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Short communication

Detection of a *Borrelia* species in questing Gulf Coast ticks, *Amblyomma maculatum*

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ABSTRACT

Borrelia spp. are agents of Lyme disease and relapsing fever, diseases which use *Ixodes* hard ticks and *Ornithodoros* soft ticks, respectively, as primary vectors. Some relapsing fever spirochetes, such as *B. miyamotoi*, are also found in hard ticks. To date, no *Borrelia* sp. is known to use the hard tick, *Amblyomma maculatum*, as a vector. However, both *B. burgdorferi* and *B. lonestari* were recently detected in *A. maculatum* removed from hosts. In our study, DNA extracts from 306 questing adult *A. maculatum* collected in Mississippi in 2009 and 2010 were tested for *Borrelia* spp. DNA by PCR amplification of *flaB* and 16S rRNA gene targets. An additional 97 *A. maculatum* collected in 2013 were tested by amplification of 16S rRNA gene target. Two ticks, one collected in 2009 and the other in 2010, were positive by PCR of the *flaB* and 16S rRNA gene targets; both were collected from the same location in central Mississippi. Interestingly, 16S rRNA gene amplicons from these two tick extracts were 98% identical to twelve *Borrelia* spp. including the reptile-associated spirochete *B. turcica* and *Borrelia* sp. "tAG158M"; *flaB* amplicons from these two ticks shared closest identity (89%) to the reptile-associated spirochete, *B. turcica*. These results demonstrate a *Borrelia* sp. in unfed *A. maculatum* ticks that is unique from other species in the NCBI database and in a clade with reptile-associated *Borrelia* species. Detection of a previously unrecognized *Borrelia* in a hard tick species generates additional questions regarding the bacterial fauna in these arthropods and warrants further studies to better understand this fauna.

Introduction

Lyme disease is the most commonly reported vector-borne disease and perhaps the most socio-politically charged infectious disease in the United States, where the etiologic agent, *Borrelia burgdorferi* sensu stricto, is transmitted by the hard tick, *Ixodes scapularis* Say (1821) (Burgdorfer et al., 1982). Relapsing fever, the other endemic borrelial disease in the United States, is most commonly caused by *B. hermsii* (Schwan and Piesman, 2002) and transmitted by soft ticks in the genus *Ornithodoros*. *Borrelia* species within the relapsing fever group have also been identified in hard ticks. For example, *B. miyamotoi*, first discovered in *I. persulcatus* ticks from Japan (Fukunaga et al., 1995), has been found in *I. ricinus* in Europe (Fraenkel et al., 2002; Richter et al., 2003), *I. scapularis* in the eastern United States (Scoles et al., 2001; Ullmann et al., 2005), and *I. pacificus* in the western U.S. (Mun et al., 2006). Interestingly, *B. miyamotoi* was recently associated with human disease in both the U.S. and Russia (Gugliotta et al., 2013; Krause et al., 2013; Platonov et al., 2011). *Borrelia lonestari*, another hard-tick relapsing fever group spirochete, was first detected in *Amblyomma americanum*, ticks common in the southeastern and south-central U.S. (Barbour et al., 1996). While this spirochete was associated with one case of human disease, "southern tick-associated rash illness," over a decade ago (James et al., 2001), additional evidence implicating *B. lonestari* as a human disease agent is lacking (Feder et al., 2011). The Gulf Coast tick, *Amblyomma maculatum* Koch (1844) is a hard tick found primarily along the Gulf and Atlantic Coasts of the U.S., with a host range that includes rodents and ground-dwelling birds for immature stages, and large mammals, such as cattle, for adult stages (Teel et al., 2010). While *A. maculatum* is

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a known vector for the human spotted fever rickettsial pathogen, *Rickettsia parkeri* (Paddock et al., 2004), and the canine protozoal pathogen *Hepatozoon americanum* (Ewing and Panciera, 2003), *A. maculatum* is not known to harbor or transmit a *Borrelia* species. However, a recent study of *A. maculatum* in Arkansas reported the presence of a *Borrelia flaB* gene amplicon (*B. lonestari* and *B. burgdorferi*) in approximately 28% of *A. maculatum* collected from canines and white-tailed deer (Fryxell et al., 2012). To our knowledge, the presence of a *Borrelia* sp. in unfed *A. maculatum* has not been shown.

The objective of this study was to evaluate questing (unfed) adult *A. maculatum* for the presence of *Borrelia* species. Based on previous evidence of *B. burgdorferi* and *B. lonestari* in *A. maculatum* removed from hosts, the possibility of acquiring these spirochetes during the blood meal and the lack of evidence for *A. maculatum* as a vector for either *Borrelia* sp., we hypothesized that *B. burgdorferi* and *B. lonestari* would not be present in questing *A. maculatum*. We tested *A. maculatum* collected in Mississippi (MS) in 2009 and 2010 as part of another study (Ferrari et al., 2012), and an additional 97 samples collected in 2013, using PCR assays and amplicon sequencing.

Materials and methods

Amblyomma maculatum collection

A subset of 306 (177 in 2009 and 129 in 2010) questing adult *A. maculatum* that were collected as part of a previous study was used in the present study (Ferrari et al., 2012). An additional 97 questing *A. maculatum* that was collected in May and June of 2013 from 2 sites in northern MS and one site in central MS were also tested. The three 2013 sites were among those included in the previous study (Ferrari et al., 2012).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using an Illustra Tissue and Cells GenomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Piscataway, NJ, USA) from the 306 whole-individual *A. maculatum* collected previously and from the 97 half tick bodies collected in 2013. *Amblyomma maculatum* collected in 2013 were first cleaned with 20% sodium hypochlorite (NaClO), 0.5% benzalkonium chloride, and 70% ethyl alcohol, rinsed in sterile PBS and then bisected sagittally with the half tick used for DNA extraction and the other half used as part of another study.

DNA extracts from 2009 and 2010 were used in 2 nested PCR assays to amplify approximately 330-base-pair (bp) and 650-bp regions of the *flaB* and 16S rRNA genes, respectively, for *Borrelia* spp. based on previously published assays (Barbour et al., 1996; Richter et al., 2003). Samples from 2013 were tested by *Borrelia* spp. 16S rRNA gene PCR only (Richter et al., 2003). All ticks were also tested in a PCR amplifying a portion of the tick mitochondrial 16S rRNA gene to ensure presence of amplifiable DNA (Black and Piesman, 1994; Ferrari et al., 2012). DNA extracted from cultured *B. burgdorferi* strain B31 and *B. hermsii* strain DAH were used as positive controls; molecular grade water was used as a non-template negative control. PCR products were detected by electrophoresis in a 1.5% agarose gel and visualized with ethidium bromide. PCR amplicons were purified using a DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA), bidirectionally sequenced (Eurofins MWG Operon, Huntsville, AL, USA), aligned using ClustalX2 (Larkin et al., 2007), and compared to available sequences in the National Center for Biotechnology Information (NCBI) database. Alignments were also used in MEGA 5 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2011) to generate phylogenetic trees.

Results

Amblyomma maculatum collection and amplicon sequence

A total of 177 adult *A. maculatum* collected in 2009, comprised of 84 females (48%) and 93 males (53%), were tested by PCR assay. Of these ticks, 78 (44%), 26 (15%), and 73 (41%) were collected from northern, central, and southern Mississippi, respectively. In 2010, 129 ticks were tested of which 81 were females (63%) and 48 were males (37%). The majority ($n = 123$; 95%) of these ticks was collected from sites in northern Mississippi, while 6 (5%) were collected from central Mississippi. In 2013, 97 ticks were tested, of which 55 were females (57%) and 42 were males (43%). The majority ($n = 95$; 98%) of these ticks were collected from northern Mississippi, while 2 (2%) ticks were collected from the central Mississippi site.

Two *A. maculatum* tick extracts out of 306 (0.65%) that were collected in 2009 and 2010 generated bands of appropriate size on PCR amplification of both the *flaB* and 16SrRNA gene targets. The positive tick samples were collected from the same location in central Mississippi (Byram, MS, USA). No tick extracts collected in 2013 were positive by PCR assay of the *Borrelia* 16S rRNA gene target. 16S rRNA gene sequences amplified from the 2 positive *A. maculatum* samples were 98% identical to 12 *Borrelia* spp. including *B. turcica* (AB473539.1), *Borrelia* sp. tAG158 M (AB529425.1) and *Borrelia* sp. Tortoise 14M1 (AB473533.1). *FlaB* amplicon sequences from these same 2 tick samples were both 89% identical to *B. turcica flaB*, isolate Tortoise 7S2 and 7S1 (Accession: AB473520.1 and AB473519.1). Phylogenetic analyses using partial 16S rRNA gene sequences (Fig. 1) and *flaB* (Fig. 2) sequences from this study with selected sequences available in GenBank showed that sequences detected from the 2 ticks in this study were not closely related to Lyme disease group or relapsing fever *Borrelia* group. Rather, they were more closely related to reptile-associated borreliae. Two additional tick extracts demonstrated *flaB* amplicons on PCR that shared 100% identity to multiple strains of *B. hermsii* including DAH. A *flaB* amplicon from a third sample shared 100% identity to *B. burgdorferi* sensu stricto including strain N40 and 99% identity to *B. burgdorferi* strain B31. However, these 3 tick extracts were negative using the 16S rRNA gene target and on subsequent PCR assays to reamplify the *flaB* gene. Given that we could not amplify a 16S rRNA gene product from these samples and both *B. hermsii* DAH and *B. burgdorferi* B31 were included as PCR controls, we suspect that the *flaB* PCR results were false-positive and likely a result of PCR contamination.

GenBank accession numbers for *Borrelia* sp. partial 16S rRNA gene sequences are KF395228 and KF395229. Accession numbers for *Borrelia* sp. partial *flaB* gene sequences are KF395230 and KF395231.

Discussion

Of *A. maculatum* extracts tested in this study, 2 out of 403 (0.5%) of the extracts were positive by PCR for *Borrelia* species. Both ticks were collected from the same central Mississippi site (Byram, MS, USA) during 2009 and 2010. Sequences of the 16S rRNA gene amplicons from the 2 samples were 100% identical to each other; *flaB* amplicon sequences for the 2 samples were also identical to each other. The identification of a *Borrelia* sp. in unfed *A. maculatum* is intriguing considering *Borrelia* spp. have been detected by PCR from only fed *A. maculatum*, and were limited to *B. lonestari* and *B. burgdorferi* (Fryxell et al., 2012). The absence of *B. burgdorferi* and *B. lonestari* amplicons in over 400 questing adult *A. maculatum* tested here suggests that the spirochetes detected previously in *A. maculatum* attached to canids and deer may have been acquired from the blood meal (Fryxell et al., 2012). Alternatively, the geographic areas sampled here differed from the previous report and there