Borrelia burgdorferi Not Confirmed in Human-Biting Amblyomma americanum Ticks from the Southeastern United States

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The predominant human-biting tick throughout the southeastern United States is Amblyomma americanum. Its ability to transmit pathogens causing Lyme disease-like illnesses is a subject of ongoing controversy. Results of previous testing by the Department of Defense Human Tick Test Kit Program and other laboratories indicated that it is highly unlikely that A. americanum transmits any pathogen that causes Lyme disease. In contrast, a recent publication by Clark and colleagues (K. L. Clark, B. Leydet, and S. Hartman, Int. J. Med. Sci. 10:915–931, 2013) reported detection of Lyme group Borrelia in A. americanum using a nested-flagellin-gene PCR. We evaluated this assay by using it and other assays to test 1,097 A. americanum ticks collected from humans. Using the Clark assay, in most samples we observed nonspecific amplification and nonrepeatability of results on subsequent testing of samples. Lack of reaction specificity and repeatability is consistent with mispriming, likely due to high primer concentrations and low annealing temperatures in this protocol. In six suspect-positive samples, Borrelia lonestari was identified by sequencing of an independent gene region; this is not a Lyme group spirochete and is not considered zoonotic. B. burgdorferi was weakly amplified from one pool using some assays, but not others, and attempts to sequence the amplicon of this pool failed, as did attempts to amplify and sequence B. burgdorferi from the five individual samples comprising this pool. Therefore, B. burgdorferi was not confirmed in any sample. Our results do not support the hypothesis that A. americanum ticks are a vector for Lyme group Borrelia infections.

The vectors and etiologic agents of Lyme-like diseases in the southeastern United States are a subject of ongoing controversy (1, 2). In the United States, most Lyme disease is caused by infection with Borrelia burgdorferi sensu stricto, a bacterium that is phylogenetically within the B. burgdorferi sensu lato “Lyme group” of spirochetes vectored by hard ticks. The Lyme group also includes genospecies implicated as the etiologic agents of Lyme disease in other geographic regions, including B. garinii, B. afzelii, B. spielmanii, and B. valaisiana in Europe. Additional Lyme group genospecies continue to be described. In the United States, these include B. americanana, B. andersonii, and B. carolinensis, all of unknown pathogenicity, and B. bissetti, which has been implicated in cases of human illness (1). In contrast to the Lyme group, the relapsing fever (RF) group spirochetes, many of which are vectored by soft ticks, are a separate phylogenetic cluster that includes agents associated with RF disease in humans. In the United States, genospecies within the RF group include B. hermsii, B. turicatae, and B. parkeri, vectored by soft ticks, and B. miyamotoi, B. davisii, and B. lonestari, vectored by hard ticks. B. hermsii is the main cause of tick-borne relapsing fever in the United States; B. turicatae and B. parkeri have also been associated with human disease (3). B. miyamotoi was recently implicated as a cause of human disease in the United States (4), and the pathogenicity of B. davisii is unknown. B. lonestari was provisionally implicated in a single case of early Lyme disease-like illness (erythema migrans), but subsequent investigation has not established it as a human pathogen (5–7).

The predominant human-biting tick throughout the Southeast is Amblyomma americanum, accounting for over 90% of tick bites in southeastern states (8, 9). Vector competency studies have concluded repeatedly that B. burgdorferi is unlikely to be transmitted by A. americanum (10–16), and a potent borreliacidal agent has been identified in A. americanum saliva (17, 18). From 1997 to 2010, the Department of Defense (DOD) Human Tick Test Kit Program (HTTKP), a tick identification and PCR testing service provided by the U.S. Army Public Health Command (USAPHC) for DOD personnel and dependents, tested 22,565 A. americanum ticks (21,245 adults and nymphs removed from humans, and 1,320 field-collected larvae) for borrelial agents of Lyme-like diseases using a series of nested- and real-time PCR assays. These ticks include representation across the majority of the geographic distribution of the species (8, 9).

HTTKP methods have evolved across the years in response to changing diagnostic capabilities and new public health threats (2, 19, 20). For example, in 2001, when B. lonestari, an RF group Borrelia species found in A. americanum, was tentatively linked to...
a case of southern tick-associated rash illness (STARI) (5), HTTKP testing was modified to include testing for *B. lonestari* (21–24). However, epidemiological studies of STARI patients did not support the hypothesized link between STARI and *B. lonestari* (6, 7, 25), so targeting of this *Borrelia* species in the HTTKP ended in 2009. As a summary of our program from 1997 to 2010, a total of 4,019 *A. americanum* adults, nymphs, and larvae were tested with nested primers expected to amplify *B. burgdorferi*, and 7,421 tests were performed to confirm that would have amplified all *Borrelia* spp. (23). Although we initially reported that seven samples (0.3%), all from 1997, produced amplicons (19), we believe contamination was involved because all occurred within a short time period in the first year of our study when we initially implemented PCR techniques, and zero positive results were obtained across large numbers of samples in all subsequent years. In contrast, over the same period, a total of 5,458 *Ixodes scapularis* and 12 *Ixodes pacificus* adults and nymphs were tested by the HTTKP using equivalent assays: 24.7% of *I. scapularis* and 8.3% of *I. pacificus* ticks tested positive for Lyme group *Borrelia* DNA. To summarize, we found no measurable prevalence of *B. burgdorferi* in an epidemiologically relevant collection of *A. americanum* ticks from across a broad geographic range over a 13-year period. In 2013, however, debate about the role of *A. americanum* in Lyme disease-like illness intensified, following a publication that reported detection of *B. burgdorferi* DNA in two *A. americanum* ticks removed from humans, and *B. burgdorferi* and *B. andersonii* DNA in humans bitten by *A. americanum* (26). Given our extensive background of PCR testing of *A. americanum*, and in view of the novelty and potential clinical significance of the suggestion by Clark et al. (26) that this tick species is contributing to unrecognized cases of human borreliosis in the southeastern United States, we used the primers and thermocycling protocol of Clark et al. (26) in an attempt to detect Lyme group spirochetes in a large sample (>1,000) of *A. americanum* ticks removed from humans throughout the eastern United States in 2013.

**MATERIALS AND METHODS**

**Tick identification and DNA extraction.** *A. americanum* ticks were removed from military personnel and their families in the mid-Atlantic and southeastern United States and submitted to the HTTKP (Table 1). Submitted ticks were identified to species morphologically. Genomic DNA was then extracted either from individual ticks or from small pools of conspecific ticks removed from the same human on the same date, using a *Zymo Genomic DNA II kit* (*Zymo Research Corporation, Orange, CA*) (27). Aliquots of DNA from five *A. americanum* samples were pooled for a screening PCR; individual samples in these pools were archived for later PCR and/or sequencing confirmation if the pool tested suspect positive. Samples removed from the same person could contain more than one tick, so some pools contained DNA from more than five ticks.

**PCR and sequence analysis.** At our USAPHC laboratory, nested PCR for the flaB gene using primers from Clark et al. (26) was performed in 25-μl reaction volumes prepared with Ready-To-Go PCR beads (*Amer sham Pharmacia Biotech, Piscataway, NJ*), containing 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 200 μM each deoxynucleotide triphosphate (dNTP), and 1.5 U *Taq* DNA polymerase. External reactions using outer reaction primers 280F and 754R contained 2.5 μl tick DNA (0.5 μl from individual ticks in pools of 5) and amplified a 475-bp product; internal reactions using inner reaction primers 301F and 737R contained 1.0 μl external PCR and amplified a 437-bp product. Of the two internal reaction primer sets published by Clark et al. (26), we used the set that provided the clearest gel results when tested with positive controls. This was the primer combination—280F, 754F and 301F, 737R—used to amplify Lyme group *Borrelia* in a subsequent study of human samples by Clark et al. (28). Published thermal cycling parameters were followed (26, 28). Three positive controls were used, consisting of *B. burgdorferi* strain B31 (a gift from R. Wirtz, CDC, Atlanta, GA) and two *B. burgdorferi*-positive *I. scapularis* samples from the HTTKP. Each PCR included at least two negative controls, consisting of a water template sample that was introduced at the time of DNA extraction and a water template sample that was introduced at the time of PCR. Amplicons were visualized on 2% agarose gel cassettes using ethidium bromide (E-Gel; Invitrogen Corp., Carlsbad, CA). When a pool produced an amplicon at the 437-bp position, we then tested DNA from the individual tick samples that comprised the pool to ascertain individual-level infection, using the same method as described above.

At our Texas A&M University (TAMU) laboratory, a blinded collection of flaB PCR-suspect-positive and -negative samples, along with positive and negative controls, was subjected to further analysis, following whole-genome amplification to increase the amount of template DNA available for testing (GenomiPhi; GE Healthcare, Pittsburgh, PA). This amplification process produces a concentrated DNA template, and therefore DNA was diluted 1:25 for subsequent analyses. First, the identification of tick species was confirmed through PCR and DNA sequencing of the 12S rRNA gene (29). Next, a nested PCR for the 16S-23S rRNA intergenic spacer region (IGS) of *Borrelia* was performed, using primer and thermal cycling parameters described previously (30). This assay amplifies both RF and Lyme group borreliae, producing approximate 500-bp and 1,000-bp fragments, respectively. PCR amplicons from positive samples were purified (ExoSAP-IT; Affymetrix, Santa Clara, CA) and bidirectionally sequenced. Sequences were manually edited, and the identification of the *Borrelia* species was ascertained through comparison to sequences published in NCBI GenBank. Finally, real-time quantitative PCR (qPCR) was run on the samples using primers and probes for the 16S rRNA gene of *Borrelia*, including a *B. burgdorferi*-specific probe and an RF group probe (31).

At Ibis Biosciences, further testing of PCR-suspect-positive samples was undertaken, using isothermal *Borrelia* enrichment performed as previously published (32). Enrichment of a negative control was also performed. Following isothermal *Borrelia* DNA enrichment, a broad-range

| TABLE 1 Origins by state of all ticks tested in this study |
|------------------------|-----------------|
| State                | No. of ticks tested |
| VA                   | 661              |
| MD                   | 151              |
| NJ                   | 53               |
| NC                   | 48               |
| KY/TN               | 40               |
| KY                   | 33               |
| KS                   | 27               |
| TN                   | 24               |
| MO                   | 22               |
| DE                   | 14               |
| SC                   | 11               |
| AL                   | 10               |
| AR                   | 10               |
| FL                   | 24               |
| GA                   | 2                |
| FL/GA               | 1                |
| RI                   | 1                |
| PA                   | 1                |
| Total                | 1,097            |

a. Ticks from Ft. Campbell, on the border of Kentucky and Tennessee, are listed as "KY/TN."

b. One tick was reported as acquired in either Florida or Georgia and is listed as "FL/GA."
PCR followed by electrospray ionization mass spectrometry (IA/PCR/ESI-MS) was performed, in which each sample was subjected to a series of eight diagnostic PCRs (32, 33). By the selection of Borrelia PCR targets that vary in sequence between Borrelia species and strains (genotypes), it is possible to use the IA/PCR/ESI-MS technology to identify the Borrelia species and distinguish its genotype even when present in mixtures of genotypes (32, 33). A volume of 10 μl of isothermal amplified nucleic acid extract was used in each PCR, and all PCRs were analyzed using an electrospray ionization mass spectrometry system (Abbott Molecular, Des Plaines, IL) as previously described (32, 33).

In silico analysis. Potential mispriming genomic loci were identified for each primer assay of Clark et al. (26) using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) default parameters against the nr database (a nonredundant NCBI database that includes all GenBank, RefSeq Nucleotides, EMBL, DDBJ, and PDB sequences, excluding HTGS0, 1, and 2, EST, GSS, STS, PAT, and WGS). The species of each potential mispriming site was noted for each assay.

The flagellin sequences of experimentally identified potential cross-reacting Borrelia species were aligned using Clustalw (http://www.gene.com/tools/clustalw/). The sequences used were from B. burgdorferi B31 (NC_001318.1), B. andersonii (D83762.1), B. americana SCW-30 h (HM802232.1), and B. lonestari MO2002-V1 (AY850063.1). The B. andersonii, B. americana, and B. lonestari genomes are not yet fully assembled, so the longest flagellin sequences deposited in NCBI Nucleotide were chosen for the alignment.

The melting temperatures (Tm) for each primer assay were determined using an online calculator (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), adjusting default parameters to represent our PCR conditions (0.8 μM primers, 200 μM dNTPs, and 1.5 mM MgCl2) and identified mismatches.

Sensitivity test. PCRs were performed at USAPHC to compare the sensitivity of the flaB primers from Clark et al. (26) and the set of nested Borrelia genus flaB primers from Barbour et al. (23) routinely used in the HTTKP. Serial dilutions of B. americana SCW-30E, B. andersonii SI-10, and B. bissetti FD-1 were tested with the two PCRs. These particular strains are from the southern United States and were chosen in order to more rigorously test the sensitivity of these primers and their ability to detect all members of these species, because these strains varied the most with the Barbour primers. To identify mismatches between the Barbour flaB primers and B. americana, B. andersonii, and B. bissetti strains from the United States, an alignment of the flagellin sequences found in NCBI GenBank was made using the MegAlign application in the DNAStar Lasergene 12 software. The protocol for PCR using the Clark primers is described above, and the PCR protocol for the Barbour primers is described by Stromdahl et al. (3). Each test contained a positive control containing B. burgdorferi strain B31 and a negative control consisting of a water template sample that was introduced at the time of the PCR.

RESULTS

USAPHC testing. PCR using the nested flaB primers from Clark et al. (26) was performed on 171 pools, comprising 1,097 individual A. americanum ticks. The geographic origin of these ticks is listed in Table 1, and the results of testing are summarized in Table 2. The B31 positive controls and the two I. scapularis samples previously PCR positive for B. burgdorferi all produced major bands at 437 bp. In contrast, in most A. americanum samples, we observed only a “ladder” effect (Fig. 1) that typically is indicative of mispriming. For the few samples that produced a distinct major band of the expected length (e.g., Fig. 1A, lane 7, and B, lane 8), we classified the sample as suspect positive and investigated further. Nine of 171 pools (5.3%)—P009, P084, P123, P171, P174, P189, P191, P194, and P211—were selected by this criterion.

Additional testing was conducted on the individual samples comprising the nine pools that produced major bands of the 437-bp size. Two of these pools (P009 and P171) yielded no suspect-positive individual samples. Accordingly, we repeated the flaB PCR on these and the remaining pools before testing the individual samples from any more pools. The pools P009 and P171, with no suspect-positive individual samples, and three additional pools, P189, P191, and P211, were negative in the repeat PCR of the pool, so the individual samples from P189, P191, and P211 were not initially tested using the flaB primers. The five individual samples from P189 were later tested (see “Follow-up testing at USAPHC and TAMU” below). Four pools (P084, P123, P174, and P194) contained individual samples that produced a 437-bp amplicon. Three pools, P084, P123, P194, contained one positive tick each, and one pool, P174, contained two positive ticks. Overall, 5 of 1,097 (0.46%) individual A. americanum ticks (130786, 131114, 131429, 131433, and 131566) produced major bands of 437 bp. These five samples were submitted from Rhode Island, Virginia, Tennessee, and New Jersey.

TAMU testing. The five individual samples (130786, 131114, 131429, 131433, and 131566) and eight pools (P009, P123, P171, P174, P189, P191, P194, and P211) produced major bands of 437 bp in the flaB PCR were sent to the TAMU laboratory for further analysis. The pool (P084) containing the individual suspect-positive sample from Rhode Island was not sent. The 12s rRNA sequence analysis revealed that all samples except one (130786 from P084), contained DNA from A. americanum. Sample 130786, was molecularly identified as I. scapularis and so had been misidentified by morphology at USAPHC as A. americanum. This tick sample, which had been removed from a human in Rhode Island, was retained as a positive control in subsequent analyses and was confirmed as positive for B. burgdorferi in all subsequent assays.

In the IGS PCR, six of eight pools (P123, P171, P174, P189, P194, and P211) produced amplicons, including five amplicons (P123, P171, P174, P194, and P211) that were approximately 500 bp (indicative of RF group spirochetes) and one very faint ampli- cion (P189) that was approximately 1,000 bp (indicative of Lyme group spirochetes) (Table 2). Additionally, all four individual A. americanum ticks (131114, 131429, 131433, and 131566) produced amplicons approximately 500 bp in size, and all four were associated with pools producing amplicons approximately 500 bp in size. DNA sequencing of these 500-bp IGS amplicons in each case revealed the presence of B. lonestari (Table 2). The sequence reaction failed for the faint 1,000-bp IGS amplicon from pool P189.

In the qPCR, six (P123, P171, P174, P189, P194, and P211) of eight pools were considered suspect positive. Consistent with the IGS results reported above, five pools (P123, P171, P174, P194, and P211) were positive with the RF group probe (CT values ranged from 32 to 35), and one (P189) was weakly positive with the Lyme group Borrelia probe (CT value of 39) (Table 2). Additionally, all four individual A. americanum ticks (131114, 131429, 131433, and 131566) were positive with the RF group probe (CT values ranged from 31 to 33), consistent with the IGS results that indicated the presence of B. lonestari DNA in these samples.

IA/PCR/ESI-MS analysis. Nine pools (P009, P084, P123, P171, P174, P189, P191, P194, and P211) and four individual samples (131114, 131429, 131433, and 131566) producing a major band of 437 bp in the flaB PCR assay and four pools (P184, P187, P20, and P211) and five individual samples (131431, 131559, 131560, 131570, and 131571) that were negative in that assay were

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sent blinded from USAPHC to Ibis Biosciences for analysis using the IA/PCR/ESI-MS assay.

The two pools (P009 and P191) that were negative in all TAMU assays were also negative in the Ibis assay. The four previously Borrelia-negative pools (P184, P187, P220, and P221) and two (131431 and 131570) of the five Borrelia-negative individual samples sent as controls were also negative using the Ibis assay. Three in analysis at Ibis Biosciences (Table 2). All five pools (P123, P171, P174, P194, and P211) and four individual samples (131114, 131429, 131433, and 131566) identified as containing B. lonestari at TAMU were also positive for this organism at Ibis Biosciences. Pool P189, which was suspect positive for B. burgdorferi at USAPHC and was later molecularly identified as I. scapularis at TAMU, was positive for B. burgdorferi at Ibis.

Follow-up testing at USAPHC and TAMU. In order to thoroughly investigate P189, the DNA of the five ticks (131355, 131536, 131537, 131538, and 151359) that comprised this pool was analyzed individually both at USAPHC and at TAMU. Two nested PCRs for Borrelia were used at USAPHC; the flaB assay from Clark et al. (26) and the Borrelia genus flaB PCR from Barbour et al. (23). In the former assay, all of the individual samples from P189 produced multiple faint bands (laddering) and none had a 437-bp major band; all were scored as negative. Similarly, all sam-

TABLE 2 Analysis of A. americanum" samples with bright bands at 437 bp in the B. burgdorferi flaB PCR of Clark et al. (26)"
mentally identified species in the generic fla assay from Clark et al. (26) at the TAMU laboratory, we also obtained a laddering effect in most samples on the gel, indicative of mispriming. One sample (1311536) produced a faint band at the diagnostic 437-bp size (in addition to at least one smaller fragment band of the same intensity). Attempts to obtain a DNA sequence from this sample using a direct approach as well as after excising the 437-bp band from the gel were not successful, and the sample was therefore scored as negative.

In silico analysis of the nested-PCR assay. NCBI Primer-BLAST analysis of the nested-PCR assay from Clark et al. (26) indicated that the flagellin genes from multiple Borrelia species could be amplified. For the external reaction, a 475-bp amplicon is expected. The BLAST results yielded amplicons of 445 to 475 bp that corresponded to 19 Borrelia species, including the experimentally identified species B. burgdorferi, B. andersonii, B. americana, and B. lonestari. For the internal reaction, a 437-bp amplicon is expected. The BLAST results yielded amplicons of 407 to 440 bp. All 19 species identified by the external reaction are also potential mispriming loci with the internal reaction.

A more detailed in silico analysis was performed comparing the flaB gene from B. burgdorferi to those of the three experimentally identified cross-reacting Borrelia species. The external and internal primers were also aligned to the sequences. The external primer assay revealed a perfect match to B. burgdorferi and B. andersonii (due to the degenerate base) and a single mismatch to B. americana. The external reaction has multiple mismatches to the B. lonestari sequence, most importantly a mismatch at the 3′-terminal nucleotide in the reverse primer and a mismatch 3 bp from the 3′ terminus of the forward primer. The internal reaction has a perfect match with B. burgdorferi, B. andersonii, and B. americana. This reaction has four mismatches between the internal reverse primer and the B. lonestari sequence, including two close to the 3′ end of the primer.

Sensitivity test. The MegAlign comparison of the flagellin sequences found in NCBI GenBank revealed that all four of the Clark primers were an exact match to B. americana SCW-30E. Three of the primers (301F, 737R, and 745R) were 100% matches to B. andersonii SI-10 and B. bissettii FD-1, although with the 301F primer, only 21/23 bases of the primer could be determined. The match of the 280F primer to B. andersonii SI-10 and B. bissettii FD-1 could not be determined as the sequences are unknown; however, the 280F primer was an exact match to all of the U.S. sequences of B. bissettii (DN127) and B. andersonii (19857, 21038, and 21123) that were available in NCBI GenBank.

The B. americana SCW-30E flagellin sequence was a 100% (24/24) match with the Barbour FlaLL primer and a 95% (21/22) match with FlaLS; its matches with FlaRS and FlaRL could not be determined. However, the FlaRS primer was a 100% match (26/26) with the one U.S. sequence of B. americana (SCW-30 h) that was available in NCBI GenBank. The B. andersonii SI-10 flagellin sequence was a 100% match (22/22) with the Barbour FlaLL primer over the 22/24 bases it could be aligned with, an 86% (19/22) match with FlaLS, a 96% (25/26) match with FlaRS, and a 92% (11/12) match with FlaRL over the 11/24 bases it could be aligned with. The B. bissettii FD-1 flagellin sequence was a 100% match (22/22) with the Barbour FlaLL primer over the 22/24 bases it could be aligned with, a 100% (22/22) match with FlaLS, and a 92% (24/26) match with FlaRS, and its matches with FlaRL could not be determined. However, the FlaRL primer was a 96% match (23/24) with the one U.S. sequence of B. bissettii (DN127) that was available in NCBI GenBank.

In the PCR comparison, the flaB primers from Clark (26) and the fla primers from Barbour et al. (23) produced identical results in amplifying increasingly dilute amounts of the three Lyme group spirochetes. The analytical sensitivity (limit of detection [LOD]) of both primer sets in amplifying B. americana DNA was determined to be ~0.3 copy/μL (1:10 M dilution). The LOD of both primer sets in detecting B. andersonii DNA was ~7 copies (1:1 M dilution), and the LOD of both primer sets in detecting B. andersonii DNA was ~6 copies (1:1 M dilution).

DISCUSSION

We were unable to confirm any Lyme group Borrelia infection in a detailed investigation of 1,097 A. americanum ticks removed from humans in the southeastern and mid-Atlantic United States.
Other recent investigations have similarly failed to reveal *B. burgdorferi* in *A. americanum*. No *B. burgdorferi* was detected during an extensive molecular characterization of the microbiome of 732 *A. americanum* adults and nymphs collected in Texas and Missouri (34), and a study of >3,000 *A. americanum* ticks from Georgia using PCR with *fla* primers from Barbour et al. (23) reported detection only of *B. lonestari* (35). The Barbour primers are designed to amplify all *Borrelia* spp., and analytical sensitivity testing of these primers in this study has indicated that they can detect the strains of *B. americana*, *B. andersonii*, and *B. bissetti* that have the most mismatches with these primers, suggesting that they can detect all members of each of these species.

Clark et al. (26) have suggested that *A. americanum* ticks are a significant source of viable *B. burgdorferi* (sensu lato or sensu stricto). In our hands, however, the *flaB* nested-PCR assay upon which that suggestion was based produced many faint nonspecific bands, likely indicative of mispriming by amplification of DNA from the tick, human DNA in the tick blood meal, or other organisms in the tick midgut microbiome. Amplification was also inconsistent since samples with major 437-bp bands failed to produce amplicons in repeat testing. Similar inconsistent results (i.e., decreasing the sensitivity of these primers so that a higher target concentration of primers and low annealing temperature specified by Clark et al. This analysis reveals that the low annealing temperatures of the Clark assay are not very stringent, increasing the probability of mispriming resulting in amplification of *B. lonestari* and other nonspecific targets. This mispriming has the result of decreasing the sensitivity of these primers so that a higher target copy number is required for amplification to occur. Although Clark et al. performed a BLAST analysis (26), the analysis would not have identified many of the 19 identified potential cross-reacting *Borrelia* species if they used the reference genome data set or only BLASTed against selected GenBank files. Many *Borrelia* genomes (including *B. lonestari*) are not yet fully sequenced. Although we chose the longest deposited *B. lonestari* flagellin sequence for the alignment to *B. burgdorferi*, we did an additional alignment with 109 *B. lonestari* flagellin sequences deposited in NCBI Nucleotide (data not shown). Only three records included the outer forward reaction, but five included the inner forward reaction, all of which showed sequence identity. All 109 records included the inner reverse reaction, and 107 records included the outer reverse reaction. Of these alignments, only two did not have sequence identity. This alignment confirmed the accuracy of the sequence we used for the original alignment.

As explained above, the assay from Clark et al. (26) would not be expected to consistently amplify *B. lonestari*; therefore, some *B. lonestari*-positive *A. americanum* samples might have been missed in the initial screen at USAPHC using only the Clark primers. Testing at TAMU and Ibis Biosciences using assays designed to amplify *B. lonestari* detected this organism in samples that had produced negative or inconsistent results using the Clark primers at USAPHC (Table 2). However, the low prevalence of infection with *B. lonestari* detected in these samples is congruent with previous surveillance (2, 8, 21, 24, 34, 35). For example, in our program from 1997 to 2010, a total of 18,546 *A. americanum* ticks were tested with nested and real-time primers that were capable of amplifying *B. lonestari*, and 195/17,226 (1.1%) of adults and nymphs and 4/1,320 larvae (0.3% minimum infection prevalence) were positive (2, 8, 20).

Ibis Biosciences reported low-level detection of *B. burgdorferi* DNA in a pooled sample of *A. americanum* DNA (sample P189) by PCR readthrough from the external reaction explains both the *B. lonestari* positive results, and the lack of repeat positive results. If the readthrough occurs during the first cycles of the external reaction, then sufficient amplicon is produced to have a high rate of amplification in the internal reaction, resulting in a major band of the expected size. The internal reaction should amplify *B. lonestari* sequences from the external reaction, since the forward internal primer has an exact match to *B. lonestari* sequence, and the reverse primer only shows mismatches at the 5′ end of the primer, which will not affect amplification. However, if readthrough does not occur until a later cycle of the external reaction, insufficient amplicons are produced, resulting in a negative reaction.

Although readthrough and mispriming occur, we would not expect this to be a common event. Therefore, we examined the PCR methods as an additional explanation. The readthrough and mispriming likelihood may have been increased due to the high concentration of primers and low annealing temperature specified by Clark et al. This analysis reveals that the low annealing temperatures of the Clark assay are not very stringent, increasing the probability of mispriming resulting in amplification of *B. lonestari* and other nonspecific targets. This mispriming has the result of decreasing the sensitivity of these primers so that a higher target copy number is required for amplification to occur. Although Clark et al. performed a BLAST analysis (26), the analysis would not have identified many of the 19 identified potential cross-reacting *Borrelia* species if they used the reference genome data set or only BLASTed against selected GenBank files. Many *Borrelia* genomes (including *B. lonestari*) are not yet fully sequenced. Although we chose the longest deposited *B. lonestari* flagellin sequence for the alignment to *B. burgdorferi*, we did an additional alignment with 109 *B. lonestari* flagellin sequences deposited in NCBI Nucleotide (data not shown). Only three records included the outer forward reaction, but five included the inner forward reaction, all of which showed sequence identity. All 109 records included the inner reverse reaction, and 107 records included the outer reverse reaction. Of these alignments, only two did not have sequence identity. This alignment confirmed the accuracy of the sequence we used for the original alignment.

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plification of the remnants of an infected blood meal from a previous life stage. All five samples in this pool were single ticks from Ft. Pickett, VA; two were nymphs, two were females, one was a male, and all were engorged and removed from humans. A few *B. burgdorferi*-infected *I. scapularis* ticks have been submitted to the HTTKP from Ft. Pickett, demonstrating that the pathogen is indeed circulating in that environment (E. Stromdahl, unpublished data). Other Lyme group *Borrelia* species, *B. americana*, *B. andersonii*, *B. bissetti*, etc., were not detected.

To summarize, we were unable to confirm any Lyme group *Borrelia* infection in *A. americanum* ticks removed from humans in the southeastern and mid-Atlantic United States. In our investigation, we utilized the *flaB* primers and thermal cycling parameters of Clark et al. because this diagnostic tool was associated with the reported finding of Lyme group *Borrelia* DNA in a small number of *A. americanum* ticks and blood samples from humans reported to have been fed upon by *A. americanum* (26). In our investigation, this *flaB* assay produced indistinct and inconsistent results in *A. americanum* ticks, and the samples that produced major bands at the expected fragment size were confirmed to be positive for *B. lonestari* DNA (but not Lyme group *Borrelia* DNA) in multiple different assays. In agreement with decades of previous research, we therefore conclude that human-biting *A. americanum* ticks are not a vector of *B. burgdorferi*. The conclusion of Clark et al. that *A. americanum* is infected with Lyme group *Borrelia* spp. is based on the detection of *flaB* gene sequences alone. Given that this finding is in striking contrast to decades of previous investigations of the topic, a more rigorous approach would be needed for more convincing evidence of *B. burgdorferi*-infected *I. scapularis* ticks to be confirmed to be positive for *B. lonestari* DNA (but not Lyme group *Borrelia* DNA) in multiple different assays. In agreement with decades of previous research, we therefore conclude that human-biting *A. americanum* ticks are not a vector of *B. burgdorferi*. The conclusion of Clark et al. that *A. americanum* is infected with Lyme group *Borrelia* spp. is based on the detection of *flaB* gene sequences alone. Given that this finding is in striking contrast to decades of previous investigations of the topic, a more rigorous approach would be needed for more convincing evidence of *B. burgdorferi*-infected *I. scapularis* ticks to be confirmed to be positive for *B. lonestari* DNA (but not Lyme group *Borrelia* DNA) in multiple different assays. In agreement with decades of previous research, we therefore conclude that human-biting *A. americanum* ticks are not a vector of *B. burgdorferi*. The conclusion of Clark et al. that *A. americanum* is infected with Lyme group *Borrelia* spp. is based on the detection of *flaB* gene sequences alone.

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Erratum for Stromdahl et al., *Borrelia burgdorferi* Not Confirmed in Human-Biting *Amblyomma americanum* Ticks from the Southeastern United States

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Volume 53, no. 5, p. 1697–1704, 2015. Page 1698, column 1, lines 10–11: “Although we initially reported that seven samples (0.3%), all from 1997, produced amplicons (19), we believe . . .” should read “Although we initially reported that seven samples (7/222 = 3.2%), all from 1997, produced amplicons in PCRs for two different gene targets (19), we believe . . . .”