describes our efforts to detect *B. lonestari* in Lone Star ticks from Missouri using 16S rRNA and *fla*B DNA

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sequence analysis. In addition, we extend the *fla*B DNA sequence of this organism.

Materials and Methods

Tick Collection and Processing. *A. americanum* was collected in June 2001 outside of Lake Wappapello within Butler County, Missouri (≈60 miles southwest of Cape Girardeau, MO), by passing white drag cloths over native vegetation. Ticks were sorted (nymphs, adults), placed in glass vials, and transported to the laboratory in which they were held at 21°C in a saturated humidity chamber before analysis. Ticks were separated into 12 pools of 1–25 ticks (1 pool of 25 nymphs, 8 pools of 20 nymphs, 1 pool of 8 nymphs, 1 pool of 20 adults, and 1 pool of 1 adult). Each pool of ticks was macerated in 1 ml of phosphate-buffered saline with at least 20 plunges using a 2-ml Ten Broeck glass grinder with 0.004- to 0.006-inch clearance (Bellco 1982-1002, Vineland, NJ). The homogenate was decanted using a 1-ml pipette tip and placed in a sterile 1.5-ml centrifuge tube. Large tick parts were discarded. Each homogenate was centrifuged at 12,000 × *g* for 10 min, the supernatant was discarded, and the pellet was stored at −20°C.

DNA Extraction. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's tissue protocol with minor modification. Each pellet was resuspended in 180 μl of buffer ATL (Qiagen) and 20 μl of vendor-supplied proteinase K. The homogenate was incubated at 56°C overnight with constant agitation and then centrifuged at 82 × *g* for 1 min to pellet tick parts that had not completely lysed. The supernatant was removed to a new 1.5-ml microfuge tube for DNA extraction, according to the remainder of the QIAamp tissue protocol. Finally, DNA was eluted with 100 μl of 10 mM Tris, pH 8.0. Once DNA was extracted from the 12 tick pools, 10 μl from each was combined, of which aliquots were used for PCR amplification (combined pool).

PCR and Sequencing. *Borrelia* genus-specific primers previously described by Barbour (Barbour et al. 1996) were used to PCR amplify the 16S rRNA gene fragment (primers 16RnaL and 16RnaR) and *fla*B gene (primers FlaLL and FlaRL) from the combined pool. Each PCR reaction of 50 μl contained: 10 mM Tris, pH 8.3, 50 mM KCl, 6.5 mM $MgCl_2$, 0.001% gelatin, 200 μM each dNTP, 0.1 μM each primer, 2.5 U of Herculase HotStart Polymerase (Stratagene, La Jolla, CA), and ≈100 ng of total tick-extracted DNA. The thermocycler program consisted of 1 cycle at 95°C for 2 min, 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension cycle at 72°C for 5 min. PCR products were viewed after TAE agarose gel electrophoresis, excised, and purified for sequencing using a QIAquick Gel Extraction kit (Qiagen). DNA sequence analysis was performed using *Borrelia* genus-specific 16S rRNA primers or *fla*B primers, DTCS Quick Start reagents (Beckman-Coulter, Fullerton, CA), and a CEQ 8000 Genetic Analysis System (Beckman-Coulter). Sequence alignment and comparisons were completed using the ClustalW algorithm of MegAlign (DNASTAR, Madison, WI).

To obtain the complete coding sequence for *B. lonestari fla*B, GenBank flagellin sequences from *Borrelia miyamotoi* (D4377), *Borrelia hermsii* (M86838), and *B. lonestari* (U26705) were aligned, and PCR and sequencing primers were designed in regions of homologous DNA. PCR amplification, DNA sequencing, and sequence alignment were performed, as described above.

Once sequence analysis was completed for the combined pool, PCR analysis of the 12 original tick pools was performed, as described above, using: 1) the Barbour *Borrelia* genus-specific *fla*B primers (Barbour et al. 1996) in a two-tube nested protocol (FlaLL/FlaRL first tube; FlaLS/FlaRS second tube); 2) *Borrelia* genus-specific 16S rRNA primers (5′ primer at position 346–366 and 3′ primer at position 964–941 of GenBank U23211); and 3) *B. burgdorferi* outer surface protein A (*osp*A) primers as a control for false positives.

Estimating Tick Infection Prevalence. A tick pool was said to contain *B. lonestari* DNA if PCR evidence of *fla*B, or 16S rRNA, or both was found using methods described above. An estimate of the proportion (with 95% confidence intervals) of all *A. americanum* ticks containing *B. lonestari* DNA was calculated based on the assumption that only a single tick in each pool needed to contain *B. lonestari* for the pool to test PCR positive.

Results and Discussion

This is the first description of the presence of *B. lonestari* in Lone Star ticks from Missouri confirmed by 16S rRNA and *fla*B gene sequence analyses and the only report to extend the *B. lonestari fla*B DNA sequence that was first described in 1996. One 16S rRNA (≈1,330 bp) and two *fla*B (≈640 and ≈1,600 bp) PCR products were obtained from the combined pool and then sequenced. Our derived 16S rRNA DNA sequence (GenBank AY166715) was aligned with published *B. lonestari* 16S rRNA gene sequence obtained from GenBank U23211. The two shared 99.7% identity over 1,336 nucleotides. The 641-bp *fla*B product shared 99.7% identity with published *B. lonestari fla*B partial coding sequence (GenBank U26705 and U26704), and 100% identity at the amino acid level. We were successful in amplifying and sequencing a 1611-nucleotide *fla*B product that contains the complete coding sequence of *B. lonestari fla*B and the 5′ and 3′ flanking sequence (GenBank AY166716). This extends the published sequence of *B. lonestari fla*B by 970 nucleotides.

Ten of twelve pools were positive for both *fla*B and 16S rRNA, two were positive for either *fla*B or 16S rRNA, and all were *osp*A negative. These data provide evidence that all 12 tick pools contained *B. lonestari* DNA, resulting in an estimate of infection prevalence of 5.6% (95% CIs 2.5%, 8.7%). We cannot eliminate the possibility that DNA from other coinfecting *Borrelia* may have been present in minute amounts because

sequence analysis was only completed for the combined pool.

The PCR positivity of all tick pools analyzed in this study does suggest that humans residing in southeast Missouri who are bitten by *A. americanum* are at risk for exposure to *B. lonestari.* Still, more evidence is needed to prove that exposure to *B. lonestari:* 1) is the cause of EM rash associated with antecedent Lone Star tick bite, or 2) poses a serious public health threat for humans.

Many human cases of EM-like rash illness reported from Missouri each year are perhaps caused by infection with *B. lonestari* following a Lone Star tick bite. Lacking the ability to cultivate this microbe, it is important that we establish its presence in biting vectors and expand our knowledge of its genetic profile to improve our ability to investigate its role as the putative causative agent of this illness. To date, we have not been successful in detecting *B. lonestari* DNA in four skin biopsy samples obtained in 2001 from patients in Missouri with EM rash illness at the site of a Lone Star tick bite. However, we believe these efforts have been thwarted by a lack of positive control DNA template for PCR optimization. With this situation now remedied, our efforts to develop more sensitive and specific genetic tools for detecting *B. lonestari* in human skin will continue in an effort to fully understand the public health impact of *B. lonestari* exposure following *A. americanum* tick bite.

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