Geographical and genospecies distribution of *Borrelia burgdorferi sensu lato* DNA detected in humans in the USA

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The present study investigated the cause of illness in human patients primarily in the southern USA with suspected Lyme disease based on erythema migrans-like skin lesions and/or symptoms consistent with early localized or late disseminated Lyme borreliosis. The study also included some patients from other states throughout the USA. Several PCR assays specific for either members of the genus *Borrelia* or only for Lyme group *Borrelia* spp. (*Borrelia burgdorferi sensu lato*), and DNA sequence analysis, were used to identify *Borrelia* spp. DNA in blood and skin biopsy samples from human patients. *B. burgdorferi sensu lato* DNA was found in both blood and skin biopsy samples from patients residing in the southern states and elsewhere in the USA, but no evidence of DNA from other *Borrelia* spp. was detected. Based on phylogenetic analysis of partial flagellin (*flaB*) gene sequences, strains that clustered separately with *B. burgdorferi sensu stricto*, *Borrelia americana* or *Borrelia andersonii* were associated with Lyme disease-like signs and symptoms in patients from the southern states, as well as from some other areas of the country. Strains most similar to *B. burgdorferi sensu stricto* and *B. americana* were found most commonly and appeared to be widely distributed among patients residing throughout the USA. The study findings suggest that human cases of Lyme disease in the southern USA may be more common than previously recognized and may also be caused by more than one species of *B. burgdorferi sensu lato*. This study provides further evidence that *B. burgdorferi sensu stricto* is not the only species associated with signs and/or symptoms consistent with Lyme borreliosis in the USA.

INTRODUCTION

Lyme borreliosis (LB), the most frequently reported arthropod-borne infection in the USA (Bacon et al., 2008), is caused by several species within the *Borrelia burgdorferi sensu lato* complex worldwide. *B. burgdorferi sensu lato* includes over 20 genospecies, at least seven of which are present in North America: *Borrelia americana, B. andersonii, B. bissettii, B. burgdorferi sensu stricto* (hereafter referred to as just *B. burgdorferi*), *B. californiensis, B. carolinensis* and *B. kurtenbachii* (Johnson et al., 1984; Marconi et al., 1995; Margos et al., 2009; Margos et al., 2010; Postic et al., 1998; Postic et al., 2007; Rudenko et al., 2009a; Rudenko et al., 2009b). *B. burgdorferi* has been widely reported as the only genospecies responsible for human disease in the USA; however, strains similar to *B. bissettii* have been identified in human cases in California (Girard et al., 2011), and both *B. americana* and *B. andersonii* strains were found in human cases with Lyme-like illness in Florida and Georgia (Clark et al., 2013) using molecular methods.

In the southern USA, a Lyme disease-like clinical presentation referred to as southern tick-associated rash illness (STARI) has been recognized in association with bites from the lone star tick, *Amblyomma americanum* (Masters et al., 2008). Despite the association of STARI with signs and symptoms very similar to Lyme disease, this mostly southern phenomenon (Feder et al., 2011) has been regarded as a clinical entity separate from Lyme disease, based on interpretations of evidence obtained from STARI patients in previous studies (Kirkland et al., 1997; Wormser et al.,...
METHODS

Human patient samples. During the period from September 2009 to September 2010, the University of North Florida Environmental Epidemiology Laboratory (UNF Laboratory) received for microbiological testing human blood or other body fluids, and skin biopsy samples, from patients suspected of having Lyme disease (UNF IRB approval #06-140) as part of ongoing studies of tick-borne diseases in the southern USA. Clinicians who submitted the samples suspected possible early localized or late disseminated Lyme disease as the explanation for the patients' clinical presentations and histories. Besides samples submitted by individual clinicians, to obtain a larger number of samples the UNF Laboratory partnered with the former Central Florida Research, Inc. (Winter Haven, FL, USA) to obtain aliquots of blood samples from patients submitted for *B. burgdorferi* tests offered by that laboratory. These samples included ones from patients throughout the USA. Another set of patient blood and skin biopsy samples of erythema migrans (EM) lesions was collected from patients in Missouri by the late Dr Edwin J. Masters, and provided to the UNF Laboratory in 2009 shortly after his death. Only limited demographic (state of residence), epidemiological and clinical data were available for most of the patients included in the present study. Many of the samples used in this study were stored refrigerated for up to several months prior to DNA extraction and PCR testing, and some were stored frozen for several years before testing.

As part of the study, we also tested blood samples from 12 control patient samples provided previously by an internal medicine cardiology specialty practice in central Florida. These control patients had diagnoses of congenital heart defects, coronary artery disease or heart valve disorders, and did not have signs or symptoms consistent with borreliosis. An additional 18 control patient samples were provided by several private practice primary care physicians in Florida and Georgia; these controls were from patients presenting for normal well-visit physical checkups, and had no history of signs or symptoms suggestive of Lyme disease or other acute or chronic health conditions. The age range of the control patients was from 21 to 74 years. The control samples were used for multiple negative-control DNA extractions utilized in PCR testing during the present study.

DNA extraction. For all samples, DNA extractions were carried out inside a class II biosafety cabinet and utilized a commercially available kit (MasterPure; Epicentre). DNA was extracted from 2 mm skin punch biopsies, 200–300 μl EDTA anticoagulated whole blood or 100–200 μl plasma, as described previously (Clark, 2004; Clark et al., 2005, 2013). Final DNA pellets from all samples were rehydrated with 100 μl TE buffer. Negative-control samples free of any template were included in each round of DNA extractions to monitor potential contamination.

PCR testing. The DNA samples extracted from the first 100 patient samples and the 30 control patient samples were screened by nested PCR using published primers (FlaL+FlaRL and FlaLS+FlaRS) designed to amplify a 350 bp portion of the conserved 41 kDa chromosomal flaB gene from *Borrelia* spp. (Barbour et al., 1996). All of these samples were also screened with nested PCR assays targeting portions of the rrf (3S–rrf) (23S) intergenic spacer (IGS) region, outer surface protein A (ospA) gene and chromosomal 66 kDa protein (p66) of *B. burgdorferi sensu lato* according to published protocols (Guy & Stanek, 1991; Rosa et al., 1991; Rijpkema et al., 1995; Clark, 2004). Additionally, all patient and control samples were screened with primers targeting a portion of the flaB gene of *B. burgdorferi sensu lato* group species: outer reaction primers 280F (5′-GCCATTCTGA-GTTAAACGG-3′, nt 280–299) and 754R (5′-TAGCAAGTGATGTA-ATCGCATCAAC-3′, nt 730–754), which amplify a 475 bp product; and inner reaction primers 301F (5′-ACATATTGCATGACAG-AGAGG-3′, nt 301–323) and 737R (5′-GATCAACCTGTGTTGGA-ACATTTACAGG-3′, nt 709–737), which amplify a 437 bp product. First-round amplifications contained 2.5 μl DNA extract per individual sample in a total reaction volume of 25 μl. All reactions utilized GoTaqGreen PCR Master Mix (Promega) resulting in a final concentration of 1.25 U *Taq* DNA polymerase, 1.5 mM MgCl₂, 200 μM each dNTP and 0.8 μM each primer, and were carried out in an automated DNA thermal cycler (PTC 200; MJ Research). Each primary PCR consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, primer annealing at 42 °C for 30 s for the ospA and p66 tests and at 52 °C for 30 s for the 5S–23S and flaB tests, and extension at 72 °C for 45 s. Each inner/nested reaction used 1 μl outer reaction product as template for 35 additional cycles, and an annealing temperature of 42 °C for the p66 primers, 50 °C for ospA and 55 °C for all others.

The causative agent of STARI is still unknown. In the north-eastern USA, *B. burgdorferi* spirochaetes are believed to be transmitted to humans only by the black-legged tick, *Ixodes scapularis* (Halperin et al., 2013). In the southern USA, where the habitat, climate and other ecological factors are different, *I. scapularis* does bite humans but not as often (Felz et al., 1996; Merten & Durden, 2000). In the south, larvae, nymphs and adult stages of the much more aggressive lone star tick, as well as adult American dog ticks (Dermacentor variabilis), more commonly bite people (Merten & Durden, 2000; Williamson et al., 2010). Several studies have demonstrated *B. burgdorferi* in *A. americanum* or *D. variabilis* by culture isolation (Teltow et al., 1991; Walker et al., 1994), specific antibody testing (Schulze et al., 1984; Levine et al., 1985; Teltow et al., 1991; Feir et al., 1994; Sonenshine et al., 1995) or PCR using *B. burgdorferi sensu lato*-specific PCR primers (Rosa et al., 1991; Teltow et al., 1991; Feir et al., 1994; Stromdahl et al., 2001; Clark, 2004; Clark et al., 2013). Yet, the ability of these tick species to acquire, maintain and transmit *B. burgdorferi* (Mukolwe et al., 1992; Piesman & Happ, 1997) remains to be demonstrated conclusively. Therefore, questions remain regarding the cause of STARI, the occurrence of Lyme disease and the tick vector species responsible for Lyme disease transmission to humans in the southern USA.

We previously described 10 cases of LB in human patients residing or with tick exposure in Florida and Georgia, USA (Clark et al., 2013). These cases were identified using highly sensitive nested PCR assays targeting the flagellin (*flaB*) gene of *B. burgdorferi sensu lato*. A few of the cases were associated directly with lone star tick bites, and DNA sequence evidence confirmed *B. burgdorferi* in the patients and the ticks removed from them. The study’s findings suggested that some LB cases in Florida and Georgia were associated with *B. burgdorferi*, *B. americana* and *B. andersonii* strains. In the present study, we investigated the presence of *Borrelia* spp. in a large number of human patients with suspected Lyme disease who resided in multiple states across the southern USA, as well as in states in other regions of the country. We used several PCR assays specific either for members of the genus *Borrelia* or only for Lyme group *Borrelia* spp. to test samples from human patients, and confirmed the positive PCR results with DNA sequence analysis.
PCRs were set up in an area separate from DNA extractions, and within a PCR clean cabinet (CleanSpot Workstation; Coy Laboratory Products) equipped with a germicidal UV lamp. Other precautions to prevent carryover contamination of amplified DNA included different sets of pipettes dedicated for DNA extraction, PCR setup and post-amplification activities, respectively, the use of aerosol barrier filter pipette tips, and exposing PCR tubes, pipettes and tips to UV light prior to PCR setup. In addition, pipettes used for handling DNA samples were soaked in 10% bleach solution after setup of each PCR. Each outer PCR included a negative-control sample with nuclease-free TE buffer instead of template, and a positive-control sample from B. burgdorferi sensu stricto strain B31 culture extract. Negative-control samples included in each round of DNA extractions were also screened to detect any possible contamination during the extraction process. PCR products were separated by electrophoresis in 2% agarose gels, which were stained with ethidium bromide and visualized and recorded with a digital gel documentation unit.

**DNA sequence analysis.** PCR products from positive samples were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA templates were sequenced using both the forward and reverse primers used in the nested PCRs. Investigator-derived sequences were compared with those obtained by searching GenBank using BLAST (Altschul et al., 1990) and aligned using CLUSTAL_X (Thompson et al., 1997). Nucleotide sequences were confirmed by comparing both the forward and reverse primer sequence data. A phylogenetic tree was reconstructed using the maximum-likelihood method based on the model of Tamura & Nei (1993) with the tree-building program MEGA5 (Tamura et al., 2011). The analysis involved a total of 36 representative sequences obtained from human patients and 11 B. burgdorferi sensu lato reference strains. To estimate node reliability, bootstrap values (Felsenstein, 1985) based on analysis of 1000 replicates were determined. All positions with less than 95% site coverage were eliminated; thus, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

**Nucleotide sequence accession numbers.** The reference strain B. burgdorferi sensu lato flaB sequences in GenBank included in the analyses are AB035613, AF264883, AF264886, CP002312, D82857, D83762, D83763, EU076498, EU081293, FJ188426, X15661, X75200 and X75203.

**RESULTS**

In the present study, we screened blood samples from a total of 277 human subjects residing in the USA and suspected of having Lyme disease using the PCR assays and methods described above (Table 1). Blood or skin samples from the first 100 patients, and whole-blood extracts from all 30 controls, were screened with all five PCR assays described in Methods. No evidence of positive PCR results was indicated from any of the negative-control patient DNA extracts or the TE buffer negative controls included in the PCR testing. Of the first 100 experimental patient samples tested, only 3% were positive with the genus Borelia flaB or B. burgdorferi sensu lato 5S–23S IGS primers, 7% were positive with the ospA primers and 5% were positive with the p66 primers (Table 1). However, 45% of the first 100 were positive with the B. burgdorferi sensu lato-specific flaB primers, including all of the samples that tested positive with the other primer sets. Clearly, the B. burgdorferi sensu lato flaB primers were the most sensitive in detecting Lyme Borelia DNA in the samples tested. Therefore, only the B. burgdorferi sensu lato-specific flaB primers were used thereafter to test additional samples. Overall, 118 (42.6%) of 277 blood or skin samples from experimental study patients tested positive with the B. burgdorferi sensu lato-specific flaB primers, and all positive results were confirmed by DNA sequencing as belonging to B. burgdorferi sensu lato, except for eight (6.8%) samples that were not sequenced (Table 1).

DNA sequences derived from all PCR-positive samples were compared with reference strain sequences in GenBank via BLAST. Partial flaB DNA sequences of approximately 430 nt (nt 312–742) derived from representative samples were then aligned and compared phylogenetically via the maximum-likelihood method (Fig. 1). Based on the flaB gene comparisons, human patient samples clustered with three different B. burgdorferi sensu lato genospecies: B. burgdorferi, B. americana, and B. andersonii (Fig. 1). Over the sequence fragment analysed, the human subject B. burgdorferi-like strains differed from reference strain B31 by 0–3 nt; sequences from only four patients were identical to B31. For comparison, B. americana SCW-30h differs from B31 by 7 nt (BLAST result 423/430=98.4%). The B. americana-like human strains differed from B. americana SCW-33 and SCW-30h by 1–4 nt, differed from B. burgdorferi strains by a minimum of 8–11 nt and clustered separately in the phylogenetic analysis (Fig. 1). The human subject B. andersonii-like sequences differed from reference strains of that species in GenBank by 0–3 nt. Several of the

### Table 1. PCR primer sensitivity for detecting B. burgdorferi sensu lato in human blood samples in the present study

<table>
<thead>
<tr>
<th>PCR assay target*</th>
<th>No. tested</th>
<th>No. (%) positive</th>
<th>Species (no. strains) identified by DNA sequence of specified target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borrelia spp. flaB</td>
<td>100</td>
<td>3 (3)</td>
<td>B. burgdorferi (3)</td>
</tr>
<tr>
<td>B. burgdorferi sensu lato flaB</td>
<td>277</td>
<td>118 (42.6)</td>
<td>B. americana (30), B. andersonii (4), B. burgdorferi (76), not sequenced (8)</td>
</tr>
<tr>
<td>B. burgdorferi sensu lato 5S–23S</td>
<td>100</td>
<td>3 (3)</td>
<td>B. burgdorferi (3)</td>
</tr>
<tr>
<td>B. burgdorferi sensu lato ospA</td>
<td>100</td>
<td>7 (7)</td>
<td>B. burgdorferi (6), B. americana (1)</td>
</tr>
<tr>
<td>B. burgdorferi sensu lato p66</td>
<td>100</td>
<td>5 (5)</td>
<td>B. burgdorferi (5)</td>
</tr>
</tbody>
</table>

*Primer sets and protocols are described or referenced in Methods.
different B. burgdorferi sensu lato sequences obtained from human subjects in this study contained unique nucleotide differences confirmed by bidirectional sequencing, compared with reference strains included in the comparison (Table 2).

The flaB sequences from 76 (64.4 %) positive patients showed between 99.3 and 100 % nucleotide similarity to B. burgdorferi B31, 30 (25.4 %) positive patients showed between 99.1 and 99.8 % nucleotide similarity to B. americana strains SCW-33 and SCW-30h, and 4 (3.4 %) positive patients showed \( \geq 99.8 \) % nucleotide similarity to B. andersonii strains (Table 3). No evidence of other Borrelia spp. (e.g. Borrelia lonestari) was obtained from any of the samples. One subject from Nebraska (NEHS-1) tested positive for both B. burgdorferi-like (based on the ospA gene) and B. americana-like (flaB gene) strains.

B. burgdorferi-like strains from patients were widely distributed geographically (Table 3, Fig. 2). Likewise, the distribution of B. americana-like strains recovered from patients in the USA included patients residing in north-eastern (MD, NJ and PA), south-eastern (FL, MO and VA), south-western (AZ, OK and NM), and north-western (OR and WA) states. Fifty-three (40 %) of 131 samples overall from Florida subjects were positive. Of the flaB PCR-positive samples from Florida patients subjected to sequencing, 31 (59%) were most like B. burgdorferi, 15 (28 %) were most similar to B. americana strains and 6 (11 %) clustered with B. andersonii. From all patients residing in southern states combined (AL, FL, GA, KY, MO, NC, OK, SC, TX and VA), 91 (42 %) of 215 tested positive. Of these flaB-positive samples, 63 (69 %) were B. burgdorferi-like, 20 (22 %) were B. americana-like and 3 (3 %) were B. andersonii-like strains (Table 3, Fig. 2).

**DISCUSSION**

It is widely accepted that multiple B. burgdorferi sensu lato spp. (at least B. afzelii, B. garinii and B. burgdorferi) cause Lyme disease in Europe (Biesiada et al., 2012). However, in the USA, despite the presence of several other genospecies (B. americana, B. andersonii, B. californiaensis, B. carolinensis, B. bissettii and B. kurtenbachi), only B. burgdorferi is recognized as causing Lyme disease (Murray & Shapiro, 2010). Certain B. burgdorferi sensu lato spp. may exist in ecological niches involving reservoir hosts and arthropod vectors that humans rarely encounter; thus, humans may rarely, if ever, become infected by these strains due to lack of incidental contact with their transmission cycles, rather than because the strains lack pathogenicity. However, recent evidence challenges the notion that only B. burgdorferi infects humans in the USA. A report by Girard et al. (2011) showed molecular evidence of B. bissettii-like strains in human patients in California. Clark et al. (2013) presented the first DNA evidence of B. americana and B. andersonii strains in several human patients with Lyme disease-like illness who lived, or had tick bite exposure, in Florida or Georgia. The present study adds additional molecular evidence suggesting that B. burgdorferi is not the only Lyme Borrelia sp. associated with human illness in the USA.

Only flaB gene fragments could be amplified from the majority of patients in the present study. This agrees with the results of a previous study of patients with EM lesions in Georgia and South Carolina, where a nested flaB PCR assay was positive for 22 % of patients (Felz et al., 1999). Unfortunately, that study did not provide DNA sequence data for the amplified flaB fragments, so genospecies determination was not presented. In the current study, additional gene targets besides flaB, comprising ospA, 5S–23S IGS and p66, could be amplified only from a few patients apparently infected with B. burgdorferi-like strains. We believe that the primary reason for our variable PCR results is a very low concentration of target organism DNA in human blood samples. Many of the LB patients in our study were past the early stage of infection, and were being tested months or years after the initial onset of symptoms. It is possible that the spirochaete DNA being detected in our tests was from dead spirochaetes being eliminated by the immune system, and the DNAs could have been highly fragmented. Previous studies have shown that the flaB gene is a highly sensitive gene target for PCR detection of Lyme borreliae (Johnson et al., 1992; He et al., 1994; Schmidt et al., 1995; Wodecka et al., 2009; Wodecka et al., 2010; Wodecka, 2011). It has also proven to be the most sensitive target in previous studies conducted by the UNF Laboratory to detect B. burgdorferi sensu lato in small mammals, ticks, lizards and human patients (Clark, 2004; Clark et al., 2005, 2013).

Notably, the genus Borrelia flaB PCR primers (Barbour et al., 1996) employed in this study amplified B. burgdorferi DNA from only 3 of 100 human subject samples. All three also tested positive with the B. burgdorferi sensu lato-specific flaB PCR primers, and all represented B. burgdorferi-like strains. This agrees with findings from a previous study (Clark, 2004) where the same genus Borrelia primers failed to amplify B. burgdorferi sensu lato strains that were identified in small mammals and ticks in Florida using a different set of Lyme Borrelia-specific flaB primers; although the genus Borrelia primers did amplify B. lonestari DNA from lone star ticks. None of the human samples in the present study that contained B. andersonii-like or B. americana-like strains tested positive with the genus Borrelia primers. These primers may simply lack the sensitivity necessary to identify low concentrations of B. burgdorferi sensu lato spirochaetes in the patients’ blood or tissue biopsy samples. This could be due at least partially to some nucleotide mismatches that these primers have with most B. burgdorferi sensu lato strains, based on BLAST comparisons (data not shown).

We found no evidence of false-positive PCR results from DNA contamination/artefacts during this study; none of the negative-control patient extraction samples or PCR

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Ixodes minor wild vertebrates from Florida and South Carolina (Clark, testing agrees with results of molecular studies of ticks and Furthermore, the pattern of PCR results from our human negative results with PCR assays for other gene targets. regions spanning the USA. The tree with the highest log-likelihood (~1070.0674) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with the superior log-likelihood value. Codon positions included were first+second+third+non-coding. All positions with less than 95% site coverage were eliminated; fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 413 positions in the final dataset. Blue circles denote B. burgdorferi-like sequences, red circles denote B. americana-like sequences and green circles denote B. andersonii-like sequences obtained from study patients. GenBank accession numbers are indicated after the strain name. B. b.s.s., B. burgdorferi sensu lato.

In the present study, the majority (64%) of the DNA sequences derived from patients was most similar (greater than 99%) to B. burgdorferi reference strains. Twenty-five per cent of patient sequences showed greater than 99% nucleotide similarity to reference strains of B. americana, and only 3% clustered with B. andersonii strains. In the portion of the flaB gene that we analysed, compared with other B. burgdorferi sensu lato ssp., B. americana reference strains and B. americana-like strains from patients in this study were most similar to B. burgdorferi strains. However, the reference strains and representative strains from patients from these two groups clustered into apparently separate genospecies in our phylogenetic analysis. Our findings therefore suggest that B. americana-like strains may be responsible for a portion of human cases of Lyme-like illness in the southern USA and elsewhere, as we identified strains of this group in patients from multiple regions spanning the USA. B. americana was originally described from Ixodes minor removed from a Carolina wren in South Carolina (Rudenko et al., 2009b). Therefore, it may be associated primarily with birds, which could explain why it has not been identified previously in some areas of the USA, as extensive studies of B. burgdorferi sensu lato in birds and ticks associated with birds have not been conducted in many areas.

Unfortunately, the lack of epidemiological information for patients in this study limits our ability to draw conclusions regarding the location of infectious exposure for all except a few endemic cases in Florida and Georgia, whose case histories have been described in detail elsewhere (Clark et al., 2013). Of the 10 cases reviewed by that study, seven had no travel history outside their state of residence and could not be explained by travel to other endemic areas. Several of the patients had a well-documented tick bite history in Florida or Georgia immediately prior to the onset of signs or symptoms. However, it is possible that a portion of the other B. burgdorferi PCR-positive patients in the present study acquired their infections while visiting or living in states other than those recorded as their present residence. Therefore, the true geographical distribution of the Lyme group Borrelia strains identified in this study is uncertain. Future studies of LB in human patients, especially cases identified in areas where Lyme disease is considered rare or non-endemic, need to obtain more specific person, place and time factor data from study subjects.

Among the patients in the present study, complete data on clinical laboratory testing for Lyme disease were available for only 10 patients with PCR- and DNA sequence-confirmed LB described in a previous report (Clark et al., 2013). Among these 10, six were tested by Lyme enzyme immunoassay: three had negative results, two had equivocal results and one patient tested positive by enzyme immunoassay. Of these same 10 patients, six also had Lyme Western blot tests performed. Of these, all six demonstrated one or more IgM or IgG antibody bands that were at least suggestive of possible borreliosis infection (Clark et al., 2013). Additionally, 45 (43.3%) of 104 patients in the present study tested positive for B. burgdorferi antigen using flow cytometry in a test conducted at the Central Florida Research Laboratory (C. Thralkeld, unpublished data). The proportion of patients who tested positive by this test was remarkably similar to the proportion positive by flaB PCR (42.6%) in the present study.

The ability to detect Lyme group Borrelia DNA in many patients in the present study, including those who live in areas where Lyme disease is considered rare or even
### Table 2. Nucleotide base comparisons among representative *flaB* gene sequences derived from human subjects and reference strains of *B. burgdorferi sensu lato*

Nucleotide position numbering based on *B. burgdorferi* B31 sequence. Bold letters represent unique nucleotide differences for a particular strain compared with others of the same genospecies included in the comparison. *B.b.s.s.*, *B. burgdorferi sensu stricto*; ND, not determined.

| Strain | Genospecies | Nt 451 | Nt 462 | Nt 470 | Nt 480 | Nt 497 | Nt 507 | Nt 514 | Nt 524 | Nt 573 | Nt 582 | Nt 594 | Nt 597 | Nt 630 | Nt 635 | Nt 639 | Nt 677 | Nt 691 | Nt 693 | Nt 697 | Nt 700 | Nt 738 |
|--------|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| B31    | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | T      |
| CAHS-1 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | G      | A      | G      | C      |
| CAHS-2 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | G      | A      | G      | C      |
| FLHS-9 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| FLHS-20| *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| ILHS-2 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| MEHS-1 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| OKHS-1 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| OKHS-9 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| NYHS-1 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| TXHS-2 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| TXHS-3 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| UTHS-1 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| VAHS-1 | *B.b.s.s.*   | C      | G      | C      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | C      | C      | A      | G      | G      | C      |
| VAHS-2 | *B.b.s.s.*   | C      | G      | A      | A      | G      | G      | A      | T      | C      | A      | T      | C      | T      | G      | A      | G      | T      | C      | A      | G      | G      | C      |
| SCW-30h*B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | T | |
| FLHS-7 | *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| FLHS-15| *B. americana* | C | A | A | T | G | T | A | C | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| FLHS-16| *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | C | T | A | A | C | C | G | G | G | C | |
| FLHS-17| *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | T | G | G | C | C | |
| FLHS-21| *B. americana* | C | A | A | T | G | T | G | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| FLHS-34| *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | A | A | C | C | G | G | G | C | |
| NEHS-1 | *B. americana* | T | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| NJHS-1 | *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| NMHS-1 | *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| OKHS-8 | *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| PAHS-2 | *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | C | |
| WAHS-2 | *B. americana* | C | A | A | T | G | T | A | T | C | G | T | C | T | T | T | A | A | C | C | G | G | G | ND |
| 19857 *B. andersonii* | C | A | A | A | G | T | A | T | C | A | T | T | T | T | A | G | C | C | A | G | G | T | |
| FLHS-65 | *B. andersonii* | C | A | A | A | G | T | A | T | C | A | T | T | T | T | A | G | C | C | A | G | A | C | |
| FLHS-120 | *B. andersonii* | C | A | A | A | G | T | A | T | T | A | A | T | C | T | G | G | C | C | A | G | A | C | |
| OHHS-2 | *B. andersonii* | C | A | A | A | A | G | T | A | T | C | A | A | T | T | T | A | G | C | C | A | G | A | C | |

*B. burgdorferi sensu stricto* B31 sequence from GenBank accession no. X15661; *B. americana* SCW-30h sequence from GenBank accession no. AF264886; *B. andersonii* 19857 sequence from GenBank accession no. D83762.
non-existent, highlights the importance of physician education and awareness. Regardless of where a patient was infected, one can present for clinical evaluation in other areas. Therefore, physicians and other healthcare providers throughout the USA need to be trained in the recognition of the clinical presentation of LB, and the limitations of currently recommended clinical diagnostic tests (i.e. two-tier antibody testing; Wormser et al., 2008).

The current

\textit{B. burgdorferi ELISA and Western blot antibody tests and test result interpretation criteria used in the USA were developed to identify \textit{B. burgdorferi} specifically, and most of the available tests are based on identifying a single strain (B31) isolated from blacklegged ticks from Shelter Island, New York (Johnson et al., 1984). It is not known if these tests would show positive results for strains of other \textit{B. burgdorferi sensu lato} spp. (e.g. \textit{B. bissettii, B. americana} and \textit{B. andersonii}) that have now been found in human patients in the USA.}

The inability previously to identify a likely cause of the Lyme-like signs and symptoms experienced by patients residing in the southern USA led to investigations of potential agents besides Lyme group \textit{Borrelia} spp. The identification of the relapsing fever group species \textit{B. lonestari} in \textit{A. americanum} ticks (Barbour et al., 1996) provided circumstantial evidence suggesting an alternative cause for the condition that came to be known as STARI or Master’s disease (Masters et al., 2008). However, besides a single human case of STARI associated with the presence of \textit{B. lonestari} DNA detected in a tick and human EM lesion skin biopsy sample (James et al., 2001), no additional evidence has been published to date that supports \textit{B. lonestari} as the cause of STARI.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
US state & No. tested & No. positive (%) & \textit{B. burgdorferi} & \textit{B. americana} & \textit{B. andersonii} & No. not sequenced \\
\hline
AK & 1 & 0 & 0 & 0 & 0 & 0 \\
AL & 1 & 0 & 0 & 0 & 0 & 0 \\
AZ & 2 & 1 (50) & 0 & 0 & 0 & 0 \\
CA & 9 & 4 (44) & 4 & 0 & 0 & 0 \\
CT & 2 & 0 & 0 & 0 & 0 & 0 \\
FL & 131 & 53 (40) & 31 & 15 & 3 & 4 \\
GA & 12 & 8 (67) & 8 & 0 & 0 & 0 \\
ID & 1 & 0 & 0 & 0 & 0 & 0 \\
IL & 5 & 3 (60) & 3 & 0 & 0 & 0 \\
KY & 1 & 0 & 0 & 0 & 0 & 0 \\
MA & 1 & 0 & 0 & 0 & 0 & 0 \\
MD & 1 & 1 (100) & 1 & 0 & 0 & 0 \\
ME & 2 & 1 (50) & 1 & 0 & 0 & 0 \\
MI & 1 & 0 & 0 & 0 & 0 & 0 \\
MO & 38 & 11 (29) & 7 & 3 & 1 & 0 \\
MN & 1 & 0 & 0 & 0 & 0 & 0 \\
NC & 9 & 6 (67) & 6 & 0 & 0 & 0 \\
NE & 3 & 2 (67) & 1 & 1 & 0 & 0 \\
NJ & 1 & 1 (100) & 1 & 0 & 0 & 0 \\
NM & 1 & 1 (100) & 1 & 0 & 0 & 0 \\
NY & 3 & 2 (67) & 1 & 1 & 0 & 0 \\
OH & 3 & 2 (67) & 1 & 1 & 0 & 0 \\
OK & 10 & 6 (60) & 4 & 1 & 1 & 0 \\
OR & 1 & 1 (100) & 1 & 0 & 0 & 0 \\
PA & 6 & 4 (67) & 1 & 2 & 1 & 0 \\
SC & 1 & 0 & 0 & 0 & 0 & 0 \\
TX & 4 & 3 (75) & 4 & 0 & 0 & 0 \\
UT & 1 & 1 (100) & 1 & 0 & 0 & 0 \\
VA & 8 & 4 (50) & 3 & 1 & 0 & 0 \\
VT & 1 & 0 & 0 & 0 & 0 & 0 \\
WA & 2 & 1 (50) & 1 & 0 & 0 & 0 \\
WI & 5 & 2 (40) & 2 & 0 & 0 & 0 \\
Total & 277 & 118 (42.6) & 76 (64.4) & 30 (25.4) & 4 (3.4) & 8 (6.8) \\
\hline
\end{tabular}
\end{table}

Lyme \textit{Borrelia} infecting humans in the USA

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Stromdahl et al., 2003). However, although a previous study conducted by the UNF Laboratory found evidence of LB in several patients with documented lone star tick bites in Florida and Georgia (Clark et al., 2013), the present study was not able to evaluate tick bite data or EM lesion evidence for the patients tested. It is possible that some other entity is the cause of the EM-like lesions experienced by some patients after being bitten by A. americanum. Nevertheless, our findings still suggest that cases of LB do occur in the southern USA, where the disease may be more common than previously recognized. Furthermore, the findings of this study provide further evidence that cases of LB in the south and elsewhere in the country may be caused by multiple species of B. burgdorferi sensu lato. In conclusion, this study provides further evidence that B. burgdorferi is not the only species associated with LB in the USA.

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