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Detection of *Borrelia burgdorferi* sensu lato species in host-seeking *Ixodes* species ticks in the United States

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Abstract

Lyme disease is the most commonly reported vector-borne disease in the United States and is transmitted by *Ixodes scapularis* in the eastern US and *I. pacificus* in the west. The causative agents, *Borrelia burgdorferi* sensu stricto (Bbss) and *B. mayonii* belong to the *B. burgdorferi* sensu lato (Bbsl) species complex. An additional eight species of Bbsl have been identified in *Ixodes* species ticks in the US, but their geographic distribution, vector associations, human encounter rates and pathogenicity in humans are poorly defined. To better understand the geographic distribution and vector associations of Bbsl spirochetes in frequent and infrequent human-biting *Ixodes* species ticks in the US, we previously screened 29,517 host-seeking *I. scapularis* or *I. pacificus* ticks and 692 ticks belonging to eight other *Ixodes* species for *Borrelia* spirochetes using a previously described tick testing algorithm that utilizes a combination of real-time PCR and Sanger sequencing for *Borrelia* species identification. The assay was designed to detect known human pathogens spread by *Ixodes* species ticks, but it was not optimized to detect Bbsl co-infections. To determine if such co-infections were overlooked particularly in ticks infected with Bbss, we retested and analyzed a subsample of 845 *Borrelia* infected ticks using a next generation sequencing multiplex PCR amplicon sequencing (MPAS) assay that can identify *Borrelia* species and Bbsl co-infections. The assay also includes targets that can molecularly confirm identifications of *Ixodes* species ticks to better inform pathogen-vector associations. We show that Bbss is the most prevalent species in *I. scapularis* and *I. pacificus*; other Bbsl species were rarely detected

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CRediT authorship contribution statement

Lynn M. Osikowicz: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Maria R. Rizzo:** Investigation, Methodology, Writing – review & editing. **Andrias Hojgaard:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Sarah E. Maes:** Investigation, Writing – review & editing. **Rebecca J. Eisen:** Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Supplementary materials

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in *I. scapularis* and the only Bbsl co-infections identified in *I. scapularis* were with Bbss and *B. mayonii*. We detected *B. andersonii* in *I. dentatus* in the Mid-Atlantic and Upper Midwest regions, *B. kurtenbachii* in *I. scapularis* in the Upper Midwest, *B. bissettiae* in *I. pacificus* and *I. spinipalpis* in the Northwest, and *B. carolinensis* in *I. affinis* in the Mid-Atlantic and Southeast, and *B. lanei* in *I. spinipalpis* in the Northwest. Twelve of 62 (19.4%) *Borrelia*-infected *I. affinis* from the Mid-Atlantic region were co-infected with Bbss and *B. carolinensis*. Our data support the notion that Bbsl species are maintained in largely independent enzootic cycles, with occasional spill-over resulting in multiple Bbsl species detected in *Ixodes* species ticks.

Keywords

Tick surveillance; Next generation sequencing; Blacklegged tick; *Borrelia burgdorferi* sensu lato

1. Introduction

Lyme disease is the most commonly reported tick-borne disease in the United States (US) and in Europe (Marques et al., 2021). It is caused by species in the *Borrelia burgdorferi* sensu lato (Bbsl) species complex and spread by *Ixodes* species ticks (Margos et al., 2011; Eisen, 2020). The Bbsl species complex currently includes 20 named and three proposed genospecies, including the agents of Lyme disease in North America (*B. burgdorferi* sensu stricto (Bbss) and *B. mayonoii*) and in Europe (*B. garinii* and *B. afzelii*) (Wolcott et al., 2021). The list of named Bbsl species has expanded rapidly, with eight species recognized since 2011 (Margos et al., 2011; Rudenko et al., 2011; Wolcott et al., 2021).

In addition to the Lyme disease causing spirochetes, Bbss (Benach et al., 1983; Steere et al., 1983) and *B. mayonii* (Pritt et al., 2016a, 2016b), there are currently an additional eight named Bbsl complex species found in *Ixodes* spp. ticks in the US: *B. americana, B. andersonii, B. bissettiae*, *B. californiensis, B. carolinensis*, *B. kurtenbachii, B. lanei*, and *B. maritima* (Wolcott et al., 2021). *Borrelia burgdorferi* s.s. has been the focus of numerous vector competence and ecological studies, but information on the geographic ranges, natural enzootic vectors and hosts, or the potential to infect humans is limited for other Bbsl species (Margos et al., 2011; Rudenko et al., 2011; Wolcott et al., 2021). *Borrelia burgdorferi* s.s. is commonly detected in *Ixodes scapularis* in the Upper Midwest, Northeast and Mid-Atlantic regions and in *I. pacificus* in the Pacific Coast States (Lehane et al., 2021; Fleshman et al., 2021, 2022). *Borrelia mayonii* has been detected at very low prevalence in *I. scapularis* only in the Upper Midwest (Lehane et al., 2021; Fleshman et al., 2022). There is limited evidence that *B. bissettiae* and *B. kurtenbachii* can cause human illness (Rudenko et al., 2011, 2016; Girard et al., 2011; Golovchenko et al., 2016). Reports of *Borrelia* sequences most similar to *B. kurtenbachii* and *B. bissettiae* strain types were detected in clinical samples from California, and these species have also been detected in European Lyme Borreliosis patients (Rudenko et al., 2011; Girard et al., 2011; Strle et al., 1997). *Borrelia kurtenbachii* has been described in *I. scapularis* at low prevalence in the Upper Midwest (Johnson et al., 2018), but *I. scapularis* is generally not considered to be the primary enzootic vector (Margos et al., 2014). *Borrelia bissettiae* is more wide-spread and has been detected in several tick species including *I. pacificus, I. spinipalpis, I. minor*, and *I. affinis* (Postic et al., 1998; Eisen et al.,

2009; Margos et al., 2011). One study reported detecting *B. andersonii* and *B. americana* in suspected Lyme disease patient samples (blood, skin biopsy) from the US by molecular methods (PCR and Sanger Sequencing); this currently is the only report of the detection of these Bbsl species in clinical samples (Clark et al., 2014).

Bbsl spirochetes are believed to be maintained in independent enzootic cycles, often involving *Ixodes* spp. vectors that bite humans less commonly than *I. scapularis* or *I. pacificus*, but in some cases there is "spill over" of Bbsl into frequent human-biting ticks that may serve as bridging vectors to humans (Oliver et al., 2003; Eisen et al., 2009; Maggi et al., 2010; Margos et al., 2011, 2014; Wolcott et al., 2021; Eisen, 2022). Although pathogenicity in humans has not been demonstrated for many Bbsl species, it is not clear if they are truly non-pathogenic in humans, or if they are indeed pathogenic but infrequently encountered and identified or differentiated in clinical specimens (Rudenko et al., 2011; Margos et al., 2011). Many previous studies that explored the geographic distributions and host associations of Bbsl were limited by using assays that could not detect Bbss and Bbsl co-infections, and therefore may under-represent the true prevalence of infections. To better understand the geographic distribution and vector associations of Bbsl spirochetes in the US, we retested host-seeking *Ixodes* spp. nymphs and adults collected across the US that were previously identified as infected with *Borrelia* spirochetes based on a TaqMan PCR tick testing algorithm (Graham et al., 2018). The assay was designed to detect known human pathogens (e.g., *Borrelia burgdorferi* s.s., *B. mayonii*, and *Borrelia miyamotoi*) spread by *Ixodes* spp. ticks, but it was not optimized to detect Bbsl co-infections. To determine if such co-infections were overlooked, we retested a subsample of ticks using a recently described multiplex PCR amplicon sequencing (MPAS) assay that can identify to species and detect co-infections with Bbsl species (Hojgaard et al., 2020). We modified the assay to include targets that can be sequenced to molecularly confirm identifications of *Ixodes* spp. ticks to better inform pathogen-vector associations.

2. Materials and methods

2.1. Source of tick samples

Host-seeking *Ixodes* spp. nymphal and adult ticks were collected by state public health partners participating in CDC's National Tick Surveillance Program (Eisen and Paddock, 2021) or through collaborative research projects from May 2012 to November 2022. Ticks were collected using dragging, flagging, or CO_2 trapping techniques from 23 states, representing five geographic regions (Tables 1 and 2). The ticks were morphologically identified by trained staff using taxonomic keys (e.g., Keirans and Clifford 1978, Durden and Keirans 1996) and were shipped in ~70 % ethanol for tick-borne pathogen testing to the CDC's Division of Vector-Borne Diseases in Fort Collins, Colorado.

2.2. Identification of Bbsl Bbss and B. mayonii positive samples in hostseeking Ixodes spp. ticks using the tick testing algorithm (TTA)

Testing results for Bbss, *B. mayonii* and *B. miyamotoi* from the majority of *I. scapularis* and *I. pacificus* ticks included in this study were reported previously (Lehane et al., 2021; Foster et al., 2023). Ticks were tested individually using a tick testing algorithm (TTA) described

by Graham et al. (2018). Briefly, total nucleic acid was extracted from each tick following previously described protocols (Lehane et al., 2021; Graham et al., 2018). The tick DNA was then screened for Bbss, *B. mayonii, B. miyamotoi, Babesia microti*, and *Anaplasma phagocytophilum* using a series of TaqMan-based multiplex real-time PCRs (Graham et al., 2018). The *Borrelia* testing portion of the TTA is summarized in Fig. 1, and the *Babesia microti* and *Anaplasma phagocytophilum* testing is detailed by Graham et al. (2018). The TTA uses a 16S rRNA pan-*Borrelia* target to detect *Borrelia* spp. If a sample tested positive for the 16S rRNA pan-*Borrelia* target, additional targets were used to identify Bbss (Bbsl *fliD*, Bbss *oppA2*), *B. mayonii (Bbsl fliD*, Bmay *oppA2*), and *B. miyamotoi* (Bm *glpQ*, Bm *purB*) infections (Fig. 1, Graham et al., 2018). One positive control (5 µl of Bbss, *B. maynoii*, or *B. miyamotoi* genomic DNA), 6 tick free extraction controls (5 µl), and one no template control (5 µl of molecular grade water) were included in each PCR. Tick specimens that were positive for the pan-*Borrelia* target, and negative for Bbss, *B. mayonii*, and *B. miyamotoi* targets were considered positive for Bbsl. Sanger sequencing was then performed on only the Bbsl positive samples using two targets capable of determining the Bbsl species (Clp protease subunit A (*clpA*) and dipeptidyl amonopeptidase (*pepX*)) (Graham et al., 2018). The 850bp region of the Clp protease subunit A (*clpA*) gene and a 668bp region of the dipeptidyl amonopeptidase (*pepX*) gene were sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) (Graham et al., 2018). If a clean sequence could not be obtained from the Sanger sequencing of *pepX* or *clpA*, the sample was designated as "*Borrelia* positive, species undetermined." In this study, *B. miyamotoi* positive samples are included in the overall *Borrelia* spp. positive count, but only samples positive for the Bbsl species complex were further analyzed. The TTA testing results reported in Tables 1 and 2 for Bbss and *B. mayonii* were based on the real-time PCR results for the targets listed above, and the Bbsl results were based the real-time PCR 16S pan-*Borrelia* result and Sanger sequencing of *pepX* and *clpA*.

2.3. Description of modified MPAS assay

A modified targeted NGS MPAS assay described by Hojgaard et al. (2020) was used to re-test selected Bbsl positive tick samples that were identified by the TTA (see Section 2.6 Sample Selection for MPAS Testing). The modifications included the use of genus level primers that produce a species-specific *Borrelia* sequence for a 335 bp region of the *flaB* gene and a species-specific tick sequence for a 135 bp region of the mitochondrial (mt) 16S rRNA gene (tick 16S mt-rRNA) (Table 3). The primary multiplex PCRs were performed in 25 µl reactions that contained 5 µl of nucleic acid sample, 12.5 µl Sso Advanced (BioRad, CA, USA), 300 nM of each primer, and molecular grade water. The primary PCRs were performed on a C1000 Touch thermal cycler (Bio-Rad, CA, USA) and included a denature step of 98 °C for 3 min, followed by 40 cycles of 98 °C for 20 s, 58 °C for 20 s and 68 °C for 1 min, and a final 5 min incubation at 68 °C (Hojgaard et al., 2020). After the primary multiplex PCR, the NGS libraries were prepared following the procedures described by Hojgaard et al. (2020). Briefly, the library preparation consisted of a purification of the initial multiplex PCR products, the addition of Nextera XT Indexes (Illumina, CA, USA) and a purification of the indexed products (Hojgaard et al., 2020). Finally, the libraries were pooled, purified, and quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific, MA, USA) (Hojgaard et al., 2020). The libraries were sequenced using the MiSeq Reagent Kit

Nano 500 cycle v2 (Illumina) on an Illumina MiSeq (Illumina) following the manufacturer's instructions. One Positive (5 μl of Bbss B31 and *I. scapularis* DNA) and two negative (5 μl molecular grade water) controls were included in each NGS library.

2.4. MPAS primer evaluation

The *Borrelia flaB* and tick 16S mt-rRNA primers were evaluated to confirm that the sequences produced from each target could distinguish known species of *Borrelia* and hard ticks, respectively. Hard tick specimens representing 20 species (Supplement A: Table S1) were used to evaluate the tick 16S mt-rRNA primers. These specimens were initially identified by trained staff using morphological keys (Keirans and Clifford, 1978; Durden and Keirans, 1996). Total nucleic acid was extracted with a KingFisher Flex MagMax CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocols. A total of 31 *Borrelia* reference DNA samples representing 14 *Borrelia* spp. (Supplement A: Table S2) were used to evaluate the *flaB* primers. The tick and *Borrelia* DNA were sequenced using the MPAS assay described above and the resulting FASTQ files were analyzed with the CLC Genomic Workbench 12.0.2 (Qiagen, Aarhus, Denmark) and DNASTAR Lasergene 17 software (DNASTAR, Madison, WI, USA). The unique tick 16S mt-rRNA and *flaB* sequences were submitted to GenBank (OQ915478- OQ915484, OQ916921-OQ916934, OQ923300, OQ923301)

2.5. MPAS sequencing analysis

The FASTQ files produced from sequencing the tick DNA samples were analyzed using a custom bioinformatics pipeline with the default parameters described by Osikowicz et al. (2023). Briefly, this pipeline first checks the quality of the FASTQ files and then the primers are trimmed, error correction is performed, read pairs are merged, and the reads are grouped into amplicon sequence variants (ASVs). The ASVs are then aligned to the reference sequences using a 95 % and 98 % sequence similarity for the *Borrelia* spp. and tick reference sequences, respectively. The reference sequences used for sequencing analysis can be found in Supplement B: Table S1. A sample was considered acceptable if it had sufficient tick reads (tick 16S mt-rRNA) and *Borrelia* reads (*flaB*). The minimum read cut-off call for a sample to be considered positive for *Borrelia* spp. was set to 50 reads, and the acceptable tick 16S mt-rRNA reads per sample must be within three times the standard deviation of the average log of tick 16S mt-rRNA reads for a sequencing run (Osikowicz et al., 2023). The identified Bbsl sequences, tick sequences, and reference sequences were analyzed with NCBI BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov)) and maximum likelihood phylogenetic tress were created using MEGA-X v10.0.5 software (Kumar et al., 2018). The Bbsl reference sequences selected for the phylogenetic analysis consisted of a combination of the *flaB* sequences generated from our *Borrelia* isolate collection (Supplement A: Table S2, Fig. S2) and the appropriate *flaB* regions from complete chromosome or complete genome *Borrelia* spp. sequences that have been uploaded to GenBank (Fig. 2). The identified *Borrelia flaB* sequence types we detected in this study were grouped into *Borrelia* genospecies based on the *flaB* phylogenetic relationship we observed in this study. The genospecies used in this study serve the purpose of discussing the ecological associations of *Borrelia* and *Ixodes* spp. ticks, not the characterization of novel *Borrelia* species.

2.6. Sample selection for MPAS testing

Ticks that were positive for *Borrelia* spp. by the TTA were re-tested with MPAS to (1) assess the rates of *Borrelia* coinfections that may have been missed with the TTA, (2) confirm tick species identifications using molecular methods and (3) identify the Bbsl genospecies. Two groups of samples were selected to be tested with the MPAS assay based on previous testing with the TTA (Fig. 1). The first group of samples consisted of adults of any *Ixodes* spp. that tested positive for *Borrelia* spp. with, at minimum, the pan-*Borrelia* 16S rRNA target with the TTA (Graham et al., 2018). This sample set was selected to assess the frequency of Bbsl and Bbss co-infections in known *Borrelia* positive samples and to confirm the morphological tick identification. Adult samples were selected to increase the likelihood of ticks being infected with Bbsl because they would have fed on two vertebrate hosts as larvae and nymphs.

The second group selected for MPAS testing consisted of samples (adults or nymphs of any *Ixodes* spp.) that were identified as positive for Bbsl with the TTA (pan-*Borrelia* 16S rRNA positive, but negative for Bbss, *B. mayonii*, or *B. miyamotoi* targets, and Sanger sequenced with *pepX* or *clpA*) (Fig. 1). These samples were amplified and sequenced with the MPAS assay to confirm the morphological tick identification and further evaluate the Bbsl sequence. Not all of the identified TTA Bbsl positive samples were available for additional MPAS sequencing, due to sample depletion during the original tick testing.

Any samples that were positive for *B. miyamotoi* are included in the overall *Borrelia* spp. positive count, but these results were not further evaluated. Results of the TTA and NGS testing are shown in Tables 1 and 2.

3. Results

3.1. Summary of TTA results

As part of CDC's national tick surveillance program or collaborative research projects, we tested a total of 28,667 *I. scapularis*, 850 *I. pacificus*, 392 *I. affinis*, 174 *I. angustus*, 80 *I. spinipalpis*, 35 *I. dentatus*, five *I. brunneus*, four *I. auritulus*, one *I. minor*, and one *I. muris* (Tables 1, 2) using the previously described TTA (Graham et al., 2018). Results from testing *I. scapularis* and *I. pacificus* for Bbss, *B. mayonii* and *B. miyamotoi* (among other human pathogens) were summarized for subsamples of these ticks in Lehane et al. (2021) and Foster et al. (2023). We separated testing results by tick species and their frequency of recorded encounters with humans (Eisen, 2022).

Ixodes scapularis and *I. pacificus* are the most common *Ixodes* species ticks to bite humans in the US (Eisen, 2022). Among the *I. scapularis* tested using the TTA, 5901 (43.2 %) of 13,662 adult and 2685 (17.9 %) of 15,005 nymphs were positive for *Borrelia* spp. and among those, the majority of infections were identified as Bbss (Table 1); 7 (0.1 %) of 6512 adults and 16 (0.2 %) of 7155 nymphs from the Upper Midwest were positive for *B. mayonii*. Of these 23 *B. mayonii* infected ticks, 11 ticks (3 adults and 8 nymphs) were co-infected with Bbss. *Borrelia kurtenbachii* was detected in five nymphs and one adult from the Upper Midwest. Among the 830 adult and 20 nymphal *I. pacificus* tested by TTA, 42 (5.1 %) adults and 1 (5.0 %) nymph were identified as *Borrelia* positive. A total of 23

adults (2.8 %) and a single nymph (5.0 %) were infected with Bbss. Twelve adults were infected with *B. miyamotoi*, five adults were identified as carrying *B. bissettiae*, and two were infected with *B. lanei* (Table 1).

Ixodes affinis was the most commonly submitted *Ixodes* spp. among the tick species that humans encounter less frequently (Table 2) (Eisen, 2022). Submissions were derived from the Mid-Atlantic and Southeast regions. *Ixodes affinis* was the only species among the less frequently encountered ticks that was infected with Bbss. Among the 392 *I. affinis* tested, 228 (58.2 %) were infected with Bbss (Table 2). Three named Bbsl species were identified: *B. andersonii* (in *I. dentatus*), *B. bissettiae* (in *I. spinipalpis* and *I. affinis*), *B. lanei* (in *I. spinipalpis*). A total of 27 *Borrelia* positive ticks were identified by the TTA, but the species could not be determined by that assay (Table 2).

A subsample of 974 ticks that were identified as *Borrelia* positive by TTA were further tested with the MPAS assay and 845 samples passed the acceptable read thresholds and were further analyzed as described in Sections 3.3–3.6.

3.2. MPAS primer evaluation

The *flaB* and tick 16S mt-rRNA primers used in the MPAS assay produced unique sequences for each species of *Borrelia* and hard tick reference DNA, respectively (Supplement A: Figs. S1 and S2). The percent similarity between the tick species sequences were all ≤97.8 %. The most similar sequences were produced from the *I. dentatus* and *I. affinis* reference DNA (97.8 % similar). The *flaB* reference sequences separated the characterized *Borrelia* species by ≤99.1 % sequence similarity, and the *flaB* sequences for *B. carolinensis* strain SW22 (GenBank OQ915480) and *B. bissettiae* strain DN127 (GenBank CP002746) were the most similar.

3.3. Molecular tick ID confirmation

In total, we tested 974 tick samples with the MPAS assay and 845 samples contained acceptable tick 16S mt-rRNA and *flaB* reads and were used in the analysis. Most of the tick DNA samples tested were molecularly identified as *I. scapularis* (741 of 845, 87.7 %). The remaining ticks were molecularly identified as *I. affinis* (64 of 845, 7.6 %), *I. pacificus* (27 of 845, 3.2 %), *I. spinipalpis* (7 of 845, 0.8 %), *I. dentatus* (5 of 845, 0.6 %), and *I. auritulus* (1 of 845, 0.1 %). The tick 16S mt-rRNA sequencing results for 99.4 % (840 of 845) of the tick DNA samples tested matched the original morphological identification and five samples contained sequences that did not match the original morphological identification (Table 4, Fig. 3). All of these tick samples were positive for Bbsl with the MPAS assay. The Bbsl genospecies detected in these samples were only found in other tick specimens with the same molecular ID and not the original morphological ID (Table 4).

3.4. MPAS sequencing analysis

The median number of reads per sample for the tick 16S mt-rRNA and *flaB* targets were 4251 (range: 789–37,782) and 389 (range: 55–5604), respectively. There was a median of 23.5 (Range: 0–214) tick 16S mt-rRNA reads and a median of 0 (Range: 0–9) *flaB* reads identified in the negative controls; and all negative control read counts fell below the assay

thresholds for calling a sample negative for a pathogen (Hojgaard et al., 2020; Osikowicz et al., 2023).

We identified a total of 22 unique *flaB* sequence types (FST 1–22), including six genospecies (*B. andersonii, B. bissettiae, B. carolinensis, B. finlandensis, B. kurtenbachii*, and *B. lanei*) from ticks tested with this MPAS assay (Table 5). All unique sequences were submitted to GenBank (OQ915485-OQ915507). The sequence types were grouped into a Bbsl genospecies based on the *flaB* phylogenetic relation to the reference sequences we used in this study (Fig. 2). The genospecies designations were used as Bbsl species calls for the detailed MPAS assay results below.

Among the 768 *I. scapularis* or *I. pacificus* ticks that were identified as *Borrelia* positive by MPAS, 733 infections were categorized as Bbss, 3 as *B. mayonii*, and a single tick was co-infected with Bbss and *B. mayonii* (Table 1). The two Bbsl infections that were detected were consistent with the TTA testing: *B. bissettiae* was detected in *I. pacificus* from the Northwest, and *B. kurtenbachii* was detected in *I. scapularis* in the Upper Midwest (Table 1). It does not appear that Bbsl species commonly co-infect *I. scapularis* or *I. pacificus* with Bbss.

Consistent with the TTA testing results (Table 2), we identified Bbss in *I. affinis* with the MPAS assay. We confirmed the Bbsl species identities revealed with TTA testing, but further resolved the Bbsl species identities with MPAS. For example, 27 specimens were identified as undetermined *Borrelia* by TTA, none were undetermined using MPAS (Table 2). All of these samples were positive for *Borrelia* spp. with the TTA, but subsequent Sanger sequencing did not produce a clean sequence that could determine the *Borrelia* species. Most of these TTA unidentified *Borrelia* spp. were found in *I. affinis* from the Mid-Atlantic and were identified as *B. carolinensis* with MPAS. The MPAS assay also identified *B. andersonii* in *I. dentatus* from the Mid-Atlantic and Upper Midwest, and *B. bissettiae, B. lanei*, and a sequence most similar to *B. finlandensis* in *I. spinipalpis* from the Northwest (Table 2).

3.5. Comparison of Bbss and Bbsl co-infections yielded by TTA and MPAS assays

The MPAS assay detected Bbss and Bbsl co-infections in 13 tick samples (Table 6). Twelve ticks molecularly identified as *I. affinis* adults from the Mid-Atlantic region were co-infected with Bbss and *B. carolinensis*. These samples were characterized as infected with only Bbss using the TTA assay; *B. carolinensis* infections were overlooked using the TTA. One adult *I. scapularis* from the Upper Midwest was positive for Bbss and *B. mayonii* based on MPAS testing, but positive for only Bbss with the TTA assay. The TTA assay detected Bbss and *B. mayonii* co-infections in 11 *I. scapularis* (8 nymphs, 3 adult) from the Upper Midwest. Only one of these samples was tested with MPAS, and this assay only detected *B. mayonii* in this sample.

3.6. Bbsl sequence variation identified by MPAS assay

The MPAS assay identified multiple *flaB* sequence types for a given genospecies (Table 5). This is potentially significant for public health or ecological studies, as Bbsl species nomenclature changes over time and some divergent clades might later be characterized as new species. The genospecies *B. carolinensis* was detected in *I. affinis* from the Mid-

Atlantic and Southeast regions. The *flaB* sequence types we observed (FST 11, 12, and 13) grouped with the *B. carolinensis* (GenBank CP124072) reference sequence (Fig. 2, Table 5), but was also similar to *B. burgdorferi* SCGT-10 (GenBank AF264895), for which there is no whole genome sequence available, and is therefore not included in our phylogenetic tree. *Borrelia burgdorferi* SCGT-10 was named a strain type of *B. bissettiae* (Lin et al., 2004), prior to the first description of *B. carolinensis*, and the *flaB* sequences are closely related (99.4 % similar).

We also observed *B. kurtenbachii* in *I. scapularis* from the Midwest, and *B. bissettiae* in *I. pacificus* and *I. spinipalpis* from the Northwest region. A single *flaB* sequence type (FST 15) detected in one *I. auritulus* from the Northwest, was 94.9 % similar to *B. garinii* Ekb712 (GenBank CP075418) and is likely an uncharacterized *Borrelia* species (Fig. 2).

The MPAS assay was able to identify multiple *flaB* sequence types of the same genospecies within a sample (Table 6). Two *I. dentatus* nymphs, one from the Mid-Atlantic (FST 3 and FST 4) and the other from the Upper Midwest (FST 5, FST 7, and FST 8), contained multiple *B. andersonii flaB* sequence types. One *I. dentatus* adult from the Mid-Atlantic contained two *B. andersonii flaB* sequence types (FST 2 and FST 6) (Tables 5, 6). Four *I. affinis* adults (3 ticks with FST 11 and FST 12 and one tick with FST 11 and FST 13) contained multiple *B. carolinensis flaB* sequence types and one *I. spinipalpis* nymph (FST 9 and FST 10) contained multiple *B. bissettiae flaB* sequence types (Tables 5, 6). Finally, one *I. spinipalpis* adult contained two *B. lanei flaB* sequence types (FST 17 and FST 21), and a single *I. spinipalpis* adult contained two *B. lanei flaB* sequence types (FST 18 and FST 19) and one *flaB* sequence type (FST 14) 97.9 % similar to *B. finlandensis* Z11 (GenBank CP124070) (Tables 5, 6). This Bbsl species has previously been reported in *I. ricinus* from Europe (Casjens et al., 2011; Kowalec et al., 2017).

4. Discussion

As part of CDC's national tick surveillance program, we tested 30,209 host-seeking *Ixodes* spp. ticks comprised of ten species collected over a decade from 23 states, representing five geographical regions. The prevalence of human pathogens detected in *I. scapularis* and *I. pacificus* were reported previously (Lehane et al., 2021; Foster et al., 2023). *Ixodes scapularis* was the most frequently submitted tick species, and it is the most commonly reported *Ixodes* spp. tick encountered by humans in the eastern US (Eisen, 2022). Consistent with previous studies, Bbss was prevalent in host-seeking nymphs and adults collected from the Northeast, Mid-Atlantic, and Upper Midwest regions (Porter et al., 2021), where *I. scapularis* serves as the primary enzootic vector in cycles involving white-footed mice and other rodents as Bbss reservoirs (Spielman et al., 1985; Tsao et al., 2021). *Borrelia mayonii* was detected only in *I. scapularis* in the Upper Midwest and at very low prevalence. Nearly half of *I. scapularis* infected with *B. mayonii* (11 of 23 ticks) were co-infected with the other North American Lyme disease agent, Bbss. The enzootic transmission cycle of *B. mayonii* is poorly defined, but the spirochete has been isolated from white-footed mice (Johnson et al., 2017). *Borrelia burgdorferi* s.s. and *B. mayonii* co-infections were detected previously in white-footed mice collected in Minnesota (Johnson et al., 2017). Together, the tick surveillance and small mammal testing data indicate a shared transmission cycle of *B.*

mayonii co-circulating with Bbss in the Upper Midwest. However, insufficient numbers of other *Ixodes* spp. ticks were tested from this region, making it unclear if there is another enzootic vector involved in the *B. mayonii* transmission cycle.

Despite retesting a subsample of *I. scapularis* with an assay capable of identifying Bbsl co-infections, we did not detect any additional co-infections in *I. scapularis*, suggesting that our TTA testing likely did not overlook significant numbers of Bbsl that co-occurred with Bbss and indeed host-seeking *I. scapularis* are rarely infected with other Bbsl species. Despite testing 28,667 *I. scapularis*, we detected only six ticks infected with *B. kurtenbachii* from the upper Midwest (four described previously by Johnson et al. 2018), but no other Bbsl species were detected in this tick species. Notably, there is limited evidence suggesting *B. kurtenbachii* infects humans (Rudenko et al., 2011), but the likelihood of human encounters with infected ticks appears to be geographically limited and uncommon. The majority of Bbsl infections were detected in other *Ixodes* spp. ticks: *B. andersonii* in *I. dentatus* in the Mid-Atlantic and Upper Midwest, *B. bissettiae* in *I. pacificus* and *I. spinipalpis* in the Northwest, *B. carolinensis* in *I. affinis* in the Mid-Atlantic and Southeast, and *B. lanei* in *I. spinipalpis* in the Northwest. These findings support the notion that Bbsl species are maintained in largely independent enzootic cycles, with occasional spill-over resulting in multiple Bbsl species detected in *Ixodes* spp. ticks. Given the fairly low infection rates and the finding that Bbsl species are more commonly found in species that bite humans less frequently than *I. scapularis*, we expect human encounters with Bbsl-infected ticks is rare, but encounter risk varies by geographic region.

Submissions of infrequent human-biting ticks were relatively few, and the TTA assay could not accurately detect Bbsl co-infections, and our MPAS sub-sampling was focused on ticks that tested positive for *Borrelia* spp. using the TTA assay. Because of these limitations, we cannot accurately estimate the prevalence of Bbsl species in these tick species. However, the MPAS assay was able to resolve the species identities of several “undetermined” or coinfected ticks detected using the TTA assay (Hojgaard et al., 2020; Osikowicz et al., 2023). By adding the molecular confirmation of *Ixodes* spp. identification to MPAS, we are confident in the identification of the Bbsl *flaB* sequence types and vector associations reported here. The Bbsl-vector associations that we describe are consistent with previous studies, as recently reviewed by Wolcott et al. (2021). Nonetheless, one limitation of the MPAS assay is that the single *Borrelia* target (*flaB*) cannot be used to accurately characterize newly identified Bbsl species. The MPAS assay was developed for high throughput testing. Therefore, we chose a single *flaB* target for its ability to separate known Bbsl species and the tick 16S mt-rRNA target for *Ixodes* species identification. Subsequent *Borrelia* multi-locus sequencing typing (MLST) or whole genome sequencing would be needed to characterize novel Bbsl species. The genospecies we identified in this study are representative of the closest identified *flaB* reference sequence to the FST at this time, and this association may change in the future as new Bbsl species are described.

Although humans reportedly encounter *I. pacificus* less frequently than *I. scapularis* at a national scale, the rate of human encounters with this tick in Pacific Coast states is significant (Nieto et al., 2018; Dykstra et al., 2020; Eisen, 2022). Based on ticks collected from Washington and Oregon, we detected Bbss in *I. pacificus* nymphs (5.0 %) and adults

(2.8 %); *B. bissettiae* was also detected at low prevalence (0.6 %) in *I. pacificus* adults. Although ticks from California were not included in this study, the reported rates of infection are consistent with those reported from California (Padgett et al., 2014; Rose et al., 2019) and ecological relationships may be similar between northern California, Oregon and Washington. Studies from northern California suggest that *B. bissettiae* is maintained in enzootic cycles involving woodrats and deer mice as reservoirs, and *I. spinipalpis* serving as the primary enzootic vector (Eisen et al., 2003, 2009). *Ixodes pacificus* may acquire infections infrequently through feeding on infected woodrats and deer mice and could serve as bridging vectors to humans (Eisen et al., 2003). In our study, we detected *B. bissettiae* in *I. spinipalpis*, but Bbss was not detected in these generally nest-associated (nidicolous) ticks. *Borrelia burgdorferi* s.s. is believed to be maintained in an independent transmission cycle in northern California. In general, deer mice and woodrats, which serve as reservoirs of *B. bissettiae*, are seldomly infected with Bbss. Instead, western gray squirrels are commonly infected with Bbss, and they are often infested with *I. pacificus* (Lane et al., 2005; Eisen et al., 2009). *Borrelia bissettiae* has been implicated as a potential human pathogen, but case reports are rare (Margos et al., 2016; Golovchenko et al., 2016; Rudenko et al., 2011, 2016). Based on the prevalence reported here and known biting behavior of *I. pacificus* and *I. spinipalpis*, human encounters with *B. bissettiae* infected ticks are likely very uncommon in the western US. In dry climates, *I. spinipalpis* is a nidicolous tick that is rarely collected by drag sampling and seldomly comes into contact with humans, but it tends to exhibit more open host-seeking behavior and will occasionally infest humans in more humid environments (Eisen et al., 2006; Dykstra et al., 2020). Although less is known about the enzootic cycle of *B. lanei* (formerly genospecies 2) (Postic et al., 1998, 2007; Margos et al., 2017), *I. pacificus* adults were infected at similar rates to *B. bissettiae* and the infection was similarly detected in *I. spinipalpis*. Additional surveillance is required to assess the likelihood of human encounters with *B. lanei*-infected ticks, but based on our limited sample, exposure probabilities appear similar between *B. bissettiae* and *B. lanei*.

Although it is generally known as a non-human biting tick, occasional records of *I. affinis* encounters have been recorded (Nadolny and Gaff, 2018; Eisen, 2022). *Ixodes affinis* is found in the southeastern US and in recent years, its geographic range has expanded northward into North Carolina and Virginia (Mid-Atlantic region), where it co-occurs with northern populations of *I. scapularis* (Brinkerhoff et al., 2014; Beati et al., 2022). In the present study, using only type strains as references, we detected *flaB* sequences closest to *B. carolinensis* in *I. affinis*. Based on further analysis we found these sequences were most similar to *B. burgdorferi* SCGT-10 (*B. bissettiae*, Lin et al., 2004), but a *flaB* fragment from a whole genome sequence was not available on GenBank for our analysis. *Borrelia carolinensis* has previously been described in *I. minor* and rodents from the southeastern US (Rudenko et al., 2009), and is closely related to *B. bissettiae*. Possibly Bbsl strain types from the southeastern US that were once categorized as *B. bissettiae*, would now be classified as *B. carolinensis* or even a new Bbsl species after additional MLST characterization or whole genome sequencing. Golovchenko et al. (2016) reported a Bbsl isolated from a patient in the southeastern US that clustered between *B. bissettiae* and *B. carolinensis* and concluded this Bbsl was more closely related to *B. bissettiae* but could also represent a novel Bbsl species. Previous studies detected Bbss and *B. bissettiae* in *I. affinis* in the

Mid-Atlantic region (Maggi et al., 2010), and in some cases individual ticks were coinfected with these pathogens. *Ixodes affinis* feeds on a wide range of hosts, including at least 15 mammalian and one avian species (Maggi et al., 2010; Nadolny and Gaff, 2018) and has been implicated as a key enzootic vector of Bbss and *B. bissettiae* in the Southeastern and Mid-Atlantic regions (Oliver et al., 2003; Maggi et al., 2010). Consistent with a previous study from coastal North Carolina (Maggi et al., 2010), we did not detect *B. bissettiae* or *B. carolinensis* in *I. scapularis* tested from the Mid-Atlantic region. However, we did detect Bbss in *I. scapularis* from the Mid-Atlantic region. Our findings, and those of Maggi et al. (2010), suggest that although there is likely overlap in the *B. bissettiae* and Bbss enzootic cycles, with *I. affinis* serving as an enzootic host, *I. scapularis* may be feeding on only a subset of infected hosts. Further studies are needed in this region to elucidate the enzootic transmission cycles of Bbss, *B. carolinensis*, and *B. bissettiae* and to determine the role of *I. affinis* and *I. scapularis* as vectors. Based on limited human encounters with *I. affinis* (Nadolny and Gaff, 2018; Eisen, 2022) and the lack of detection of *B. bissettiae* or *B. carolinensis* in *I. scapularis* in this study and in Maggi et al. (2010), we expect the rate of human encounters with Bbsl infected ticks to be low in the Mid-Atlantic and Southeast regions.

Using the MPAS assay, we also detected *B. andersonii* in *I. dentatus* submitted from the Upper Midwest and provide the first report of a sequence most closely related to *B. finlandensis* in the US (detected in an *I. spinipalpis* adult submitted from the Northwest). The numbers of ticks were too few to draw conclusions about enzootic maintenance, but their rarity and detection in species that seldomly bite humans suggest low probabilities of human encounters with ticks infected with these species. Perhaps it is not surprising that the few additional potential human pathogens (*B. bissettiae* and *B. kurtenbachii*) described thus far in the US (Girard et al., 2011; Rudenko et al., 2011, 2016; Golovchenko et al., 2016) are the Bbsl species most commonly detected in ticks collected by drag sampling and most commonly encountered by humans (*I. scapularis* and *I. pacificus*). Continued surveillance and testing with an assay such as the MPAS used in this study that accurately detects and identifies Bbsl and confirms species identities of ticks will aid in elucidating geographic variation in human risk of exposure to Bbsl spirochetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclaimer

The findings and conclusions of this study are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Data availability

Data will be made available on request.

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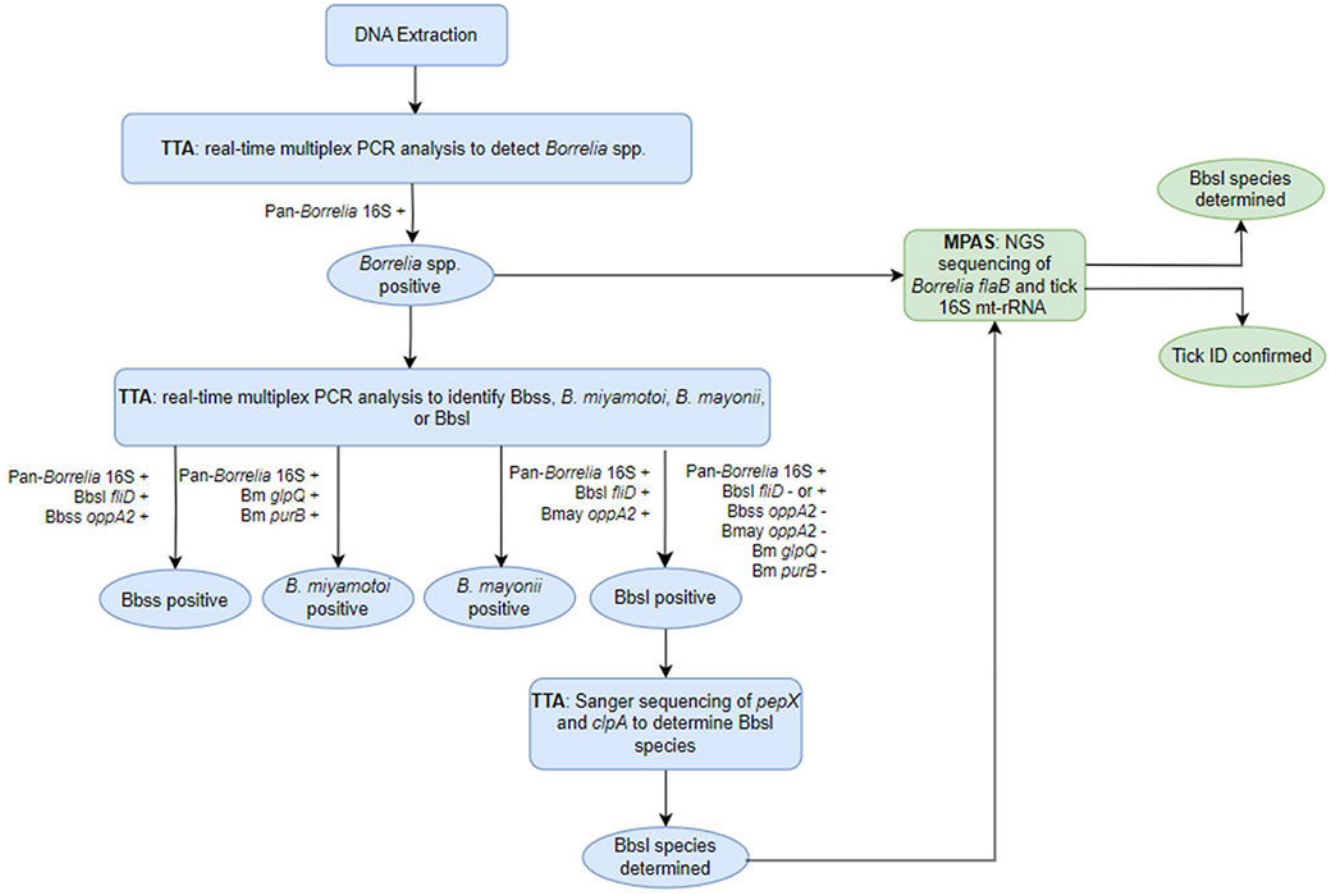

Fig. 1.

Flowchart of the previously performed tick testing algorithm (TTA; blue) and the MPAS assay, which was performed in this study (green). This flowchart only shows the *Borrelia* testing workflow for the TTA. The samples that were selected for the MPAS testing were identified based on the *Borrelia* TTA results. Bbsl: *B. burgdorferi* sensu lato, Bbss: *B. burgdorferi* sensu stricto target, Bm: *B. miyamotoi* targets, Bmay: *B. mayonii* targets.

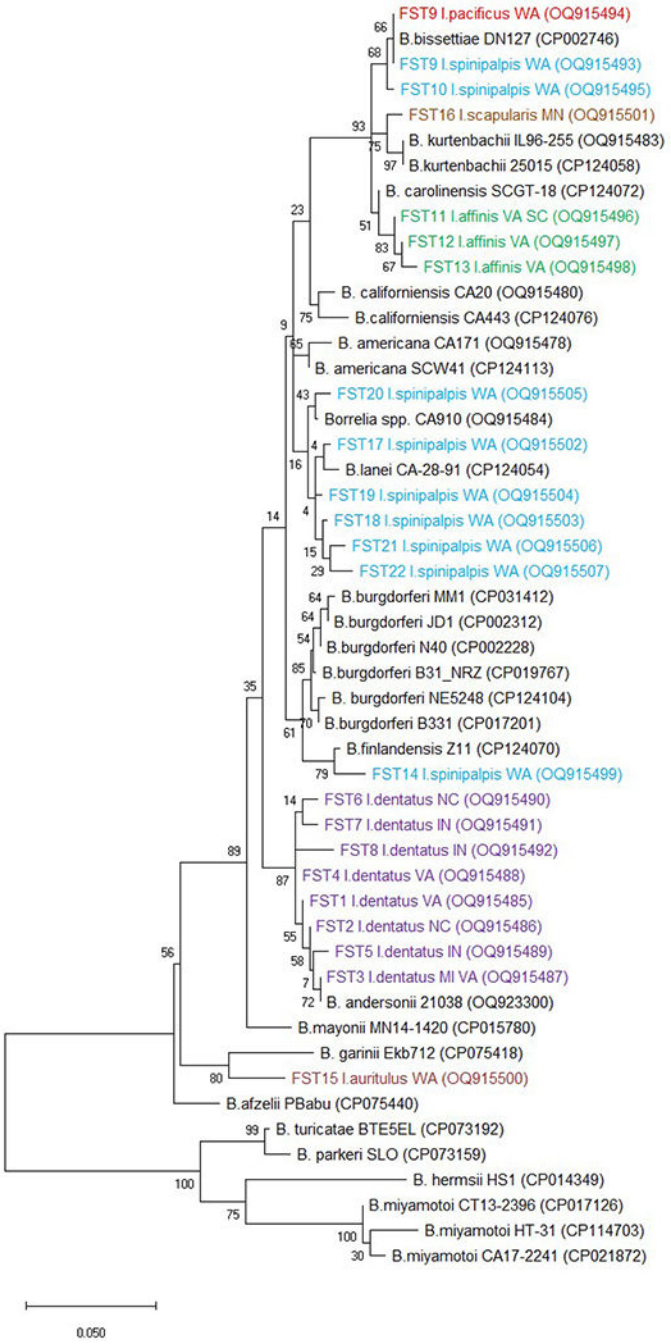

Fig. 2.

The *flaB* phylogenetic tree of representative Bbsl sequence types detected in the *Ixodes* spp. DNA samples. The representative sequences are color coded by the tick species in which the sequences were found. The *flaB* sequence type (FST), tick species, and state of the representative sequence are included in the sequence name. The GenBank Accession numbers for the reference sequences and unique sequences detected in this study are in parenthesis. Bootstrap values are based on 1000 replicates. Red: *I. pacificus*, Blue: *I. spinipalpis*, Green: *I. affinis*, Orange: *I. scapularis*, Purple: *I. dentatus*, Pink: *I auritulus*.

*The GenBank reference sequences used in this phylogenetic tree belong to the *Borreliaceae* family within the genus *Borrelia* (sometimes referred to as *Borreliella* for Bbsl species).

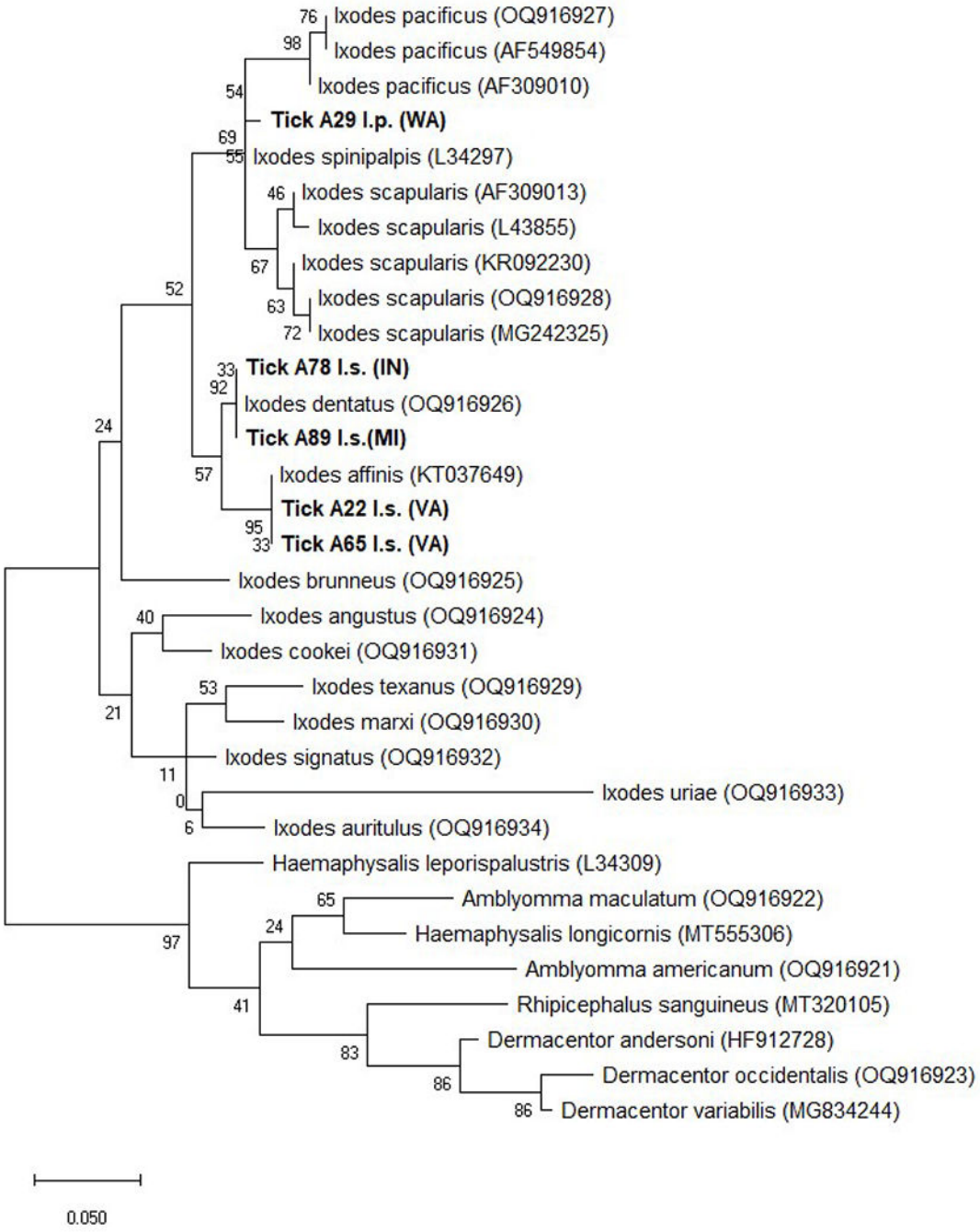

Fig. 3.

The tick 16S mt-rRNA phylogenetic tree of *Ixodes* spp. samples incorrectly morphologically identified (bold). The GenBank Accession numbers for the reference sequences are in parenthesis. Bootstrap values are based on 1000 replicates. The sequence name for incorrectly identified tick samples include the sample number, abbreviation of the morphological ID, and state in parenthesis. I.p.: *I. pacificus*, I.s.: *I. scapularis*.

Table 1

The number of *I. scapularis* and *I. pacificus* samples positive for Bbss and Bbsl with the TTA and MPAS assays. Ticks tested with the TTA assay included ticks submitted to CDC's National Tick Surveillance program for pathogen detection or through collaborative research projects. The tick species for samples tested with MPAS was based on molecular identification. Not all samples positive for Bbsl with the TTA assay were available for testing with the MPAS assay. Bbss: *B. burgdorferi* sensu stricto; Bbsl: *B. burgdorferi* sensu lato.

Region	*Ixodes* Tick spp.	Life stage	Total No. Tested		No. of Bbsl[¶] infected ticks									
					Bbss		*B. mayonii*		*B. bissettiae*		*B. kurtenbachii*		*B. lanei*	
			TTA[//]	MPAS[//]	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS
Mid-Atlantic [‡]	*I. scapularis*	Adult	1274	221	464	215	0	0	0	0	0	0	0	0
		Nymph	5864	0	966	0	0	0	0	0	0	0	0	0
Upper Midwest [‡]	*I. scapularis*	Adult	6512	396	2256	381	7	4	0	0	1	0	0	0
		Nymph	7155	1	1091	0	16	0	0	0	5	1	0	0
Northeast [‡]	*I. scapularis*	Adult	5273	119	3069	116	0	0	0	0	0	0	0	0
		Nymph	1950	0	455	0	0	0	0	0	0	0	0	0
Northwest [‡]	*I. pacificus*	Adult	830	27	23	18	0	0	5	1	0	0	2	0
		Nymph	20	0	1	0	0	0	0	0	0	0	0	0
Southeast [‡]	*I. scapularis*	Adult	603	4	5	3	0	0	0	0	0	0	0	0
		Nymph	36	0	1	0	0	0	0	0	0	0	0	0
Total			**29,517**[§,†]	**768** [†]	**8331** [*]	**733**	**23**	**4**	**5**	**1**	**6**	**1**	**2**	**0**

[*] The TTA assay detected Bbss and *B. mayonii* co-infections in 11 *I. scapularis* (8 nymphs, 3 adult) from the Upper Midwest.

[†] All tick samples were tested for *B. miyamotoi*, but the results are not reported in this study.

[‡] Mid-Atlantic: Kentucky [KY], Maryland [MD], North Carolina [NC], Virginia [VA], West Virginia [WV]; Upper Midwest: Iowa [IA], Indiana [IN], Michigan [MI], Minnesota [MN], Nebraska [NE], Ohio [OH], Oklahoma [OK], Wisconsin [WI]; Northeast: Maine [ME], New York [NY], Pennsylvania [PA], Vermont [VT]; Northwest: Oregon [OR], Washington [WA]; Southeast: Alabama [AL], Mississippi [MS], South Carolina [SC], Tennessee [TN].

[§] Ticks were not all tested for *B. mayonii* with the TTA assay. The total number of ticks tested for *B. mayonii* by region if differed from the table; Mid-Atlantic: 5103 *I. scapularis* nymphs, Northeast: 1494 *I. scapularis* nymphs, Southeast: 529 *I. scapularis* adults, Northwest: 491 *I. pacificus* adults, 31 *I. pacificus* nymphs.

[//] Adults and nymphs tested with the TTA assay included all ticks submitted to CDC for pathogen detection from May 2012 to November 2022. Ticks that tested positive for *Borrelia* spp. by TTA were retested with MPAS, when remaining nucleic acid was available, to determine if Bbss and Bbsl co-infections were missed with TTA testing.

¶The Bbsl genospecies was determined based on the closest *flaB* reference sequence from the CDC's Bacterial Disease Branch, Division of Vector-Borne Diseases *Borrelia* isolate collection (Supplement A: Table S2, Fig. S2) or the appropriate *Borrelia flaB* region from complete chromosome or complete genome GenBank accessions.

Table 2

Bbsl testing results for *Ixodes* spp. that humans occasionally encounter. The tick species for samples tested with MPAS was based on molecular identification. Not all samples positive for Bbsl with the TTA assay were available for testing with the MPAS assay. Bbss: *B. burgdorferi* sensu stricto; Bbsl: *B. burgdorferi* sensu lato.

Region	Tick spp.	Life stage	Total No. Tested		No. of Bbsl[¶] infected ticks															
					Bbss		*B. andersonii*		*B. bissettiae*		*B. carolinensis*		*B. finlandensis*		*B. lanei*		*Borrelia* spp.		Undetermined	
			TTA[//]	MPAS[//]	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS	TTA	MPAS
Mid-Atlantic [‡]	*I. affinis*	Adult	365	62	223	43	0	0	0	0	0	30	0	0	0	0	0	0	20	0
		Nymph	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	I. dentatus	Adult	2	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	0
		Nymph	27	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	I. minor	Adult	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Upper Midwest [‡]	*I. dentatus*	Nymph	6	2	0	0	2	2	0	0	0	0	0	0	0	0	0	0	1	0
Northwest [‡]	*I. angustus*	Adult	163	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
		Nymph	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	I. auritulus	Nymph	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
	I. spinipalpis	Adult	4	4	0	0	0	0	0	0	0	0	0	1	3	4	0	0	1	0
		Nymph	76	3	0	0	0	0	1	2	0	0	0	0	4	1	0	0	1	0
Southeast [‡]	*I. affinis*	Adult	24	2	5	1	0	0	3	0	0	1	0	0	0	0	0	0	0	0
	I. brunneus	Adult	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	I. muris	Adult	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total			**692**[§,†]	**77** [†]	**228**	**44**	**3**	**5**	**4**	**2**	**0**	**31**	**0**	**1**	**7**	**5**	**0**	**1**	**27**	**0**

[†]All tick samples were tested for *B. miyamotoi*, but the results are not reported in this study.

[‡]Mid-Atlantic: Kentucky [KY], Maryland [MD], North Carolina [NC], Virginia [VA], West Virginia [WV]; Upper Midwest: Iowa [IA], Indiana [IN], Michigan [MI], Minnesota [MN], Nebraska [NE], Ohio [OH], Oklahoma [OK], Wisconsin [WI]; Northwest: Oregon [OR], Washington [WA]; Southeast: Alabama [AL], Mississippi [MS], South Carolina [SC], Tennessee [TN].

[§] Ticks were not all tested for *B. mayonii* with the TTA assay. The total number of ticks tested for *B. mayonii* by region if differed from the table; Northwest: 151 *I. angustus* adults, 9 *I. angustus* nymphs.

[//] Adults and nymphs tested with the TTA assay included all ticks submitted to CDC for pathogen detection from May 2012 to November 2022. Ticks that tested positive for *Borrelia* spp. by TTA were retested with MPAS, when remaining nucleic acid was available, to determine if Bbss and Bbsl co-infections were missed with TTA testing.

[¶] The Bbsl genospecies was determined based on the closest *flaB* reference sequence from the CDC's Bacterial Disease Branch, Division of Vector-Borne Diseases *Borrelia* isolate collection (Supplement A: Table S2, Fig. S2) or the appropriate *Borrelia flaB* region from complete chromosome or complete genome GenBank accessions.

Table 3

MPAS Primer used in this study.

Organism	Target	Size[†] (bp)	Forward primer	Reverse primer	Concentration (nM)	Refs.
Borrelia spp.	*flaB*	335	GAGCTTGGAATGCARCCTGC	TCAAGTCTATTTTGRAAAGCAC	300	Hojgaard et al. (2020)
Ixodes spp.	tick 16S mt-rRNA	135	CTGCTCAATGATTTTTTAAATTGCTGTGG	AATTCWTAGGGTCTTCTTGT	300	This paper[*]

[*] The tick 16S mt-rRNA primers were adapted from Black and Piesman (1994).

[†] The amplicon size after the primers are removed.

Table 4

The identified tick specimens that had conflicting morphologic and molecular identifications. FST: *flaB* sequence type.

Tick ID	State*	Morphologic ID	Molecular ID	Life stage	Bbsl† genospecies	FST
A-22	VA	*I. scapularis*	*I. affinis*	Adult	*B. carolinensis*	11 & 12
A-29	WA	*I. pacificus*	*I. spinipalpis*	Adult	*B. lanei*	22
A-65	VA	*I. scapularis*	*I. affinis*	Adult	*B. carolinensis*	13
A-78	IN	*I. scapularis*	*I. dentatus*	Nymph	*B. andersonii*	5, 7, 8
A-89	MI	*I. scapularis*	*I. dentatus*	Nymph	*B. andersonii*	3

*State: Indiana [IN], Michigan [MI], Virginia [VA], Washington [WA].

†The Bbsl genospecies was determined based on the closest *flaB* reference sequence from the CDC's Bacterial Disease Branch, Division of Vector-Borne Diseases *Borrelia* isolate collection (Supplement A: Table S2, Fig. S2) or the appropriate *Borrelia flaB* region from complete chromosome or complete genome GenBank accessions.

Table 5

The Bbsl *flaB* sequence types detected in this study. Sequences were grouped into genospecies based on the phylogenetic clades observed from the maximum likelihood tree (Fig. 2). FST: *flaB* sequence type.

FST	Tick spp.	State*	No. ticks with FST	GenBank Accession	Nearest reference sequence (GenBank Accession)	% Similarity	Bbsl§ genospecies
1	*I. dentatus*	VA	1	OQ915485	*B. andersonii* 21,038 (OQ923300)	99.4	*B. andersonii*
2	*I. dentatus*	NC	1	OQ915486	*B. andersonii* 21,038 (OQ923300)	99.7	*B. andersonii*
3	*I. dentatus*	MI, VA	2	OQ915487	*B. andersonii* 21,038 (OQ923300)	100	*B. andersonii*
4	*I. dentatus*	VA	1	OQ915488	*B. andersonii* 21,038 (OQ923300)	99.1	*B. andersonii*
5	*I. dentatus*	IN	1	OQ915489	*B. andersonii* 21,038 (OQ923300)	99.1	*B. andersonii*
6	*I. dentatus*	NC	1	OQ915490	*B. andersonii* 21,038 (OQ923300)	98.8	*B. andersonii*
7	*I. dentatus*	IN	1	OQ915491	*B. andersonii* 21,038 (OQ923300)	98.5	*B. andersonii*
8	*I. dentatus*	IN	1	OQ915492	*B. andersonii* 21,038 (OQ923300)	97.6	*B. andersonii*
9	*I. spinipalpis*, *I. pacificus*	WA	2	OQ915493, OQ915494	*B. bissettiae* DN127 (CP002746)	100	B. bissettiae
10	I. spinipalpis	WA	2	OQ915495	*B. bissettiae* DN127 (CP002746)	99.7	*B. bissettiae*
11	*I. affinis*	SC, VA	12	OQ915496	*B. carolinensis* SCGT-18 (CP124072)	99.4	*B. carolinensis*
12	*I. affinis*	VA	20	OQ915497	*B. carolinensis* SCGT-18 (CP124072)	99.1	*B. carolinensis*
13	*I. affinis*	VA	3	OQ915498	*B. carolinensis* SCGT-18 (CP124072)	98.5	*B. carolinensis*
14	*I. spinipalpis*	WA	1	OQ915499	*B. finlandensis* Z11 (CP124070)	97.9	*B. finlandensis*
15	*I. auritulus*	WA	1	OQ915500	*B. garinii* Ekb712 (CP075418)	94.9	*Borrelia* spp.
16	*I. scapularis*	MN	1	OQ915501	*B. kurtenbachii* 25,015 (CP124058)	98.8	*B. kurtenbachii*
17	*I. spinipalpis*	WA	2	OQ915502	*B. lanei* CA-28–91 (CP124054)	99.1	*B. lanei*
18	*I. spinipalpis*	WA	1	OQ915503	*B. lanei* CA-28–91 (CP124054)	99.1	*B. lanei*
19	*I. spinipalpis*	WA	1	OQ915504	*B. lanei* CA-28–91 (CP124054)	99.1	*B. lanei*
20	*I. spinipalpis*	WA	1	OQ915505	*Borrelia* spp. (OQ915484)	99.4	*B. lanei*
21	*I. spinipalpis*	WA	1	OQ915506	*Borrelia* spp. (OQ915484)	99.1	*B. lanei*
22	*I. spinipalpis*	WA	1	OQ915507	*Borrelia* spp. (OQ915484)	98.5	*B. lanei*

*States: Indiana [IN], Michigan [MI], Minnesota [MN], North Carolina [NC], South Carolina [SC], Virginia [VA], Washington [WA].

§The Bbsl genospecies was determined based on the closest *flaB* reference sequence from the CDC's Bacterial Disease Branch, Division of Vector-Borne Diseases *Borrelia* isolate collection (Supplement A: Table S2, Fig. S2) or the appropriate *Borrelia flaB* region from complete chromosome or complete genome GenBank accessions.

Table 6

The number of *Ixodes* spp. samples that were positive for Bbss and *B. bissettiae*, *B. lanei* and *B. finlandensis*, and multiple Bbsl sequence types within a Bbsl species detected with the MPAS assay. Not all samples positive for Bbsl with the TTA assay were available for testing with the MPAS assay. Bbss: *B. burgdorferi* sensu stricto; Bbsl: *B. burgdorferi* sensu lato.

Region	Tick spp.	Life stage[†]	No. tested	No. of samples co-infected with Bbss & *B. carolinensis*[§]	No. of samples co-infected with *B. lanei*[§] & *B. finlandensis*[§]	No. of samples co-infected with multiple Bbsl[§] *flaB* sequence types			
						B. andersonii	*B. bissettiae*	*B. carolinensis*	*B. lanei*
Mid-Atlantic [‡]	*I. affinis*	Adult	62	12	0	0	0	4	0
	I. dentatus	Adult	2	0	0	1	0	0	0
		Nymph	1	0	0	1	0	0	0
Upper Midwest [‡]	*I. dentatus*	Nymph	2	0	0	1	0	0	0
Northwest [‡]	*I. spinipalpis*	Adult	4	0	1[*]	0	0	0	1
		Nymph	3	0	0	0	1	0	0
Total			**74**	**12**	**1**	**3**	**1**	4	1

[*] This sample contained two *B. lanei flaB* sequence types and one *B. finlandensis flaB* sequence type.

[†] Adults and nymphs tested with the TTA assay included all ticks submitted to CDC for pathogen detection from May 2012 to November 2022 Ticks that tested positive for *Borrelia* spp. by TTA were retested with MPAS, when remaining nucleic acid was available, to determine if Bbss and Bbsl co-infections were missed with TTA testing.

[‡] Mid-Atlantic: Kentucky [KY], Maryland [MD], North Carolina [NC], Virginia [VA], West Virginia [WV]; Upper Midwest: Iowa [IA], Indiana [IN], Michigan [MI], Minnesota [MN], Nebraska [NE], Ohio [OH], Oklahoma [OK], Wisconsin [WI]; Northwest: Oregon [OR], Washington [WA].

[§] The Bbsl genospecies was determined based on the closest *flaB* reference sequence from the CDC's Bacterial Disease Branch, Division of Vector-Borne Diseases *Borrelia* isolate collection (Supplement A: Table S2, Fig. S2) or the appropriate *Borrelia flaB* region from complete chromosome or complete genome GenBank accessions.