Borrelia burgdorferi not confirmed in human-biting *Amblyomma americanum* ticks from the southeastern United States

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ABSTRACT

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, 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 and by using it, and other assays, to test 1,097 *A. americanum* collected from humans. Using the Clark assay, in most samples we observed non-specific amplification, and non-repeatability 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,

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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 vector Lyme group *Borrelia* infections.

INTRODUCTION

The vectors and etiologic agents of Lyme-like diseases in the southeastern United States are a subject of ongoing controversy (1, 2). In the US, most Lyme disease is caused by infection with *Borrelia burgdorferi* sensu stricto (ss), a bacterium that is phylogenetically within the *B. burgdorferi* sensu lato (sl) '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 US, these include *B. americana, B. andersonii* and *B. carolinensis,* all of unknown pathogenicity, and *B. bissettii*, 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 US, 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 US; *B. turicatae* and *B. parkeri* have also been associated with human disease (3). *B. miyamotoi* was recently implicated as the cause of human disease in the US (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, 6, 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 - 2010, the Department of Defense (DOD) Human Tick Test Kit Program (HTTKP), a tick identification and PCR testing service provided by the US 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*, a RF group *Borrelia* 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 1997-2010, a total of 4,019 *A. americanum* adults, nymphs and larvae were tested with nested primers expected to amplify *B. burgdorferi*, and 7,421 were tested with primers that would have amplified all *Borrelia* spp. (23). Although

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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* tested positive for Lyme group *Borrelia* DNA. To summarize, we found no measureable prevalence of *B. burgdorferi* in an epidemiologically relevant collection of *A. americanum* from across a broad geographic range over a 13-year time 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* 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* removed from humans throughout the eastern US in 2013.

MATERIALS AND METHODS

Tick identification and DNA extraction. *A. americanum* were removed from military personnel and their families in the mid-Atlantic and southeastern US 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, California) (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 (Amersham Phamacia Biotech, Piscataway, N.J.), containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 µM each dNTP and 1.5 units *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 reaction 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, 754R 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 (gift of Dr. R. Wirtz, CDC Atlanta) and two *B. burgdorferi* - positive *I. scapularis* samples from the HTTKP. Each PCR included at least two negative controls, consisting of a water

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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, Calif.). 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 primers and thermal-cycling parameters described previously (30). This assay amplifies both RF and *Lyme group Borreliae*, producing approximate 500-bp and 1000-bp fragments, respectively. PCR amplicons from positive samples were purified (ExoSAP-IT, Affymetrix, Santa Clara, CA) and bi-directionally 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 a RF-group probe (31).

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153 At Ibis Biosciences, further testing of PCR-suspect-positive samples was undertaken,
154 using isothermal *Borrelia* enrichment performed as previously published (32). Enrichment of a
155 negative control was also performed. Following isothermal *Borrelia* DNA enrichment, a broad-
156 range PCR followed by electrospray ionization mass spectrometry (IA/PCR/ESI-MS) was
157 performed, in which each sample was subjected to a series of eight diagnostic PCR reactions
158 (32, 33). By the selection of *Borrelia* PCR targets that vary in sequence between *Borrelia*
159 species and strains (genotypes) it is possible to use the IA/PCR/ESI-MS technology to identify
160 the *Borrelia* species and distinguish its genotype even when present in mixtures of genotypes
161 (32, 33). A volume of 10ul of isothermal amplified nucleic acid extract was used in each PCR
162 reaction and all PCR reactions were analyzed using an electrospray ionization mass
163 spectrometry system (Abbott Molecular, Des Plaines, IL) as previously described (32, 33).
164 ***In silico* analysis.** Potential mispriming genomic loci were identified for each primer
165 assay of Clark et al. (26) using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-
166 blast) default parameters against the nr database (a nonredundant NCBI database that includes
167 all GenBank + RefSeq Nucleotides + EMBL + DDBJ + PDB sequences, excluding HTGS0, 1, 2, EST,
168 GSS, STS, PAT, and WGS). The species of each potential mispriming site was noted for each
169 assay.
170 The flagellin sequences of experimentally identified potential cross-reacting *Borrelia*
171 species were aligned using Clustalw (http://www.genome.jp/tools/clustalw/). Sequences used
172 were *B. burgdorferi* B31 (NC_001318.1), *B. andersonii* (D83762.1), *B. americana* SCW-30h
173 (HM802232.1), and *B. lonestari* MO2002-V1 (AY850063.1). *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 T_m for each primer assay were determined using an online calculator (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), adjusting default parameters to represent our PCR reaction conditions (0.8uM primers, 200uM dNTPs, and 1.5mM $MgCl_2$) 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 *fla* primers from Barbour et al. (23) routinely used in the HTTKP. Serial dilutions of *B. americana* SCW-30E, *B. andersonii* SI-10, and *B. bissettii* FD-1 were tested with the two PCRs. These particular strains are from the southern US 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 *fla* primers and *B. americana*, *B. andersonii*, and *B. bissettii* 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. Protocol for PCR using the Clark primers is described above, and PCR protocol for the Barbour primers is described in 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 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.* The geographic origin of these ticks is listed in Table 1, and 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; Fig. 1B, lane 8), we classified the sample as suspect-positive and investigated further. Nine of 171 pools (5.3% of pools) -- P009, P084, P123, P171, P174, P189, P191, P194, P211 -- were selected by this criterion.

Additional testing was conducted on the individual samples comprising the nine pools that produced major bands of 437-bp size. Two of these pools (P009, 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 of P189, P191, and P211 were not initially tested using the *flaB* primers. The five individual samples from P189 were later tested (see section below: "Follow-up testing at USAPHC and TAMU"). Four pools (P084, P123, P174, 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, five of 1,097 (0.46%) individual *A. americanum* ticks (130786, 131114, 131429, 131433, 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, 131566) and eight pools (P009, P123, P171, P174, P189, P191, P194, P211) producing 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, P211) produced amplicons, including five amplicons (P123, P171, P174, P194, P211) that were approximately 500-bp (indicative of RF group spirochetes) and one very faint amplicon (P189) that was approximately 1000-bp (indicative of Lyme group spirochetes; Table 2). Additionally, all four individual *A. americanum* ticks (131114, 131429, 131433, 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 1000-bp IGS amplicon from pool P189.

In the qPCR, six (P123, P171, P174, P189, P194, P211) of eight pools were considered suspect-positive. Consistent with the IGS results reported above, five pools (P123, P171, P174, P194, P211) were positive with the RF-group probe (C_T values ranged from 32 to 35), and one (P189) was weakly positive with the Lyme group *Borrelia* probe (C_T value of 39; Table 2).

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Additionally, all four individual *A. americanum* ticks (131114, 131429. 131433, 131566) were positive with the RF-group probe (C_T 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, P211) and four individual samples (131114, 131429, 131433, 131566) producing a major band of 437-bp in the *flaB* PCR assay, and four pools (P184, P187, P20, P211) and five individual samples (131431, 131559, 131560, 131570, 131571) that were negative in that assay, were sent blinded from USAPHC to Ibis Biosciences for analysis using the IA/PCR/ESI-MS assay.

The two pools (P009, 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, 131570) of the five *Borreli*a-negative individual samples sent as controls were also negative using the Ibis assay. Three of the individual samples (131559, 131560, 131571) determined to be negative at USAPHC produced weak positives for *B. lonestari* in analysis at Ibis (Table 2). All five pools (P123, P171, P174, P194, P211) and four individual samples (131114, 131429, 131433, 131566) identified as containing *B. lonestari* at TAMU were also positive for this organism at Ibis. Pool P189, which was suspect-positive for *B. burgdorferi* in two assays at TAMU was also positive for *B. burgdorferi* in the Ibis assay using their IA/PCR/ESI-MS assay, but for only three of eight primers: BCT 3515 (rplB gene), BCT3517 (flagellin gene) and BCT 3519 (hbb gene). The pool (P084) containing the *B. burgdorferi*-positive *I. scapularis* molecularly identified at TAMU was positive for *B. burgdorferi* as well as *B. lonestari* at Ibis.

Follow-up testing at USAPHC and TAMU. In order to thoroughly investigate P189, the DNA of the five ticks (131535, 131536, 131537, 131538, 151359) that comprised this pool was

analyzed individually both at USAPHC and at TAMU. Two nested PCRs for *Borrelia* were used at USPAHC: the *flaB* assay from Clark et al. (26) and the *Borrelia* genus *fla* PCR from Barbour et al. (23). In the former assay, all 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 samples were negative in the generic *fla* assay. Using the same *flaB* assay from Clark et al. at the TAMU laboratory, we also obtained a laddering effect in most samples on the gel, indicative of mispriming. One sample (131536) 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 gene from multiple *Borrelia* species could be amplified. For the external reaction, a 475-bp amplicon is expected. The BLAST results yielded amplicons of 445-475-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 407-440-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 *flaB* from *B. burgdorferi* to 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

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283 to *B. americana*. The external reaction has multiple mismatches to the *B. lonestari* sequence,
284 most importantly a mismatch at the 3' terminal nucleotide in the reverse primer, and a
285 mismatch 3 bp from the 3' terminus of the forward primer. The internal reaction has a perfect
286 match with *B. burgdorferi*, *B. andersonii*, and *B. americana*. This reaction has four mismatches
287 between the internal reverse primer and the *B. lonestari* sequence, including two close to the 3'
288 end of the primer.

289 **Sensitivity test.** The MegAlign comparison of the flagellin sequences found in NCBI
290 Genbank revealed that all four of the Clark primers were an exact match to *B. americana* SCW-
291 30E. Three of the primers (301F, 737R, 745R) were 100% matches to *B. andersonii* SI-10 and *B.*
292 *bissettii* FD-1, although with the 301F primer only 21/23 bases of the primer could be
293 determined. The match of the 280F primer to *B. andersonii* SI-10 and *B. bissettii* FD-1 could not
294 be determined as the sequences are unknown, however, the 280F primer was an exact match
295 to all of the US sequences of *B. bissettii* (DN127) and *B. andersonii* (19857, 21038, and 21123)
296 that were available in NCBI Genbank.

297 The *B. americana* SCW-30E flagellin sequence was a 100% (24/24) match with the
298 Barbour FlaLL primer, 95% (21/22) match with FlaLS, and its match with FlaRS and FlaRL could
299 not be determined. However, the FlaRS primer was a 100% match (26/26) with the one US
300 sequence of *B. americana* (SCW-30h) that was available in NCBI Genbank. The *B. andersonii* SI-
301 10 flagellin sequence was a 100% match (22/22) with the Barbour FlaLL primer over the 22/24
302 bases it could be aligned with, 86% (19/22) match with FlaLS, 96% (25/26) match with FlaRS,
303 and a 92% (11/12) match with FlaRL over the 11/24 bases it could be aligned with. The *B.*
304 *bissettii* FD-1 flagellin sequence was a 100% match (22/22) with the Barbour FlaLL primer over

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the 22/24 bases it could be aligned with, 100% (22/22) match with FlaLS, 92% (24/26) match with FlaRS, and its match with FlaRL could not be determined. However, the FlaRL primer was a 96% match (23/24) with the one US 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 copies/uL (1:10M dilution). The LOD of both primer sets in detecting *B. andersonii* DNA was ~7 copies (1:1M dilution), and the LOD of both primer sets in detecting *B. andersonii* DNA was ~6 copies (1:1M 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 US. 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 (36), and a study of >3,000 *A. americanum* from Georgia using PCR with fla primers from Barbour et al. (23) reported detection only of *B. lonestari* (37). 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. bissettii* that have the most

mismatches with these primers, suggesting that they can detect all members of each of these species.

Clark et al. (62) have suggested that *A. americanum* ticks are a significant source of viable *B. burgdorferi* (sl or ss). In our hands, however, the the *flaB* nested PCR assay upon which that suggestion was based produced many faint non-specific 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., negatives when previously suspect-positive, or inconsistent band sizes and numbers) have been reported in another trial of the Clark et al. *flaB* assay using *I. scapularis* ticks (34); in that study, as in ours, sequencing attempts of the PCR products of the unknown tick samples failed. This stochastic fluctuation in PCR outcome may be attributable to the low annealing temperature and/or the high primer concentration. The Clark assay utilizes an annealing temperature of 52°C for the external reaction and 55°C for the internal reaction (26). The optimal annealing temperature in a PCR should be determined empirically, but must be below the melting temperature (T_m) of the oligonucleotide primers. Results of analysis using the T_m calculator indicate that the T_m for range for the *flaB* primers (26) is 60°C-62°C. However, primer mismatches to the DNA template, such as to the *B. lonestari* flagellin gene, reduce primer T_m. Including the *B. lonestari* mismatches into the T_m calculator resulted in a T_m range of 57°C-64°C (the T_m for some primers increased due to an increase in GC percentage once A/T mismatches were removed). The same analysis could be used for all other potential cross-reacting non-target species, which may explain the laddering effect of this nested assay. We

did not take steps to optimize the PCR primers or protocols described by Clark et al. because our aim was to closely replicate the conditions by which *B. burgdorferi* DNA was reported detected in that study. Although these *flaB* primers were described as being specific for Lyme group *Borrelia* spp., we used an independent PCR for a different genetic region (30) to amplify and sequence *B. lonestari* from samples that appeared to be suspect-positive in the *flaB* assay (26).

Nucleotide mismatches at the 3' terminus of a primer significantly reduce polymerase extension. However, a low level of polymerase readthrough can still occur (35), and because PCR results in exponential amplification of a template, even a low level of readthrough can become significant after sufficient PCR cycles. The ability of the Clark primers to amplify *B. andersonii* and *B. americana* has already been experimentally identified (26, 28) but the *B. lonestari* result is unexpected. We hypothesize that 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

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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 non-specific 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 dataset, 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 5 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 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).

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393 However, the low prevalence of infection with *B. lonestari* detected in these samples is
394 congruent with previous surveillance (2, 8, 21, 24, 36, 37). For example, in our program from
395 1997-2010 a total of 18,546 *A. americanum* ticks were tested with nested and real-time primers
396 that were capable of amplifying *B. lonestari*, and 195/17,226 (1.1%) of adults and nymphs, and
397 4/1,320 larvae (0.3% minimum infection prevalence) were positive (2, 8, 20).
398 Ibis reported low-level detection of *B. burgdorferi* DNA in a pooled sample of *A.*
399 *americanum* DNA (sample P189) by three of the eight primer pairs capable of detecting *B.*
400 *burgdorferi*. This pool had also been weakly positive for *B. burgdorferi* in the two assays at
401 TAMU. The PCR signal was very different from that provided by *B. burgdorferi*-infected *I.*
402 *scapularis* ticks, furthermore *B. burgdorferi* sequences were not detected in any of the
403 individual samples in the pool. We suggest the weak result in our assays may reflect low-level
404 contamination of P189, possibly introduced at the DNA isolation and pool-forming step, or the
405 amplification of the remnants of an infected blood meal from a previous life stage. All five
406 samples in this pool were single ticks from Ft. Pickett, VA; two were nymphs, two were females,
407 one was a male, all were unengorged and removed from humans. A few *B. burgdorferi*-infected
408 *I. scapularis* have been submitted to the HTTKP from Ft. Pickett, demonstrating that the
409 pathogen is indeed circulating in that environment (E. Stromdahl, unpublished data). Other
410 Lyme group *Borrelia* species, *B. americana*, *B. andersonii*, *B. bissetti*, etc., were not detected.
411 To summarize, we were unable to confirm any Lyme group *Borrelia* infection in *A.*
412 *americanum* ticks removed from humans in the southeastern and mid-Atlantic US. In our
413 investigation, we utilized the *flaB* primers and thermal-cycling parameters of Clark et al.
414 because this diagnostic tool was associated with the reported finding of Lyme group *Borrelia*

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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* 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 have been to more fully characterize the organism through the amplification and sequencing of multiple genes, or verification by culturing of the organism, to provide more convincing evidence of identification (38). Conclusions based on inadequate evidence exacerbate public confusion; findings of *Borrelia* spp. in novel tick species should in future always be supported by characterization of multiple gene targets.

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DISCLAIMER

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FIGURE LEGEND

Fig. 1. Agarose gels after nested PCR to amplify the *Borrelia flaB* gene (26) in *Amblyomma americanum* ticks. Nonspecific binding was present in all tick samples, but absent in the negative control lane (#2) and the *B. burgdorferi* B31 positive control lane (#12). Only samples with bright bands at 437bp were considered as suspect-positive in our study. (A) Lanes 3 – 11 correspond to pooled samples of ticks; (B) Lanes 3 – 8 correspond to individual tick samples from previously tested pools; lanes 9 – 11 are re-tests of pooled samples. Pooled sample P123 (Gel A, lane 7) and individual sample 131114 from P123 (Gel B, lane 8) were confirmed to be infected with *B. lonestari* by PCR and sequencing of the IGS PCR product and by IA/PCR/ESI-MS (Table 2).

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TABLE 1 Origins of all ticks tested, by state

State	No. ticks tested
VA	661
MD	151
NJ	53
NC	48
KY/TN [a]	40
KY	33
KS	27
TN	24
MO	22
DE	14
SC	11
AL	4
AR	2
FL	2
GA	2
FL/GA[b]	1
RI	1
PA	1
Total	1097

[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

TABLE 2. Analysis of *A. americanum*[a] samples with bright bands at 437-bp in *B. burgdorferi flaB* PCR of Clark et al. (26)[b]

Pool #	Sample # of PCR-positives in pool[c]	USAPHC *flaB* PCR[d] (1st)	USAPHC *flaB* PCR[d] (2nd)	TAMU *Borrelia* qPCR[e]	TAMU IGS PCR[f]	TAMU IGS sequencing	Ibis *Borrelia* genotyping[g] (primers pos/primers)	Follow-up testing of samples in P189			
								USAPHC *flaB* PCR[d]	USAPHC *fla* PCR[h]	TAMU *flaB* PCR[d]	TAMU *flaB* sequencing
P009		Pos	Neg	Neg	Neg	nd	Neg	nd	nd	nd	nd
P084[a]		Pos	nd	nd	nd	nd	*Bb*, 5/8; *Bl* 1, 3/5	nd	nd	nd	nd
	130786[a]	Pos	nd	nd	1000bp	nd	nd	nd	nd	nd	nd
P123		Pos	nd	RF	500bp	nd	*Bl* 1, 3/5	nd	nd	nd	nd
	131114	Pos	nd	RF	500bp	*B. lonestari*	*Bl* 1, 5/5	nd	nd	nd	nd
P171		Pos	Neg	RF	500bp	*B. lonestari*	*Bl* 1, 4/5	nd	nd	nd	nd
P174		Pos	Pos	RF	500bp	nd	*Bl*, 4/5; *Bl* 1, 4/5	nd	nd	nd	nd
	131429	Pos	nd	RF	500bp	*B. lonestari*	*Bl*, 5/5; *Bl* 1, 5/5	nd	nd	nd	nd
	131433	Pos	nd	RF	500bp	*B. lonestari*	*Bl* 1, 5/5	nd	nd	nd	nd
P189		Pos	Neg	*Bb* (very high C_T)	1000bp (faint)	Failed	*Bb*, 3/8	nd	nd	nd	nd
	131536	nd	nd	nd	nd	nd	nd	Neg	Neg	Pos (very faint)	Failed
P191		Pos	Neg	Neg	Neg	nd	Neg	nd	nd	nd	nd
P194		Pos	Pos	RF	500bp	nd	*Bl*, 5/5	nd	nd	nd	nd
	131559	Neg	nd	nd	nd	nd	*Bl*, 1/5	nd	nd	nd	nd
	131560	Neg	nd	nd	nd	nd	*Bl*, 2/5	nd	nd	nd	nd
	131566	Pos	nd	RF	500bp	*B. lonestari*	*Bl*, 5/5	nd	nd	nd	nd
	131571	Neg	nd	nd	nd	nd	*Bl*, 1/5	nd	nd	nd	nd
P211		Pos	Neg	RF	500bp	*B. lonestari*	*Bl*, 5/5	nd	nd	nd	nd

[a] Sample 130786 in P084 had been initially misidentified as *A. americanum* at USAPHC and was later molecularly identified as *I. scapularis* at TAMU

[b] USAPHC, US Army Public Health Command; TAMU, Texas A&M University; Ibis, Ibis Biosciences; nd, not determined.

[c] PCR negative samples not shown

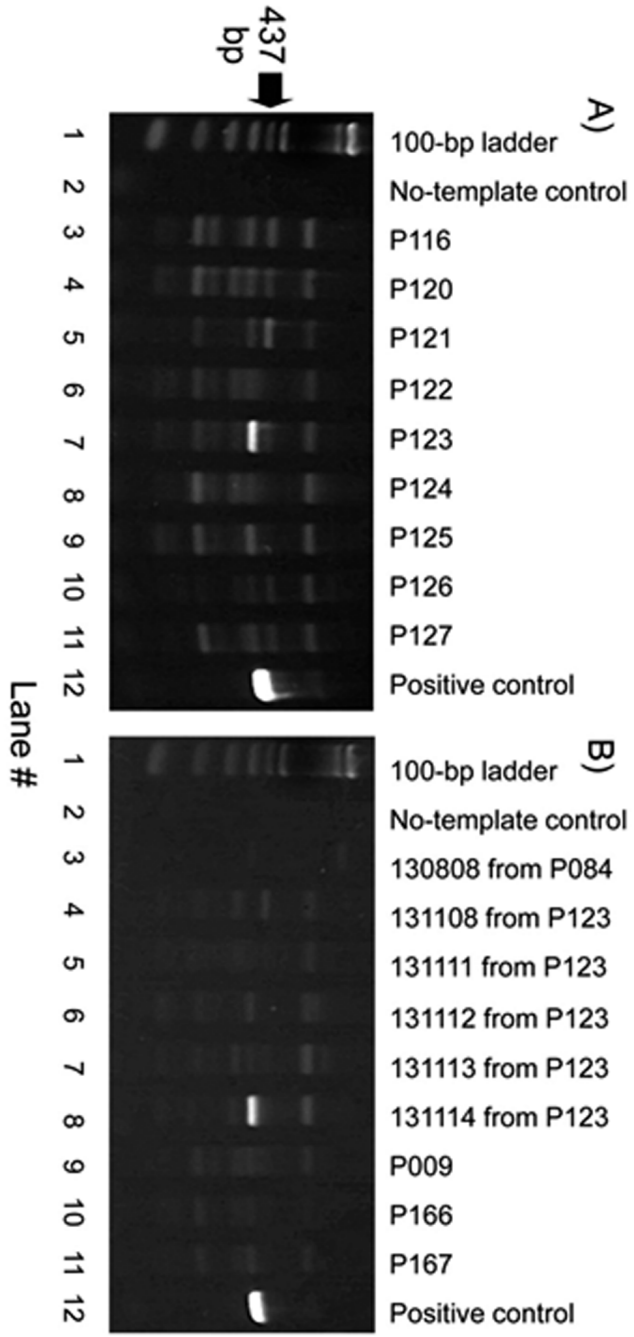
[d] *flaB* PCR for *B. burgdorferi* sl (26).

[e] 16S rRNA qPCR for *Borrelia* spp (31). C_T, cycle threshold.

[f] 16S-23S rRNA intergenic spacer region (IGS) PCR for *Borrelia* spp. (30). RF, relapsing fever group *Borrelia*; *Bb*, *B. burgdorferi*.

[g] PCR, electrospray ionization mass spectrometry (IA/PCR/ESI-MS) for *Borrelia* (32, 33). *Bl*, *B. lonestari*; *Bl* 1, *B. lonestari* var.1.

[h] *fla* PCR for *Borrelia* spp. (23).


437 bp
A)
100-bp ladder
No-template control
P116
P120
P121
P122
P123
P124
P125
P126
P127
Positive control
B)
100-bp ladder
No-template control
130808 from P084
131108 from P123
131111 from P123
131112 from P123
131113 from P123
131114 from P123
P009
P166
P167
Positive control
Lane #
1 2 3 4 5 6 7 8 9 10 11 12